Takeover of host ribosomes by divergent IRES elements

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Abstract
The ribosome is the macromolecular machinery in the host cell for which all viruses have to compete. Early in infection, the viral mRNAs have to compete with the host for both the ribosomes and for the limited pool of eukaryotic initiation factors that are needed to facilitate the recruitment of ribosomes to both viral and cellular mRNAs. To circumvent this competition, certain viruses have evolved to recruit ribosomes to IRESs (internal ribosome entry sites), highly specialized RNA elements that are located at the 5′-end of the viral genomes. Here, we discuss how divergent IRES elements can recruit ribosomes and start protein synthesis with only a minimal set of eukaryotic translation initiation factors, and how this mode of translation initiation aids viral gene amplification during early onset of innate immune responses.

Introduction
The mechanisms of translation initiation in mammalian cells are still incompletely understood. There are at least two major mechanisms by which ribosomal subunits can reach the initiator AUG codon: cap-dependent initiation and internal initiation. Cap-dependent initiation is mediated by an interaction of the cap-binding protein complex eIF4F (eukaryotic initiation factor 4F), composed of factors eIF4E, eIF4A and eIF4G, with the m7GpppG cap structure present at the 5′-end of mRNAs. Subsequently, 40 S subunits are recruited to the 5′-end of the mRNA via interactions of the 40 S-associated factor eIF3 with eIF4G (reviewed in [1,2]). The 40 S subunit, carrying the initiator tRNA^Met–eIF2-GTP complex, is then thought to scan the mRNA in a 5′–3′ direction until an appropriate AUG codon is encountered. At the AUG start codon, the 40 and 60 S subunits join to form an 80 S ribosome with the AUG positioned in the ribosomal P-site [1,2]. Several studies have suggested that the initial binding of eIF4F to the mRNA is mediated by an interaction of the factor eIF4E with the cap structure itself. This step can be regulated by the phosphorylation status of 4E-BPs (eIF4E binding proteins; reviewed in [3]). Specifically, dephosphorylated 4E-BPs have a high affinity for eIF4E and compete with the binding of eIF4G to eIF4E that holds it in place in the eIF4F complex; thus, dephosphorylation of 4E-BPs results in a decreased rate of translation initiation. Conversely, pathways that lead to the phosphorylation of 4E-BPs, such as those induced by growth factors or mitogens, lead to the dissociation of 4E-BPs from eIF4E, assembly of eIF4F into eIF4F complexes, and increased rates of translation initiation (reviewed in [3]). The discovery that the polyadenosine-binding protein (PABP) and eIF4G interact with each other, and that this interaction has a stimulatory role in translation initiation, provides another mechanism to stimulate the recruitment of 40 S initiation complexes to the 5′-end of capped mRNAs [4–6].

Viruses that interfere with host translation have frequently been used to study translation mechanisms in mammalian cells (reviewed in [6]). For example, infection of mammalian cells with poliovirus, a cytoplasmic RNA virus, results in the selective inhibition of translation of host RNAs but not viral RNAs (reviewed in [7]). A model to explain the mechanism of selective inhibition of host protein biosynthesis was based on the finding that poliovirus mRNA contains a 5′-terminal pU residue instead of a cap structure. Consequently, the translation of poliovirus RNA must proceed by a cap-independent mechanism. Subsequently, it was shown that the cap-independent translation of all picornaviral mRNAs proceeds by a mechanism whereby ribosomes are recruited to the mRNAs by IRESs (internal ribosome entry sites) [8,9]. Subsequent studies have shown that IRES elements are highly structured and can recruit ribosomal 40 S subunits without the need for free 5′-ends in IRES-harbouring mRNAs [10]. Because internal initiation of translation could be performed by the cellular translational apparatus in uninfected cells [11], the question arose whether cellular mRNAs could be translated cap-independently as well. This question was somewhat heretical as it was in opposition to current dogma and the fact that all eukaryotic mRNAs appear to be capped. However, the low requirement of the intact cap-binding protein complex eIF4F for translation of certain capped mRNAs, notably late adenoviral mRNAs (reviewed in [12]) and heat-shock mRNAs (reviewed in [13]), has provided examples of cap-independent translation of capped mRNAs. Subsequently, our laboratory and others showed that certain cellular mRNAs contain IRES elements in their 5′-non-coding regions (reviewed in [10]). Therefore some capped mRNAs can

Key words: eukaryotic initiation factor 4E (eIF4E), eukaryotic initiation factor 4E-binding protein (4E-BP), hepatitis C virus (HCV), insect virus, internal ribosome entry site (IRES), translation.

Abbreviations used: eIF, eukaryotic initiation factor; 4E-BP, eIF4E-binding protein; CrPV, Cricket paralysis virus; cyroEM, cryoelectron microscopy; IRES, internal ribosome entry site; IGR, intergenic region; IRES, internal ribosome entry site; PK, pseudoknot; TSV, Taura syndrome virus.

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potentially be translated both by a 5′-cap-dependent scanning mechanism [14] and by an internal ribosome-binding mechanism like picornaviral RNAs (reviewed in [10]).

Assembly of translation-competent IRES–ribosome complexes

Until recently, it has been difficult to study the mechanisms by which ribosomal subunits are recruited onto mRNAs and by which the appropriate start site AUG codon is selected for the initiation of protein synthesis. However, the landmark discoveries of Pestova and Hellen [15,16], demonstrating that 40 and 80 S subunits can be assembled onto mRNAs using preparations that contain salt-washed ribosomal subunits and purified initiation factors, have made it possible to study distinct steps in cap-dependent and -independent translation initiation pathways. Specifically, toeprinting analysis on the HCV (hepatitis C virus) IRES was used to demonstrate that salt-washed 40 S subunits could be recruited directly to the HCV IRES without requiring known initiation factors. In contrast, neither the EMCV (encephalomyocarditis virus) IRES element nor capped β-globin mRNA could form specific binary complexes under such conditions. In the latter experiments, initiator tRNA\(^{\text{Met}}\)–eIF2-GTP, eIF3, eIF4A and eIF4B were all needed to recruit 43 S complexes on to the mRNA [15,16].

More recently, the domains in the HCV IRES that can assemble binary 40 S–IRES complexes with high affinity (\(K_i = 2 \text{nM}\)) have been elucidated [17–20]. Therefore the HCV IRES is functionally reminiscent of prokaryotic mRNAs, which can form binary complexes with 30 S ribosomal subunits by base-pairing between Shine–Dalgarno sequences and the 16 S rRNA, prior to engaging codon–anticodon interactions at the ribosomal P-site (reviewed in [21]).

Assembly and structure of divergent IRES–ribosome complexes

More recently, unusually divergent IRES elements have been discovered in the Dicistroviridae family of insect viruses [22–25]. For example, the CrPV (Cricket paralysis virus) contains a single positive-stranded RNA genome that encodes two non-overlapping reading frames, each initiated by an IRES [22]. Curiously, it was shown that the IGR (intergenic region) IRES elements located between the two cistrons in both CrPV and PSIV (Plautia stali intestine virus), another member of Dicistroviridae, did not require the initiator Met-tRNA, for translation initiation [22,23,26]. In the case of the CrPV IGR IRES, mutagenesis and toeprinting analyses of IRES–ribosome complexes revealed that the ribosomal P-site was occupied by a CCU triplet [23], which is base-paired with 5′-upstream sequences present in a conserved secondary-structure element (Figure 1). The ribosomal A-site was occupied by a GCU triplet that encodes alanine, the N-terminal amino acid of the structural precursor protein (Figure 1). Pairing between the sequences in the P-site and 5′-IRES sequences resulted in the formation of a PK (pseudoknot)-type structure whose integrity was essential for IRES activity, because mutations that disrupted the PK structure essentially abolished IGR IRES activity, whereas compensatory mutations rescued IRES activity [23,27].

Like the HCV IRES [23,27], the CrPV IRES can recruit 40 S ribosomal subunits in the absence of the initiator tRNA\(^{\text{Met}}\)–eIF2-GTP complexes (Figure 1) with high affinity (\(K_i = 24 \text{nM}\)). Unlike the HCV IRES, however, neither GTP hydrolysis nor initiator tRNA\(^{\text{Met}}\)–eIF2-GTP complexes were required for the assembly of 80 S ribosomes [23]. To examine whether binary IRES–40 S complexes can directly recruit 60 S subunits without the aid of additional canonical translation initiation factors, the assembly of IRES–ribosome complexes was monitored in a novel composite agarose–polyacrylamide gel system in which the kinetics of IRES–40 S and IRES–80 S complexes could be examined [28]. As shown in Figure 1, it was found that addition of purified 60 S subunits to binary 40 S–IRES complexes resulted in the formation of 80 S–IRES complexes, with the CCU triplet of the IRES located in the ribosomal P-site and the GCU triplet in the ribosomal A-site [28]. Similarly, preformed 80 S subunits were capable of being directly recruited by the IRES without the aid of additional canonical translation factors [29].

Peptide synthesis of assembled IGR IRES–80 S complexes in a reconstituted system

To determine whether the \(\text{in vitro-}\)assembled 80 S–CrPV IRES RNA complexes could support translation elongation, we monitored the translation products synthesized from mRNAs that contained the CrPV IGR IRES, or the closely related IGR IRES in TSV (Taura syndrome virus) [30,31], linked to a small open reading frame. Indeed, addition of radiolabelled total tRNAs and elongation factors EF1A and EF2 (Figure 1) resulted in the synthesis of radioactivity products of the predicted size of the open reading frame [28,32]. In contrast, the EMCV IRES, which requires canonical initiation factors to start translation initiation [33], did not direct the synthesis of small peptides [28,32]. Accumulated products were resistant to RNase A treatment, but sensitive to proteinase treatment, demonstrating that the radiolabelled bands represented proteins [28,32]. Furthermore, inclusion of cycloheximide in the translation reactions abolished accumulation of the peptides, strongly suggesting that the peptides were synthesized by elongating ribosomes [28,32]. In summary, these results show that the CrPV and TSV IGR IRES elements can start protein biosynthesis from the A-site of the ribosome without initiator tRNA and canonical initiation factors that are known to mediate cap-dependent translation.

Anatomy of IRES–ribosome complexes

Because cryoEM (cryoelectron microscopy) structures of high-affinity 40 S–HCV IRES complexes have been obtained [20], we wondered whether structural analyses of 40 S–CrPV IRES, or even 80 S–CrPV IRES, complexes were feasible.
In collaboration with Christian Spahn and Joachim Frank (Wadsworth Center, Albany, NY, U.S.A.), we obtained cryoEM structures of 40 S–IRES (20 Å; 1 Å = 10^{-10} m) and of 80 S–IRES (17 Å) complexes, assembled from purified 40 and 60 S subunits [34]. Comparison of 40 S–HCV IRES [20] and 40 S–CrPV IRES complexes revealed that the IRES elements occupied different sites on the ribosomal subunit. Specifically, the HCV IRES predominantly occupied the ribosomal E-site in the head of the subunit and extended into the solvent site [20]; in contrast, the CrPV IRES occupied the ribosomal E-site and protruded into the ribosomal P- and A-sites [34]. Although the IRESs occupied different parts of the ribosomes, they remarkably induced very similar conformational changes in the ribosomal subunit, resulting in the clamping of the IRESs into the mRNA-binding cleft of the 40 S subunit.

In 80 S–CrPV IRES complexes, the IRES retracts from the A-site into the P- and E-sites, generating space for the incoming A-site located elongator tRNA [34]. Furthermore, the ribosomal protein L1 and the stalk in the 60 S subunit became ordered in 80 S–IRES complexes [34], a phenomenon seen to be induced by elongation factor eEF2 in the yeast ribosome [35,36]. This structural change may prime the 80 S ribosome for the subsequent events of elongation. These
exciting observations suggest that the IGR IRES functions like an RNA-based translation initiation factor that triggers the associated ribosome into elongation mode.

Conclusions
Why are the IGR IRES elements of the Dicistroviridae so diverse from other IRES elements [10] that need many or most of the translation initiation factors to mediate translation initiation? Translational initiation without initiator tRNA from the A-site of the ribosome suggests the hypothesis that the IGR IRES elements should be active when initiator tRNA levels are low in infected cells. Initiator tRNA accumulates at low levels when phosphorylation of eIF2 ensues as a consequence of the activation of eIF2 kinases [2]. Thus phosphorylation of eIF2 is a hallmark of diminished activity of the translation machinery. Indeed, it was found that induction of eIF2 kinases GCN2 or PERK resulted in the overall inhibition of mRNA translation. In contrast, the IGR IRES elements should be active when initiator tRNA from the A-site of the ribosome suggests the hypothesis that initiation? Translational initiation without initiator tRNA most of the translation initiation factors to mediate translation diverge from other IRES elements [10] that need many or

Due to space constraints in this minireview, we apologize for not citing many relevant contributions of other investigators in the viral IRES field. Work in our laboratory was supported by grants from the NIH (National Institutes of Health).

References

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