Structural insights into viral IRES-dependent translation mechanisms

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A diverse group of viruses subvert the host translational machinery to promote viral genome translation. This process often involves altering canonical translation initiation factors to repress cellular protein synthesis while viral proteins are efficiently synthesized. The discovery of this strategy in picornaviruses, which is based on the use of internal ribosome entry site (IRES) elements, opened new avenues to study alternative translational control mechanisms evolved in different groups of RNA viruses. IRESs are *cis*-acting RNA sequences that adopt three-dimensional structures and recruit the translation machinery assisted by a subset of translation initiation factors and various RNA binding proteins. However, IRESs present in the genome of different RNA viruses perform the same function despite lacking conservation of primary sequence and secondary RNA structure, and differing in host factor requirement to recruit the translation machinery. Evolutionary conserved motifs tend to preserve sequences impacting on RNA structure and RNA–protein interactions important for IRES function. While some motifs are found in various picornavirus IRESs, others occur only in one type reflecting specialized factor requirements. This review is focused to describe recent advances on the principles and RNA structure features of picornavirus IRESs.

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Introduction

Initiation of translation in a diverse group of RNA viruses differs from the bulk of cellular mRNAs in many ways [1,2]. Most cellular mRNAs initiate translation by a mechanism that depends on the recognition of the m⁷G(5')ppp(5')N structure (termed cap) located at the

5' end of mRNAs. In this mechanism, the 5' cap structure is recognized by a translation initiation factor (eIF) composed of the cap-binding protein eIF4E, the scaffolding protein eIF4G, and the RNA helicase eIF4A. In turn, the 43S complex that comprises the ternary complex (consisting of the initiator methionyl-tRNA_i and eIF2-GTP) bound to the 40S ribosomal subunit is recruited to the mRNA along with eIF1A, eIF1, eIF3, and eIF5. The 43S complex scans the 5' untranslated region of the mRNA until the first initiation codon in the proper context is encountered, leading to the formation of the 48S initiation complex. Base pairing between the start codon and the tRNA anticodon triggers a conformational change in the 43S complex, leading to 48S scanning-incompetent conformation. eIF5 promotes GTP hydrolysis by eIF2 in the 48S complex, followed by phosphate release and displacement of eIF1 from its binding site on the 40S subunit. GTP hydrolysis lowers the affinity of eIF2 for the Met-tRNA_i, eIF2-GDP dissociates and eIF5B replaces it on the Met-tRNA_i. Then, eIF5B and eIF1A promotes the recruitment of the 60S subunit. Finally, ribosomal subunit joining promotes GTP hydrolysis by eIF5B, leaving a competent 80S ribosome with a Met-tRNA_i in the P-site ready for translation elongation (reviewed in [1]).

In contrast to this mechanism used by the vast majority of cellular mRNAs, various viral RNAs have evolved alternative mechanisms to initiate translation [2,3,4]. A paradigmatic example is provided by picornaviruses, which subvert the host translational machinery to promote translation of the viral genome using a cap-independent mechanism. Recruitment of the ribosomal subunits to initiate translation of the viral RNA is governed by a *cis*-acting region designated internal ribosome entry site (IRES) element [5,6]. Indeed, the picornavirus genomic RNA does not contain a cap structure at the 5' end. Instead, a viral protein (VPg) is covalently linked to the 5' end of the viral genome. Moreover, cleavage of host factors by viral-encoded proteases profoundly alter several processes critical for cell viability, including transcription, nucleo-cytoplasmic transport, RNA granules composition, and global protein synthesis [7]. Specifically, cellular mRNA translation is inhibited by the proteolysis of eIF4G, eIF3a, eIF5B, or PABP, dephosphorylation of 4E-BPs and phosphorylation of eIF2α [1,7].

Dicistrovirus and hepatitis C IRESs

In addition to picornaviruses, IRESs have been found to drive translation initiation in various groups of viral

RNAs, including dicistroviruses, hepatitis C virus (HCV) and pestiviruses [3,8]. While all IRESs perform the same function, they possess different structural organization and promote internal initiation by using distinct mechanisms. Regarding factor requirement, the intergenic region (IGR) of dicistroviruses appears to use the simplest mechanism, assembling a 48S complex in the absence of any initiation factor [9]. The IGR is about 200 nt long organized in three-pseudoknotted (PKI, PKII and PKIII) RNA structure [10]. Structural analysis of complexes assembled with the IGR IRES on the 40S ribosomal subunit and the 80S ribosome has shed light on the process leading to translation initiation governed by this peculiar IRES element. The three-dimensional structure of the IGR bound to the ribosome indicates that the pseudoknot PKI mimics a tRNA/mRNA interaction in the decoding center of the A site of the 40S ribosomal subunit. The IGR binding mimics a pretranslocation rather than initiation state of the ribosome. Translocation of the IRES by elongation factor 2 (eEF2) is required to bring the first codon of the mRNA into the A site and to allow the start of translation [11^{••}]. By mimicking the elongation cycle step, these IRESs exploit the dynamic properties of the ribosome, bypassing the regulation of the initiation step.

A higher complexity of IRESs is exemplified by the HCV IRES element, which requires eIF3 and the ternary complex to assemble 48S initiation complexes in reconstitution assays [12]. This IRES element is organized in three domains, designated II, III and IV. Mechanistic studies have shown that the HCV IRES binds directly the 40S subunit, then eIF3 and the ternary complex bind, promoting initiation in the absence of factors required for cap-dependent initiation and for scanning (eIF4A, eIF4B, eIF4E or eIF4G, eIF1, eIF1A) [3]. Subsequent GTP hydrolysis, eIF release and binding of a 60S subunit yield an 80S ribosome placed at the start codon. The function of HCV IRES is conferred by its RNA structure [13], in which specific domains drive different steps of 80S ribosome formation. Domain III binds the 40S subunit and eIF3; domain IV harbors the AUG initiation codon; the pseudoknot places the AUG in the 40S subunit decoding groove; and domain II is involved in eIF2-catalyzed GTP hydrolysis, 60S subunit joining and the configuration of RNA in the decoding groove. Recent studies have shown that the apical loop of domain II contacts the ribosomal protein RPS5, stabilizing the ribosome in a conformation that leads to translocation. Delivery of aminoacyl-tRNA to the ribosome by eukaryotic elongation factor 1A (eEF1A) and subsequent peptide bond formation are then followed by eEF2-catalyzed translocation [14^{••}].

Picornavirus IRESs

Picornaviruses induce a shutdown of cap-dependent translation in infected cells [7]. Furthermore, these RNA viruses hijack the translation machinery and

evade translation inhibition taking advantage of IRES elements [5,6]. These RNAs recruit the 40S ribosomal subunit internally by a process guided jointly by RNA structural motifs, a subset of eIFs and a number of RNA-binding proteins (RBPs). Translation of picornavirus RNA is, therefore, resistant to cap-dependent inhibition.

IRESs, initially reported in the genome of poliovirus (PV) and encephalomyocarditis virus (EMCV) [5,6], drive internal initiation of translation in the mRNA of all members of the *Picornaviridae* family. Picornavirus IRESs are characterized by the presence of ignored AUGs upstream of the functional start codon, and by heavy RNA structure with high GC content [15]. Yet, viral IRESs differ in primary sequence, RNA structure and *trans*-acting factors requirement [8]. A distinctive feature of picornavirus IRESs is their relative long length. According to RNA secondary structure, picornavirus IRESs have been classified into five types designated I, II, III, HCV-like and AV-like (aichivirus-like) (Figure 1). Although there is conservation of the core secondary structure within each group of IRES, there is little similarity among the different types. IRES activity depends on specific sequence motifs located on the RNA architecture in such a way that functionally related elements in different viruses harbor conserved sequence motifs and have a common RNA structure core maintained by evolutionary conserved covariant substitutions.

Host factors critical for picornavirus IRES activity

Picornavirus IRES-dependent protein synthesis rely on the recognition of the IRES element by specific eIFs, which however, depend upon the type of IRES (Table 1). One of these factors is the proteolytic C-terminal fragment of eIF4G that, despite being unable to direct cap-dependent translation, it is fully efficient in type I and II IRES-driven translation initiation. Reconstitution assays have shown that assembly of 48S initiation complexes into IRESs belonging to types I and II require eIF4A, eIF1, and eIF3, in addition to the C-terminal end of eIF4G resulting from the proteolytic cleavage induced by the L protease of foot-and-mouth disease virus (FMDV) or the 2A protease of enteroviruses, but no eIF4E [16,17[•],18]. Unlike types I and II, the AV-like IRESs depend on the RNA helicase DHX29, in addition to eIF4G, eIF4A, eIF4B, eIF1, eIF1A, eIF3 and eIF2 to assemble 48S complexes [19]. Type III IRES requires intact eIF4G [20]. In contrast, the HCV-like elements differ from all the picornavirus in that they do not need eIF4G to assemble 48S complexes in *in vitro* reconstitution assays [21] (Table 1).

It was shown long ago that, in addition to eIFs, auxiliary factors present in extracts of mammalian cells were needed for IRES activity [22]. These factors, subsequently termed IRES-transacting factors (ITAFs) are functional proteins

Figure 1

Schematic of the secondary structure of picornavirus IRESs. The location of domains, sub-domains and RNA motifs referred to in the text is indicated.

associated in ribonucleoprotein complexes involved in RNA biology processes, including transcription, splicing, RNA transport, or RNA stability. The multifunctional nature of these proteins such as polypyrimidine-binding

protein (PTB), poly(rC)-binding protein 2 (PCBP2), SR splicing factor (SRp20), erbB-3-binding protein 1 (Ebp1), upstream of N-ras (unr), hnRNPA1, far upstream element binding protein 2 (FBP2) or Gemin5, among

Table 1

Host proteins controlling picornavirus IRES activity

Type of IRES	I (PV)	II (FMDV)	III (HAV)	HCV-like (PTV)	AV-like (AV)
Factors required for 48S assembly	eIF3, eIF2, eIF4G-Ct, eIF4A, eIF4B, eIF1, eIF1A, PCBP2	eIF3, eIF2, eIF4G-Ct, eIF4A, eIF4B, eIF1, eIF1A	eIF3, eIF2, eIF4G	eIF3, eIF2	eIF3, eIF2, eIF4G-Ct, eIF4A, eIF4B, eIF1, eIF1A, DHX29
ITAFs enhancing IRES activity	PTB, SRp20, PCBP2, Unr, hnRNPA1	PTB, Ebp1			PTB
Negative ITAFs	DRP76:NF45, FBP2	Gemin5	GAPDH		

others [23–26,27*,28,29], exemplify the capacity of ITAFs to act at various steps of gene expression control (Table 1).

PTB (also known as hnRNP I) was reported as an ITAF soon after the discovery of IRESs [23]. This protein recognizes U/C-rich sequences of both type I and type II IRESs, and its binding constrains the RNA structure presumably facilitating the interaction of the IRES with the ribosomal subunits [30,31]. Notwithstanding, recent *in vitro* reconstitution studies showed that PCBP2 is the only ITAF absolutely required to assemble 48S initiation complexes on type I IRESs using purified components [17*]. Complementing this information, studies carried out in living cells have suggested that IRES-dependent translation is enhanced through the concerted action of two or more factors (for instance, SRp20 and PCBP2, or Ebp1 and PTB for type I or type II IRESs, respectively) [27*,32]. In support of the modulating effect of host factors on IRES activity, and consistent with the fact that not only translation but the entire picornavirus replication cycle occurs in the cell cytoplasm, many ITAFs are nuclear proteins that shuttle to the cytoplasm in infected cells [33]. In this way, factors that normally participate in nuclear events associated to RNA metabolism become delocalized and participate in translation modulation of a viral RNA lacking nuclear localization. A well-documented case is SRp20, a splicing factor that has the capacity to stimulate IRES activity and shows a cytoplasmic localization in enterovirus-infected cells [27*]. However, not all factors interacting with the IRES element enhance internal initiation. In the case of enterovirus 71 (EV71) delocalization of FBP2 to the cytoplasm of infected cells negatively regulates enterovirus IRES activity [28]. Similarly, GAPDH [34] and DRP76:NF45 heterodimer [35] negatively impacts on HAV and human rhinovirus (HRV) IRES function, respectively (Table 1). Moreover, the IRES-binding factor Gemin5 negatively regulates IRES-driven translation of both FMDV and HCV [36].

A characteristic of many ITAFs is their recognition as substrates of picornavirus-encoded proteases, which in some cases, leads to generation of truncated peptides with different capacity to modulate translation than the unfragmented polypeptide. For instance, proteolysis of PTB in PV infected cells results in truncated polypeptides that repress IRES activity [37], and proteolysis of Gemin5 in FMDV infected cells [38] leads to the appearance of C-terminal fragments that harbor a noncanonical bipartite RNA-binding motif which downregulates translation [39*]. In contrast, the FBP2 fragment lacking the C-terminal region behaves as an IRES stimulator [28].

In summary, most ITAFs are proteins that shuttle between the nucleus and the cytoplasm, and in many cases are targets of viral proteases or suffer post-translational modifications leading to the reprogramming of gene

expression in infected cells. Although the composition of the IRES-assembled complexes needs to be elucidated in more detail, the available data reveal that ITAFs are not just passengers. Instead, the specific binding of proteins to the different IRES domains suggests an active role of these factors during internal initiation of translation.

RNA motifs critical for picornavirus IRES activity

As mentioned above, IRESs govern initiation of translation of all picornavirus RNAs. Despite a similar general genome organization, the untranslated regions of picornavirus genomes differ in length, sequences and structural elements. These differences also apply to the IRES element, which in most picornavirus genomes precede the single open reading frame encoding the viral polypeptide. Only in the case of cadicivirus A structural and nonstructural proteins are encoded by different open reading frames [40], and some members of the genus cardiovirus encode the L* protein in an alternative reading frame to the polypeptide [41].

Evolutionary conserved motifs tend to preserve sequences impacting on both, RNA structure and RNA-protein interactions eventually determining the activity of the IRES element. However, while some motifs are found in various picornavirus IRESs, others are found only in one type suggesting specialized requirement of factors. The RNA structure of type I IRESs, occurring in enteroviruses (*e.g.* poliovirus), is organized in modular domains (designated II–VI) (Figure 1). These IRESs span about 450 nts in which conserved pyrimidine tracts occur at the base of domain II, and in the central and basal part of domain V [42]. Domain IV harbors a C-rich loop and a GNRA motif (N stands for any nucleotide, and R for purine) [43]. These motifs are conserved in IRESs classified as type II and AV-like [19,44*,45]. Domain V, which harbors a determinant of PV neurovirulence [46], provides the binding site for PTB partially overlapping with eIF4G and eIF4A binding sites during 48S complex assembly [17*]. The 3' border of type I IRES harbors the Yn-Xm-AUG motif in which Yn (a pyrimidine tract of 8–10 nts) is separated by a spacer (Xm, 10–20 nts) from an AUG triplet. This motif has been proposed to be the ribosome entry site, which in turn is separated from the functional initiator codon by a non-conserved spacer (about 150 nts in PV), which is scanned by the initiation complex [47].

The RNA structure of the type II IRESs, exemplified by EMCV and FMDV, is arranged in modular domains (H to L, or 2–5) (Figure 1). Domain 2 (or H) contains a conserved pyrimidine tract that provides a binding site for PTB [23]. Domain 3 (or I) is a self-folding cruciform structure that harbors the conserved motifs GNRA, RAAA, and the C-rich loop [48]. The GNRA motif is essential for IRES activity in both FMDV and EMCV

Figure 2

Secondary RNA structure of the FMDV C-S8 IRES. Accessibility to DMS, RNase T1 and T2 was previously described [40]. For SHAPE reactivity, RNA was folded in HEPES pH 8, 140 mM KCl, 6 mM MgCl₂. 3D RNA model of distal stem-loops was generated using the MC-Fold and MC-Sym pipeline [68] incorporating SHAPE reactivity data as constraints (low: 0.1–0.29; medium: 0.3–0.69, high ≥0.7). MC-Fold input RNA sequences: domain 2 (nt 29–84), subdomain 3a (nt 159–194), subdomain J (nt 321–365), subdomain K (nt 367–397) and domain 5 hairpin (nt 419–440). For simplicity, IRES nucleotides are numbered 1 to 462, such that position 1 of the IRES element corresponds to nt 576 in the GeneBank sequence (Accession DQ409183).

[49,50], adopts a tetraloop conformation [48], and participates in long-range interactions [51]. Domain 4 (corresponding to subdomains J and K) consists of a Y-shape RNA structure and provides the binding-site for eIF4G [52,53]. Finally, domain 5 (or L), which consists of a short hairpin and a conserved pyrimidine tract on its 3' end, provides the binding site for eIF4B, PTB and other RNA-binding proteins [36,54]. Type II IRESs also have a 3' terminal Yn-Xm-AUG motif, but in contrast to type I IRESs, the ribosome entry site is at this AUG codon without any spacer sequence [55].

The relationship between RNA structure and biological function of picornavirus IRESs has been inferred from mutational analysis and RNA probing. Overall, the data derived from functional analysis were in agreement with the conservation of structural motifs in highly variable viral genomes [15]. In particular, covariation data in conjunction with computational RNA modeling [44*,56*] have contributed to define the RNA secondary structure of the FMDV IRES (Fig. 2). Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) reactivity [57*] provided data on the susceptibility of different residues, reinforcing the localization of loops and internal bulges within the IRES structure in solution. Additionally, the lack of attack to several residues within the folded RNA observed after RNase digestion were in accordance with the stems defined by covariation data [44*]. Likewise, incorporation of SHAPE reactivity as constraints to MC-Fold/MC-Sym software generates three-dimensional RNA models of distal stem loops (Figure 2), which are in agreement with RNA accessibility to chemical attack and enzymatic digestion [58]. It is interesting to note that the local RNA flexibility of specific IRES regions, including the apical stem-loop of domain 3, the 10-nt loop of domain 4 (subdomain J), and the basal region of domain 5, are sensitive to the concentration of Mg^{2+} ions in the RNA folding buffer [57*]. Along this line of evidences, recent NMR studies carried out with synthetic oligoribonucleotides corresponding to short stem-loops of the EMCV IRES have shown that Mg^{2+} ions affect the conformation adopted by the GNRA tetraloop and the C-rich loop [59].

In contrast to IRESs belonging to type I and II, little is known about the RNA structural organization of type III IRES, present in Hepatitis A virus (Figure 1). The boundaries of this element, about 410 nts long, were mapped by deletion analysis. In addition, a model of the secondary structure was proposed based on RNA probing and sequence covariation data [60]. The core element contains two principal domains (IV and V) followed by a Yn-Xm-AUG motif, although the upstream 175 nts (encompassing domains II and III) enhanced its activity.

Concerning the growing and diverse group of picornavirus IRESs resembling the HCV IRES, secondary structure

model for the porcine teschovirus 1 (PTV-1) IRES element (Figure 1) predicted domains II and III but domain IV was lacking [61]. On the basis of sequence conservation among HCV-like IRESs it has been proposed that these elements may have arose by horizontal gene transfer [62], and that IRES evolution may involve addition of modular domains to a preexisting core. As in the case of HCV IRES, reconstitution of 48S complexes *in vitro* on the PTV-1 IRES only required 40S subunit, eIF3, eIF2/GTP/met-tRNA [21] (Table 1). Nonetheless, a group of the HCV-like picornavirus IRES showed partial requirement for eIF4F components [63], suggesting mechanistic differences with the HCV IRES. The PTV-1 IRES structure model derived on the basis of sequence covariation and thermodynamic features have been validated by chemical probing using free RNA, 40S-IRES and 48S complexes [64], and the RNA structure of domain IIa of the Seneca Valley virus has been resolved by X-ray crystallography [65].

The RNA structure of the fifth group of picornavirus IRES, the AV-like, is arranged in four domains (H, I, J-K, L) (Figure 1) that share some features with type I, and others with type II. Domain H and the apical region of domain J resemble type I but domain K is essential for eIF4G binding and resembles type II. However, domain I, the basal region of domain K, and domain L are unique to this group [19]. Binding sites for PTB are located in domain I and in the basal region of domain K. Domain L differs from other picornavirus in containing the AUG codon embedded in a stem. This specific feature explains the requirement of the DHX29 helicase to accommodate the start codon in the ribosomal-mRNA binding cleft.

Concluding remarks

IRES elements consist of a modular organization with a distribution of functions among the different domains. Deciphering the RNA structure organization of two groups of viral IRESs, the dicistrovirus intergenic region and the HCV-like IRES, has made great progress in recent years [11**,14**,66*]. However, the three-dimensional structure of picornavirus IRESs is still unknown, likely due to their long RNA sequence, and high RNA flexibility [8,15]. Given the diversity of primary sequences and secondary RNA structures there is still much to learn about how different picornavirus IRESs achieve their function. Although a conserved tertiary structure element unique to these RNA regulatory sequences has not been found, understanding the structural organization of specific domains could contribute to predict hidden IRESs in other genomes by using inverse folding [67]. IRESs seem to operate at multiple levels, from primary sequence to three-dimensional structure assembled in ribonucleoprotein complexes. Thus, elucidating the function of IRES-dependent translation demands a deep understanding of the RNA structural domains that determine their three-dimensional fold, and of how proteins modulating internal initiation recognize this RNA structure.

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