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Gene regulation: translational initiation by internal ribosome binding

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During the past year, several examples of cellular mRNAs have been described in which translational initiation occurs by internal ribosome binding, a mechanism hitherto thought to be restricted to picornaviral RNAs. New insights into the molecular mechanism of internal ribosome entry have been provided by the structural and functional analyses of both the internal ribosome entry sites and the protein factors that stimulate translation mediated by these elements.

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Introduction

Translation initiation of most mammalian mRNAs is initiated by a 'scanning mechanism' [1]. In this mechanism, the 43S ternary complex, composed of the 40S ribosomal subunit carrying the initiator tRNA as well as a set of eukaryotic initiation factors [2,3], binds at the 5′ end of capped cellular mRNAs and moves linearly, scanning the primary sequence of the mRNA, until an AUG codon in the context of a PuNNAUGPu (where Pu indicates A or G, and N any nucleotide) consensus motif is encountered. Subsequently, the 60S ribosomal subunit joins the complex and protein synthesis commences [1,3].

While the scanning mechanism can easily accommodate translational initiation on most known mammalian mRNAs, the efficient translation of certain mRNAs containing long 5′ non-coding regions (5′ NCRs) burdened with numerous AUGs and embedded in consensus motifs, is not easy to envisage [4]. Most notably, mRNAs of picornaviruses contain 5′ NCRs that lack a 5′ m^7GpppG cap, are 600 to 1200 nucleotides in length and harbor many AUG codons [5]. In addition, picornavirus infection results in the specific inhibition of host cell translation [6]. This observation indicated that viral mRNA translation must be initiated by a mechanism that is different from the cap-dependent scanning mechanism used by cellular mRNAs. Indeed, in 1988, Pelletier and Sonenberg [7], and Jang *et al.* [8] showed that the mRNAs of two picornaviruses, poliovirus and encephalomyocarditis (EMC) virus, are translated by the unusual mechanism of internal ribosome binding. Specifically, they showed that internal ribosome entry site (IRES) sequences could be used to create functional dicistronic transcripts [7,8]. This was the first indication that eukaryotic ribosomes can in principle utilize an initiation mechanism resembling the internal initiation mechanism used in prokaryotes.

Over the past year, other viral as well as cellular mRNAs were shown to harbor IRES elements that can be used for internal initiation of translation. This review details studies that seek to answer how widely IRES elements are used, and what the molecular mechanism of internal ribosome binding is.

New strategies for the functional identification of internal ribosome entry sites

Dicistronic mRNAs produced *in vitro* or *in vivo*

As first demonstrated by Pelletier and Sonenberg [7], and Jang *et al.* [8], most investigators have employed dicistronic mRNAs to identify IRES sequences. As diagramed in Fig. 1a, the first cistron of a capped dicistronic mRNA can be translated by a cap-dependent scanning mechanism. The second cistron should not be translated unless preceded by either sequences that mediate internal ribosome entry, or sequences that allow ribosomal reinitiation or ribonuclease cleavage followed by cap-independent translation of this now-monocistronic mRNA.

To demonstrate that translation of the second cistron is indeed due to internal ribosome binding, as opposed to a reinitiation mechanism [9••], it is necessary to show that the translation of the second cistron in intact dicistronic mRNAs is independent from the translation of the first cistron in the same RNA. This was done in several instances by demonstrating three occurrences: firstly, that intact IRES-containing dicistronic mRNA was associated with polysomes in poliovirus-infected cells under

Abbreviations

Antp—*Antennapedia*; **eIF**—eukaryote initiation factor; **EMC**—encephalomyocarditis; **FMD**—foot and mouth disease; **HCV**—hepatitis C virus; **IBV**—infectious bronchitis virus; **IRES**—internal ribosome entry site; **5′ NCR**—5′ non-coding region; ***Tfm***—testicular feminization.

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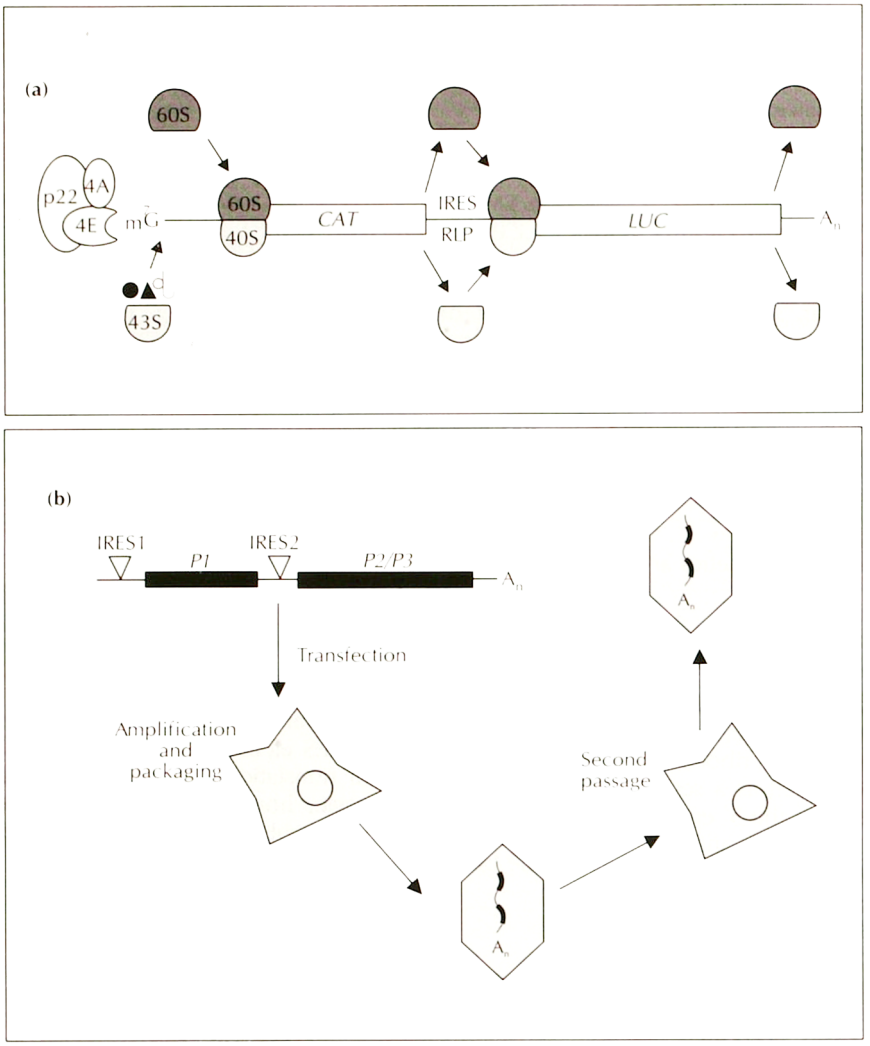

Fig. 1. Strategies for the functional identification of internal ribosome entry sites (IRESs). **(a)** The suggested mechanism for the translation of capped, dicistronic mRNAs containing sequences between the two cistrons (here, the *CAT* and *LUC* genes) that mediate internal ribosome binding. Interaction of the three components of the cap-binding protein complex (p220 and eukaryotic initiation factors 4A and 4E) with the 5′-terminal cap, and the 43S ternary complex, composed of the 40S ribosomal subunit, eukaryotic initiation factors and initiator-tRNA^{Met}, are shown. Sequences mediating internal ribosome binding, termed the IRES or ribosome landing pad (RLP), and the association and disassociation of ribosomal subunits at the beginning and the end of the coding regions, respectively, are indicated. **(b)** Poliovirus as a vehicle for the delivery of dicistronic mRNAs. Dicistronic polioviral transcripts synthesized *in vitro* and containing the 5′ noncoding region of poliovirus (IRES1) and a putative IRES (IRES2) inserted between sequences encoding the viral capsid (P1) and viral non-structural proteins (P2 and P3) are transfected into tissue culture cells (shaded). IRES2 mediates the translation of the non-structural proteins required for amplification of the recombinant viral genomes. High-titer viral stocks can be obtained and used to quantitatively deliver the recombinant RNA into cells for further analysis. This strategy was introduced by Molla *et al.* [16••].

conditions in which cap-dependent translation was inhibited [7,10,11••]; secondly, that direct transfection of uncapped dicistronic RNA into tissue culture cells resulted in the translation of the second but not the first cistron [11••,12] (C Wang, P Sarnow and A Siddiqui, unpublished data); and thirdly, that translation of the first cistron in dicistronic mRNAs was inhibited by an analog of the m^{7}GpppG cap without affecting the translational efficiency of the second cistron [13].

Demonstrating that translation of the second cistron of a dicistronic mRNA does not result from the generation of monocistronic transcripts, produced by nucleases, is a more difficult problem. It has been argued that dicistronic mRNAs are not conclusive tools to identify IRES elements because one can not be certain that the dicistronic transcript is the only transcript produced [14]. One can, of course, never conclusively demonstrate a zero concentration of smaller, uncapped transcripts present in cells that mediate translation of the second cistron. However, it is striking that small deletions in IRES elements have been shown to abolish translation of the second cistron in a dicistronic mRNA without inducing a detectable increase in cleavage of the dicistronic mRNA [8,15,16••].

A further argument against the use of dicistronic RNAs to demonstrate IRES function was that IRES elements, which one could imagine to be position-independent, function with different efficiencies depending on their location in the RNA [14]. This is not really surprising; it is expected that the functional highly structured IRES elements [17,18] may be affected by long-range tertiary interactions between the IRES and other parts of a long RNA molecule. This may explain the deleterious effect on IRES function of certain small mutations located outside the IRES element [15,19,20]. Furthermore, ongoing translation of the first cistron in dicistronic mRNAs may affect the structure of the IRES in a dicistronic context, and thus result in altered translational efficiency of the second cistron. Such effects, termed 'translational attenuation', are known in prokaryotes [21].

Dicistronic mRNAs carried in poliovirions

Very recently, the elegant genetic approach of Molla *et al.* [16••] has provided further evidence of IRES function in the 5′ NCR of EMC virus. A dicistronic poliovirus RNA genome was constructed containing the EMC virus IRES inserted into the normally contiguous poliovirus coding region (Fig. 1b). Transfection of the dicistronic RNA con-

taining two IRESs, into human HeLa cells resulted in the production of polioviruses that had packaged the recombinant genome. Because translation of the P1 coding region was terminated by an introduced stop codon, it was concluded that IRES2 (Fig. 1b) was mediating translation of the P2 and P3 non-structural proteins by an internal ribosome-binding mechanism. Also a deletion in IRES2 abolished the synthesis of P2 and P3 proteins, arguing against the possibility that translation of the P2 and P3 proteins was mediated by a reinitiation mechanism after translation had terminated at the P1 stop codon. A similar result might have been obtained if virus particles were produced that harbored subgenomic P2 and P3 RNA molecules, in addition to full-length viral RNAs containing the two IRES sequences. Because the number of plaque-forming units was linearly dependent on virus stock concentration, it could be concluded that each individual plaque was the result of infection by a single poliovirus particle [16••]. Using viral vehicles as carriers for dicistronic RNAs will be a valuable approach for the identification of IRES elements and for the delivery of dicistronic RNAs with high efficiency into cells.

RNA circles

The use of single-stranded RNA circles to identify and characterize IRES elements is currently being pursued. It has been shown that eukaryotic ribosomes do not bind to circular RNAs composed either of 110 polyadenosine residues [22] or of 73 nucleotides derived from a RNase T1 resistant (and thus lacking G residues) fragment of tobacco mosaic virus [23]. However, both kinds of RNA circles [22,23] could bind to prokaryotic ribosomes. The prediction is that eukaryotic ribosomes should bind to RNA circles containing IRES elements, because a free 5′ end in the RNA should not be needed for the internal ribosome-binding conferred by these elements.

The recent report that RNA molecules, when held together with a DNA 'splint', can be ligated to each other by T4 DNA ligase [24•], makes it possible to construct IRES-containing RNA circles that are up to 1000 nucleotides in length (C-Y Chen and P Sarnow, unpublished data). Upon addition of translation extracts and in the presence of translation elongation inhibitors, an 80S ribosome should form at an AUG codon located downstream of the IRES in such circular RNA molecules. Indeed, preliminary experiments have indicated that intact EMC virus IRES-containing circles sediment at 80S (C-Y Chen and P Sarnow, unpublished data). Therefore, circular RNAs should prove to be useful in the elucidation of the mechanism of internal ribosome binding.

New information on internal ribosome entry sites

Viral elements

IRES elements, usually hundreds of nucleotides in length, have been identified in viral genomes from all genera of the *Picornaviridae*, including poliovirus (genus *Enterovirus*) [7], rhinovirus (genus *Rhinovirus*) [25••], EMC virus (genus *Cardiovirus*) [8] and foot and mouth disease (FMD) virus (genus *Aphthovirus*) [26,27•]. The IRES elements of poliovirus and rhinovirus are very similar, located upstream of the AUG initiator codon [25••]. From this, and experiments in which additional AUGs were added between the IRES and the initiator AUG, it was concluded that ribosomal subunits bind to the IRES and subsequently scan in a 5′ to 3′ direction until the next AUG codon is encountered. There is little similarity between the polioviral/rhinoviral IRESs and those found in the EMC or FMD viruses. It has been found that the EMC and FMD viral IRESs are both located at the initiator AUG codon, suggesting that the ribosomal subunits are recruited directly to the initiator AUG codons in these viruses [27•,28].

An essential feature of the picornaviral IRES element is the presence of a conserved oligopyrimidine sequence located upstream of an AUG codon [29••–31••]. Mutations in the oligopyrimidine sequence abolish IRES function, and the proper spacing between the oligopyrimidine sequence and the AUG codon is also important for the maintenance of a functional IRES [29••–31••]. Because part of the oligopyrimidine sequence reveals complementarity to the 3′ end of ribosomal 18S RNA, it has been suggested that this sequence may function in a manner similar to the Shine-Dalgarno sequence [15,31••]. However, it has not been reported whether the oligopyrimidine–AUG sequence motif can function as an IRES on its own, as predicted by this model.

Much work has been devoted to the identification of viral and cellular proteins that mediate ribosome entry to viral IRES elements [32,33•,34••]. In particular, two cellular proteins, p52 [35] and p57 [30••,36,37], have been identified by their ability to be crosslinked by ultraviolet light to multiple sites in viral IRES elements. Further functional assays are needed to reveal the role of these proteins in internal initiation. In addition, poliovirus encodes a transactivator protein, 2A, that can stimulate IRES usage [38••]. More recently, the eukaryotic initiation factor (eIF)-4F has been shown to stimulate translation of the second cistron in a dicistronic mRNA [39••,40•]. This finding is intriguing, because eIF-4F, also known as the cap-binding protein complex [2,3], is also involved in cap-dependent translational initiation. Furthermore, there seems to be competition for eIF-4F between the cap-dependent and the cap-independent (by internal ribosome binding) initiation pathways [39••]. It is possible that one of the reasons that poliovirus encodes a function that modifies eIF-4F by proteolytically cleaving the p220 component of eIF-4F [41] is to alter eIF-4F to enable the viral RNA to compete for it more efficiently. The proteolyzed form of eIF-4F is known to moderately stimulate internal initiation and inhibit cap-dependent translation *in vitro* [42]. In contrast, EMC virus does not induce the cleavage of the p220 component of eIF-4F and, therefore, may compete more efficiently with cellular mRNAs for eIF-4F.

Two recent reports have described the presence of IRES elements in viruses outside the *Picornaviridae* family. First, hepatitis C virus (HCV), tentatively assigned to be a flavivirus, contains a 5′ NCR whose sequence and predicted secondary structure are more similar to picornaviral 5′ NCRs than to the 5′ NCRs of RNAs from other

flaviviruses. Both the poliovirus and the HCV genome contain long 5′ NCRs with several AUG triplets, some preceded by oligopyrimidine sequences. Two studies reported that the 5′ NCR of HCV, when placed into the intercistronic spacer of a dicistronic mRNA, promoted internal initiation as efficiently as the EMC virus IRES in *in vitro* translation systems [43•] (C Wang, P Sarnow and A Siddiqui, unpublished data). A third study did not find evidence that the HCV 5′ NCR could function as an IRES [44•]. In this last study, additional non-viral sequences were present between the HCV 5′ NCR and the initiator AUG triplet; these extra sequences could have changed structures in the RNA resulting in an abrogation of a functional IRES (C Wang, unpublished data). An IRES was also discovered in mRNA3 of infectious bronchitis virus (IBV), a coronavirus [45••]. The capped mRNA3 is functionally tricistronic encoding 3a, 3b and 3c proteins [45••]. It was argued that 3a is likely to be produced by a cap-dependent scanning mechanism, 3b by a leaky scanning mechanism, in which the 3a initiator AUG is bypassed, and 3c by internal ribosome entry [45••]. The IRES thought to mediate translation of 3c is located within the 3a and 3b coding sequences; the first example of an IRES located within a coding region. It will be very interesting to study the effects of ribosomes engaged in the synthesis of 3a or 3b on IRES usage for 3c translation.

Cellular elements

Because internal initiation mediated by picornaviral IRES is efficient in uninfected cells [46,47], it was clear that the host cell translation apparatus was able to perform this function without the help of viral gene products. This led to the idea that cellular mRNAs, that could escape the inhibition of cap-dependent translation in poliovirus-infected cells [48], may contain functional IRES elements. In fact, it was found that the 5′ NCR of the mRNA encoding the immunoglobulin heavy chain binding protein, whose translation continues in poliovirus-infected cells [48], could be translated by internal initiation [10].

A second example of a cellular IRES came from the examination of the mRNA of the murine androgen receptor. Mice bearing the testicular feminization (*Tfm*) mutation in this gene display altered androgen responsiveness [49]. Curiously, the *Tfm* androgen-receptor mRNA contains a single-nucleotide deletion in the coding region, resulting in short-lived mRNA that produces carboxyl-terminal androgen-receptor peptides by internal ribosome binding [49].

In a search for additional cellular IRES elements, it was noted that 42% of known *Drosophila* genes contain one or more AUG triplets in their 5′ NCRs [50]. The average length of a *Drosophila* gene 5′ NCR is 250 nucleotides [50], five times longer than the average mammalian gene 5′ NCR [51]. One striking example of such a *Drosophila* gene is the homeotic gene *Antennapedia* (*Antp*) whose 5′ NCR is either 1512 or 1727 nucleotides in length, depending on whether transcription was initiated from the *P1* or *P2* promoter [52,53]. A 252 nucleotide sequence element in exon D, common to mRNAs from both transcription units, was found to contain an IRES element [11••]. Moreover, within this IRES is a 55 nucleotide sequence element that is highly conserved among different *Drosophila* species [54]. When placed into the intercistronic region of a dicistronic mRNA, the 55 nucleotide sequence alone functioned as an IRES in cultured *Drosophila* cells (S-K OH and P Sarnow, unpublished data). The function and potential regulation of the *Antp* IRES in *Drosophila* is currently being explored.

Conclusions

Over the past year IRES elements have been discovered in mRNAs from viruses outside the *Picornaviridae*, such as the HCV and coronaviruses, and in cellular mRNAs, such as the homeotic *Antp* mRNA. Novel experimental systems involving dicistronic polioviruses and circular RNAs will serve as useful genetic and biochemical tools to elucidate the mechanism of internal ribosome binding. In addition, the fruitfly *Drosophila* may be the choice for genetic approaches to identify key players in internal initiation and to study their regulation during cell growth.

The surprising finding that the coding region of mRNA3 of coronavirus can harbor an IRES element demonstrates that eukaryotic mRNAs can be functionally polycistronic, opening the possibility of controlling translational initiation within the coding region as well as at the 5′ end of mRNAs. IRES elements within coding regions may provide an interesting way to control gene expression at the cotranslational level.

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This study shows that, in addition to eIF-4F (see [38••]), factor eIF-4E stimulated cap-dependent as well as internal initiation of translation. This finding is surprising because eIF-4E is the protein in the eIF-4F complex that interacts directly with the cap structure present at the 5′ ends of mRNAs. Both [38••,39••] indicate that eIF-4F (composed of eIF-4A, eIF-4E and p220) is a multifunctional translation factor which is involved in different modes of translational initiation.

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