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Minireview

Molecules and Cells

Cap- and IRES-Independent Scanning Mechanism of Translation Initiation as an Alternative to the Concept of Cellular IRESs

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During the last decade the concept of cellular IRES-elements has become predominant to explain the continued expression of specific proteins in eukaryotic cells under conditions when the cap-dependent translation initiation is inhibited. However, many cellular IRESs regarded as cornerstones of the concept, have been compromised by several recent works using a number of modern techniques. This review analyzes the sources of artifacts associated with identification of IRESs and describes a set of control experiments, which should be performed before concluding that a 5′ UTR of eukaryotic mRNA does contain an IRES. Hallmarks of true IRES-elements as exemplified by well-documented IRESs of viral origin are presented. Analysis of existing reports allows us to conclude that there is a constant confusion of the cap-independent with the IRES-directed translation initiation. In fact, these two modes of translation initiation are not synonymous. We discuss here not numerous reports pointing to the existence of a cap- and IRES-independent scanning mechanism of translation initiation based on utilization of special RNA structures called cap-independent translational enhancers (CITE). We describe this mechanism and suggest it as an alternative to the concept of cellular IRESs.

INTRODUCTION

We decided to write this review since in our opinion the cellular IRES story is very instructive and hence deserves attention of a broad scientific society, in particular, of young researchers. The story shows in a clear way how a scientific hypothesis, owing to its attractiveness, enters textbooks before being proven. And this did not happen hundreds years ago. This happened at our times, the times of the absolute domination of the peer-review system. Without being noticed by many specialists in the field (including the authors of this article), the concept actually became the only idea explaining the most important mechanisms of cellular response to viral infection, stress conditions and environmental changes (for review see Holcik and Sonenberg, 2005; Komar and Hatzoglou, 2005; Silvera et al., 2010; Spriggs et al., 2008; Stonely and Willis, 2004). This misconception certainly slowed down appearance of alternative concepts and, unfortunately, resulted, albeit non-intentionally, in dissemination of wrong ideas among investigators who are not specialists in eukaryotic translation. Nevertheless, we do not intend to deny in principle the existence of cellular IRESs. This review analyzes the sources of artifacts associated with identification of IRESs and describes a set of control experiments, which should be performed before concluding that a 5′ UTR of eukaryotic mRNA does contain an IRES. Hallmarks of true IRES-elements as exemplified by well-documented IRESs of viral origin are also presented. Our chief intention is to stimulate here new ways of thinking, to show that even the scanning mechanism of translation initiation, regarded to be classical, is still a poorly understood process, especially in mammalian cells. And, hence, we poorly understand how it can be regulated.

Scanning model of the translation initiation in eukaryotes and its consequences

The overwhelming majority of eukaryotic mRNAs initiates the synthesis of the corresponding proteins by the cap-dependent model proposed by Kozak (1989). According to the current version of this mechanism (Fig. 1A and see a detailed scheme in the recent review by Sonenberg and Hinnebusch, 2009), which was developed by combined efforts of many labs, the 40S ribosomal subunit, already loaded with initiation factors eIF1, eIF1A, eIF3, eIF5 and the ternary complex eIF2 · GTP · Met-tRNA$_f^{Met}$, binds strictly at the 5′ end of mRNA near the m^{7}G-cap. The cap recognition is performed by factor eIF4F consisting of three subunits: eIF4E (cap-binding subunit), eIF4G (the subunit that plays the role of a scaffold) и eIF4A (RNA-helicase). A specific interaction between eIF4G, bound to the 5′ end of mRNA via the cap-binding subunit eIF4E, and eIF3, bound to the 40S ribosome, plays a major role in the recruitment of the 40S ribosome to the 5′ end of eukaryotic mRNA. Following this step, the 40S subunit starts to move to the 3′ end of mRNA, searching (scanning) on its way for an AUG codon in an optimal nucleotide context (….A/GNNAUGG/A… in the case of vertebrates). The correct location of the initiation codon is controlled by factors eIF1 and eIF1A. This scanning process is accompanied by unwinding of stem-loop structures within 5′ UTRs of mRNAs by helicase eIF4A (the third subunit of eIF4F).

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This process requires ATP hydrolysis and assisted by factor eIF4B. Once the 40S subunit reaches the initiation codon, it stops there, eIF5 triggers a hydrolysis of the GTP molecule bound to eIF2 and the latter quits the ribosome. The following joining of the 60S ribosomal subunit and release of all initiation factors bound to the small ribosomal subunit is assisted by factor eIF5B. The formation of the 80S ribosome competent for the elongation of polypeptide chain requires hydrolysis of the second molecule of GTP.

This model implies that 40S ribosomal subunits cannot bind to internal sites of the mRNA polynucleotide chain. In other words, once the ribosome terminates the synthesis of a protein it dissociates from the mRNA and can rebind it only at its 5′ end. Sliding after the polypeptide synthesis termination in the 3′ end direction and reinitiation at a downstream cistron are possible but this is known to be a very inefficient process, with the exception of short uORFs or a few specific cases. These features of translation initiation in eukaryotes explain why the overwhelming majority of eukaryotic mRNAs is monocistronic. Another implication of the model which is important for our further discussion is that a base-pairing within 5′ UTRs of mRNAs has always been thought to cause an adverse effect on the scanning process.

Some viruses replicating in the cytoplasm can use the internal initiation that can be revealed by method of dicistronic constructs

The data obtained in 80s using cap-dependent artificial and natural mRNAs were in an excellent agreement with the scanning model (Kozak, 1989). However, the model was unable to explain how some animal viruses which replicate in the cytoplasm and do not have their own system of mRNA capping synthesize viral proteins. Moreover, their mRNAs are capable of actively competing with mRNAs of host cells, at least under conditions of viral infection. The 5′ terminal nucleotides for some of these viral genomic RNAs (e.g. from picornaviruses) are covalently linked to special proteins called VPg. In addition, the 5′ UTRs of picornavirus RNAs are very long, have numerous upstream AUG (uAUG) codons and very stable stem-loop structures. It was quite obvious that these RNAs could not employ the standard mechanism of initiation and somehow allowed ribosomes to get to internal regions of 5′ UTRs.

The existence of internal ribosome entry was proven in two laboratories simultaneously, by Sonenberg (MacGill, Canada) and Wimmer (Stony Brook, USA) (Jang et al., 1988; Pelletier and Sonenberg, 1988). In both cases, the approach termed *method of dicistronic constructs* was employed. The first group used a dicistronic cDNA under control of SV40 promoter where the two reporters were separated by the 5′ UTR of poliovirus. The control construct had just a polylinker sequence between the same reporters. Expression from the first and second cistrons in transfected cells was measured for both constructs and it was found that the significant translation of the second cistron was observed only for the construct with the polio 5′ UTR in the intercistronic position. Similar results but for a dicistronic RNA with the 5′ UTR of encephalomyocarditis virus RNA between two reporters were obtained in the cell-free translation system by Wimmer and co-workers (Jang et al., 1988). These experiments demonstrated for the first time the existence of ribosomal entry to internal regions of RNAs and the corresponding structures allowing such an entry were termed IRES-elements (Internal Ribosome Entry Sites). Since then, the dicistronic approach has remained the main primary test to check whether a sequence possesses an IRES activity. Thus, if a polynucleotide sequence is unable of directing translation of the downstream cistron to a significant extent, one can be sure that there are no IRES within this sequence. However, the opposite is not correct. Moreover, the comprehension of the word “significant” is rather subjective since 100-fold higher than a negative control is undoubtedly significant, but no one can say the same for a 3-fold stimulation. As we shall see below, if the downstream cistron is expressed, many additional experiments should be performed before claiming the discovery of a *bona fide* IRES-element.

Distinctive features of viral IRES-elements

Soon after discovery of picornavirus IRESs, such elements were found in other families of viruses replicating in the cytoplasm. At present, we have a rather detailed information on mechanisms utilized by IRESs from the encephalomyocarditis virus (EMCV) RNA, foot-and-mouth disease virus (FMDV), Theiler’s Murine Encephalitis Virus (TMEV), hepatitis C virus (HCV), Classical Swine Fever Virus (CSFV) and Bovine Viral Diarrhea Virus (BVDV) (see Pestova et al., 2001), porcine teschovirus 1 (PTV-1) (Pisarev et al., 2004), *Rhopalosiphum padi* virus (RhPV) (Terenin et al., 2005), Simian picornavirus type 9 (SPV9) (de Breyne et al., 2008) and the IRES from the intercistronic region of the natural dicistronic RNA from Cricket Paralysis virus (CrPV) (Wilson et al., 2000). The functionally important stem-loop structures in these IRESs were identified, in some cases we even know what protein factors they bind (see Belsham and Jackson, 2000; Pestova et al., 2001). A successful reconstitution of translation initiation complexes from purified components on these IRESs was a real breakthrough in our understanding as to how some of these structures work (Pestova et al., 1996). Using this approach, it was shown that the EMCV and, by analogy, FMDV IRES-elements have a high affinity binding site within domain J-K for the complex of central part of factor eIF4G and helicase eIF4A (Kolupaeva et al., 1998; Lomakin et al., 2000). The presence of such a high affinity site for eIF4G+eIF4A nicely explains why the 40S ribosomal subunit is directed to the IRES rather than to other regions of viral RNA. Using the same reconstitution approach, IRESs from HCV, CSFV and PTV RNAs, which are structurally unrelated to those mentioned above, were shown to require just two factors, eIF2 and eIF3, to form a proper initiation complex with the 40S ribosomal subunit (Pestova et al., 1998; Pisarev et al., 2004). The reason is that these IRES-elements recognize a highly specific binding site on the surface of the small ribosomal subunit. Owing to this site, they can form a binary complex with the 40S ribosomal subunit in which the initiation codon is placed directly in its P-site (Fig. 1B). The CrPV IRES forms a specific initiation complex with the ribosome not only without any initiation factor but also without the initiator tRNA (Spahn et al., 2004; Wilson et al., 2000).

Some of the IRESs listed above require additional auxiliary factors for efficient translation initiation. They are termed IRES Trans-Acting Factors (ITAFs) (for recent review see Pacheco and Martinez-Salas, 2010). For instance, Pyrimidine Tract Binding protein (PTB) stimulates formation of the 48S initiation complex on the EMCV IRES (Borovjagin et al., 1994), whereas the FMDV IRES in addition to PTB requires ITAF45 (Pilipenko et al., 2000). Although structures of IRES-elements from other picornaviruses, in particular, from poliovirus and rhinovirus are also well studied, the attempts to reconstitute 48S initiation complexes on them were unsuccessful to date. That is why the information on requirement for ITAFs for these IRES-elements is still very contradictory (Belsham and Jackson, 2000; Costa-Mattioli et al., 2004). Anyway, from the data existing for much better studied picornavirus IRES-elements (EMCV, FMDV), one can conclude that ITAFs are needed to stabilize or modu-

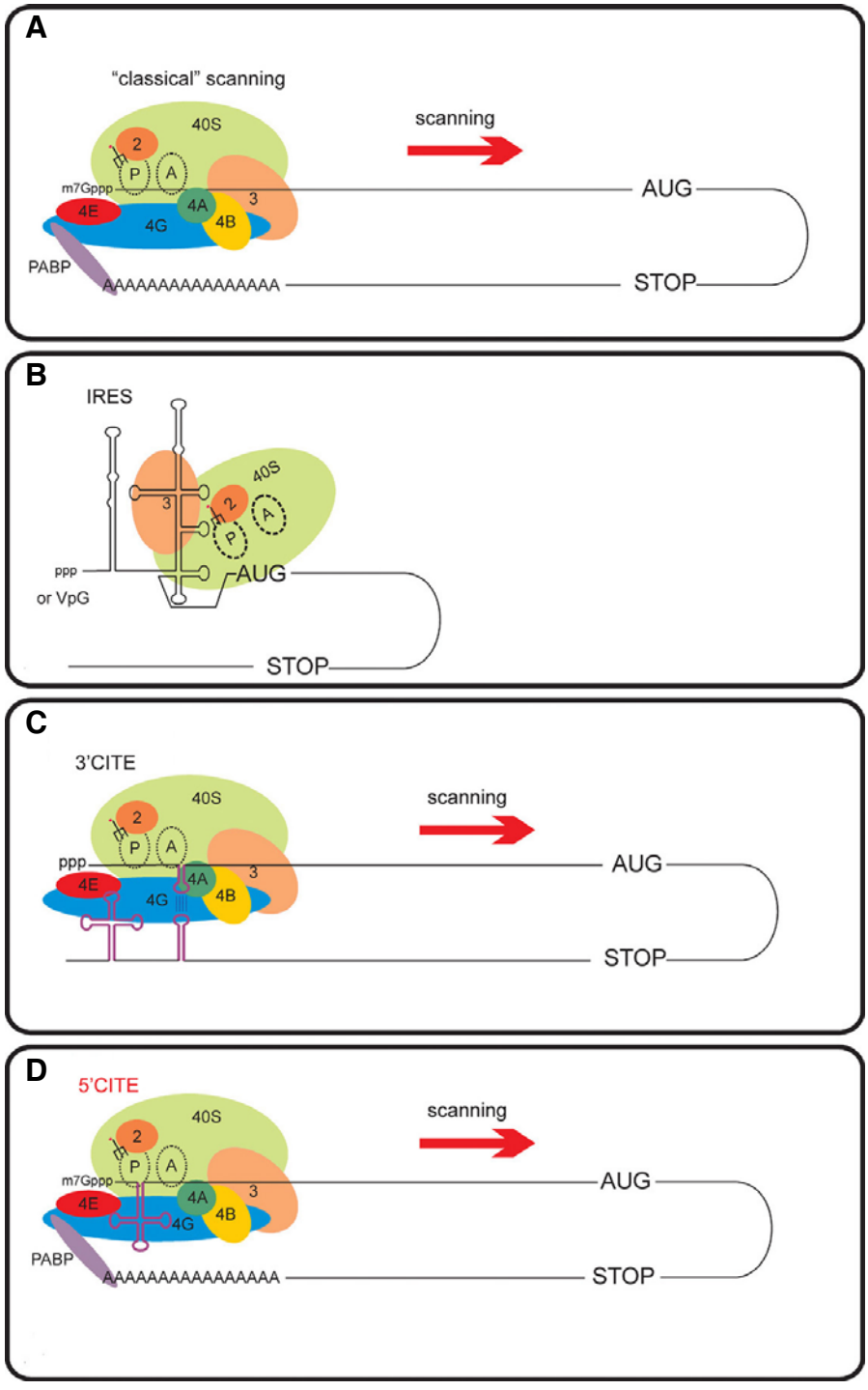

Fig. 1. Modes of translation initiation in eukaryotes. (A) Classical cap-dependent scanning mechanism. In this mechanism, the interaction of the cap with factor eIF4E plays a critical role in the recruitment of the translation apparatus to the 5′ end of mRNA. "P" and "A" denote P- and A-sites of the 40S ribosomal subunit. PABP - poly (A) binding protein. (B) IRES-dependent translation initiation. Here, this mode of initiation is exemplified by HCV-like IRES-elements. (C) 3′ CITE-assisted initiation. 3′ CITEs are shown in violet color. In this particular case, eIF4E and eIF4G bind by means of CITEs to the 3′ UTR of an mRNA. A long distance interaction of stem-loop structures results in circularization of the mRNA and positioning of the scanning machinery near its 5′ end. (D) 5′ CITE-assisted initiation. In this case, a CITE is located within the 5′ UTR and is capable of additional, presumably rather weak interactions with initiation factors of the scanning machinery. Although the recognition of the cap by eIF4E still plays a major role in the mRNA recruitment, the primary binding of the mRNA is still possible in the absence of this interaction, solely due to some interactions of key initiation factors (or the 40S ribosomal subunit iteself) with 5′ CITEs.

late their complex secondary and tertiary structures, thereby promoting the formation of 48S translation initiation complexes. However, they are neither components that directly recruit 40S ribosomal subunits onto these IRES-elements, no components that bridge them with the IRESs.

Properties common to all viral IRES-elements

In spite of a large variety of structures of viral IRESs, they are nevertheless amenable to classification. For instance, the secondary (but not the primary) structure of EMCV IRES (cardioviruses) is similar to that for the FMDV IRES (aphthoviruses); the poliovirus IRES (enteroviruses) resembles the Human Rhino Virus (HRV) IRES (rhinoviruses) (for references see Belsham and Jackson, 2000). The HCV IRES (hepacivirus), distinct structurally and functionally from just mentioned picornavirus IRESs, is quite similar to that from CSFV RNA (pestivirus) and the both are similar to the IRES from Porcine Teschovirus 1 (PTV-1) (see Pestova et al., 1998; Pisarev et al., 2004), though

the latter belongs to the picornavirus family since the organization of the coding part of PTV-1 is typical of picornavirus RNAs. However, there is one property of well-studied viral IRES-elements which is common to all of them, regardless of their organization and mechanism of functioning. As clear from the data described above, they all have at least one specific site within their structure with a high affinity to a key translational component. The EMCV IRES (and FMDV IRES, as well) has a high affinity site for the central part of eIF4G + eIF4A. The binding constant of this protein complex is 100-fold higher than that for the interaction of eIF4G + eIF4A with the 5′ UTR of standard cap-dependent β-globin mRNA (Lomakin et al., 2000). The HCV, CSFV and PTV-1 IRES-elements have a highly specific site on the 40S ribosome allowing them to form stable binary complexes (Pestova et al., 1998; Pisarev et al., 2004). In addition, the same IRESs specifically bind initiation factor 3 (Sizova et al., 1998).

These highly specific and strong binding sites for key ribosome recruiting components or ribosome itself are real hallmarks of viral IRESs. Their existence helps to understand how and why 40S ribosomes are directed to a defined internal region of the 5′ UTR of an mRNA rather than to the 5′ end or any other sequence within this mRNA. Consequently, even point mutations in these sites, let alone deletions, dramatically affect the activity of the IRESs. Some of such mutations completely inactivate it.

The existence of specific and strong binding sites for initiation factors or ribosomes within 5′ UTRs is mandatory but not the only specific property of true viral IRES-elements. Another property is a highly specific secondary and tertiary structure of these IRES-elements which are probably needed for the sequences in vicinity of the initiation codon to be accommodated in the ribosome mRNA-binding channel. The mechanism of this accommodation is still poorly understood, especially for picornavirus IRESs. Probably, it involves a change of conformation of the small ribosomal subunit. Some information on this point is available for HCV-like IRES-elements (see below).

History of cellular IRESs

It was suggested that the novel mechanism of initiation might explain the disparate translation of several other eukaryotic messenger RNAs, including cellular mRNAs which are known to be all capped in eukaryotes. To search for new IRESs, researchers used the dicistronic test (initially mostly in its DNA format) and cells infected by poliovirus (Johannes and Sarnow, 1998). The poliovirus propagation in infected cells results in a cleavage of factor eIF4G into two fragments, a smaller N-terminal possessing the binding site for the cap-recognizing subunit eIF4E and a larger fragment (p100) harboring binding sites for eIF3 and helicase eIF4A. This cleavage disconnects eIF4E from eIF4G and, hence, from other interactions involving eIF3 and the 40S subunit (Fig. 2), thereby resulting in a strong inhibition of the standard cap-dependent translation. On the other hand, as described above, picornaviral IRES-elements do not need eIF4E and most of them can manage with p100 + eIF4A to recruit the viral mRNA onto the 40S ribosomal subunit. Some of the picornaviruses (e.g. EMCV) do not cleave eIF4G. Instead, they induce in infected cells activation of eIF4E repressors, so-called 4E-Binding Proteins 1-3 (4E-BP1, 2, 3) (Lin et al., 1994; Pause et al., 1994; Poulin et al., 1994). The activated (dephosphorylated) 4E-BPs sequester eIF4E and prevent it from binding to eIF4G (Beretta et al., 1996; Pause et al., 1994). Again, this should inhibit the cap-dependent but not affect the picornavirus IRES-directed translation. That is why the ability of an mRNA to be efficiently translated in poliovirus infected cells (or extracts prepared from such cells) started to be regarded as an indication of an IRES-directed translation (Johannes and Sarnow, 1998). In fact, as we discuss below, IRES-driven and cap-independent are not synonymous definitions.

The first cellular IRES was discovered by Macejak and Sarnow (1991). These authors showed that the mRNA encoding the immunoglobulin heavy-chain binding protein (BiP) can be translated in poliovirus-infected cells and that the 5′ leader of the corresponding mRNA can directly confer the internal ribosome binding to an mRNA in mammalian cells. That publication catalyzed appearance of many other reports of cellular IRESs. Papers on new internal ribosome binding regions within 5′ UTRs of eukaryotic mRNAs soon flooded not only Molecular Biology literature but also articles by medical doctors. The overwhelming majority of cellular IRESs were found in rather long and highly structured 5′ UTRs of mammalian mRNAs (Vagner et al., 2001). As the mRNAs with such 5′ leaders are very often code for regulatory proteins (transcription, growth, differentiation, apoptosis factors etc.), it was concluded that cellular IRESs exist to play a crucial role at some critical moments of cell life when the cap-dependent translation initiation is compromised. The number of cellular IRESs went on multiplying even after it was realized that at least some of them turned out to be artifacts. Unfortunately, the appearance of poorly identified IRESs still continues.

The artifacts in identification of cellular IRESs were mostly revealed for those of them which were identified by transfection of cells with dicistronic DNAs. The first alarm rang out when Han and Zhang (2002) found a cryptic promoter in the IRES from the 5′ UTR of the mRNA encoding translation initiation factor eIF4G. Therefore, the expression of the downstream cistron could occur from some amount of monocistronic mRNAs transcribed from this cryptic promoter rather than from the dicistronic DNA. In addition, monocistronic mRNAs can be formed because of cryptic acceptor splice sites within the intercistronic region or even within the first reporter of the dicistronic DNA construct (Baranick et al., 2008; Van Eden et al., 2004). To check whether the monocistronic mRNAs containing the downstream cistron are formed, Lloyd and co-workers (Van Eden et al., 2004) applied the siRNA interference test against the first cistron (Rluc) of their standard cDNA dicistronic construct, Rluc-5′ UTR-Fluc (where Rluc and Fluc are Renilla and firefly luciferases, respectively). If Rluc and Fluc are translated exclusively from an intact dicistronic mRNA, then both Rluc and Fluc activities should be proportionally reduced. Conversely, if the downstream cistron is expressed, to any extent, from a monocistronic mRNA(s), then Fluc values should be less affected or not affected at all. The analyses performed by this approach indicated that aberrantly spliced transcripts occurred in cells transfected with the XIAP dicistronic DNA construct. The 5′ UTR of XIAP mRNA (X-linked inhibitor of apoptosis) had earlier been suggested to comprise an IRES which works under apoptosis conditions (Holcik et al., 1999). This contributed to the unusually high levels of apparent IRES activity exhibited by the XIAP 5′ UTR *in vivo*, when the most popular bicistronic reporter system, Rluc/Fluc, was used (Van Eden et al., 2004).

Transfection of dicistronic RNAs to cultured cells is free from mentioned above artifacts related to dicistronic DNAs. When some of the putative cellular IRESs were placed in the intercistronic position and analyzed by RNA transfection, their activity proved to be negligibly low (Andreev et al., 2009; Bert et al., 2006; Mardanova et al., 2008a; Young et al., 2008) as compared to the activity of the same 5′ UTRs in the natural situation, i.e. in the capped monocistronic mRNAs. In spite of the fact that many 5′ UTRs with putative IRES-elements are very long and

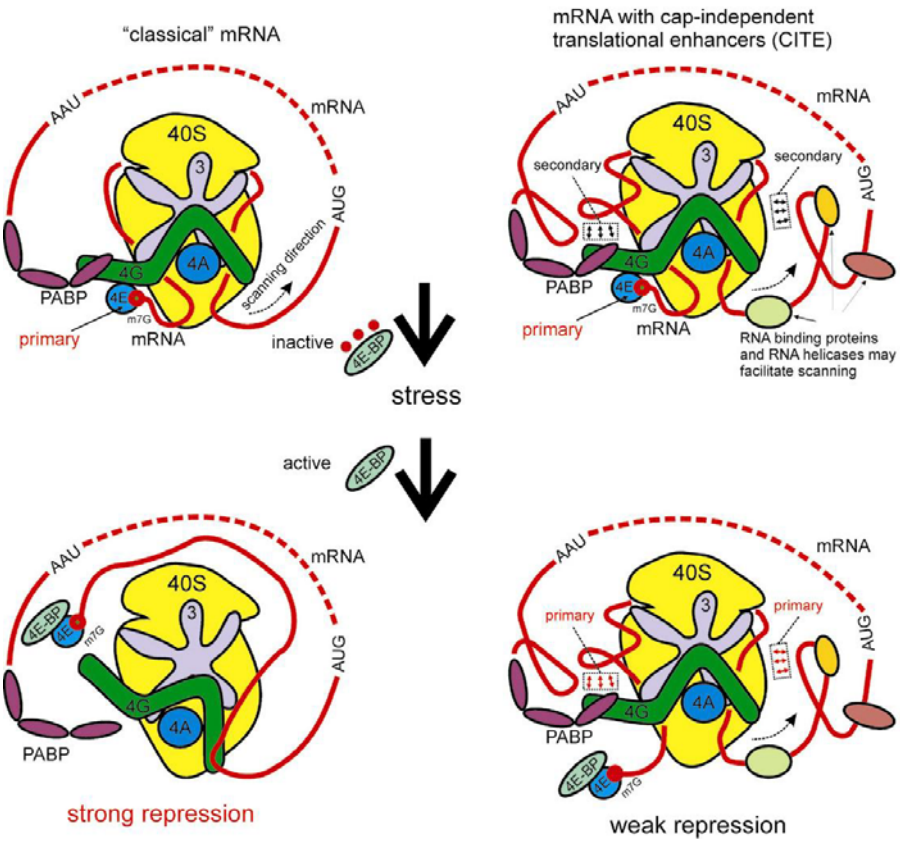

Fig. 2. Effect of stress conditions on the translation initiation of mRNAs lacking (left pictures) and harboring (right pictures) 5′ CITEs in their 5′ UTRs. The activation of translation repressors 4E-BPs by dephosphorylation induced by stress blocks the interaction of eIF4E with eIF4G. In the first case, this dramatically inhibits a recruitment to and stable association of the mRNA with the ribosome resulting in a "strong repression". However, the mRNA with CITEs (shown with small arrows) is still capable of recruiting and retaining the translation apparatus demonstrating a "weak repression" under stress conditions which affect the interaction of eIF4E with eIF4G.

highly structured, their activity in transfected cells was surprisingly high in monocistronic format and comparable with the activity of short 5′ UTRs from standard cap-dependent mRNAs (β-globin or β-actin) (Andreev et al., 2009; Dmitriev et al., 2009). Therefore, the comparison of absolute levels of expression of a 5′ UTR in the intercistronic *vs.* 5′ terminal position (in the capped and polyadenylated mRNA) after normalization to the control vector is a crucial test for determination of whether a mechanism of internal initiation is utilized by this particular 5′ UTR under these particular conditions. If the 5′ UTR reveals just a background activity in the dicistronic mRNA being compared to its activity in the monocistronic context, such an "IRES" is unlikely to play any physiological role.

It is not surprising that the efficiency of such a background translation can vary for different 5′ UTRs. Moreover, it is not surprising that this residual activity can be somewhat stimulated under some stress or starvation conditions. Such stimulation is often interpreted as the activation of an IRES. In fact, this is a predictable effect: stress conditions in most cases suppress the cap-dependent translation initiation through inhibiting the activity or availability of the cap-binding factor eIF4E. In turn, this relieves a strong competition of the highly efficient cap-dependent initiation with the inefficient background activity of putative IRESs in the intercistronic position. The same non-specific effects are usually observed with classical cap-dependent leaders of a similar length inserted into intercistronic position - the necessary control that is often missed by researchers. It should be noted, however, that even under abnormal conditions the activity of many putative cellular IRESs in the intercistronic position remains very low, both in absolute terms and in comparison with the activity of the corresponding monocistronic mRNAs [Mardanova et al., 2008b; Andreev et al. (submitted to publication)]. Anyway, investigators should directly show that such a low activity is of physiological significance, at least under certain conditions. To the best of our knowledge, nobody has ever addressed this problem.

Thus, comparison of the absolute efficiencies of a 5′ UTR in the intercistronic position *vs.* its natural position in the monocistronic mRNA is one of the key tests to conclude that this 5′ UTR does utilize an IRES-dependent mechanism. In the overwhelming majority of reports claiming the discovery of cellular IRESs, this test was not carried out. Another important test is the effect of mutations within the putative IRES on its translation initiation activity. The only known ways for internal ribosome entry in eukaryotes imply the presence of a highly specific structure or sequence to recruit one of the key translation factors bound to the ribosome (e.g. eIF3 or eIF4G) or the 40S ribosome itself so deletions or even point mutations within this specific signal should completely kill the ability for the internal initiation of the entire cellular 5′ UTR as it is observed for viral 5′ UTRs (see above). When such a "mutation" test was applied to the very first cellular IRES from the BiP mRNA, it failed (Yang and Sarnow, 1997). At least, such test mutations should inactivate the translation activity of cellular 5′ UTRs under stress conditions when the cap-dependent translation is suppressed. However, it should be emphasized again that such an inactivation should be seen for natural monocistronic positions of these 5′ UTRs. Unfortunately, as a rule, such tests have not been performed for most reported cellular IRESs.

These and other criteria to conclude that a sequence under study is a true cellular IRES have been discussed in several papers published during last four years (Andreev et al., 2009; Baranick et al., 2008; Bert et al., 2006; Young et al., 2008). They are summarized in our recent report (Andreev et al.,

2009). Application of these criteria to some of the most popular cellular IRESs did not confirm the IRES activity for the following cellular 5′ UTRs: c-Myc (Andreev et al., 2009; Bert et al., 2006), Apaf-1 (Andreev et al., 2009), BiP (Young et al., 2008), HIF-1α (Bert et al., 2006; Young et al., 2008), VEGF (Bert et al., 2005; Young et al., 2008), XIAP (Baranick et al., 2008; Bert et al., 2006; Van Eden et al., 2004), La (Young et al., 2008), RUNX2 (Elango et al., 2006) and other. The case of factor HIF-1α is especially interesting. The HIF-1α mRNA is frequently cited as an example of an mRNA that is preferentially translated via IRES-dependent mechanism under hypoxic conditions, in particular, in cancer cells (for recent reviews, see Silvera et al., 2010). However, Young et al. (2008) showed that hypoxic stress failed to activate cap-independent synthesis, indicating that it is unlikely that this is the primary mechanism for the maintenance of the translation of HIF-1α mRNA under low O_2.

Attractive features of the concept of cellular IRESs

The concept of cellular IRESs became very popular because it helped to explain why some mRNAs with highly structured 5′ UTRs are nevertheless efficient in translation. Indeed, stem-loop structures within 5′ UTRs are regarded as detrimental for the scanning mechanism. Conversely, with one exception (Terenin et al., 2005), well-documented IRES-elements of viral origin have highly structured domains (Balvay et al., 2009). However, unlike viral IRESs, research on cellular IRESs and the involvement of auxiliary factors in their function has so far revealed a puzzling diversity of structure and mechanism precluding any classification and comparative analysis of these structures (Baird et al., 2006), and for none of the putative cellular IRESs the mechanism of the internal ribosome entry has been confirmed by initiation complex reconstitution from purified components. On the other hand, we have recently shown that translation of mRNAs with highly structured 5′ UTRs may be quite efficient, and mRNAs with 5′ UTRs of different length and containing different number of stem-loop structures may be translated with similar efficiencies, at least in mammalian systems (Dmitriev et al., 2007; 2009). We came to conclusion that once the scanning complex is accommodated at the 5′ terminal part of mRNA, it starts to unwind one stem after another rather than to unfold at once all of them. Therefore, the current practice to calculate cumulative energies for secondary structures of various mammalian 5′ UTRs makes no sense and says very little of the translation initiation potential of an mRNA.

The IRES concept is also appealing as an explanation of a higher resistance of translation of some mRNAs to special conditions of cell stress, mitosis or apoptosis when the cap-dependent translation is compromised (Holcik and Sonenberg, 2005). Regulation of protein synthesis is effected, as a rule, at the level of initiation by modulating the activity of key protein factors involved either in recruitment of mRNA onto ribosomes (eIF4E, eIF4G) or initiator tRNA delivery (eIF2). The signal transduction pathways by which the activity of these translation initiation factors is repressed or activated is now under intense investigation (Cully and Downward, 2009; Proud, 2009; Sonenberg and Hinnebusch, 2009).

As mentioned above, the availability of active eIF4E, and therefore formation of eIF4F, is regulated by a family of translation repressors, the eIF4E-binding proteins (4E-BPs) (Lin et al., 1994; Pause et al., 1994; Poulin et al., 1998), which bind to eIF4E and prevent its association with eIF4G. In turn, the capacity of the 4E-BPs to block eIF4E is determined by their phosphorylation status. When 4E-BPs are hypophosphorylated, they form the complex with the eIF4E and inhibit the cap-dependent translation initiation, while the cap-independent translation through viral IRESs (internal ribosome entry sites) is usually unaffected (Beretta et al., 1998; Pause et al., 1994; Poulin et al., 1994). However, when cells are stimulated with serum, growth factors or hormones, 4E-BP1 becomes hyperphosphorylated, which releases eIF4E from 4E-BP1 and allows the formation of the eIF4F complex. Several stress conditions (serum deprivation, hypoxia, heat shock, viral infection, apoptosis) can block mTOR kinase pathway and cause dephosphorylation of 4E-BPs, which in turn sequester eIF4E away from eIF4G and inhibit cap-dependent translation initiation. The conclusion has been drawn that the mRNAs which remain to be active under abnormal conditions listed above use the cap-independent translation. This conclusion is probably correct. Incorrect is an opinion that the cap-independent and IRES-directed translations are synonyms.

The cap-independent translation does not need to be directed by an IRES, it can be the 5′ end- and scanning-dependent

Studies recently performed in our lab have demonstrated that it is not mandatory to invoke the IRES hypothesis, at least for some mRNAs, to explain their preferential translation when eIF4E is partially inactivated. Using transfection of cells with capped and uncapped reporter mRNAs, we found that the contribution of the cap was not similar for various 5′ UTRs of cellular mRNAs, though none of the tested cellular 5′ UTRs had an IRES (Andreev et al., 2009). Some of them were stimulated by the 5′ cap much stronger than the other. Notably, there was no correlation between the magnitude of the stimulation effect and the length or the overall stability of the 5′ UTR secondary structure. Among tested 5′ UTRs, the 5′ UTR of mRNA encoding the Apoptosis Protease Activating Factor 1 (Apaf-1) revealed the lowest cap-dependence: in HEK293 cells, the stimulation of translation by the cap of the RNA containing the Apaf-1 5′ UTR was no more than 7-fold. Insertion of additional upstream AUG codons in the uncapped Apaf-1 5′ UTR close to the 5′ end resulted in a dramatic inhibition of translation of the corresponding mRNA. A similarly strong effect was observed for the capped version of the same mRNA but under conditions of a profound apoptosis when the cap-dependent translation was severely suppressed (Andreev et al., submitted to publication). This means that even in the absence of the eIF4E-cap interaction, the translational machinery is able to recognize the 5′ end, bind there and then scan to the 3′ end in a search for the initiation codon. Our observation is supported by Gunnery et al. (1997). In the paper entitled “Translation of an uncapped mRNA involves scanning” these authors demonstrated a correct translation of the mRNA produced in living cells by RNA polymerase III which is known to form uncapped RNAs. An additional support of our conclusions can also be found in the data obtained *in vitro* (Ali et al., 2001; De Gregorio et al., 1998). In the first of these reports, the authors observed stimulation of the *in vitro* translation by C-terminal fragments of eIF4G, i.e. lacking the binding site for eIF4E. Although the stimulation effect was cap-independent, it still required 5′ end-dependent ribosome binding.

Importantly, being in the uncapped state, some of mammalian 5′ UTRs directs translation with much higher efficiency than others (Andreev et al., 2009). Therefore, there must be some elements within eukaryotic mRNAs that can promote a cap-independent translation initiation but are not truly IRESs.

Alternative modes of cap-independent translation: lessons from plant viruses

In attempt to get an idea about the features that could allow cellular mRNAs to be preferentially translated under stress

conditions we addressed some cases of translational regulation of plant viral RNAs. The mRNAs of a large portion of all plant viruses, including those in the *Potyviridae*, *Comoviridae*, *Tombusviridae* and *Luteoviridae* families and the *Sobemovirus* and *Umbravirus* genera, lack the m^7GpppN-cap structure. To be efficiently translated without the cap, these viruses employ a strategy alternative to the internal ribosome entry. They use special elements termed Cap Independent Translation Enhancers (CITE) (reviewed in Dreher at al., 2006; Kneller et al., 2006; Miller et al., 2007). The majority of CITEs is located in 3′ UTRs (so called 3′ CITEs) and can be classified in several structural classes with no apparent sequence or structural similarity to each other. CITEs is thought to recruit components of translational apparatus and then to deliver it to the 5′ end of mRNA through long distance base pairing between 5′ and 3′ UTRs (Fig. 1C). Without making attempt to describe all of them we shall address the most studied cases, where the factors recruited by CITEs are identified. The CITE located in the 3′ UTR of Satellite Tobacco Necrosis Virus (STNV) RNA binds eIF4F and eIF(iso)4F through interactions with both eIF4E and eIF4G subunits (Gazo et al., 2004), the 3′ CITE of Barley yellow dwarf virus (BYDV) RNA recruits eIF4F mainly through interaction with eIF4G subunit (Treder et al., 2008; Wang et al., 1997), CITE in the 3′ untranslated region of Pea Enation Mosaic Virus (PEMV) RNA 2 interacts with eIF4E (Wang et al., 2009), the 3′ CITE of Maize necrotic streak virus (MneSV) RNA binds to both the eIF4E and eIF4G subunits of eIF4F, with eIF4E contributing a specificity to the interaction (Nicholson et al., 2010) and the 3′ CITE of Turnip crinkle virus (TCV) RNA binds directly to 60S′ and 80S′ ribosomes (Stupina et al., 2008).

It should be noted that the initiation on the 5′ UTR of BYDV mRNA directed by the 3′ CITE is cap-independent, but 5′ end dependent and requires scanning to locate the authentic initiator codon: insertion of a stable stem-loop at the very 5′ end of mRNA inhibited the cap-independent translation (as well as the cap-dependent translation) (Guo et al., 2001), and insertion of uAUG codon out of frame also strongly inhibited translation (Rakotondrafara et al., 2006). Moreover, 3′ CITEs from BYDV, TNV (Tobacco Necrosis Virus) and STNV RNAs can direct the cap-independent translation when placed in 5′ UTRs (Meulewaeter et al., 1998; Shen et al., 2004; Wang et al., 1997). Interestingly, mutations in the 3′ CITE of BYDV mRNA that disrupted base-pairing with its 5′ UTR completely inactivates the CITE in the context of 3′ UTR but not in the context of 5′ UTR. This observation indicates that the cap-independent element and the element which allows the communication between the 3′ and 5′ ends of BYDV mRNA function independently on each other (Rakotondrafara et al., 2006). Finally, the BYDV CITE cannot work as an IRES when placed between two cistrons (S. Wang and E. Allen, unpublished, cited in Kneller et al., 2006). Thus, at least for the CITE of BYDV RNA, we can summarize that this CITE: a) can recruit eIF4F, b) can stimulate a cap-independent, but 5′ end-dependent translation when placed at the 5′ end of mRNA, c) cannot direct an internal initiation. This allows us to hypothesize that some kind of CITE-like elements may exist within 5′ UTRs of mammalian mRNAs (5′ CITEs) as illustrated in Fig. 1D. They allow these mRNAs to be preferentially translated under conditions when translation of "canonical" cap-dependent mRNAs are strongly inhibited.

CONCLUSION

Why the 5′ CITE hypothesis looks so attractive for us? First, we think that a mere binding of translational factors or ribosomes to internal sequences within 5′ UTRs is not sufficient for the internal initiation of translation. Most well studied viral IRESs comprise hundreds of nucleotides and possess a very complex organization. Some mutations completely inactivate them without altering the affinity of the key components required for translation initiation. For instance, deletion of domain II of HCV IRES only slightly changes its affinity to 40S ribosomal subunits and eIF3 [K_d(40S) 1,9 ± 0,3 nM (wt) vs. 3,1 ± 0,2 nM (mut); K_d(eIF3) 35 ± 3 nM (wt) vs. 35 ± 4 nM (mut)], but completely abolishes the IRES-driven translation (Kieft et al., 2001). Moreover, it has been shown that the HCV IRES (and most probably other HCV-like IRESs) actively changes conformation of 40S ribosome to allow the internal ribosome entry (Locker et al., 2007; Spahn et al., 2001). Thus, it looks like such complex IRES elements can be poorly substituted for by binding sites for canonical translation initiation factors or ribosomes (De Gregorio et al., 1999; 2001). Instead, some binding of key translation initiation components to 5′ UTRs (or in some cases to 3′ UTRs) more likely leads to stimulation of the 5′ end dependent initiation due to an increase of their local concentration near the 5′ end of mRNA. In this connection, one can recall that CITEs of plant viruses described earlier are much shorter than IRESs of mammalian viruses.

In addition, one should not forget that unlike many uncapped viral mRNAs, all cellular mRNAs possess the cap structure and thus do not need to use IRESs to be translated under normal conditions. As discussed above, the influence of the secondary structure of 5′ UTRs on the efficiency of scanning should be revised since, at least in some cases, 900-nt long UTRs can be scanned with an efficiency similar to that of 50-nt long ones (Dmitriev et al., 2007). However, under stress conditions, the binding of initiation factors to CITE-like elements can give some advantage to the mRNAs that possess them. This is illustrated in Fig. 2. Several CITE-like "modules" presented in the 5′ UTRs of mammalian RNAs may provide a cumulative effect on resistance of translational initiation to stress inducers affecting the activity of cap-binding factor eIF4E and 4E-BP. Therefore, the mechanism may be regarded as an alternative to the concept of cellular IRESs. The work on verification of this mechanism is now in progress.

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