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Towards a structural understanding of IRES RNA function

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Abstract

Protein synthesis of an RNA template can initiate by two different known mechanisms: cap-dependent translation initiation and cap-independent translation initiation. The latter is driven by RNA sequences called internal ribosome entry sites (IRESs) that are found in both viral RNAs and cellular mRNAs. The diverse mechanisms used by IRESs are reflected in their structural diversity, and this structural diversity challenges us to develop a cohesive model linking IRES function to structure. With more direct structural information available for the viral IRESs, data suggest an inverse correlation between the degree to which an IRES RNA can form a stable structure on its own, and the number of factors that it requires to function. Lessons learned from the viral IRESs may help understand the cellular IRESs, although more structural data is needed before any strong links can be made.

Introduction

In translation, a messenger RNA (mRNA) serves first as the platform for assembly of the ribosome (initiation), then as the template for protein synthesis (elongation), and finally signals the end of the protein-coding sequence and ribosome disassembly (termination). In eukaryotes, the majority of translation initiation occurs by a complex, multi-step process that requires a modified nucleotide cap on the mRNA's 5' end. Recognition of this cap leads to binding of eukaryotic initiation factor (eIF) proteins and the small (40S) subunit of the ribosome, which then scans the message until it finds the appropriate start codon, at which the large (60S) ribosomal subunit is recruited to form a translationally competent ribosome (review: [1]). This canonical cap- and scanning-dependent initiation does not account for all of the protein synthesis in a eukaryotic cell: translation initiation can also occur by a cap-independent process in which sequences within the mRNA recruit the translation machinery, often elimination the need for many eIF proteins (Figure 1a). This type of translational initiation occurs independent of the 5' end of the RNA, it is called internal initiation of translation and the RNA sequences responsible are called "internal ribosome entry sites (IRESs)" (reviews: [2–5]).

IRESs were first discovered in the 5' untranslated regions (5' UTRs) of two picornavirus RNAs where they comprise highly conserved RNA sequences with a large amount of secondary structure, suggesting that these IRESs work by functionally replacing the cap and some eIFs with structured RNA. Efforts to understand the structural basis for the function of these viral

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IRESs began almost immediately after their discovery. Subsequent discovery of IRESs in many other viral RNAs and in a variety of cellular mRNAs uncovered substantial diversity in IRES-driven translation and IRES structure. What have we learned about the structural basis of IRES function? Are general trends emerging as more IRES-containing RNAs are identified and more structural information is gathered? Can we apply what has been learned about the structures of viral IRESs to cellular IRESs? Where should ongoing IRES structure-function studies be focused? In this short review we offer our perspective on these questions using some illustrative examples and recent discoveries, without attempting a comprehensive review of the field.

The structural diversity of viral IRES RNAs reflects functional diversity

Viral IRESs are defined by their ability to drive cap-independent translation, but the origin of the IRES RNA and the context in which it functions can vary dramatically (Figure 1a & b), correlating to a rich diversity of structures. In many cases, the viral IRES can respond to different cellular conditions. For example, the positive sense (+), single stranded RNA (ssRNA) *Dicistorviridae* viruses are activated by cellular conditions associated with the cell's own antiviral response [6] (review: [7]). Retroviruses such as lentiviruses use a mix of cap- and IRES- driven translation to regulate protein synthesis during the cell cycle, indicating an ability to respond to cellular conditions [8,9]. Much information regarding the structural basis of IRES function comes from a mix of structural, biochemical, biophysical, and functional studies of IRESs from (+) ssRNA viruses. These IRESs can be divided into fairly distinct mechanistic classes based on their protein factor requirements and secondary structures (Figure 2 & Figure 3), as well as their three-dimensional structures and folding architectures (Figure 4 & Figure 5). This existing functional data in combination with structural information provides an emerging correlation between the folding characteristic of different IRES RNAs and their factor requirements, which we describe below.

Compactly folded IRES RNAs—The most highly structured IRES RNAs yet identified are the (+) ssRNA *Dicistroviridae* intergenic region (IGR) IRESs (hereafter referred to as the IGR IRESs) that fold into a specific and compact three-dimensional structure [10]. In fact, these IRES RNAs have biophysical characteristics that are similar to catalytic RNAs, emphasizing their tightly folded character. These highly structured IRESs do not require any initiation factors, and operate essentially as an all RNA-based ribosome recruitment apparatus (for review: [6]). The folding characteristics of the IGR IRESs make them amenable to high-resolution structural studies; detailed structural information has come from a combination of X-ray crystallographic studies of the unbound IRES RNA (Figure 5a) [11,12] and cryo-EM studies of IRES RNA bound to the ribosome (Figure 4a,d) [13,14]. Even before encountering the ribosome, the IGR IRESs "prefold" into a conserved conformation that is required for binding both ribosomal subunits in a process that, remarkably, does not require GTP hydrolysis [10,15–18]. The IRES occupies part of the space within the ribosome used by tRNAs, changes the conformation of both ribosomal subunits, and directs translation initiation from a non-AUG codon in the ribosome's A-site, suggesting that the IRES actively manipulates the ribosome (for review: [6,19,20]) possibly by mimicking a hybrid state tRNA [12,21].

Extended IRES RNAs with compact regions—The hepatitis C virus (HCV) IRES (and structurally related IRESs such as classical swine fever virus, CSFV) are part of a second structural class of IRESs that are mostly extended but maintain some structured and tightly packed regions [22] (review: [23,24]). The extended nature of the this class makes high-resolution structural studies of large portions of the RNA difficult, mandating a "divide and conquer" approach in which the structures of individual secondary structural elements are solved by NMR and crystallography (Figure 5b) [25–32], and then docked into cryo-EM reconstructions [33]. Like the IGR IRESs, the HCV IRES binds directly to the 40S ribosomal subunit and alters its conformation [34], but unlike the IGR IRESs, it also binds directly to

eIF3 and it requires initiator tRNA, eIF2, and GTP hydrolysis to generate 80S ribosomes [35] (although recent reports show that under certain *in vitro* conditions, the HCV and CSFV IRESs can bypass some factor requirements [36,37]). Some reports implicate a role for IRES trans-acting factors (ITAFs; proteins not part of the canonical translation initiation machinery but important for the function of a specific IRES) in enhancing of inhibiting translation from the HCV IRES [38–44], but their role is unclear and there is no evidence they alter the global fold of the RNA or are necessary for 40S subunit binding [45,46]. Detailed mechanistic studies using a combination of genetic and biochemical analysis show that different parts of the HCV IRES structure are involved in different steps of the preinitiation complex assembly process. Specifically, one part is important for binding the 40S subunit [47–50], another promotes binding of eIF2 and tRNA [51,52], and yet another promotes phosphate release from eIF2 [30] (for review: [23,24]). Thus, by remaining extended even when bound to the ribosome (Figure 4b, c, e) [33,34,53], the HCV IRES correctly positions various structural elements to interact with, direct, and coordinate the action of different components of the translation initiation machinery in a process that is more complex than that used by the IGR IRESs.

Extended and largely flexible IRES RNAs—The final structural class of (+) ssRNA viral IRESs considered here do not fold into globally compact structures, but retain some conformational flexibility before binding to the ribosome and other factors. They comprise mostly picornavirus IRESs such as the foot-and-mouth disease virus (FMDV) (review: [54, 55]), but some picornaviruses have HCV-like IRES [56,57] (review: [58]). Using the FMDV IRES as an example, there is evidence for long-range RNA-RNA interactions [59–61] but no direct evidence of tightly packed regions. Specifically, solvent-accessibility probing experiments with the IGR IRESs and HCV IRESs reveal protected areas of tight RNA backbone packing [10,22]; however, no such protections have been found in the FMDV IRES (unpublished data). This does not mean that the FMDV IRES is unstructured, but indicates it has a very different biophysical character than do the IGR and HCV-like IRESs. The FMDV IRES cannot bind directly to the 40S subunit, but requires the C-terminal half of eIF4G, eIF4A, eIF5B, eIF3, and two ITAFs: polypyrimidine binding protein (PTB, also called hnRNP-I), and ITAF45 [62–65]. Poliovirus, encephalomyocarditis virus (EMDV), and hepatitis A virus (HAV) IRESs also require several eIFs and often ITAFs in order to recruit the ribosome (review: [4]). The requirements of these less-structured IRES RNAs for more factors suggests that the IRES is part of a multi-component ribonucleoprotein (RNP) complex with eIFs and ITAFs organized on an RNA scaffold that then recruits the ribosome. Because there are no high-resolution structures or cryo-EM reconstructions of these IRESs, the three-dimensional structures of IRES RNPs and any conformational changes in the ribosome that occur within the preiniation complex are unknown.

When the biophysical characteristics of the IGR, HCV, and FMDV IRES RNAs are compared, we see a trend: the amount of inherent folded structure in the unbound IRES RNA is inversely correlated with the need for ITAFs and eIFs (Figure 2). We readily admit that this observation is based on a limited number of examples, and that once the three-dimensional structures, biophysical characteristics, and factor requirements of many other viral RNAs are known, this view could change.

What are the structural roles of ITAFs?

The need for ITAFs on the less-structured viral IRES RNAs raises the question of ITAF function. One hypothesis is that they stabilize a specific IRES RNA conformation that enables binding of other factors or the ribosome. Support for this “chaperone model” is found in the fact that the more structured IRESs such as the IGR IRES and the HCV-like IRES do not need ITAFs to bind the ribosome or eIFs, but that binding of PTB and ITAF45 to the FMDV IRES induces structural changes in the IRES, and synergistically enhances eIF4G and eIF4A binding

[62,66]. The chaperone model is appealing and clearly explains the role of ITAFs on some IRESs, but it does not preclude the possibility that in some cases ITAFs could make direct contact to the ribosome or other factors (Figure 6). It also elicits the question of what components of the IRES RNA make up the ribosome-binding surface.

Are structures of viral IRESs teaching us anything about cellular IRESs?

Unlike viral IRESs, which do not originate in the cell, cellular IRESs perform many roles associated with differentiation, mitosis, stress, proliferation, and other conditions where cap-dependent translation initiation is diminished [3]. For example, hypoxia inhibits cap-dependent protein synthesis but leads to translation of growth factors that promote blood vessel formation, including IRES-driven proteins such as the alpha subunit of hypoxia-inducible factor-1 (HIF-1) [67,68] and fibroblast growth factor 2 [69]. HIF-1 transcriptionally upregulates vascular endothelial growth factor-A, which itself uses two IRESs [70]. These examples illustrate the idea that cellular IRESs, much like viral IRESs, have evolved to respond to the state of the cell and to tightly regulate translation from individual messages, possibly in a variety of ways.

Given advances in understanding viral IRES structure, it is worth asking if we can apply what we have learned about the structural basis of viral IRES function to cellular IRESs. One area in which viral and cellular IRESs are similar is the factors they employ. The factor requirements for only a few cellular IRES have been determined, but for those it is clear they also use many eIFs and ITAFs. For example, the c-*myc* and N-*myc* IRESs use the C-terminal part of eIF4G, eIF4A, eIF3, and eIF2; while the L-*myc* IRES uses eIF4F complex, poly-(A) binding protein (PABP), and eIF3 [71]. Human immunoglobulin heavy-chain binding protein IRES uses the C-terminal part of eIF4G that binds eIF4A [72], and the *S. cerevisiae YMR181c* IRES binds PABP and recruits eIF4G [73]. Also, many of the same proteins identified as viral ITAFs are used by cellular IRESs, inlcuding PTB, hnRNP-A1 (and other hnRNP proteins), La, and unr.

The rate at which cellular IRESs have been discovered has far outpaced the rate at which they have been structurally characterized. In many cases it is not clear that a defined secondary structure exists or plays any role in the activity of the IRES, hence we hesitate to draw strong conclusions. However, the structures of the c-*myc* and apoptotic peptidase activating factor **1** (Apaf-1_ IRESs are illuminating. In the c-*myc* IRES, the tertiary structure contains several pseudoknots; interestingly, destabilization of certain structures within the IRES increases its activity, suggesting these structures are inhibitory [74]. Likewise, ITAF binding to the Apaf-1 IRES RNA causes part of the RNA to become less structured and increases IRES activity, probably by providing access for ribosomal subunits or other factors to bind [75]. Recently, it has been shown that the activity of certain yeast and *Drosophila* IRESs is inversely correlated to the stability of any secondary structure [76]. The pattern of less structure = higher IRES activity seen in these cellular IRESs seems very different from the functional requirements of viral IRESs to form very specific and stable structures.

The recognition that ITAFs can induce structural changes in cellular IRESs (and viral IRESs as well) leads to an appealing model of how these IRESs precisely response to changing cellular conditions. Specifically, as a cell's state changes, the localization and cellular concentration of proteins are altered, and this is sensed by structural changes and activation of a subset of cellular IRESs, leading to translation of specific messages (review: [77]). Evidence for this model is found in studies showing that known ITAFs are regulated by changes in subcellular localization [78], but there is little direct evidence linking these localization changes to IRES RNA structure.

Conclusions

It is clear that "diverse" is the word that best describes IRES structure. No universally conserved IRES sequences or structural motifs have yet been identified (although structures such as pseudoknots appear often). In some cases, specific and stable RNA structure is necessary for IRES activity; in other cases stable structure is inhibitory. In viral IRESs, there appears to be a correlation between the biophysical characteristics of the folded architectures of IRES RNAs and their eIF/ITAF requirements. In most instances it is difficult to extend what we have learned from viral IRES structure to cellular IRES structure. However, we suspect common features will emerge in how they use some ITAFs and eIFs as more IRES structures and IRES RNP structures are studied.

The gaps in knowledge help chart a path for future structural studies of IRESs: to date there is direct structural data for only two viral IRESs; exploring the global architecture and high-resolution structures of more IRES RNAs and also eIF- and ITAF-containing IRES RNPs will be challenging but rewarding. In addition, studies to understand the dynamics of IRES RNA structures and IRES RNP composition will be critical to develop models describing how IRESs manipulate the translation machinery and how they operate within the changing conditions of the cell. Finally, as structural information is amassed on more cellular IRESs, we should gain a better understanding of the similarities and differences when compared to viral IRES.

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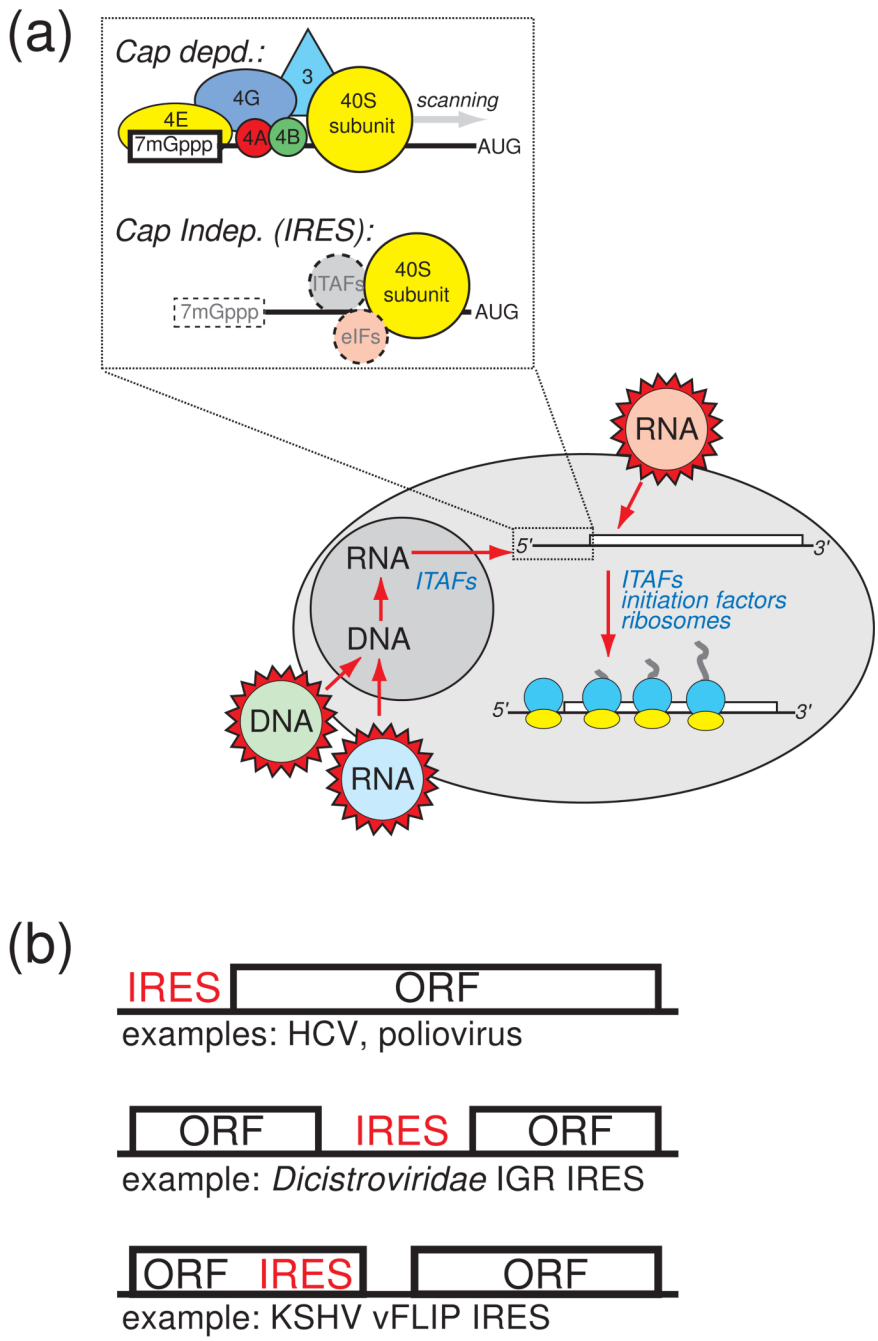

Figure 1. Cap- and IRES- dependent translation initiation

(a) At upper left is a cartoon illustrating a simplified mechanism for IRES-driven initiation compared to cap-dependent initiation. In cap-dependent translation, the 7-methyl guanosine cap is bound by eIF4E, and this leads to the binding of many other factors, recruitment of the ribosome, and scanning to the start codon. IRESs do not use the cap structure, although IRES-containing messages can be capped (dashed box), and they may or may not use canonical eIFs and ITAFs to recruit the ribosome to the message (dashed circles). The diagram below illustrates the sources of IRES RNAs in the cell. They can come from RNA viruses that introduce RNA directly into the cytoplasm (red), and thus never experience the nuclear environment. IRESs that do have a “nuclear history” include those from RNA viruses whose

genetic information is reverse transcribed and integrated into the host's genome (blue), DNA viruses (green), and cellular IRESs. The degree to which nuclear history plays a role in the binding of specific factors to certain IRESs, and the effect it may have on the structure of the IRES, is a very important question under exploration. **(c)** Some mRNA contexts in which IRESs are found. Most are found in the 5' untranslated region of the mRNA or viral RNA (top), but some are found between open reading frames in intergenic regions (middle), and they can also reside within (or partially within) coding regions (bottom). Viral IRES examples are provided for each.

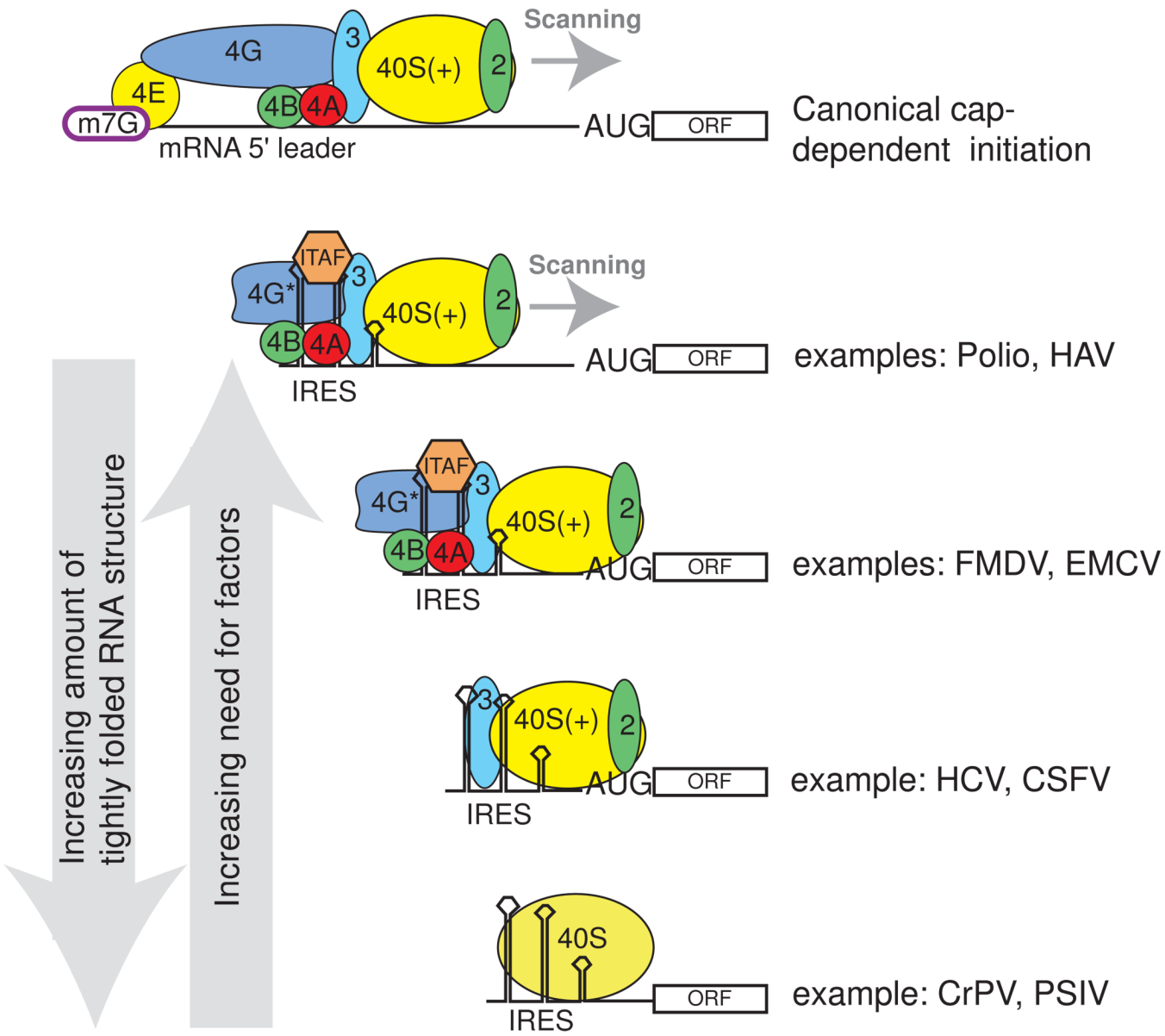

Figure 2. Examples of the diversity of viral IRES factor requirements

Canonical initiation requires the full complement of translation eIFs (top), while IRES initiation can use subsets of these factors as well as ITAFs (below). Shown are examples of some viral IRESs with the factors each requires. For simplicity, all the factors associated with the 40S subunit are not shown. As described in the text, we note a trend in which IRES RNAs with the most inherent stably folded structure (left arrow) are those that require the fewest factors, and as the IRES become less inherently structured, more ITAFs and eIFS are needed (right arrow). The degree to which this trend will prove predictive, or can be extended to cellular IRESs, is unknown.

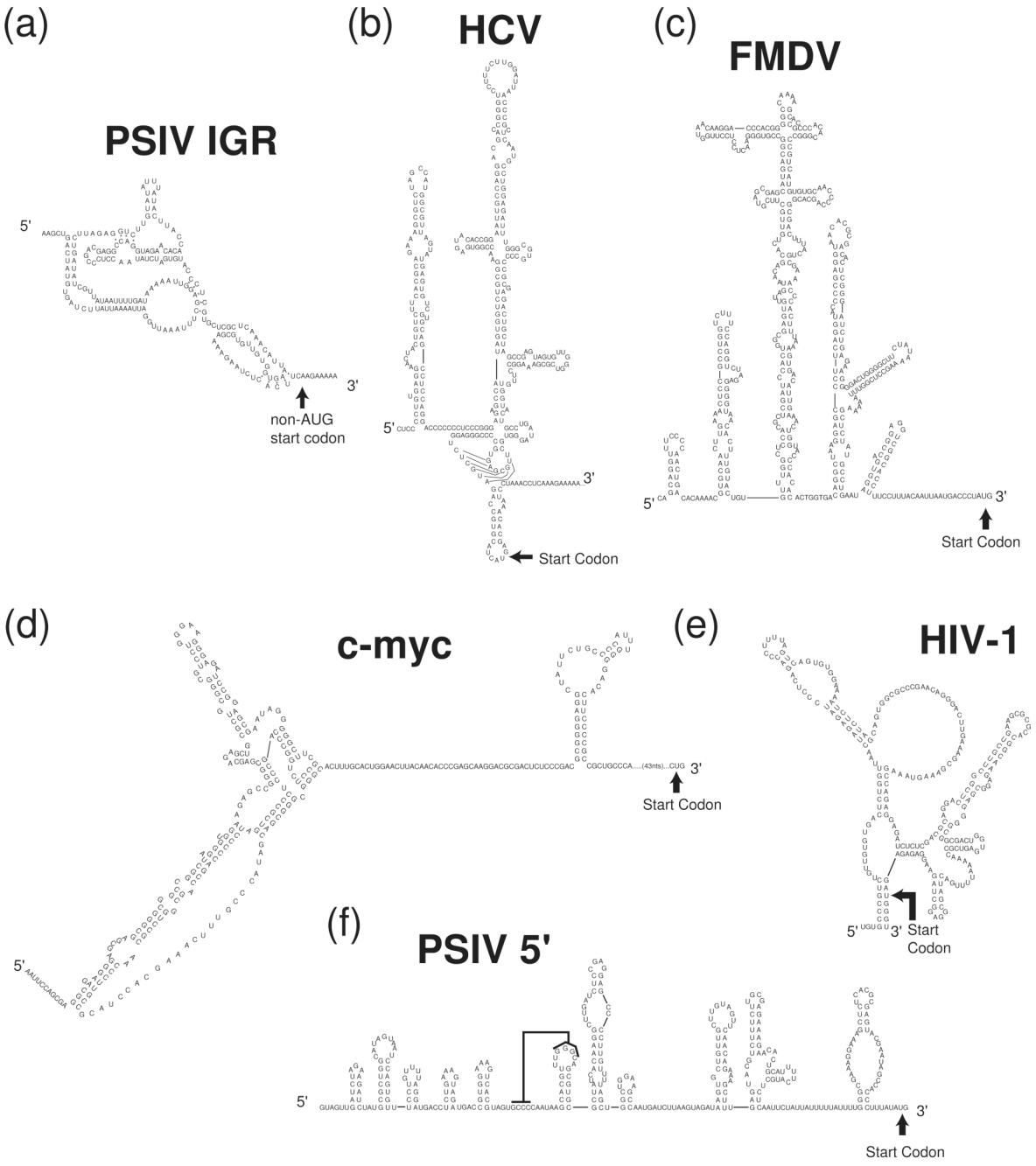

Figure 3. Examples of viral and cellular IRES secondary structures

Experimentally tested secondary structures of several diverse viral and cellular IRES RNAs are shown. **(a)** Plautia stali intestine virus (PSIV) IGR IRES. **(b)** HCV IRES **(c)** FMDV IRES **(d)** c-*myc* IRES **(e)** Human immunodeficiency virus-1 **(**HIV-1) gag- IRES **(f)** PSIV 5' IRES, the black line indicates a proposed pseudoknot interaction. Note that these secondary structures may be revised as more information becomes available regarding differences between RNA made and folded *in vitro* versus that made *in vivo*, which folds co-transcriptionally.

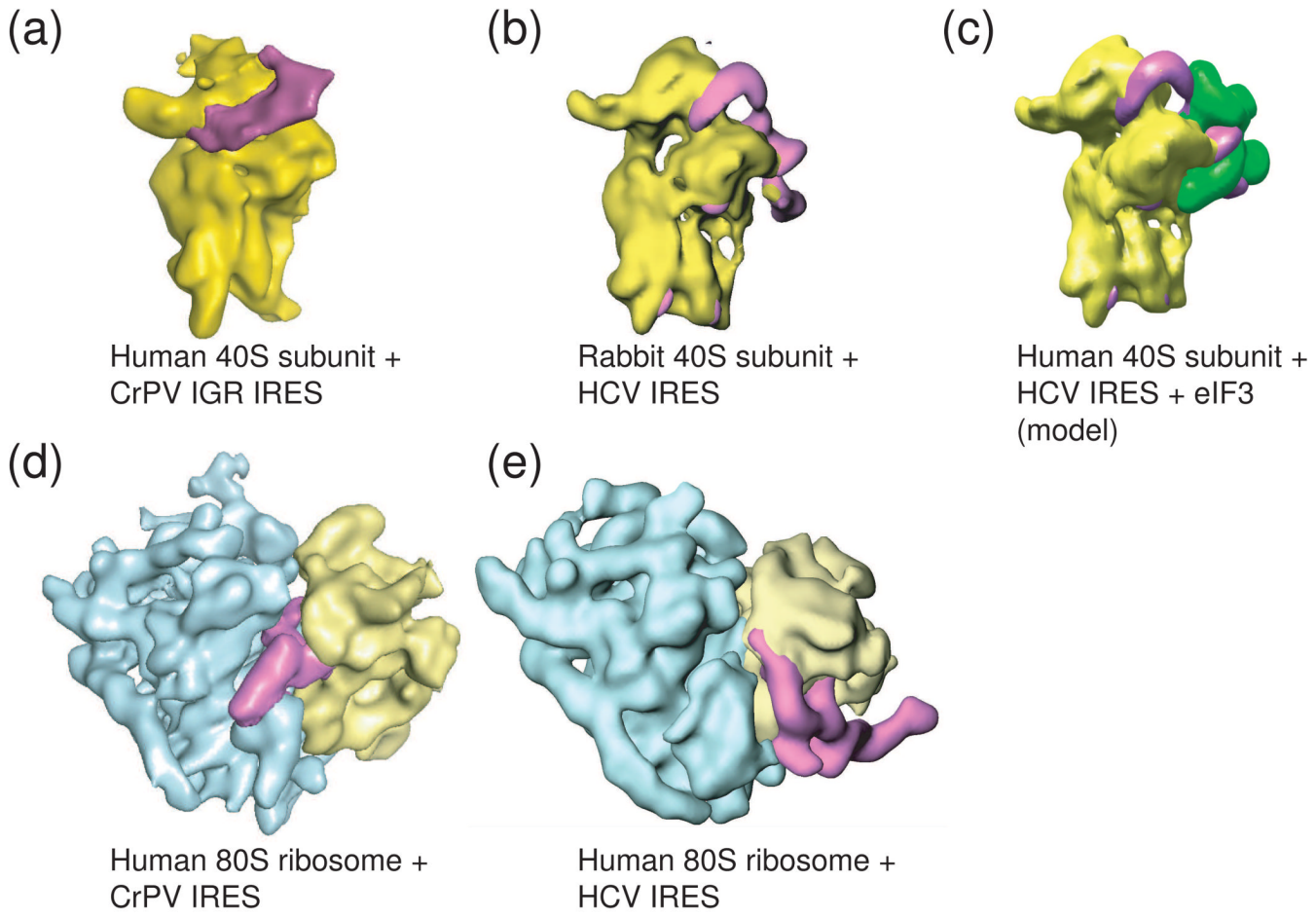

Figure 4. Cryo-EM reconstructions of two different viral IRESs bound to the ribosome

To date, only the HCV IRES (and related) and the *Dicistroviridae* IRES RNAs have been shown to bind directly to ribosomal subunits, and these complexes have been studies by cryo-EM. **(a)** The Cricket paralysis virus (CrPV) IGR IRES (magenta) bound to the 40S subunit (yellow). **(b)** The HCV IRES (magenta) bound to a 40S subunit (yellow). These two IRES types occupy different sites on the subunit, but both induce a similar conformational change in the 40S subunit when they bind, suggesting some mechanistic convergence. **(c)** Model of the HCV IRES bound to the 40S subunit and eIF3 (green), built from several cryo-EM reconstructions. **(d)** The CrPV IGR IRES bound to an 80S ribosome, the view is rotated 90 degrees from the view in panel (a). Note that higher resolution reconstructions of this complex have been published, but to allow more direct comparison with the HCV IRES-bound ribosome, the middle resolution structure is shown. **(e)** The HCV IRES bound to an 80S ribosome. Again, the different binding modes of these two IRESs to the ribosome are obvious, as are differences in the overall folded architectures of the IRESs; the CrPV IGR IRES is more compact, while the HCV IRES is extended. Cryo-EM reconstructions of preiniation complexes containing other IRESes have not been reported.

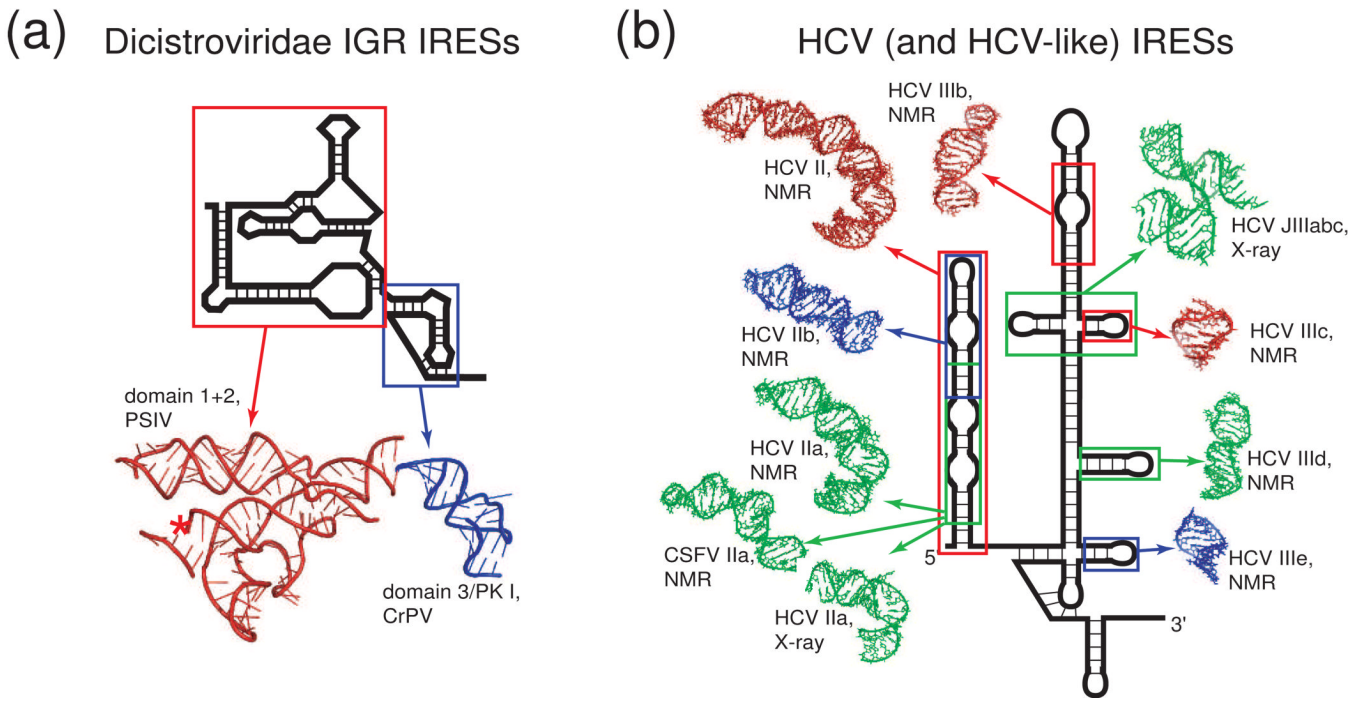

Figure 5. IRES RNA domains whose structures have been solved by X-ray crystallography or NMR

The structures of only a handful of IRES RNA domains have been determined. **(a)** Secondary structure cartoon of a *Dicistroviridae* IGR IRES with ribbons diagrams of the two structural domains of this IRES that have been solved by X-ray crystallography. This is the only IRES for which a complete structural picture exists. **(b)** Secondary structure cartoon of the HCV IRES surrounded by structures of various domains (shown in various colors) from both it and the CSFV IRES that have been solved by NMR or crystallography [25–31]. For both the HCV and the *Dicistroviridae* IGR IRESs, these structures have been combined with cryo-EM reconstructions to develop models for how the IRES RNAs interact with the ribosome (not shown).

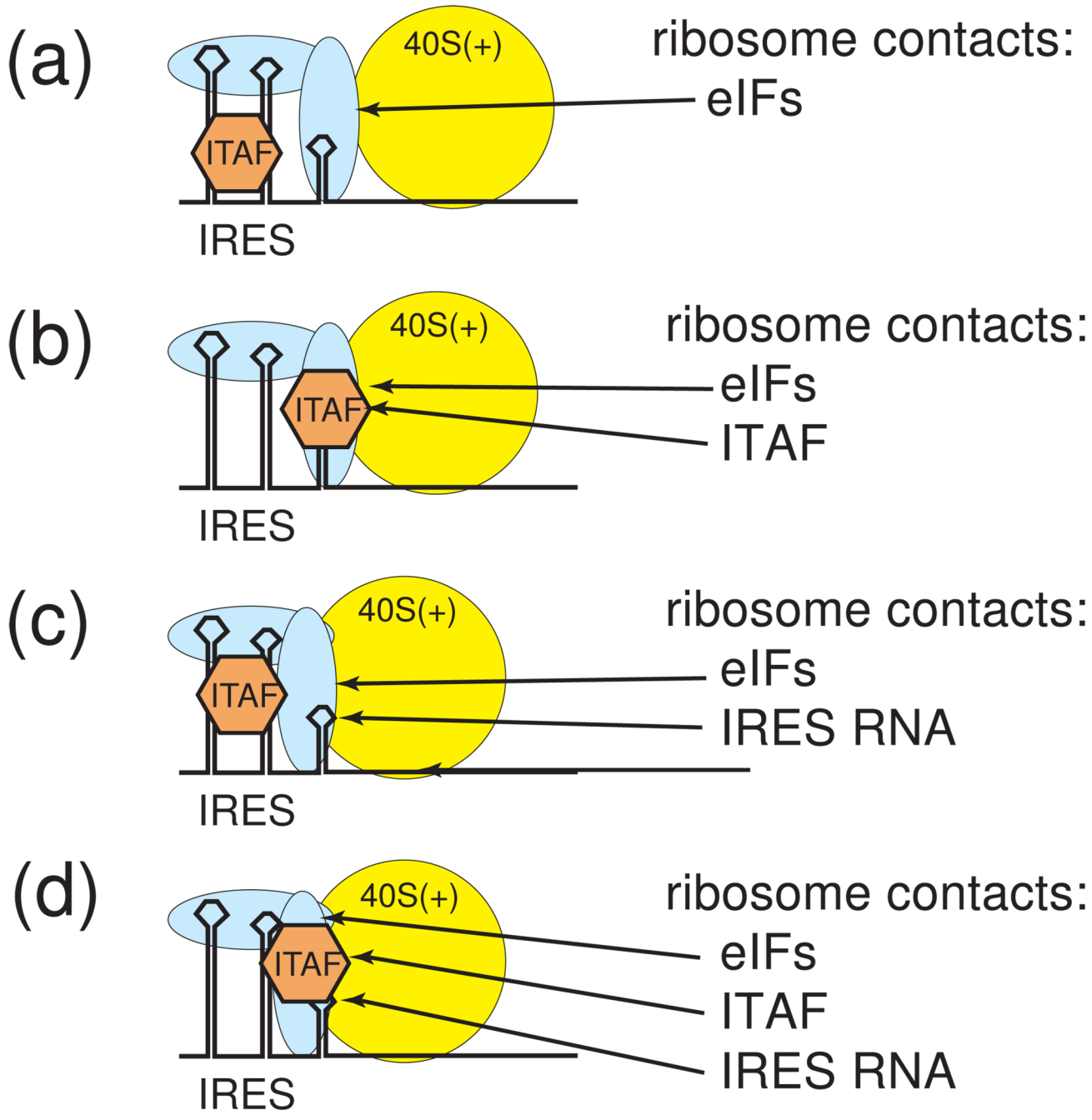

Figure 6. Models for ribosome recruitment by IRES•eIF•ITAF complexes

Within complex "IRES RNPs," does the ribosome contact only the bound eIFs, a combination of eIFs and the IRES RNA, a combination of eIFs and ITAFs, etc.? The answer to this question may vary depending on the IRES, and awaits more structural information. A few possibilities are diagrammed here. **(a)** The ITAF could stabilize the active conformation of the IRES RNA, which is bound by eIFs that interact with the ribosome, but without direct interaction between the IRES RNA and the ribosome. **(b)** The ITAF and eIFs could both interact directly with the ribosome, again with no direct IRES RNA-ribosome interactions. **(c)** The ITAF could stabilize the active conformation of the IRES RNAs, and both the IRES and eIFs could contact the

ribosome. **(d)** The ITAF, the eIFs and the RNA could all directly contact the ribosome. Note that other combinations could occur and that these are not mutually exclusive possibilities.