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Review

Structural and functional diversity of viral IRESes

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ABSTRACT

Some 20 years ago, the study of picornaviral RNA translation led to the characterization of an alternative mechanism of initiation by direct ribosome binding to the 5′ UTR. By using a bicistronic vector, it was shown that the 5′ UTR of the poliovirus (PV) or the Encephalomyelitis virus (EMCV) had the ability to bind the 43S preinitiation complex in a 5′ and cap-independent manner. This is rendered possible by an RNA domain called IRES for Internal Ribosome Entry Site which enables efficient translation of an mRNA lacking a 5′ cap structure. IRES elements have now been found in many different viral families where they often confer a selective advantage to allow ribosome recruitment under conditions where cap-dependent protein synthesis is severely repressed. In this review, we compare and contrast the structure and function of IRESes that are found within 4 distinct family of RNA positive stranded viruses which are the (i) Picornaviruses; (ii) Flaviviruses; (iii) Dicistroviruses; and (iv) Lentiviruses.

1. Introduction

Viruses are intracellular parasites that rely on the components of the host cell for gene expression and replication. Although viral replication greatly varies from one virus to another, all known eukaryotic viruses have to produce messenger RNAs (mRNA) that can be translated by the cellular ribosomes. The Baltimore classification system of all viruses in six classes, based on the strategies adopted to produce mRNA from their genomes, emphasises this dependency on the cell's translational machinery [1]. Soon after infection, the host cell often tends to limit viral production and replication by shutting-off global translation [2]. This regulatory mechanism generally targets events from the initiation phase because it is the rate limiting step that determines overall protein production for most mRNAs. Many viral genomes have evolved to bypass this general inhibition of translation by developing mechanisms of initiation independent of the classical recognition of an m^7G cap structure at the 5′ end of the mRNA. These mechanisms imply the utilization of internal ribosome entry sites (IRESes) which can promote 5′ end independent initiation.

1.1. The cap-dependent mechanism of translation initiation

In eukaryotes, protein synthesis is a complex process by which polypeptides are produced from mRNA templates read by ribosomes in a 5′ to 3′ direction. Translation can be divided in three distinct stages: initiation, elongation and termination. Initiation is the process that allows assembly of a constituted 80S ribosome on an mRNA in which the first translated codon is based paired with the anticodon of the aminoacylated initiator methionyl transfer RNA ($tRNA_i^{Met}$). Elongation is the phase during which the ribosome selects an aminoacylated tRNA and catalyzes the formation of a peptide bond between the polypeptide chain already synthesized and the incoming amino acid. Termination takes place when a stop codon enters the A site of the 80S ribosome, this triggers the release of the neo synthesized protein and ribosome dissociation into both 40S and 60S subunits. In eukaryotes the initiation phase of protein synthesis is generally the most controlled step and determines overall protein production, for recent reviews see [3,4].

The vast majority of eukaryotic mRNAs (except those found in mitochondria) begins with a 5′ terminal 7-methyl-guanosine (m^7G) cap structure linked to the second nucleotide by a 5′–5′ phosphodiester bond. The cap is followed by a 5′ untranslated region (5′ UTR) which may vary in length and nucleotide composition but is typically comprised between 50 and 120 nucleotides [5]. The open reading frame encodes the protein and is ended by a 3′ untranslated region (3′ UTR) of variable length followed by a stretch of adenylate residues

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known as the poly(A) tail. In the canonical mechanism for translation initiation or "5′ scanning model" initially described by Marilyn Kozak [6,7], the 40S ribosomal small subunit binds to the 5′ terminal cap moiety and linearly scans the 5′ UTR in a 5′ to 3′ direction until it locates and recognizes the initiation codon. This process requires at least 12 different eukaryotic initiation factors (eIFs). The first event is the recruitment of the eukaryotic translation initiation factor 2 (eIF2)–GTP–$tRNA_i^{Met}$ ternary complex to the 40S ribosome to form a 43S preinitiation complex; this is also catalyzed by eIF5 which interacts with both eIF2 and eIF3 [8]. This complex formation is stabilized by eIF3, the largest initiation factor composed of at least 11 subunits [9–11], eIF1 and eIF1A [12–15]. The 43S preinitiation complex can be recruited to the 5′ capped end of the mRNA by its interaction with the heterotrimeric eIF4F factor which is constituted by eIF4E, the cap binding protein [16,17] associated with eIF4G [18], and eIF4A an ATPase RNA helicase which unwinds local RNA secondary structures [19]. This process is stimulated by a physical and functional interaction between the poly (A) binding protein (Pab1P) at the 3′ end and the initiation factor eIF4G located at the 5′ end [20–23] resulting in the pseudo circularization of the mRNA. Translation is also stimulated by the homodimer eIF4B which enhances the RNA helicase enzyme activity of eIF4A [24,25]. After initial binding to the 5′ end of the mRNA and scanning [6,26], the ribosome will then locate an AUG start codon surrounded by a good nucleotide consensus which is defined by a purine at the −3 position and a G at the +1 position [27]. Computer based analysis of different transcripts has shown that the first AUG codon encountered is usually selected (in a percentage varying between 90% and 60% depending on the studies and the surrounding context [28,29]). Accuracy of initiation codon selection is promoted by both eIF1 and eIF1A, which trigger the formation of a "closed" conformation when a proper codon–anticodon base pairing between the mRNA and the $tRNA_i^{Met}$ is found [30]. At this stage, eIF5 induces hydrolysis of eIF2-bound GTP to release some eIF2-GDP. Then the dissociation of initiation factors is promoted by the GTPase factor eIF5B and allows the association of the 60S subunit to form the 80S ribosome with the $tRNA_i^{Met}$ in the P site marking the end of the initiation phase.

1.2. Internal ribosomes entry sites

The study of poliovirus (PV) replication in the early 70's revealed that the viral mRNA was structurally distinct from cellular mRNAs as it does not contain a 5′ terminal cap structure but a VPg protein covalently linked to the 5′ end [31]. In addition, the 5′ UTR of PV is unusually long, rich in secondary RNA structures [32,33] and contains multiple upstream AUG codons. Finally, the finding that PV entry into the host cell is accompanied by a severe shut-off of host cell translation [34] with no impact on viral translation, suggested the use of a novel mechanism for translation initiation. Thus, it was hypothesized that the ribosome could bind to the viral mRNA in an internal position rather than at the mRNA 5′ end. By using bicistronic mRNA assays it was shown that the highly structured 5′ UTR of poliovirus was able to promote internal entry of the 43S ribosomal subunit on its mRNA, characterizing the first viral IRES [35]. This discovery was almost immediately extended to EMCV [36], and to other classes of RNA viruses such as the Hepatitis C Virus (HCV) [37], and to some DNA viruses [38,39].

Internal entry of ribosomes has also been evidenced in some eukaryotic cellular mRNAs that have the ability to remain associated with polysomes in poliovirus-infected cells when cap-dependent translation of host cell mRNAs was severely repressed ([40,41], review [42]).

Just about 20 years later, internal initiation has now emerged as an alternative way to bind a 43S ribosome at the correct initiation codon in a manner independent of the 5′cap structure of the viral or cellular mRNA considered. However, the exact order of events and the set of proteins required for efficient ribosomal binding is different between different IRESes.

2. Experimental demonstration of IRES activity

A peculiar characteristic of viral IRESes is the absence of a consensus or motif in their primary sequence, rendering a computer-based search very difficult [43,44]. Nevertheless, viral IRES elements often share several common structural characteristics such as long and structured 5′ UTRs, several upstream AUG triplets located before the authentic start codon, and, often but not always, the absence of a cap structure at the 5′ end of the RNA. These features are not compatible with an efficient cap-dependent translation and reflect the use of an alternative mechanism for translation initiation. However, only a thorough, functional assessment of IRES activity can validate a candidate sequence. Historically internal ribosome binding was first tested by measuring protein production from the second open reading frame (ORF) of mRNAs containing two ORFs called bicistronic mRNAs [35,36] or by looking at the translation of circular mRNAs [45].

2.1. Bicistronic constructs

This test needs the construction of a synthetic mRNA which contains two open reading frames (ORFs) separated by the candidate IRES, so that this single bicistronic RNA molecule codes for two reporter genes (see Fig. 1). The first cistron is translated in a 5′ cap-dependent manner whereas efficient production of the second one can only take place if ribosomes are recruited internally by the candidate IRES sequence located in the intercistronic spacer. The ratio of protein production measured from translation of both cistrons gives an indication of the relative strength of the IRES.

This bicistronic assay has rapidly been considered as the gold standard to define internal initiation and it is the most widely used method for testing putative IRES sequences. It can be utilised both *in vitro* in the reticulocyte lysate or cell extracts but also *ex vivo* in cultured cells. However, its utilization *ex vivo* should be thoroughly controlled as it has caused several drawbacks and misinterpretations. In fact, this test has been criticized [46,47] on the basis that even low amount of alternative monocistronic mRNA transcripts can result in a misinterpretation of an IRES activity. These constructs may be generated by different ways: i.e. the presence of a cryptic promoter in the sequence of interest, or by a splicing event that would skip completely the first ORF. These mRNAs, in very low concentration, could be poorly detectable but highly translatable and could distort the interpretation. This has been demonstrated in the case of the *Renilla* and *Firefly luciferase* reporter genes (widely used in bicistronic constructs) which have been shown to contain a splice-donor sequence (Renilla) or a cryptic promoter (Firefly) respectively [48]. Thus when testing IRES activity by transfection of bicistronic DNA test constructs in cells, it is essential to check for the presence of alternate transcripts by stringent RNA tests, such as that proposed by Van Eden et al. [49], which is based on RNAi combined to RT-PCR detection of aberrant RNAs. Nevertheless, one easy way to circumvent the presence of cryptic promoters or splicing sites, is to use direct transfection of *in vitro* transcribed bicistronic RNA [50–52]. This method rules out the intervention of any possible nuclear processing events but may also prevent proper association with nuclear ITAFs (IRES trans-acting factors) involved in RNP complex formation and full IRES activity, and it should be noted that the influence of a nuclear event for efficient translation of cellular IRESes was pointed out by Willis et al. [53].

2.2. Monocistronic RNAs

Virtually all eukaryotic genes, even those containing IRESes, are transcribed as monocistronic messenger RNAs. Thus, one should bear in mind that candidate IRESes must also be assessed as monocistronic entities (i.e. with no 5′ flanking sequence and a free 5′ end) as it is the relevant physiologically context. Although not useful when demon-

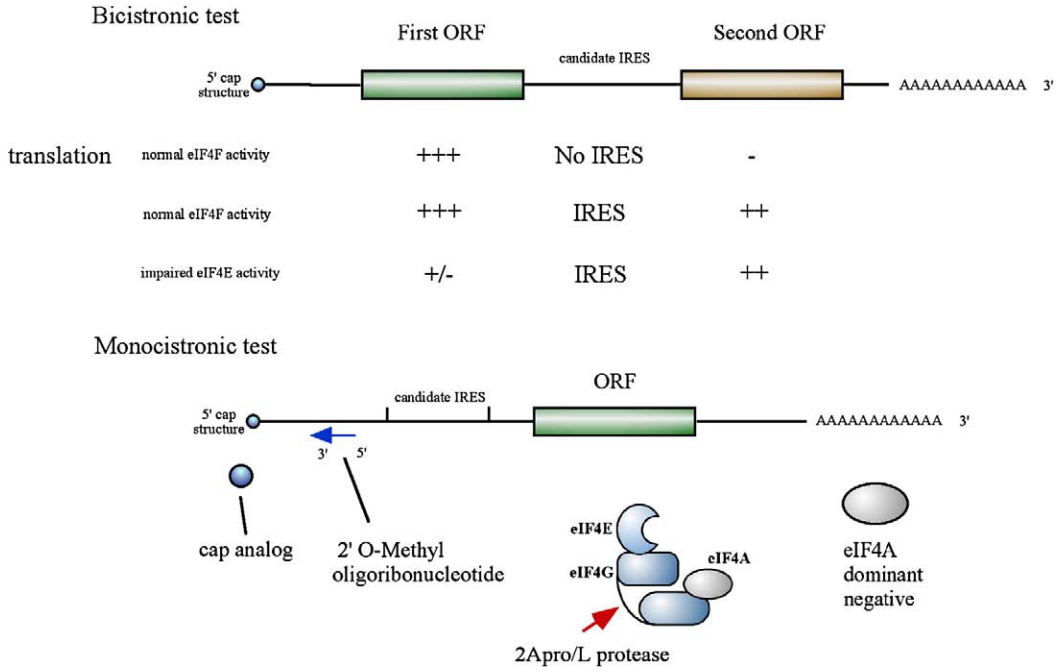

Fig. 1. Experimental demonstration of IRES activity. The Bicistronic assay: the candidate sequence is inserted between two open reading frames (ORF). Expression of the first ORF is driven by the 5′ cap structure whereas translation of the second ORF takes place only if the intergenic tested sequence is able to recruit ribosomes internally. IRES activity can also be identified on monocistronic constructs. Translation of monocistronic RNAs containing the putative IRES sequence in 5′ can be assessed under conditions where cap-dependent protein synthesis is disrupted by: (i) hybridization of 2′-O-methyl oligoribonucleotides on the 5′UTR; (ii) cleavage of endogenous eIF4G by viral proteases; (iii) addition of cap analog; (iv) addition of eIF4A dominant negative mutants (in the case of Hepaciviruses and Dicistroviruses).

strating IRES activity, monocistronic RNAs are essential to study translational regulation of a given 5′ UTR, and notably to evaluate the respective contributions of IRES-driven translation and 5′ dependent translation (either cap or cap-independent) in mRNAs in which a dual translation mechanism could coexist (see later, Lentivirus IRES). This is of particular interest for mRNAs supposed to switch from one mechanism to another depending on changes in the cellular environment [54–56]. However, to examine the relative contribution of a cap/IRES mechanism on monocistronic RNAs, it is essential to use experimental settings to limit cap-dependent entry from the 5′ of the RNA. Thus, translation efficiency can be evaluated: (i) on capped and uncapped transcripts; (ii) with 2′-O-methyl oligoribonucleotides hybridized to the 5′ UTR; (iii) in the presence of viral proteases that cleave eIF4G; (iv) upon addition of cap analog; (v) in the presence of a dominant negative form of eIF4A in the case of Hepaciviruses or dicistroviruses (see Fig. 1).

Nowadays more than 80 cellular and 56 viral IRES sequences have been characterized [44,57] amongst a broad range of different organisms including viruses, yeast, plants [55,58] and higher eukaryotes. Thus, it would be too simplistic to consider one mechanism of internal initiation and one would rather consider mechanisms that are shared between different families of viruses or genes. This review article will focus exclusively on the diversity of IRES sequences amongst viruses. As such, it will not deal in great detail with the mechanism of internal initiation but rather provide a non-exhaustive view of the diversity of viral IRESes that are found in four major viral families that are namely: Picornaviruses, Flaviviruses, Dicistroviruses and Lentiviruses.

3. Picornavirus IRESes

3.1. Structure of genomic RNA in picornaviruses

Picornaviruses are a large family of non-enveloped positive single-stranded RNA viruses, which replicate entirely in the cytoplasm [59]. Their non-segmented genome is composed of a single linear RNA molecule of 7000 to 8500 nucleotides in length. The 5′-end of the genomic RNA has a covalently linked protein named VPg and the 3′-terminus exhibits a poly(A) tail (Fig. 2). All picornaviruses share common features such as a long and structured 5′ UTR (600 to 1200 nt depending of the virus) containing multiple upstream AUG triplets that are never used to start translation [60]. Computer based analysis of the 5′ UTR region has evidenced structural constraints that should totally inhibit 40S ribosomal scanning [32,61], and this was further demonstrated by experimental evidence [62]. The picornaviral 5′ UTR is implicated in two different functions with the extreme 5′-end motifs devoted to RNA replication whereas downstream sequences serve for translation. Nevertheless, the boundary is not so clear with evidence of crosstalks between replication and translation RNA sequences [63,64].

3.2. Internal initiation in picornavirus

Based on biochemical, structural and phylogenetic studies it has been proposed to divide the picornaviral IRESes into two major groups [65,66] and a minor group. Major type I IRESes are found in the mRNAs of enteroviruses (i.e. PV) and rhinoviruses, major type II are found in the mRNAs of aphthoviruses (i.e. FMDV), cardioviruses (i.e. EMCV), whereas the HAV IRES exhibits a very distinct architecture that justifies a group on its own which will not be detailed herein. All picornaviral IRESes share some characteristics in common which is the presence of a GNRA motif in the central domain which adopts a tetraloop conformation and a small polypyrimidine rich tract that lies about 20 to 25 nucleotides upstream of an AUG triplet. In type I IRESes this AUG codon is not utilised to start translation and the ribosome has to scan some 100 to 150 nucleotide downstream to initiate protein synthesis at the next AUG (Fig. 2) (review[67]). Studies on EMCV type II IRESes [68,69] have shown that the distance between the oligopyrimidine tract and the initiator AUG is critical and that the 40S ribosome seems to be loaded directly, or, at the immediate vicinity of this AUG without any scanning process. Finally, it has to be mentioned that some picornavirus IRES elements share very similar

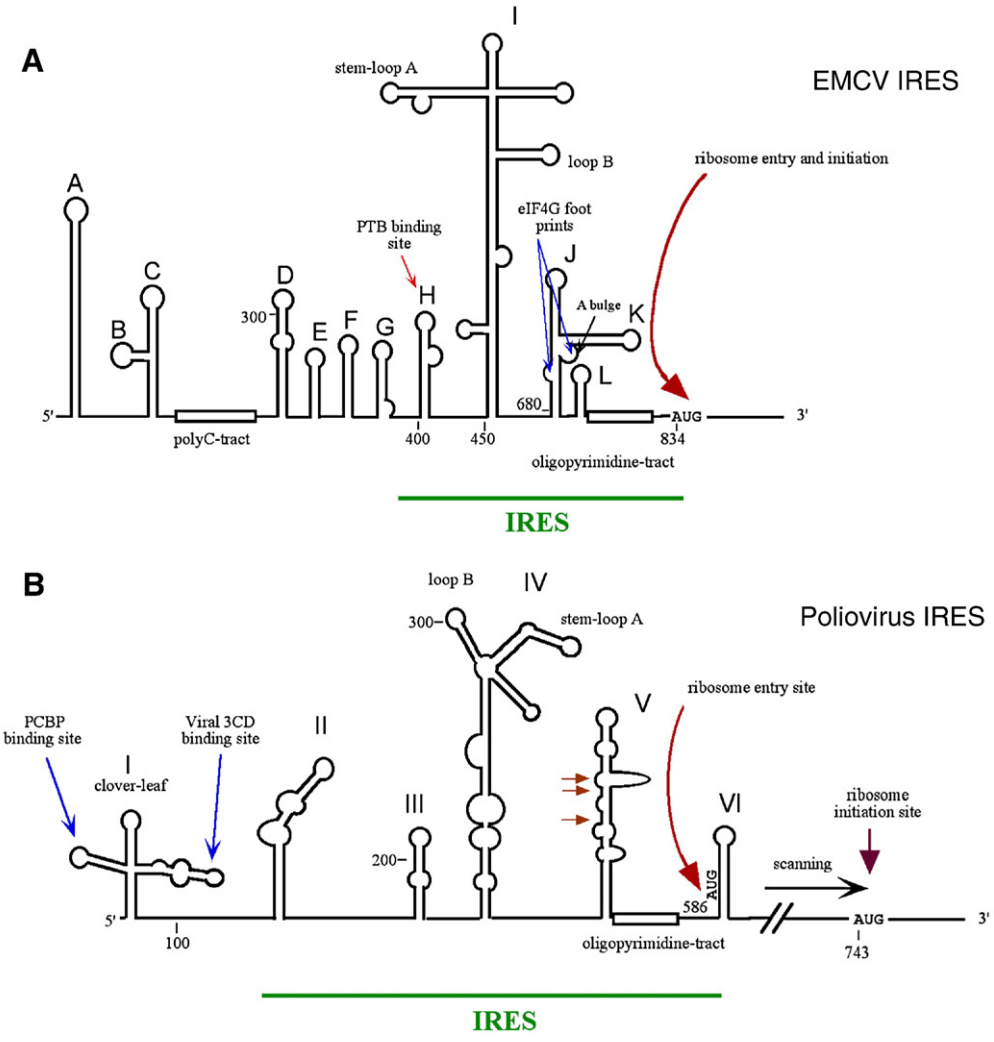

Fig. 2. Schematic representation of two picornaviral IRESes (adapted from [53] and [67]). (A) Structures of the 5′UTR of EMCV (type II IRES). The core of the IRES within the 5′UTR is underlined in green. The binding site for PTB and eI4G are indicated. The ribosome lands and initiates at the AUG located at the end of the polypyrimidine tract. (B) Structures of the 5′UTR of PV (type I IRES). The body of the IRES within the 5′UTR is underlined in green. The binding sites for the viral proteins PCBP and 3CD on the clover leaf structure are indicated. The ribosome enters the RNA at nucleotide 586 just after the oligopyrimidine tract and then scan down until it reaches the authentic initiation site located at position 743. Sites of mutations in Sabin vaccine strains of poliovirus are indicated on domain V by arrows.

characteristics to those found in HCV and pestivirus elements [70,71], for a review see [72].

3.3. Requirement for eukaryotic initiation factors (eIFs) and cellular proteins (ITAFs)

In 1996, using the FMDV L protease as a tool to cleave eIF4GI, we showed that the carboxy-terminal domain of eIF4GI was sufficient to support IRES driven translation from EMCV even in the absence of eIF4E [73]. At the same time, Pestova and Hellen had just developed a reconstituted translation assay in which all the components required for translation initiation: initiation factors, amino acids, ribosomes, mRNA and tRNAs are added sequentially to form a preinitiation complex. Assembly of this preinitiation complex to the initiation site of the mRNA is monitored by the arrest of primer extension imposed by the 43S stalled at the initiation codon. In agreement with our results, they show that virtually all of the canonical translation factors were needed to recruit the 40S subunit, on the EMCV IRES, with the exception of eIF4E and the amino-terminal of eIF4G [74,75]. It is somehow intriguing to consider that functional eIF4A factor is required for translation of EMCV despite the fact that there is probably no involvement of ribosome scanning to locate the AUG codon [69]. This discrepancy was partly solved when it was shown that the association of eIF4A with eIF4GI increases the affinity of this latter factor for the EMCV IRES, and induces conformational rearrangements of the RNA [76,77]. The initiation factor eIF4G also appears to be a key player in the recruitment of the 43S complex onto the IRES sequence. It has been shown that eIF4G has the ability to directly contact the IRES sequence of EMCV at the J–K domain [78] and the stem–loop V of PV in association with eIF4B (see Fig. 2). It is particularly interesting if one considers that this domain of PV is mutated in the vaccinal Sabin strain of poliovirus [79,80].

If some picornaviral IRESes such as EMCV are able to efficiently drive translation in the rabbit reticulocytes lysate (RRL) with the sole set of canonical initiation factors, this is clearly not the case for members of the polio-/rhino-virus genera. Translation of these type I IRESes in the RRL results in a very low yield of protein production which is often below the threshold of detection and is accompanied by multiple aberrant initiation events at cryptic AUGs within the coding region. Interestingly, such profile can be corrected by supplementation of the reticulocyte lysate with HeLa cell extracts [81] suggesting that some cellular factors that are present in HeLa but absent from the RRL are needed for ribosomal entry. These

factors were called IRES trans-acting factors (ITAFs) [82] and were identified by cross-linking and band shift assays between the IRES and cell extracts. The Lupus antigen protein (La) was the first one to be characterized [83,84], and was soon followed by the identification of the polypyrimidine tract binding protein (PTB) [85]. Both La and PTB were later involved in the modulation of translation of other IRESes and many other ITAFs have been identified since such as ITAF45/Mpp1, hnRNP E2/PCBP2, and unr or nucleolin (for a review see [86]).

Picornavirus IRES translation is also modulated by the presence of its poly(A) tail that can act in synergy with the 5′ UTR of the genome. Thus, it was reported that the 3′ poly(A) tail stimulates translation of PV and EMCV [87–89], this effect is dependent on eIF4G since its cleavage abrogates the stimulatory effect of poly(A) tail on translation. If this stimulation could be partly explained by eIF4G–PABP interactions via a synergistic enhancement comparable to the classical cap-poly(A) tail cooperation, some data show the maintenance of such an activation in PABP depleted cell free extracts [90], suggesting the existence of a more complex mechanism.

3.4. The "shut-off" of cellular translation during infection

All picornavirus genomes encode a single polyprotein that is rapidly processed by virally encoded proteases. In the case of rhino/polio and aphtho-virus, the expression of the viral protease 2A ($2A^{pro}$) or L, respectively, results in the proteolytic destruction of the cellular initiation factors eIF4GI and eIF4GII. Complete cleavage of eIF4GI generally occurs within 2 to 3 h after PV entry and severely disrupts the eIF3 and eIF4E binding domains which results in the inhibition host cellular translation [34,91]; this has also been reported for eIF4GII [92]. In addition to its proteolytic action on eIF4G, ($2A^{pro}$) is also able to cleave a small cellular fraction of PABP, with the help of another viral protease ($3C^{pro}$) [93]. This provokes a very strong cytopathic effect due to a rapid and complete shut-off of cap-dependent protein synthesis following PV infection. Interestingly, infection with the cardiovirus EMCV does not result in any proteolytic event but rather induces dephosphorylation of the eIF4E-binding protein (eIF4E-BP) which sequesters eIF4E to inhibit cap recognition [94]. Thus, most picornaviruses seem to have evolved strategies to shut-off cellular translation allowing IRES-dependent initiation to take place. In fact, the down regulation of capped cellular translation often results in the enhancement of IRES-driven translation as shown upon addition of rapamycin [95] or protease in *in vitro* assays [96,97], and in cells [98,99].

3.5. C-IRESes of Flaviviridae

Members of the Flaviviridae are enveloped single-stranded linear positive-sense RNA viruses that comprise the Hepatitis C Virus (HCV), GB virus B, Bovine Viral Diarrhea Virus (BVDV) or the Classical Swine Fever Virus (CSFV), all of them were shown to contain an IRES in their 5′ UTR [100–103]. The lifecycle of HCV is exclusively cytoplasmic with an IRES-mediated translation of the genomic RNA which occurring shortly after virus entry and giving rise to a large polyprotein that is later cleaved by different viral and cellular proteases (for a review see [104]).

The 9500 nucleotides-long single-stranded positive RNA HCV genome is composed of a non-capped highly structured 5′ UTR implicated both in translation and replication. Interestingly, the virus does not possess a poly(A) tail but, instead, harbours a highly structured 3′ UTR composed of a short variable region containing a poly(U/UC) of approximately 80 nucleotides length, ended by an invariant region of 98 nucleotide called the X-tail [105,106]. This 3′ UTR is important both for replication of the virus, and for IRES activity [88,107,108].

3.6. Structure

The prototype of this family is represented by the HCV IRES which is composed of a 5′ UTR of 341 nt long (up to the AUG codon) and folds into four major structural domains numbered I to IV. An interesting feature of the HCV IRES is the fact that the sequences required for IRES activity are located between domains II and III and IV and extend beyond to the AUG codon on the first 30 nucleotides of the ORF [109,110]. Domain I has been shown to be important for replication but appears to be dispensable for translation activity. Secondary structures of the HCV IRES have been extensively studied by biochemical methods, and are summarized in Fig. 3. Domain II is a 75 nt long hairpin structure with 3 internal and a terminal loop that enhances significantly IRES activity although it is not absolutely necessary [37,111,112]. Domain III is the largest RNA structure and comprises several helices and hairpins named IIIa to IIIf. Domains IIIa–IIIc and IIIe–IIIf form a three-way helical junction with the IIIf stem–loop being part of the pseudoknot that is essential for ribosomal recruitment (see Fig. 3). The correct folding of the whole HCV RNA structure depends on the presence of divalent cations [113]. The tertiary structure of the different parts of this IRES has been determined by various biophysical approaches including cryo-electro microscopy [114,115] and NMR [116], which show that this IRES has an extended conformation and is able to coil up on multiple sites of the 40S subunit [117]. The 5′ UTRs of HCV, GBV-B and pestiviruses share similar, but not identical, secondary structures [118]. For instance, GBV-B contains two additional hairpins in domain II and pestiviruses an additional hairpin in domain III; nevertheless, key structural elements are conserved in all "HCV-like" IRESes [119]. Finally, although the architecture of this IRES seems to be critical for its function, it should be noted that recent data have reported some naturally occurring mutations found in the HCV 5′ UTR that do not disrupt the overall structure but were detrimental to translation suggesting that structure and function can be uncoupled [120].

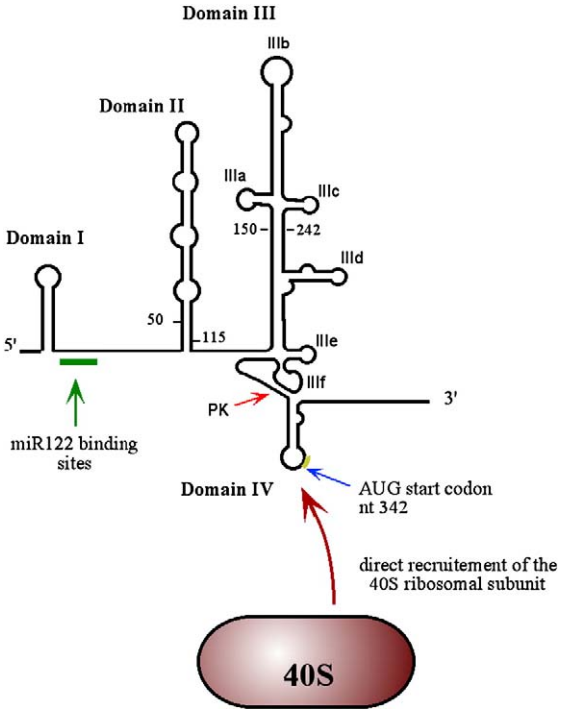

Fig. 3. Secondary structure of the 5′UTR of HCV (adapted from [130]). The IRES is composed of domains II–III–IV whereas domain I is dispensable for IRES activity. The two miR122 binding sites located in the 5′UTR are located just downstream of domain I and are close to each other. The ribosome is directly recruited at the AUG initiation codon which is part of the loop of domain IV extending on the first nucleotides of the coding sequence.

3.7. Mechanisms of internal initiation and host cell factors requirement

The mechanism of ribosomal recruitment onto the HCV IRES is quite different from that described for the picornaviruses (see above). Once again, using the reconstituted translational assay, Pestova and Hellen have investigated in detail the factor requirements [121,122] and they could show that assembly of the 48S initiation complex was independent of eIF4A, eIF4B, and eIF4F. In fact, a crude 40S ribosomal subunit can directly bind to stem–loops IIId or IIIe of the HCV IRES, and intermolecular contact zones have been reported on most of the IRES sequence [123]. Crosslinking experiments further showed that these contacts are due to interactions between RNA motifs from the IRES and ribosomal proteins that constitute the 40S subunit [117,124]. As a result of this interaction, the positioning of the HCV IRES on the surface of the 40S ribosomal subunit induces a conformational change [115] that clamps the mRNA and positions the AUG codon in the P site of the ribosome with no need for ribosomal scanning. Furthermore, by using biochemical methods, it was shown that the IRES can also directly bind to the initiation factor eIF3 via loops IIIa and IIIb and the junction domain IIIabc [123,125,126] and this was later confirmed by cryo-EM reconstitution [127]. This interaction is essential for the recruitment of the eIF2–$tRNA_i^{Met}$ ternary complex and the initiation of translation [128]. Thus, direct interaction between the IRES and the 43S ribosome results in the correct positioning of the initiator AUG in its P site, in a "prokaryotic mode" as it has been initially described by Pestova and Hellen [122]; after recruitment of the ternary complex stabilized by eIF3, the hydrolysis of eIF2-bound GTP (catalyzed by eIF5) allows the joining of the 60S subunits [129]. Translation elongation begins with the IRES at the vicinity of the E site of the ribosome, where the deacylated tRNA is positioned after translocation of the 80S ribosome. Detailed review on the molecular mechanism of HCV translation can be found in this recent review [130].

Interestingly, it has been shown that the HCV IRES could support the reduced availability of eIF2–GTP–$tRNA_i^{Met}$ ternary complex that is found in stress conditions, by switching to an eIF2 independent mechanism when this factor is inactivated by phosphorylation [131]. Thus, this low requirement in initiation factors certainly represents a selective advantage for efficient replication of HCV even under unfavourable physiological conditions. Recently, *in vitro* studies have shown evidence for the direct recruitment of 80S ribosomes competent for elongation that were observed at high (5 mM) concentrations of Mg^{2+} [132]. However at physiological concentrations of Mg^{2+} (2.5 mM), eIF2 independent translation initiation on the classical swine fever virus (CSFV) IRES was still observed but dropped to only 10% [133].

Translation efficiency driven by the HCV IRES can be modulated in different ways. Firstly, some cellular proteins, such as hnRNP-L, the La antigen, PTB or NSAP-1, [134–138] can enhance IRES-dependent translation through different interactions, even if the role of some of these factors is still a matter of debate [139]. Secondly, despite some early contradictory studies [88,140,141], it now appears that the non-polyadenylated 3′ UTR region of HCV acts as a translational enhancer as demonstrated by adding it in *cis* on minigene reporters [142,143], or in *trans* [139]. Molecular basis for this enhancement remains obscure and may involve a 5′ to 3′ communication or a local effect on translation termination [142,144].

Finally, HCV translation is also controlled by the effect of a cellular microRNA (miR122) and this is probably one of the main determinant aspect of cellular tropism. The hybridization of miRNAs on mRNAs usually results in the inhibition of gene expression of the target gene (for review see [145]). However, the effect of miRNAs on IRES-driven translation is still unclear with several discordant reports showing no effect on translation [146], whereas others argue for a pronounced inhibition of gene expression [147]. The genome of HCV contains sites for the liver specific miRNA-122 that are located both in the 5′ UTR (2 sites) and in the 3′ UTR (1 site). It is noteworthy that miR122 is one of the major microRNA expressed in liver cells, representing approximately 70% of the total liver miRNA content [148]. In a very elegant set of experiments, Sarnow et al. showed that binding of miR122 to the target sites contained in the HCV genome resulted in the stimulation of HCV replication. This was particularly convincing by using the miR122 expressing cell line HuH7 as compared to the HepG2 one which is devoid of miR122 [149]. Very recently, further work has now shown that binding of the miR122 to the 5′ target sites (but not the 3′ one) was responsible for the enhancement of HCV translation [150]. This effect seems to be due to a stimulation of the association of the 40S subunit with the viral RNA although more work is needed to fully understand this mechanism.

4. IRES of dicistrovirus

A subfamily of the Picornavirales: the discistroviridae family of insect viruses is non-segmented single-stranded linear positive-sense RNA genome. The viral genomic RNA has a size comprised between 8500 and 9500 nucleotides and contains a VPg protein linked at its 5′ terminal end and a poly(A) tail at its 3′ extremity [151]. The dicistroviridae family is composed of 11 members amongst which are found the Cricket paralysis virus (CrPV) [152], the Drosophila C virus (DCV) [153], Rhopalosiphum padi virus (RhPV) and the Plautia stali intestinal virus (PSIV) [154]. The genome is organized in two large open reading frames, ORF1 and ORF2, that are translated as long polyproteins which are later processed by a virally encoded protease to give the non-structural (NS) and structural proteins respectively. These two ORFs are separated by an intergenic region (IGR) of approximately 200 nucleotides in length (Fig. 4A) and are expressed by two distinct IRESes [155]. The first one lies within the 5′ UTR and was shown to be functional in a wide range of translational systems [156,157] whereas the second IRES sequence is located in the intergenic region and has the unique ability to recruit a ribosome in the absence of initiation factors and without the $tRNA_i^{Met}$ (see below) [116,158].

4.1. Structure of dicistrovirus IGR IRES

By introducing mutations in the PSIV genomic RNA, Sasaki and Nakashima showed that the intergenic region (IGR) IRES could initiate translation at a CAA codon by the insertion of a glutamine amino acid. Further complementary mutations/deletions in the sequence revealed the presence of a pseudoknot secondary structure (PKI) implicating a CUU triplet immediately upstream of the CAA initiator codon and a 29 nt stem–loop structure [159,160]. Although the length of the intergenic region varies among dicistroviruses, the overall secondary and tertiary structures of the IGR IRESes appear to be conserved [161]. The structure can be split in three different domains, which all contain a pseudoknot domain (PK); PKII and PKIII overlap to form a stable folded domain whilst PKI is attached to them by a linker and forms a more independent domain 3 which is able to dock into the ribosome decoding groove (see Fig. 4B) [162–164]. Different methods that include structural probing, Cryo-EM analysis [165], and crystallography [164] indicate that the CrPV IGR IRES occupies the region involved in tRNA binding in the P and E sites. This is mainly due to domain 3 which seems to be positioned in the P site and able to adopt a tRNA–mRNA conformation which mimics the conformation of a $tRNA_i^{Met}$ bound to its initiator codon [155,166,167]. In the 80S ribosome the IGR IRES gets positioned in the precise hybrid state that can be found during the transition between a P/E hybrid tRNA and a P site tRNA. This P/E hybrid state mimicry could explain how the initiation can start with no requirement for a peptide bond formation with the amino acid loaded in the A site [164].

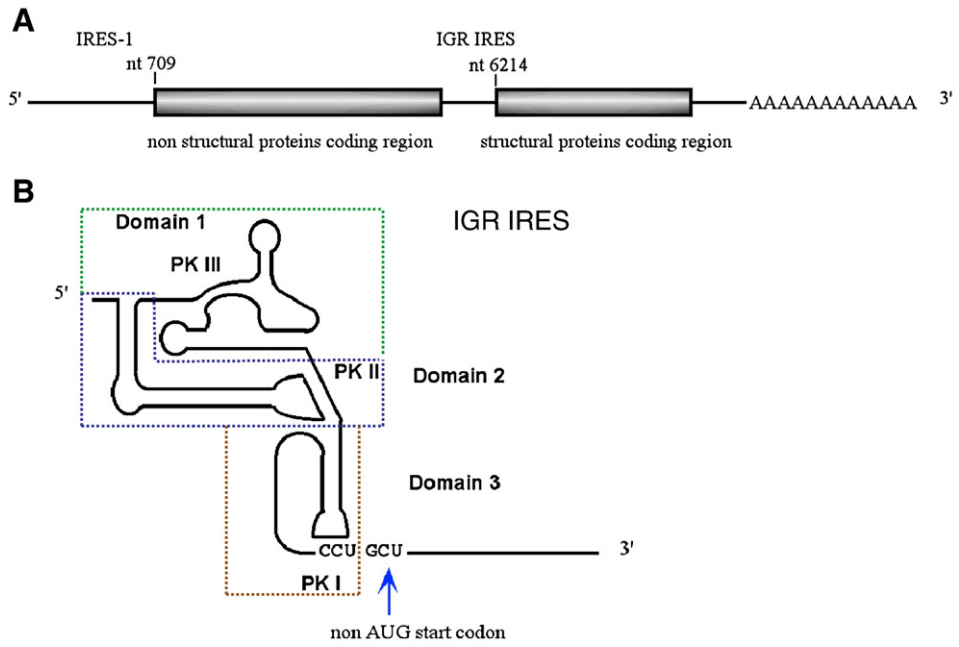

Fig. 4. (A) Genome organization of CrPV. The first IRES is located on the 5′UTR part of the molecule. The first ORF ends at nucleotide 6022 when begins the intergenic region and the IGR IRES. (B) CrPV IGR IRES secondary structure (adapted from [171]). Domains 1, 2 and 3 are limited by coloured dashed lines. The CCU triplet at position 6214–6216 fits in the P site of the ribosome and determines the open reading frame used, the following GCU is the first codon to be translated.

4.2. Unique properties of the IGR IRES

The insect IGR IRESes use a unique and remarkable mechanism for translation as they are able to directly bind to a 40S subunit and to assemble the 80S ribosome without any requirement for any initiation factor, $tRNA_i^{Met}$ or hydrolysis of GTP [166]. Experiments in yeast demonstrate that initiation from these IRESes was inhibited by ternary complex availability ($tRNA_i^{Met}$–eIF2–GTP) or by high concentrations of $tRNA_i^{Met}$ [168]. Finally, *in vitro* assembled 80S–CrPV IRES complexes are able to support amino-acid incorporation into polypeptide after addition of elongation factors and aminoacyl-tRNAs [169–171]. The total absence of requirement for initiation factors confers to this IRES the ability to drive viral protein production in extreme cellular physiological conditions that can be found after induction of the unfolded protein response with a high level of eIF2 phosphorylation [166] or following amino acid starvation [172]. Thus, these IRESes are well adapted to the physiological status of the cell that is usually found during the infection by dicistroviruses [173,174]. It should be remembered that the non-structural proteins must also be produced under these conditions from ribosome entry at the first IRES which requires a complex set of initiation factors [157]. Thus, it is not surprising that expression of structural proteins is in molar excess compared to the production of the non-structural proteins [173,174]. Uncoupling synthesis of these structural and non-structural proteins has been explained as a process to facilitate the production of the large amount of structural proteins required for the formation of the viral capsid [155].

5. IRESes in Lentivirus

Since the original discovery of an IRES in the Murine leukemia virus (MLV) genomic RNA, internal initiation has been shown to occur in several simple and complex members of the retrovirus family [175–178]. Interestingly, IRESes have now been characterized in most members of the lentiviral family and they have been found both in the 5′ UTR and in the coding region where they are used to express N-truncated isoforms of the major structural Gag polyprotein.

5.1. Structure of genomic RNA in Lentiviruses

The Lentivirus family is composed of nine different species and representative members of the genus include the human immunodeficiency viruses types 1 (HIV-1) and 2 (HIV-2), the simian immunodeficiency virus (SIV) and the feline immunodeficiency virus (FIV). Their genome is composed of a single-stranded positive RNA molecule of about 9000 nt in length that encodes the three main retroviral genes *gag*, *pol* and *env*. The genome of these complex retroviruses also codes for a number of regulatory and accessory proteins that are implicated at different levels in the viral cycle. Infection begins with interaction with receptors and viral entry in the cytoplasm. The viral genome is not directly used as an mRNA at this step, but is converted by a virally encoded reverse transcriptase into a double strand DNA molecule that reaches the nucleus and incorporates in the host cell genome. Once integrated, the provirus is transcribed to give the capped and polyadenylated unspliced genomic RNA (gRNA) by the cell RNA polymerase II, and exported in a Rev-dependent manner in the cytoplasm where it becomes translated. The Gag and Gag-pol polyproteins are synthesized from the unspliced RNAs by translation of the first reading frame (gag) and a frame-shifting event that allows about 5% of the ribosomes to continue decoding of the second gene (Pol) to yield a Gag-Pol fusion protein (for a recent review, see [56]). Both Gag and Gag-Pol polyproteins are then processed by the virally encoded protease. For some simple retroviruses such as MLV, packaging and translation are mutually exclusive [179], whereas it is not the case for the HIV-1 [180] were the same gRNA molecule is competent for both processes. Although these are controlled by RNA elements present within the same leader region (5′ UTR) of full-length unspliced RNA, one has to keep in mind that translation and encapsidation probably compete for the same RNA molecule [181].

The 5′ UTR of Lentiviruses genomic RNA is long and contains several structured domains. These elements are detailed in Fig. 5 and are: the TAR element (only found in Lentiviruses) implicated in transcriptional trans-activation, the poly-adenylation stem–loop responsible for 3′ processing, the primer binding site (PBS) and the

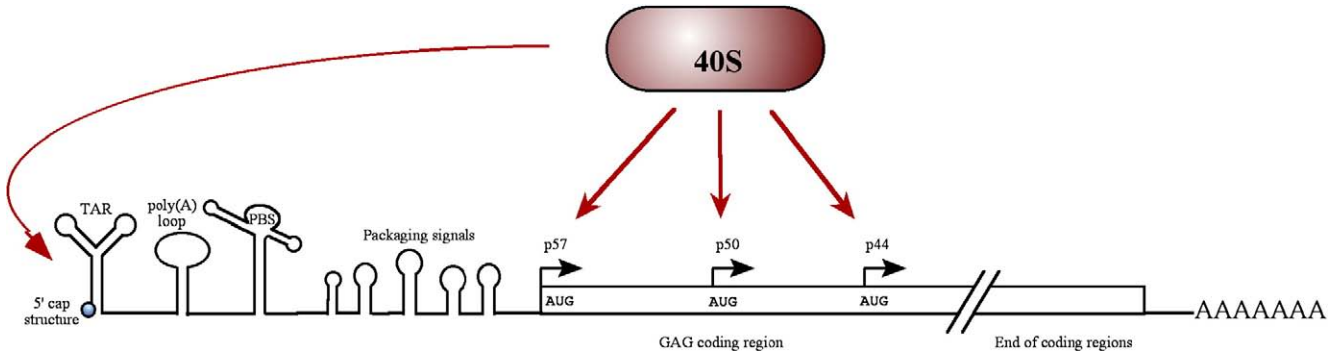

Fig. 5. Secondary structure of the 5′UTR of HIV-2 (adapted from [51] BST). Elements of the 5′UTR of HIV-2 implicated in transcriptional transactivation (TAR), 3′ processing (Poly(A) loop), reverse transcription (PBS) and packaging signals are indicated. The ribosome can be recruited at the 5′ capped end or at 3 distinct IRES elements within the gag coding region.

dimerization and packaging signals [182]. Their stability (mainly TAR) could represent barriers for ribosomes and it fact different *in vitro* and *ex vivo* approaches have first pointed out a negative role for this RNA structure in translation initiation [183–186].

5.2. IRES elements in the lentiviral genomic RNA

The first attempt to characterize an IRES activity within the 5′ UTR of the HIV-1 failed to identify any putative sequences that would allow internal ribosomal entry [187]. However, by studying the Simian Immunodeficiency Virus (SIV), we were able to show that the 5′ UTR of this virus was able to recruit ribosomes in an internal position both *in vitro* and *ex vivo* [188]; this was soon extended to other members of the lentiviral family [189,190] (Recently reviewed in [56]). Interestingly, internal initiation from the SIV IRES was active both *in vitro* and in cell culture whereas translational activity from the HIV-1 and FIV IRESes was only detectable under specific physiological conditions such as the G2/M transition phase of the cell cycle (for HIV-1) or under cell stress conditions for FIV [189,190]. Such a specific control may explain the apparent discrepancies between the results of Miele et al. with other workers. Translational control of Lentiviruses presents an additional layer of complexity as IRES sequences have also been characterized in the *gag* coding region of HIV-1 [191], HIV-2 [51,192], SIV [193] and FIV [190]. Internal ribosome entry on the gag coding region produces shorter N-truncated isoforms of the Gag polyprotein from alternative AUG codons (see Fig. 5). These isoforms can be encapsidated in the viral particle and were shown to be important for the replication cycle [192]. Interestingly, the synthesis of these N-truncated isoforms can occur independently from the production of the main Gag polyprotein ([51,194]). In the case of HIV-2, it has even been shown that the IRES element can drive translation from an initiator AUG located upstream of its core domain [192]. This discovery was of particular interest because it adds a new way of thinking IRESes as global attractors of ribosomes that once poised on them, would be able to pounce onto an RNA "landing zone" located close to the AUG codon. This concept fits well with the results of Juneman et al. showing that translation of the upstream gene of a bicistronic construct is affected by the level of stimulation of the downstream gene [195].

5.3. Alternative use of a cap and an IRES mechanism

All spliced and unspliced lentiviral transcripts contain a 5′ cap moiety and a poly (A) tail at their 3′ extremity [196]. Thus, they are structurally 'designed' to produce proteins by a 5′ cap-dependent mechanism. This is also in agreement with the fact that the lentiviral 5′ UTR rarely contains upstream AUG codons. In the case of HIV-1, HIV-2 and FIV, it has clearly been shown that the genomic RNA can very efficiently produce proteins by a cap-dependent scanning mechanism in the absence of ongoing viral infection [190,194]. Moreover, the strong requirement of HIV-1 for the RNA helicase A, may suggest a role for the latter in the unwinding of the RNA structures that are encountered between the cap and the AUG codon [197].

The oncourse of viral infection is characterized by profound modifications of the cellular environment by the expression of lentiviral accessory proteins such as Vpr of HIV-1, HIV-2 and SIV [198], and Orf-A of FIV [199] that were shown to modulate cell cycle progression by inducing G2 arrest, a context in which cap-dependent translation is known to be repressed [200]. In this context, an important production of the Gag polyprotein from the genomic RNA is still observed [201] suggesting the exclusive use of an IRES mechanism. It is also noteworthy that the viral genome also codes for an aspartyl protease which processes the Gag and the Gag-Pol proteins but also targets a number of cellular polypeptides such as eIF4GI and PABP for HIV-1 and HIV-2 and eIF4GII for SIV [202–206].

The paradox of lentiviral translation comes from the fact that some data argue in favour of a cap-dependent mechanism whereas others report evidence for internal initiation. These apparent discrepancies in the literature may be possible to reconcile after further consideration. First of all, it has to be kept in mind that some lentiviral IRESes require special conditions to be active [189,190]. Secondly it is difficult to appreciate respective contributions of IRES and cap-dependent translation initiation in the course of viral replication, especially if they function in synergy. Finally, lentiviral infection does not induce a complete shut-off of host translation [206], and proteolysis of both eIF4GI and PABP occurs at a late time during infection when most of protein production has already occurred. Therefore, the potential ability to use both cap- and IRES-dependent translation seems to be an opportunistic and sophisticated strategy to ensure efficient and robust protein synthesis when required. Under normal conditions, ribosome recruitment probably takes place by binding to the 5′ end cap. Then, later during infection, when the cell cycle is arrested in G2/M or when Gag polyprotein accumulates and binds to the 5′ UTR, cap-dependent translation is blocked and the viral RNA switches to an IRES-dependent mechanism that can load ribosomes onto the 5′ UTR or directly to the gag coding region.

6. The role of the IRES in the viral life cycle

6.1. Maintenance of viral protein synthesis

In order to replicate and propagate, all viruses identified so far are strictly dependent on the host translational cell apparatus for protein production. Not surprisingly, they have evolved ways to hijack the eukaryotic translational machinery for their own purpose. In response, many antiviral strategies have been developed by the

cell to fight against viral replication. These cellular strategies mainly target the very first steps of protein synthesis, more particularly the attachment of the ribosome to the cap structure. (for a recent review see [207]).

By acquiring an IRES through evolution, viruses have evolved a means to circumvent this cellular control by promoting internal entry of ribosomes in a way that is completely independent from eIF4E [73–75]. Virtually all viral IRESes have the ability to bind a 43S ribosomal subunit which possesses all of the canonical cap-dependent initiation factors but eIF4E; such a complex does not exhibit affinity for the cellular cap structure but conserves all the other factors required for attachment, scanning and selection of the AUG codon [208]. This strategy is used mostly by polio- and rhino- viruses that have the ability to cleave the initiation factor eIF4G which results in the loss of the Nt domain of eIF4G associated to eIF4E from the 43S complex (see Fig. 6A). As a consequence, 43 ribosomal subunits that contain all initiation factors and the Ct domain of eIF4G can no longer be used by cellular cap-dependent mRNAs but have a greater affinity for the polio- and rhino- IRES sequences [209]. This results in a drastic inhibition of cellular translation and the enhancement of viral protein production. Interestingly, the cardiovirus EMCV targets a different key regulator of translation initiation by promoting dephosphorylation of 4E-BP which, in turn, precludes most of the eIF4E from cellular RNA–protein synthesis [95].

In the case of the HCV IRES and relative members, the ability to recruit a 43S subunit in the absence of any of the eIF4F factors provides a real selective advantage over cellular mRNAs that ensures efficient translation (see Fig. 6B) [122]. Finally, the case of the dicistroviruses is even more astonishing as they can produce proteins in the total absence of any initiation factors and even without a tRNA rendering translation possible under extreme stress conditions [166,167,171] (see Fig. 6C).

A glance at the structural diversity of IRESes shows a great conservation of RNA structures of the IRESes that bind directly to the ribosome: HCV, CrPV and relatives [116]. However, picornaviruses and Lentiviruses exhibit a greater diversity of size, sequence, and mechanism to contact and attract the ribosome. This raises the interesting question of can we—, and, how to—classify viral IRESes? Some workers have proposed to split viral IRESes into four groups [116], depending on primary sequence, secondary structure conservation, the location of the initiation codon relative to the IRES, and translational activity in different systems. These classifications could be completed by analysis of their requirement towards cellular initiation factors separating a first group comprising picornaviruses and Lentiviruses in which a central role has to be given to eIF4G which seems essential for their function, and a second group composed of the flavi- and IGR dicistroviruses IRESes that have the ability to recruit ribosomes with a limited set of initiation factors.

6.2. How to reconcile viral translation and replication?

One of the structural elements shared by the 4 classes of RNA viruses described herein is the complex RNA secondary structures that are present in their 5′ UTR. Many of these RNA structures are used for viral replication as it is the case of the Tar structure, the primary binding site or the dimerization loops in Lentiviruses (see Fig. 5) or the 5′ cloverleaf stem–loop found at the very 5′ of the poliovirus genome [64,182,210] (see Fig. 2). Structure and stability of these RNA elements were shown to interfere with scanning of the ribosome [185,211]. Thus, the presence of an IRES in the 5′ UTR of these viruses may be a way to reconcile translation and replication. Interestingly, it has been reported that translation and replication are mutually exclusive on the same poliovirus gRNA [64,212] and that the cloverleaf structure located upstream of the IRES element right in the beginning of the 5′ UTR plays a pivotal role [213]. This structure is able to interact with two cellular proteins: the poly(rC) binding protein 1 and 2 (PCBP1 and PCBP2), and the $3CD^{pro}$ viral protease. During early stages of infection, PCBP1 and PCBP2 interact with the stem–loop IV of the IRES where they serve as an ITAF to enhance IRES activity [214,215]. This results in the increase of the cellular concentration of the $3CD^{pro}$ viral protein which stimulates the binding of PCBP1 and 2 to the cloverleaf structure [216]; formation of such a complex between the viral $3CD^{pro}$, the cloverleaf and PCBP2 is responsible for the molecular switch from translation to replication [64,217]. As the ITAF activity of PCBP2 probably functions by promoting a circularization of the gRNA [218] through its dual interaction between PABP and the IRES [219], it is tempting to speculate that inhibition of translation may be caused by disruption of this 5′–3′ interaction. In agreement with this hypothesis, Toyoda et al. found that the spacing between the cloverleaf and the IRES was an important determinant in the transition from translation to replication [220]. The viral $3CD^{pro}$ protease is also able to cleave PTB, a protein known for its PV IRES ITAF activity. The cleavage products of PTB were shown to inhibit polioviral IRES-dependent translation and may contribute to the molecular switching from translation to replication [221,222].

In the case of members of the Lentivirus family, there is no conflict between translation and replication as both events take place in different cell compartments (nucleus for replication and cytoplasm for translation). However, the unspliced lentiviral genomic RNA is the template for protein synthesis and also serves as a support for genetic information once encapsidated in newly formed virions. Furthermore, there is no distinct subpopulations of unspliced viral RNAs devoted to distinct fates as it is the case in simple retroviruses [179]. Upon arrival of the gRNA in the cytoplasm, translation probably takes place by a cap-dependent mechanism as it is the most efficient way to rapidly build on the stock of viral proteins. Once a sufficient amount has been produced, notably the viral protease and the Gag polyprotein, this will impact the cellular environment in which the gRNA is translated. First of all, the viral protease will cleave the initiation factor eIF4GI [204,206] and PABP ([202] for a review see [56]) resulting in the inhibition of cellular protein synthesis. Secondly, production of the Gag polyprotein results in the preferential binding of the latter to its cognate 5′-UTR creating a scaffold of RNA–Gag complex which progressively occludes the accessibility of the 5′-UTR for ribosomes by steric hindrance [51,223]. At this stage, viral protein production is probably taking place from internal ribosomal entry from the IRES, ensuring robust and continuous synthesis during the initial steps of viral assembly.

7. Is it possible to define an IRES prototype?

The 130 viral and cellular IRES sequences described so far show quite a large divergence in terms of structure, length and function. In hindsight would it be possible to consider the IRES mechanism as a link between a prokaryotic mode of initiation and the classical cap-dependent eukaryotic one?

In prokaryotes, 30S ribosomes are brought to the messenger by mRNA–rRNA base pairing between the Shine–Dalgarno sequence and the 3′end of the 16S ribosomal RNA [224]. In eukaryotes such contacts between the 18S ribosomal RNA and the mRNA have been suggested in some case [225,226] but it is now clear that crude ribosomes cannot directly bind to the mRNA. Instead, RNA binding proteins and/or initiation factors can bridge the 40S ribosomal subunit to the mRNA either at the 5′ capped end or at different sites in the IRES sequence. More recently, it has been shown that a naturally A-rich cellular IRES was able to interact with PABP and eIF4G to contact the 40S subunit [227]. Interestingly, experiments from Hentze et al. have shown that a bicistronic mRNA in which the iron responsive element (IRE) RNA sequence could bind to a chimeric protein formed of the iron responsive element binding protein-1 (IRP-1) fused to eIF4GI was able to promote internal

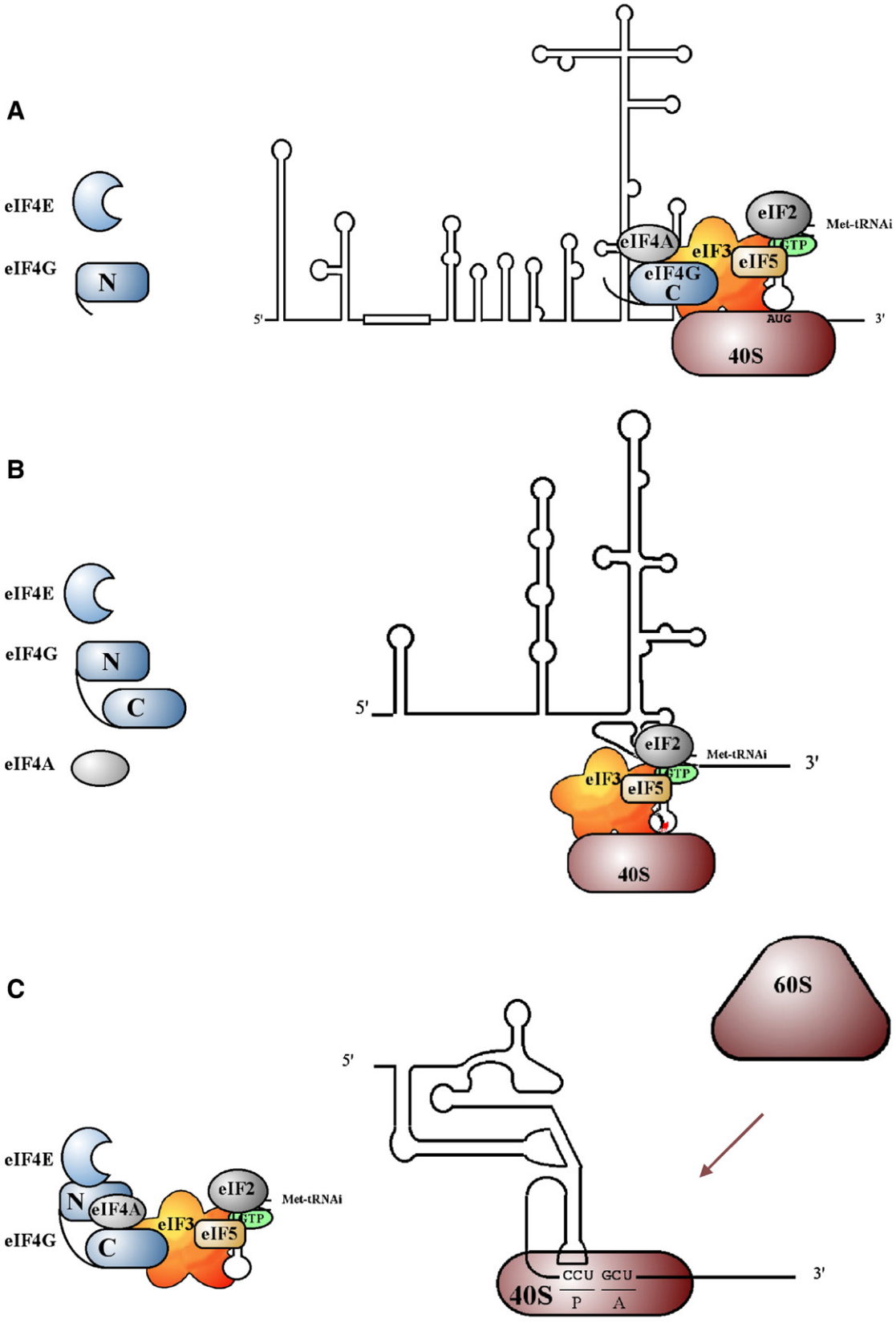

Fig. 6. Factor requirement of different viral IRESes. (A) Most viral IRESes including Picornaviruses and Lentiviruses. All canonical initiation factors are required with the exception of eIF4E and the Nt of eIF4G. (B) Flaviviral/pestiviruses IRES. Ribosome entry does not require eIFs 4F/4A/4B/1/1A, but need eIF5, eIF2 and eIF3. (C) Dicistrovirus IGR IRESes. This 200 nt long IRES is able to contact directly a 40S subunit and to assemble an 80S elongation competent ribosome without any of the initiation factors.

ribosomal entry from the intercistronic region and translation [228]. Although these experiments brought significant insights into the mechanistic of IRES-driven translation, it should be noted that translational efficiency was very low compared to viral standards such as EMCV or HCV [228] suggesting that additional factors are required to enhance ribosomal entry and/or scanning. Nevertheless, these results showed that the tethering of eIF4GI was both necessary and sufficient to promote the recruitment of a 43S ribosomal subunit onto a mRNA in an initiation competent manner.

The structural and functional diversity of the viral IRESes also raises the interesting question of their genetic origin. Our point of view would be that the broad diversity of size, structure and mechanism of ribosome recruitment would argue for a polyphyletic origin. Furthermore, different classes of IRESes can be distinguished but one cannot find the trace for an 'intermediate ancestor' that would be in favour of a linear evolution. One of the strategies generally used to draw a phylogenetic trend is to take a look at the most simple and primitive organisms to search for common features that could reflect early mechanisms. Thus, it is tempting to speculate that the primitive eukaryote *Giardia lamblia* which exhibits some unique features with most of its mRNAs being composed of extremely short 5′ UTR of about 14 nucleotides in length and its genome lacking some key eukaryotic initiation factors such as eIF4G, eIF4B, and eIF4H [229] could be a good primitive candidate. Interestingly, its trophozoites can be infected by an RNA virus: the Giardiavirus (GDV) [230] which drives translation with a 500 nucleotide-long IRES that extends both sides of the initiation codon and does not fall into any of the IRES families described above. Although the mechanisms by which ribosomes can be recruited onto this IRES remain to be determined, it clearly appears that multiple structured stem–loops domains and two reported pseudoknots are necessary for activity [231,232]. Such a peculiar architecture of an IRES is not found in any of the other viral families and would argue against a common origin.

Let us now have a closer look at the differences between IRESes from related viral families such as Flaviviridae and Picornaviridae. For the IRESes found in the pestivirus genus such as the classical swine fever virus (CSFV) or the bovine viral diarrhea virus (BVDV), they both are very similar to the HCV prototype in terms of sequences and structures [119]. For the picornaviral family, IRESes can be divided in different groups based on sequence similarity and structure conservation with entero-/rhino- and cardio-/aphtho-virus being clearly distinct although they all contain an oligopyrimidine tract at the 3′ end of the IRES body [211]. However, the porcine teschovirus-1 (PTV-1) which belongs to the picornavirus family exhibits a different RNA structure that is closer to an HCV-like shaped IRES [71]. Interestingly this structural resemblance also reflects functional properties as internal ribosomal entry on the PTV-1 can take place without any members of the eIF4F group by direct binding of the 43S to the IRES [71]. These data were confirmed and extended by the work of Hellen and de Breyne who have searched and found picornaviruses containing HCV-like IRESes [70]. On a functional point of view, they identify minor differences such as a moderate stimulation of translation by addition of eIF4F and eIF4B in the case of Simian picornavirus type 9 (SPV9) [233]. These results suggest an evolutional process that could be due to recombination events between distinct viruses belonging to the Flaviviridae and Picornaviridae families. This does not suggest the existence of a "missing link" between these two families, which does not favour a gradual linear evolution theory. It rather suggests that IRESes can be exchanged by recombination between different viruses, similar to the "exon shuffling" theory that was previously proposed to explain gene evolution [234].

8. Conclusion

Since their seminal discovery in 1989, IRESes have now been characterized in many viral families and cellular genes. Despite their differences, all viral IRESes can be defined as an entity which is functionally capable of recruiting ribosomes in a 5′ independent manner. This occurs by either promoting direct contact between the mRNA and the small subunit of the ribosome, as it is for the extreme cases of the Classical Swine Fever Virus (CSFV), Hepatitis C Virus (HCV) and Cricket Paralysis Virus (CrPV), or by establishment of an RNA–protein–ribosome interaction via a set of initiation factors and/or by the use of specific IRES trans-acting factors (ITAFs). The importance of internal initiation is now reflected by the fact that it is conserved across the animal kingdom and it allows translation to continue in various cellular stress conditions. However, despite the relatively large number of IRES sequences that have now been identified, a structural 'prototype' for internal initiation is still lacking suggesting that there is not one but several RNA motifs that enable ribosomal entry.

Viral IRESes have been largely exploited as molecular tools for gene expression as they have the ability to drive translation in stringent cellular conditions such as heat shock, cell cycle arrest, hypoxia [44,235]. Moreover, the unique ability of IRESes to express two genes from the same transcriptional unit makes it a valuable tool in gene replacement in mice to monitor the expression of the transgene when fusion proteins must be avoided, or to co-express a gene of interest with a selectable marker in gene therapies strategies [236].

Finally, it is also interesting to consider that the binding of the ribosome to an IRES element is a critical step of gene expression of many viral pathogens, thus making this RNA element as a promising target for new antiviral strategies. The proof of concept of such a strategy has been demonstrated with HCV as a model system [237,238]. Various RNA-based strategies are currently under development to control IRES-driven translation by the use of RNA aptamers [239], small interfering RNAs [240,241], ribozymes, DNAzymes [242] or morpholinos [243]. In parallel, approaches targeting the cellular proteins necessary for the function of viral IRESes (ITAFs) are also considered. Therefore, a better understanding of the structural basis of these RNA elements together with the mechanism by which they attract and bind ribosomes would certainly help to develop new therapeutical strategies against many pathogenic viruses.

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