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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.

Current Opinion in Genetics and Development 1993, 3:295-300

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' m7GpppG 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 can 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.
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 Samow and A Siddiqui, unpublished data); and thirdly, that translation of the first cistron in dicistronic mRNAs was inhibited by an analog of the m7GpppG 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 long. It has been shown that circular RNAs containing up to 1000 nucleotides in length (C.Y. Chen and P. Sarnow, unpublished data). Upon addition of translation extracts and IRES-containing RNA circles that are up to 1000 nucleotides long, 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 crosslink to 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.

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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 were present between the HCV 5' NCR and the initiator AUG triplet; these extra sequences could have changed.

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 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.

**Acknowledgements**

We are grateful to Karla Kirkegaard for critical reading of the manuscript and to Valerie Vaden for the artwork. We also thank Chang-You Chen, Susan Hooper, Naomitsu Yizuka and Susan McBratney for helpful comments. The authors' work was supported by grants from the National Institutes of Health (AI25105, AG 07347) and The Council for Tobacco Research, USA. P Sarnow acknowledges the receipt of a Faculty Research Award from the American Cancer Society.

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Identification of an IRES located in the mRNA of a homeotic gene. This finding suggests that internal initiation may possibly be used to regulate gene expression during development in Drosophila.

12. BANDYOPADHYAY PK, WANG C, LIPTON HL: Cap-Independent Translation by the 5' Untranslated Region of Theiler’s Murine Encephalomyelitis Virus. *J Virol* 1992, 66:6249-6256.

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Describes an efficient method for synthesizing site-specifically modified RNA molecules by joining two RNA molecules, held together by a 'DNA splint', with T4 DNA ligase. This strategy can be applied to produce large quantities of circular RNA. This study showed that an oligopyrimidine motif and an AUG triplet, located upstream of the initiator AUG codon, are used for initiation of translation.

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The picornavirus 5' non-coding region harbors at least seven RNA hairpin structures. Interactions of proteins with two of these structures were investigated in this work. Interestingly, these RNA-protein interactions are conserved among certain picornaviruses, implying that this conservation has functional significance.

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