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Tricks an IRES uses to enslave ribosomes

Sunnie R. Thompson

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294, USA, sunnie@uab.edu

Abstract

In eukaryotes, mRNAs are primarily translated through a cap-dependent mechanism whereby initiation factors recruit the 40S ribosomal subunit to a cap structure at the 5' end of the mRNA. However, some viral and cellular messages initiate protein synthesis without a cap. They use a structured RNA element termed an internal ribosome entry site (IRES) to recruit the 40S ribosomal subunit. IRESs were discovered over 20 years ago but only recently have studies using a model IRES from dicistroviruses expanded our understanding of how a three dimensional RNA structure can capture and manipulate the ribosome to initiate translation.

Keywords

IRES; *Dicistroviridae*; translation initiation; ribosome; IGR IRES

Mechanisms of translation initiation in eukaryotes

In eukaryotes the majority of mRNAs initiate protein synthesis through a cap-dependent mechanism that requires a free 5' end that has a modified nucleotide called a 5' cap-structure (m^7GpppG). The 5' cap is recognized by a cap-binding complex (composed of initiation factors eIF4E, eIF4G, and eIF4A) that recruits the 40S ribosomal subunit as a 43S pre-initiation complex (containing the 40S subunit, eIF3, eIF1, eIF1A, eIF5 and eIF2•GTP•Met-tRNA^{Met}). These factors function to bring the 40S ribosomal subunit to the 5' end of the mRNA and load the mRNA onto the 40S ribosome. Then, the 40S subunit scans the mRNA in a 5' to 3' direction until the AUG start codon is recognized, at which point GTP is hydrolyzed, eIF1 is released, and a conformational change in the 40S subunit occurs that clamps down on the mRNA to prevent further scanning. Lastly, the 60S subunit joins facilitated by eIF5B, and GTP hydrolysis triggers release of eIF1A and eIF5B to form a fully competent 80S ribosome (for a review see [1]). The ribosome has three tRNA binding sites, the peptidyl (P-site), aminoacyl (A-site) and exit (E-site). During initiation the AUG start codon is positioned in the P-site with the Met-tRNA^{Met}. The next aminoacyl tRNA (aa-tRNA) comes into the A-site of the ribosome. Following peptide bond formation, the P-site tRNA is translocated to the E-site prior to release from the ribosome. The function of the E-site has been controversial, but it appears to be important for maintaining the reading frame [2].

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Correspondence to: Sunnie R. Thompson.

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During viral infection or under certain cellular conditions, such as stress, cap-dependent translation is downregulated and an alternate cap-independent pathway is used to synthesize proteins. mRNAs using a cap-independent mechanism to initiate protein synthesis have an internal ribosome entry site (IRES) located in the 5' untranslated region (UTR) that can recruit ribosomes internally to the mRNA. IRESs were first discovered in viral RNAs [3, 4]. The fact that an IRES is able to recruit the ribosome internally and does not require a free 5' end was demonstrated by placing the IRES on a circular RNA that had all the stop codons removed. Ribosomes were recruited onto the RNA circle and translated around the entire circle multiple times [5]. Using this approach it was demonstrated that the circles were intact and the IRES recruited the ribosomes internally. Importantly, circular RNAs that had a mutant IRES were not translated demonstrating that only circles with a functional IRES could recruit ribosomes internally. IRESs cannot be identified bioinformatically [6], rather they must be identified using a functional assay that demonstrates that an RNA element can recruit a ribosome internally to the RNA [7]. Since IRESs are identified by a functional assay they can vary with respect to size, sequence, and secondary and tertiary structures.

Certain viruses use IRESs to translate their viral proteins. Our best understanding of how IRESs function comes from the study of viral IRESs, since they are more structured and only initiate translation through a cap-independent mechanism. In contrast, IRES-containing cellular mRNAs can often use both cap-dependent and cap-independent mechanisms to initiate protein synthesis [8, 9]. Viral IRESs can be grouped into different types that differ with respect to their secondary structure, the translation initiation factors they require, and whether or not they recruit the ribosome upstream of the AUG start codon or directly at it (Figure 1) [10-15]. Many viral IRESs require translation initiation factors (Figure 1; Types 1-3). Furthermore, the activity of many of these other viral IRESs are enhanced by IRES transacting factors (ITAFs), which are cellular proteins that bind to the IRES and modulate its structure to enhance IRES activity (for a review see [16]). The dicistrovirus intergenic region (IGR) IRES is exceptional in that it does not require any translation initiation factors. While a comprehensive list of all the different types of viral IRESs is not presented in Figure 1, it does demonstrate the variety of viral IRESs and relative complexity compared to the IGR IRESs (Figure 1, compare Type 4 with Types 1-3). The IGR IRES has been amenable to genetic, biochemical and structural studies due to its highly structured nature and simplicity of its mechanism of initiation since it can bind directly to 40S ribosomal subunits and form translationally competent 80S complexes in the absence of any other factors [17-22].

Disicetrovirus IGR IRESs as a model IRES

Dicistroviridae viruses are positive stranded RNA viruses that infect insects and crustaceans. The genome is naturally dicistronic, meaning they have two open reading frames (ORFs) on the same mRNA (Figure 2a). The ORFs are translated by two independent IRESs. The IGR IRES drives expression of the downstream ORF encoding the structural proteins in supramolar excess over the non-structural proteins, which are translated by the 5' IRES [23]. However, in some honeybee viruses, translation of the two ORFs may be coordinated resulting in a suppression of translation of the structural proteins until later in infection when translation of the first ORF is decreased [24]. Specifically, melting of a stem loop structure at the end of the upstream ORF by translating ribosomes reduces translation by the downstream IGR IRES. To subvert host ribosomes to translate the viral proteins the virus causes shut down of host protein synthesis by preventing the interaction between eIF4G and eIF4E [25].

Most viral and cellular IRESs have been shown to function only in mammalian translation extracts or cells. However, the IGR IRESs are also active in wild-type yeast, mammalian

tissue culture cells, and plant extracts [17, 26-29] with demonstrated activities of 15 to 950-fold over background depending on the IGR IRES [27]. Consistent with IRES activities increasing under different cell stresses when cap-dependent translation is downregulated, the IGR IRES demonstrates a 4 to 20-fold increase in activity when eukaryotic initiation factors are mutated or deleted in yeast [22]. The efficiency of IGR IRES translation is decidedly lower than other viral IRESs such as hepatitis C virus (HCV), poliovirus or encephalomyocarditis virus (EMCV), however it is sufficiently robust to support growth on plates [22]. The IGR IRES functions in so many different eukaryotic cell types because it requires only the highly conserved ribosomal subunits to initiate protein synthesis. In contrast, other viral IRESs rely on eukaryotic translation initiation factors or ITAFs, which are not as well conserved.

The diverse systems that the IGR IRES functions in has facilitated biochemical studies in purified systems that have been important for establishing steps involved in initiation by the IGR IRES. In addition, genetic studies in yeast have provided a tool that is not available for other viral IRESs [28, 30]. Perhaps one of the underappreciated characteristics of the IGR IRESs is that a two-nucleotide mutation in pseudoknot I (PKI; Figure 2b, see PKI mutant) can be made in the IGR IRES that completely eliminates IRES activity [17, 27]. This allows clear delineation within a system for what is true IRES activity and what may be background due to random initiation or cap-dependent artifacts [7]. The IGR IRES is less than 180 nucleotides and is highly structured (Figure 2b). This has enabled crystallization and cryo-electron microscopy (cryo-EM) studies that have revealed that the IGR IRES forms a compact core structure and binds in the intersubunit space between the ribosomal subunits [31-34]. An additional advantage of the IGR IRES is that it does not initiate translation with an AUG, therefore any background reporter expression (such as low level cryptic promoter activity) can be eliminated by removing any AUG start codons from the reporter [22, 27, 28]. Since the IGR IRES is amenable to biochemical, genetic, and structural studies work on this IRES has resulted in a fairly clear picture of how the IGR IRES initiates protein synthesis.

Structure of the *Dicistroviridae* IGR IRES and interactions with ribosomes

More than 15 dicistroviruses have been identified (for a phylogenetic comparison of *Dicistroviridae* see [35, 36]), and while the *Dicistroviridae* IGR IRESs have considerable sequence variation they form highly conserved secondary structures. The IGR IRES is a compact highly structured RNA, with three PKs (Figure 2b and 2d) [33, 34, 37]. The IGR IRES consists of two domains, the ribosome binding domain (Domains 1 and 2) and PKI (Domain 3), which docks into the P-site of the ribosome. These structures fall into two groups, Class I or Class II that exhibit minor variations in structure, with Class II IGR IRESs having an extra stem-loop in Domain 3 and a longer bulge region (Figure 2c) [38]. Interestingly, these domains can function independently and be interchanged between various IGR IRESs including being swapped between Class I and Class II IGR IRESs and still retain IRES activity [27, 39]. The activity of the chimeric IGR IRES tracks with the ribosome binding domain, suggesting that this domain is the major determinant for IRES activity [27, 39]. Interestingly, Class I and Class II IGR IRESs activities are comparable in yeast [27]. However, in human cells the Class II IGR IRESs are significantly more active than the Class I IGR IRES. This difference likely reflects a better fit between the IGR IRESs and the human ribosomes compared to the yeast ribosomes because the reduced IRES activity can be overcome in yeast at higher temperatures where ribosomes are predicted to be more flexible [27, 40].

The IGR IRES binds on the intersubunit surface of the 40S ribosome (Figure 3a), with the ribosome binding domain (Domains 1 and 2) in the E-site of the 40S subunit and PKI

(Domain 3) positioned into the P-site (Figure 2b and 2d) [31, 32]. For recent reviews of the IGR IRES structure and its interaction with the ribosome see [13, 41]. The crystal structure of the IGR IRES was shown to fit into the cryo-EM density of the IGR IRES bound to a 40S subunit with good agreement. This suggests that the IGR IRES is pre-formed and undergoes very little structural changes upon ribosome binding [32-34]. The three-dimensional structure of the ribosome binding domain revealed that stem loop 2.3 (SL2.3) is turned upwards and is positioned close to SL2.1 (Figure 2d), and both are predicted to interact with the E-site of the ribosome [31, 32]. There are two small subunit proteins, RPS25 and RPS5 that are located adjacent to one another in the E-site (Figure 3b). Both have been implicated in IGR IRES interactions with the E-site [28, 31, 42, 43]. The cryo-EM structural model suggests that RPS5 may interact with SL2.1, but confirmation awaits genetic, biochemical or higher resolution structural studies. One study suggested that the N-terminal extension of the yeast RPS5 affected IGR IRES activity [43], however, further experiments are required to establish a role for RPS5 in IGR IRES translation. RPS25 crosslinks to the IGR IRES [44] and is required for the IGR IRES binding to the 40S subunit [28, 42]. It is not known whether RPS25 contacts SL2.3, SL2.1 or both. In addition, deletion or knockdown of RPS25 results in a loss of IGR IRES activity with no significant decrease in cap-dependent translation or ribosome function [28]. RPS25 was also shown to be required for HCV IRES translation as well [28], suggesting that it may play a general role in IRES translation. Interestingly, domain IIb of the HCV IRES and SL2.3 of the CrPV IGR IRES both have a terminal loop structure with similar sequences. However, not all the IGR IRESs that have been shown to be RPS25 dependent share this exact sequence [27], suggesting that the important interaction between the IRES and RPS25 may be the RNA structure rather than the sequence. Studies on different IRES types are needed to determine if RPS25 is only required for IRESs that bind directly to the ribosome as the IGR and HCV IRESs do, or is also required for cellular and Type 1 and 2 viral IRESs. The bulge region does not appear to contribute to 40S binding affinity, but rather plays a role in 60S subunit joining or stability of the 80S ribosome [34, 45, 46]. In addition, mutations in PKI have no effect on the IGR IRES affinity for the 40S subunit [18, 47]. Rather, PKI is positioned into the P-site of the ribosome and establishes the reading frame for translation [17, 19, 24, 33]. Significantly, changes in the IGR IRES sequences that do not affect the IRES structure do not dramatically reduce the IGR IRES affinity for the 40S subunit [18]. This suggests that the IGR IRES makes a number of contacts with the 40S ribosome and the sum of these contacts contribute to the high affinity binding that is observed ($K_d = 5-15$ nM) [28, 30, 39, 45]. Nonetheless, mutations in the IGR IRES that impact events following 40S binding result in a dramatic reduction in IRES activity. For example, mutations in the bulge region result in a loss of IRES activity and a defect in 80S formation without affecting 40S binding [45].

IGR IRES mechanism of initiation

The IGR IRES has been instrumental in understanding IRES mechanisms because of its highly structured nature and its simplicity. The IGR IRES can bind directly to purified 40S subunits, form 80S complexes, and initiate translation in the absence of any initiation factors (Figure 4, left) [17-22]. The major challenge for the IGR IRES is how it assembles a complex ready for protein synthesis without the aid of the eukaryotic initiation factors. Figure 4 illustrates the steps for cap-dependent initiation and contrasts how the IGR IRES either maneuvers around or performs each step.

The IGR IRES binding to the 40S subunit induces a significant conformational change in the 40S subunit that is similar to the conformational change that 'opens' the mRNA channel upon eIF1 and eIF1A binding to the 40S ribosomal subunit (Figure 5) [31, 48]. Interestingly, the HCV IRES, which also binds directly to 40S subunits induces the same conformational change in the 40S subunit upon binding [49]. Deleting the region of the HCV IRES (domain

Iib) that contacts the E-site of the 40S subunit has little effect on the binding affinity but results in the 40S subunit remaining in the 'closed' conformation [49]. This suggests that HCV and the IGR IRESs can induce a conformational change in the 40S subunit to open the mRNA binding channel through interactions with the E-site. A 40S subunit without any factors bound to it is in a closed conformation (Figure 5, left), so it stands to reason that IRESs that bind directly to the 40S subunit in the absence of initiation factors would need to open the mRNA channel in order to load the RNA for translation (Figure 5b). Since binding of the IGR IRES to the 40S subunit can occur even when PKI is disrupted, assembly of a IGR IRES•40S complex appears to be a two-step process [47]. First, the ribosome binding domain binds to the E-site of the 40S ribosome and induces the conformational change to open the mRNA channel. Then, the second step can occur, that positions PKI (Domain 3) into the P-site of the ribosome.

Once the IGR IRES has bound the 40S subunit, the 60S subunit can join to form an 80S ribosome in the absence of eIF5B, which normally facilitates this joining step during cap-dependent translation [22] (Figure 4). In cap-dependent translation, the AUG start codon is positioned in the P-site where the initiator Met-tRNA_i^{Met} recognizes the start codon (Figure 4, right). However, PKI of the IGR IRES occupies the P-site, which explains the atypical initiation of the IGR IRES at a non-AUG codon [17]. PKI mimics the interaction between the initiator tRNA anticodon loop and the AUG start codon and establishes the reading frame for translation [19, 24, 33, 50]. Some IGR IRESs have the potential to extend the base-pairing in PKI by one base pair which results in a portion of the ribosomes translating in the +1 reading frame [24]. The IGR IRES initiates protein synthesis from the A-site where the elongation factor 1A (eEF1A) brings in an aminoacylated tRNA (aa-tRNA) to decode the first codon [17-20, 51-53]. Therefore, the first codon is translated without eIF2•GTP•Met-tRNA_i^{Met} (Figure 4) [17, 19, 22, 26, 51]. The IGR IRES is the only known IRES that does not require the initiator Met-tRNA_i^{Met} to initiate translation. The first codon translated primarily encodes alanine (GCU, GCA, GCC) with 12 out of 14 IGR IRESs using the GCU codon. Only rarely are glutamine (CAA) or glycine (GGC) used as the first codon [38]. [The structure of PpSRV (*Pteromalus puparum* small RNA virus) IGR IRES has not been described so the first translated codon has not been predicted]. Mutational analysis of the A-site codon has revealed that any codon other than a stop codon can be placed in the A-site and translation can occur [17, 54]. However, the activity of the IGR IRES is affected such that alanine codons result in higher levels of translation whereas glutamine and glycine codons yield less active IRESs [27, 54]. Interestingly, substitution of the glycine codon with the GCU alanine codon in the IAPV (Israeli acute paralysis virus) IGR IRES, which normally uses the glycine codon, resulted in a ten-fold increase in IRES activity. Consistently, swapping a GCU alanine codon with the glycine codon in the KBV (Kashmir bee virus) IGR IRES, which normally uses the alanine codon, resulted in a three-fold decrease in IRES activity [27]. These results suggest that IGR IRESs function most efficiently with a GCU codon in the A-site, which may explain the bias for the GCU codon [38].

Typically in translation initiation, prior to the first peptide bond forming, the P-site initiator Met-tRNA_i^{Met} is in the P/P position meaning that it is in the P-site for both the 40S and 60S subunits. The codon in the A-site is decoded by the elongation factor eEF1A, which brings in the aa-tRNA. As soon as the A-site tRNA is accommodated and peptide bond formation occurs, the deacylated tRNA spontaneously moves into the P/E hybrid state with the codon-anticodon end of the tRNA in the P-site of the 40S and the acceptor end of the tRNA in the E-site of the 60S. Normally, a deacylated tRNA is required in the P-site for eEF2 (eukaryotic elongation factor 2) to stimulate translocation (Figure 4, right). However, the IGR IRES is positioned on the ribosome in a P/E hybrid state upon binding, which is the natural state of a tRNA just prior to translocation [33, 50, 55]. This pre-positioning of the

IGR IRES into the P/E hybrid state may prime translocation such that as soon as the aa-tRNA is accommodated it rapidly moves into the A/P hybrid state ready for translocation [50, 55]. Once the aa-tRNA is in the A-site of the ribosome, eEF2 promotes translocation of the mRNA in the absence of peptide bond formation (termed pseudotranslocation) (Figure 4) [17, 19]. In the cryo-EM structure, PKI is at a lower resolution and is not fully in the P-site, which suggests it may be more dynamic and may not be fully docked until the aa-tRNA is delivered to the A-site [33]. In fact, binding of the aa-tRNA and eEF1A to the A-site is unstable and only becomes stabilized with eEF2, suggesting that the aa-tRNA binding is only stable after pseudotranslocation into the P-site [55]. The first codon that is translated by the IGR IRES resembles an elongation step rather than an initiation step of protein synthesis. In this way the IGR IRES manipulates the ribosome to mimic a scenario that is reminiscent of elongation rather than an initiation event.

Concluding remarks and future perspectives

Studies of the IGR IRES have revealed how an RNA structure can capture a 40S ribosomal subunit and bind with high affinity. The IGR IRES single-handedly (without any initiation factors) opens the ribosome's mRNA binding channel and loads the PKI domain into the P-site of the 40S [17, 19, 31, 50]. 60S subunits then readily join to form translationally active 80S complexes that are primed for elongation [19, 55]. In this manner the IGR IRES circumvents the entire initiation process by directly assembling an elongation complex. Because of the unique properties of the IGR IRES, it has proved to be an invaluable research tool for understanding other molecular mechanisms such as mechanisms of translational repression and ribosome function (Box 1). The IGR IRES as a model has allowed us to make great progress in understanding IRES structure, internal recruitment of a ribosome, and IRES-ribosome interactions, yet many important questions still remain.

The resolution of currently available structural and genetic studies is limited. To get the crystal structure of the IGR IRES ribosome binding domain, the loop regions were mutated to form tetraloops, in order to increase the stability of the folded RNA. Even so, the bulge region was disordered in the crystal structure, suggesting that it is dynamic. Unfortunately, it is precisely these loop and bulge regions that are the most conserved between the dicistroviral IGR IRESs, suggesting that they make key interactions with the ribosome or are essential for IRES function. Mutational analysis has revealed that small mutations in the bulge or loop regions that do not disrupt IRES structure can result in a loss of IRES activity [45-47, 56]. Additionally, the cryo-EM structure of the wild-type IGR IRES is at a 7.3 Å resolution [32], which is sufficient to gain a general understanding of how the IRES interacts with the ribosome and which proteins and ribosomal RNA regions are in proximity to the IRES, but is too low to identify specific interactions between specific nucleotides of the IRES and the ribosome. Now that the crystal structure of the eukaryotic ribosome has been solved [57] a high-resolution structure of the IGR IRES bound to 80S ribosomes should be obtainable.

In the genetic studies, most of the IGR IRES mutations are large and either delete or mutate an entire loop region or PK structure. It is notable that many of these mutations have had only a modest effect on ribosome affinity but often completely inactivate the IRES, suggesting that there is much more to IRES activity than recruitment of the ribosome [45]. Therefore, to really understand how the IGR IRESs are manipulating the ribosome to trigger opening of the mRNA channel, to initiate 60S joining, and to promote pseudotranslocation, a detailed mutagenesis of the conserved IGR IRES sequences is needed. In addition, more assays are needed to probe steps following ribosome binding, such as FRET analysis (fluorescence resonance energy transfer analysis) to detect the conformational change in the 40S subunit that occurs upon IRES binding. The crystal structure and the genetic data

together would define the key interactions between the IGR IRES and the ribosome. In particular, as ribosomes lacking RPS25 are unable to bind to the IGR IRES, contacts between the IGR IRES and RPS25 are likely to be important for ribosome affinity. The IGR IRES is a very approachable IRES to study in order to understand how an RNA structure (IGR IRES) can internally recruit ribosomes and initiate translation. The studies on the IGR IRESs are likely to impact our understanding of how other IRES types initiate translation. There is no doubt that some properties of the IGR IRES will be unique this IRES type, but the study of the IGR IRES may reveal how an RNA structure can manipulate the 40S ribosome to initiate protein synthesis and this may be shared amongst other IRES types. Twenty-two years after the discovery of IRESs and over a decade after the IGR IRES was first characterized we have begun to understand how an RNA structure can manipulate the ribosome to initiate proteins synthesis, a process normally requiring 10-13 initiation factors and an specialized cap-structure at the 5' end of a mRNA.

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Box 1**The IGR IRES as a research tool**

The unique properties of the IGR IRES have made it a powerful research tool for understanding other cellular processes. Below are just a few examples, by no means a complete list, of how the IGR IRES has been used as a tool to reveal molecular mechanisms in other systems.

Pseudouridylation of ribosomal RNA (rRNA)

rRNA has two types of modifications: pseudouridylation and methylation. These modifications are highly conserved between prokaryotes and eukaryotes, and essential as a whole. However, only when several modifications are missing at a time is there a significant phenotypic consequence for the cell; if a single modification is absent, little to no defect is observed. Therefore, the role of pseudouridylation in ribosome function remained unclear until 2006, when Yoon *et al.* demonstrated that cells from patients with defective pseudouridylation exhibited a specific decrease in IRES-mediated translation (both cellular and IGR IRESs) with no significant concomitant decrease in cap-dependent translation [58]. The IGR IRES was used to demonstrate that the decrease was a direct result of the loss of ribosomal pseudouridylation, because the IGR IRES uses only the ribosomal subunits to initiate translation. A later study confirmed this and showed that human and yeast ribosomes with reduced pseudouridylation have decreased binding affinity for the IGR IRES [30].

mRNA turnover and translational repression

When mRNAs are degraded in the cell they are translationally inactivated and decapped. Dhh1p (yeast) or RCK/p54 (human) translationally inactivates mRNAs, stimulating their turnover. The IGR IRES was used to show that RCK/p54 represses translation by acting directly on the mRNA, possibly by promoting assembly of a complex that targets the mRNA for degradation or inhibiting the 40S subunit [59]. Using an IGR IRES reporter Collier and Parker demonstrated that translational repression occurred when Dhh1p was added to the *in vitro* translation rabbit reticulocyte lysate (RRL). This effectively ruled out any possibility that Dhh1p was inhibiting translation through interactions with any of the initiation factors, as IGR IRES uses only the ribosomal subunits to initiate translation.

Inhibition of translation initiation by a 3' UTR element

There are numerous examples of elements within the 3' UTR that regulate translation, an event that involves initiation of protein synthesis at the other (5') end of the mRNA. A good example is observed in the reticulocyte 15-lipoxygenase (LOX) mRNA, which is translationally silenced in erythroid precursor cells until it is needed to mediate mitochondrial breakdown just prior to erythrocyte maturation. A differentiation control element (DICE) in the 3' UTR of LOX is bound by heterogeneous nuclear ribonucleoprotein (hnRNP) K and hnRNP E1, preventing initiation of translation at the 5' end of the mRNA. To determine which step in initiation is blocked in the translational repression, reporters were assayed containing the 3' DICE element paired with either a Type 2, 3 or 4 IRES (Figure 1). The results demonstrated that DICE, hnRNP K and hnRNP E1 were able to mediate repression of Type 2 and 3 IRESs, but not the Type 4 IGR IRES, which is translation factor independent [60]. Additionally, 48S (mRNA•40S) complexes were able to form, with the ribosome positioned at the AUG start codon, but 80S complexes did not assemble. Therefore, repression occurs after 40S recruitment but before 60S joining. More precisely, since the Type 4 IGR IRES was immune to the translational repression, this suggests that the repression is mediated through an initiation

factor that is involved in 60S joining since the IGR IRES can join 60S subunits in the absence of eIFs (eukaryotic initiation factors).

Mechanism of translational repression by microRNAs (miRNAs)

miRNAs are short (~21 nucleotide) RNAs that anneal to a mRNA through imperfectly complementary basepairing, resulting in translational repression and, potentially, mRNA degradation. The IGR IRES bypasses the normal steps of initiation and directly assembles an elongation complex. Thus, it was used to demonstrate that miRNA inhibition of translation occurred at a step following initiation, as the IGR IRES reporter was also repressed by miRNAs [61].

Figure 1.

Viral internal ribosome entry sites (IRESs) can be classified into different types. Here Types 1-4 are illustrated. The Type 1 and 2 IRESs such as the poliovirus (top) and encephalomyocarditis virus (EMCV) IRES (middle), respectively use the central domain of eIF4G and eIF4A to recruit the 43S pre-initiation complex (eIF1, eIF1A, eIF3, eIF2/GTP/Met-tRNA^{Met}) subunit upstream of the AUG start codon, which can be enhance by eIF4B [62, 63]. *In vitro* reconstitution of the Type I IRES initiation has not been performed but the assumption is that the factors are similar between the Type 1 and Type 2 IRESs. Following ribosome recruitment at a Type 1 IRES, the 40S subunit scans down the RNA in a 5' to 3' direction to the start codon to initiate protein synthesis. Type 1 IRESs do not function efficiently in rabbit reticulocyte *in vitro* translation lysate (RRL) unless it is supplemented with extracts from human cells that provide necessary ITAFs (IRES transacting factors).

However, Type 2 IRESs function efficiently in the RRL and can initiate at the site of ribosome binding. Type 3 IRESs represented by the hepatitis C virus (HCV) IRES can bind to 40S subunits in the absence of any initiation factors but require eIF3 and a factor (eIF2-GTP, eIF2D (ligatin), or eIF2A) to bring in the initiator Met-tRNA^{Met} [64-66]. They recruit the ribosome directly to the AUG start codon within the IRES. Type 4 IRESs such as the intergenic region (IGR) IRES do not require any initiation factors to initiate translation. They initiate translation from the A-site of the ribosome at a non-AUG codon and therefore, do not require initiator Met-tRNA^{Met}. The hepatitis A viral, Aichivirus-like, and lentiviral *gag* IRESs constitute unique IRES types (not depicted) [10, 11, 14, 15].

Figure 2.

The intergenic region (IGR) internal ribosome entry site (IRES). **(a)** A diagram of the *Dicistroviridae* RNA genome. Dicistroviruses are naturally dicistronic with two open reading frames that are translated by independent IRESs. The non-structural proteins are translated by the 5' IRES while the structural proteins are translated by the IGR IRES. A genome linked viral protein (Vpg) and the poly(A) tail (A_n) are indicated. **(b)** The secondary structure of the cricket paralysis virus IGR IRES showing the three domains (Domains 1-3) and three pseudoknot structures (PKI-III). Stem loop 2.1 (SL2.1) and stem loop 2.3 (SL2.3) contact the E-site of the 40S ribosome [28, 31, 32, 42]. The bulge region is indicated, as is the codon in PKI that occupies the P-site (P) of the ribosome. The first translated codon is positioned in the A-site (A) during initiation. A PKI mutation that eliminates IRES activity is indicated. **(c)** Line diagrams of the two classes of IGR IRESs: Class I and Class II. Both classes are structurally similar except that Class II IGR IRESs have an extended bulge region and an extra stem-loop in Domain 3. **(d)** The 3-dimensional structure of a Class I IGR

IRES generated using 2NOQ from the Protein Data Bank (PDB) using PyMOL [32]. The domains are color coded to match the domains in (a) and the regions of the IGR IRES are label for clarity. Domains 1 and 2 (ribosome binding domain) form a compact core with Domain 3 folding independent of the ribosome binding domain. SL2.3 is turned upwards and is in close proximity to SL2.1.

Figure 3.

The intergenic region (IGR) internal ribosome entry site (IRES) binding to the 40S ribosomal subunit. **(a)** The cryo-electron microscopy structure of the IGR IRES (purple) bound to the intersubunit surface of the 40S subunit (yellow). Reprinted by permission from Macmillan Publishers Ltd: *Nature Structural & Molecular Biology* [32], copyright (2006). The labels are as follows: body (b), helix 44 (h44), shoulder (sh), platform (pt), head (h), ribosomal protein S18 (rpS18), and pseudoknot I (PKI). The ribosome binding domain occupies the E-site where RPS25 and RPS5 reside and PKI is in the mRNA channel. **(b)** The crystal structure of the 40S ribosome subunit generated using PyMOL (PDB 3IZ6 and 3IZ7) [67]. RPS25 (red) and RPS5 (green) are located in the E-site where the IGR IRES contacts the 40S subunit. The P-site tRNA (blue) and mRNA (pink) are shown.

Figure 4.

A diagram of intergenic region (IGR) internal ribosome entry site (IRES) and cap-dependent mechanisms of translation initiation. A 40S ribosomal subunit that is not bound to any factors is in a 'closed' conformation (top center). During cap-dependent translation initiation (right), factors eIF1 (1) and eIF1A (1A) induce a conformational change in the 40S ribosomal subunit that 'opens' the mRNA binding channel. Opening of the channel is required for loading the mRNA and scanning to the start codon [48]. Then, the 40S subunit is recruited to the mRNA through interactions between eIF3 on the 43S pre-initiation complex and eIF4G of the cap-binding complex [68]. The IGR IRES (left) in contrast does not use eIF1 or eIF1A and induces the conformational change upon IGR IRES binding to the 40S subunit (see Figure 5). The IGR IRES positions pseudoknot I (PKI) into the P-site and

does not rely on scanning. PKI mimics the initiator tRNA codon anti-codon interaction and establishes the reading frame for translation in the A-site of the ribosome [17, 33]. The first codon translated is in the A-site of the ribosome. In contrast to cap-dependent translation, the IGR IRES does not require any initiation factors for IGR IRES•40S complex formation and 60S joining (left). During cap-dependent translation (right), following recognition of the AUG start codon, eIF5B-GTP is recruited to the pre-initiation complex through interactions with eIF1A. eIF5B accelerates the rate of 60S subunit joining to form an 80S complex. Then eIF5B hydrolysis of GTP triggers eIF5B and eIF1A release following subunit joining, which is required for elongation to proceed [69-71]. During IGR IRES initiation (left), subunit joining occurs spontaneously and does not require eIF5B [17, 22]. Presumably because the initiation factors eIF1, eIF1A and eIF3 are not bound to the IGR IRES ribosome complex so they do not need to be released by eIF5B in order for elongation to proceed. However, if these initiation factors are present on an in vitro assembled complex, then eIF5B stimulates 60S subunit joining on the IGR IRES [21]. Both cap-dependent and IGR IRES-mediated translation rely on elongation factor 1A (eEF1A) to bring in the first aa-tRNA to decode the codon in the A-site. For cap-dependent translation (right), as soon as the aa-tRNA is accommodated, peptide bond formation occurs and the tRNAs move into the P/E and A/P hybrid states (acceptor ends of the tRNAs move into the 60S E- and P-sites while the anti-codons remain in the P- and A-sites on the 40S). Then eEF2 promotes translocation (movement of the mRNA by one codon) bringing the tRNAs into the E/E and P/P sites with a vacant A-site for the next round of elongation (right). Translocation for the IGR IRES (left) is fairly similar except that initiation occurs without Met-tRNA_i^{Met} so there is no peptide bond formed following accommodation of the A-site tRNA. Therefore, translocation that occurs in the absence of peptide bond formation is termed pseudotranslocation (left).

Figure 5. Binding of the intergenic region (IGR) internal ribosome entry site (IRES) to the 40S ribosome induces an open conformation similar to that observed when the initiation factors eIF1 and eIF1A bind to the 40S subunit. The cryo-electron microscopy (cryo-EM) structures of the 40S subunits are viewed from the 60S interface side. **(a)** An APO 40S subunit from yeast has the latch for the mRNA binding channel in the 'closed' position (left). However, binding of the initiation factors eIF1 and eIF1A induces a conformational change in the 40S subunit that 'opens' the mRNA binding channel (right). Opening of the mRNA binding channel also corresponds to an additional contact (see *) between the shoulder and the head region on the solvent side of the 40S subunit that involves helix 16 and ribosomal protein S3 (RPS3). The figure was adapted with permissions from [48]. **(b)** A cryo-EM structure of a human APO 40S subunit in the closed conformation (left). A cryo-EM structure of the IGR IRES bound to the 40S subunit that has the IGR IRES density subtracted reveals a similar conformational change that resembles the 'open' conformation (right). Specifically, the latch

to the mRNA binding channel is open and the additional interaction between the head and shoulder regions is visible (see *), thus resembling the same conformational change induced by the initiation factors eIF1 and eIF1A. The landmarks for the 40S subunit are labeled as follows: n, neck; b, body; bk, beak; h, head; pt, platform; and sh, shoulder. The entry and exit channels for the mRNA are designated “mRNA entry” and “mRNA exit.” Five 18S rRNA helices (h16, h18, h30, h34, and h44) and ribosomal protein S5 (rpS5) are indicated. The figure was adapted with permissions from [31].