Structural and mechanistic insights into hepatitis C viral translation initiation

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Abstract | Hepatitis C virus uses an internal ribosome entry site (IRES) to control viral protein synthesis by directly recruiting ribosomes to the translation-start site in the viral mRNA. Structural insights coupled with biochemical studies have revealed that the IRES substitutes for the activities of translation-initiation factors by binding and inducing conformational changes in the 40S ribosomal subunit. Direct interactions of the IRES with initiation factor eIF3 are also crucial for efficient translation initiation, providing clues to the role of eIF3 in protein synthesis.

Initiation of protein synthesis in eukaryotes requires the ordered assembly of ribosomal pre-initiation complexes, beginning with the association of the small (40S) ribosomal subunit with a messenger RNA (mRNA). Cap-dependent translation involves initiation-factor protein association with the 7-methyl guanosine moiety at the 5′ end of mRNA, leading to 40S ribosome binding and scanning to the initiation codon. This is followed by association with the 60S ribosomal subunit to form an active 80S ribosome (BOX 1). An alternative pathway, called internal translation initiation, is a cap-independent mechanism of recruiting, positioning and activating the eukaryotic protein-synthesis machinery driven by structured RNA sequences called internal ribosome entry sites (IRESs) that are located in the 5′-untranslated region (UTR) of mRNA. These sequences have been identified in numerous viral RNAs, and there is evidence that certain cellular mRNAs also contain IRES elements.

Structural and biochemical studies of the IRES found in hepatitis C virus (HCV) have provided the most detailed information so far for the mechanism of IRES-driven translation. These data offer exciting insights into the mechanism of protein-synthesis initiation during viral infection and indicate functional parallels with cap-dependent translation initiation in host cells. In this Review, we discuss recent advances in understanding HCV IRES structure, function and interactions with translation-initiation complexes, providing a foundation for developing therapeutics to prevent viral protein synthesis.

Architecture of the HCV IRES

The positive-strand RNA genome of the flavivirus HCV has been characterized in detail[1], leading to the initially surprising discovery of conserved sequences in the 5′ and 3′ UTRs of the viral mRNA that flank the single open reading frame[2]. The 5′ UTR has two independent functions that are necessary for the virus life cycle. The recognition of a short sequence by a microRNA that is highly expressed in the liver enhances replication[3,4] whereas the 5′ UTR also contains an IRES further downstream that allows protein synthesis to occur in a cap-independent manner. Following the identification of an IRES within the 5 UTR of the HCV mRNA[5,6], the predicted secondary structure of the IRES (FIG. 1) revealed structural and functional conservation with other members of the *Flaviviridae* family of viruses despite significant primary nucleotide sequence differences[7–10]. The HCV IRES has three main structural domains comprising ~340 nucleotides that adopt a tertiary fold under physiological salt conditions[11,12] (FIG. 1). Mutational analysis of the IRES domains showed that structural integrity is required for efficient protein synthesis both *in vitro* and *in vivo*[13–16]. The demonstration that many domain mutations resulting in reduced protein synthesis can be restored by compensatory mutations[17] showed that the IRES structure is required to initiate protein synthesis. Also, mutations in the loop regions of domains IIId and IIIe indicate that the function of the HCV IRES relies to some degree on the primary sequence[11,18]. Following these initial experiments, much attention focused on determining how the HCV IRES promotes correct positioning of the small 40S ribosomal subunit at the initiation codon of a viral or engineered mRNA.

Functional roles of the HCV IRES domains

The reconstitution of eukaryotic protein synthesis *in vitro* has been possible for many years using purified ribosomes and initiation factors[19,20]. A reconstituted system that is similar to those early studies was used to

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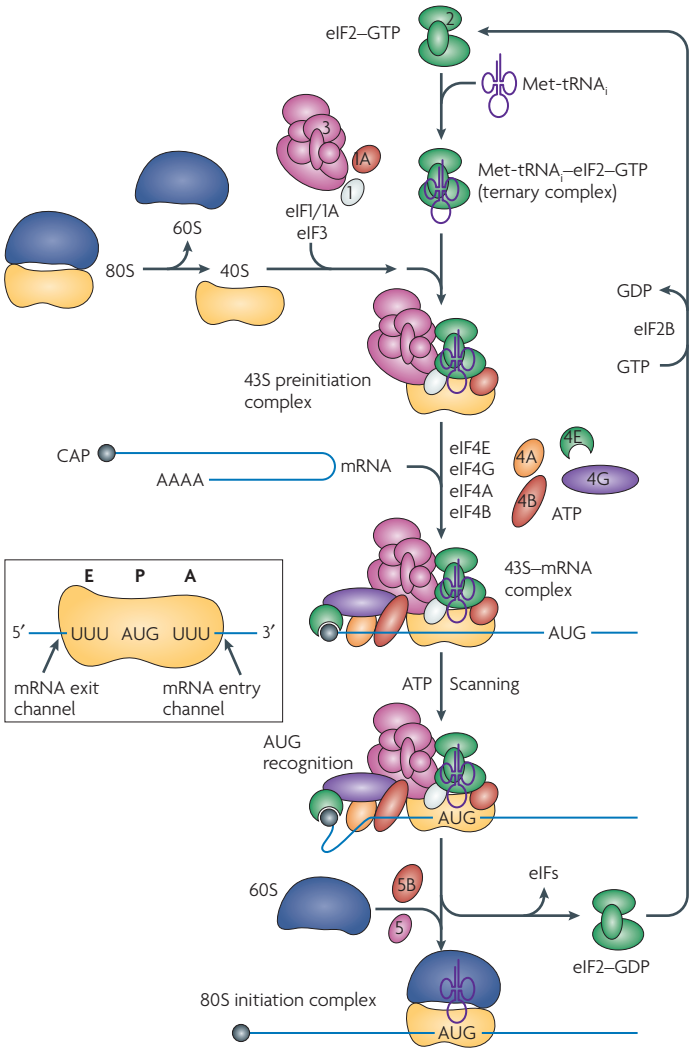
Box 1 | **Cap-dependent translation initiation**

In most cellular mRNAs, recognition of the start site for protein synthesis occurs by ribosome scanning, a proposed model of which is shown in the figure (reviewed in REFS 67,69,87–89). This mechanism requires all of the canonical initiation factors that have been characterized so far and, in contrast to the HCV IRES, also requires the 5′-cap structure found on all cellular mRNAs. Translation initiation begins on the 40S (small) ribosomal subunit, which is composed of an mRNA binding cleft, an aminoacyl (A) site, peptidyl (P) site, and exit (E) site. The mRNA enters the 40S ribosomal subunit through the mRNA entry channel, passes through the A, P and E sites and leaves through the mRNA exit channel. To initiate translation, a free pool of 40S ribosomal subunits, stabilized by association with the large multisubunit initiation factor eIF3, binds to Met-tRNA$_i$ and mRNA (reviewed in REFS 50,90,91). The Met-tRNA$_i$ is brought to the 40S ribosomal subunit as part of an eIF2–GTP complex, and together with two small initiation factors, eIF1 and eIF1A, forms the 43S preinitiation complex. Assembly of the 43S preinitiation complex on an mRNA by this pathway requires the 5′end of the mRNA, as ribosomes are unable to bind an mRNA that has been covalently circularized[92]. 43S preinitiation complex formation is enhanced by the presence of the cap-binding protein eIF4E, which in turn associates with the scaffold protein eIF4G. The interaction between eIF4G and eIF3 stabilizes the 43S preinitiation complex, leading to its migration (scanning) to the AUG start codon by the unwinding of any RNA structure present in the 5′-untranslated region (UTR). On pairing between the Met-tRNA$_i$ anticodon and the AUG start codon, eIF2 hydrolyses its GTP with the help of the GTPase activating protein eIF5. The eIF2–GDP complex dissociates from the 40S ribosomal complex and is recycled by a guanine nucleotide exchange factor, eIF2B, so that it can associate with a new Met-tRNA$_i$ and take part in another round of initiation. The exchange of GDP for GTP by eIF2B is highly regulated in eukaryotic cells, by a mechanism involving the phosphorylation of eIF2 by one of at least four distinct kinases, preventing the efficient recycling of this initiation factor[90,93,94]. Finally, a second GTPase, eIF5B, promotes the joining of the 60S ribosomal subunit to form an 80S initiation complex and the removal of the remaining initiation factors (reviewed in REFS 90,93).



see how different domains of the HCV IRES are involved in capturing the translation machinery. An elegant set of experiments determined that the HCV IRES, and the closely related classical swine fever virus (CSFV) IRES, can capture the small 40S ribosomal subunit in the absence of any known initiation factors that had previously been identified as requirements for a ribosome to bind to an mRNA[21]. This finding was important as at the time it was thought that only the prokaryotic 30S ribosomal subunit could recognize and bind directly to an mRNA through the Shine–Dalgarno sequence[22]. Furthermore, by the use of a toe-print assay to determine the position of the ribosome on the mRNA, the HCV IRES was found to recruit the small ribosomal subunit directly to the initiation codon, indicating that ribosome scanning to the initiation codon would not be required to initiate protein synthesis. Furthermore, the introduction of an initiation codon that is 7 nucleotides upstream, or 8 nucleotides downstream, of the correct initiation codon is not recognized by the ribosome, indicating that the structure of the HCV IRES is highly specialized in placing the correct initiation codon close to the 40S ribosomal peptidyl (P) site[23–25].

Further studies indicated that a large binding surface comprising two important junctions between domains IIIabc and domains IIIef, as well as domain IIId, are

Shine–Dalgarno sequence
(AGGAGG) This sequence is located 5′ of the AUG codon on bacterial mRNAs and functions as the signal for the initiation of protein synthesis.

Peptidyl (P) site
The site on the small ribosomal subunit that holds the tRNA molecule that is linked to the growing end of the polypeptide chain.

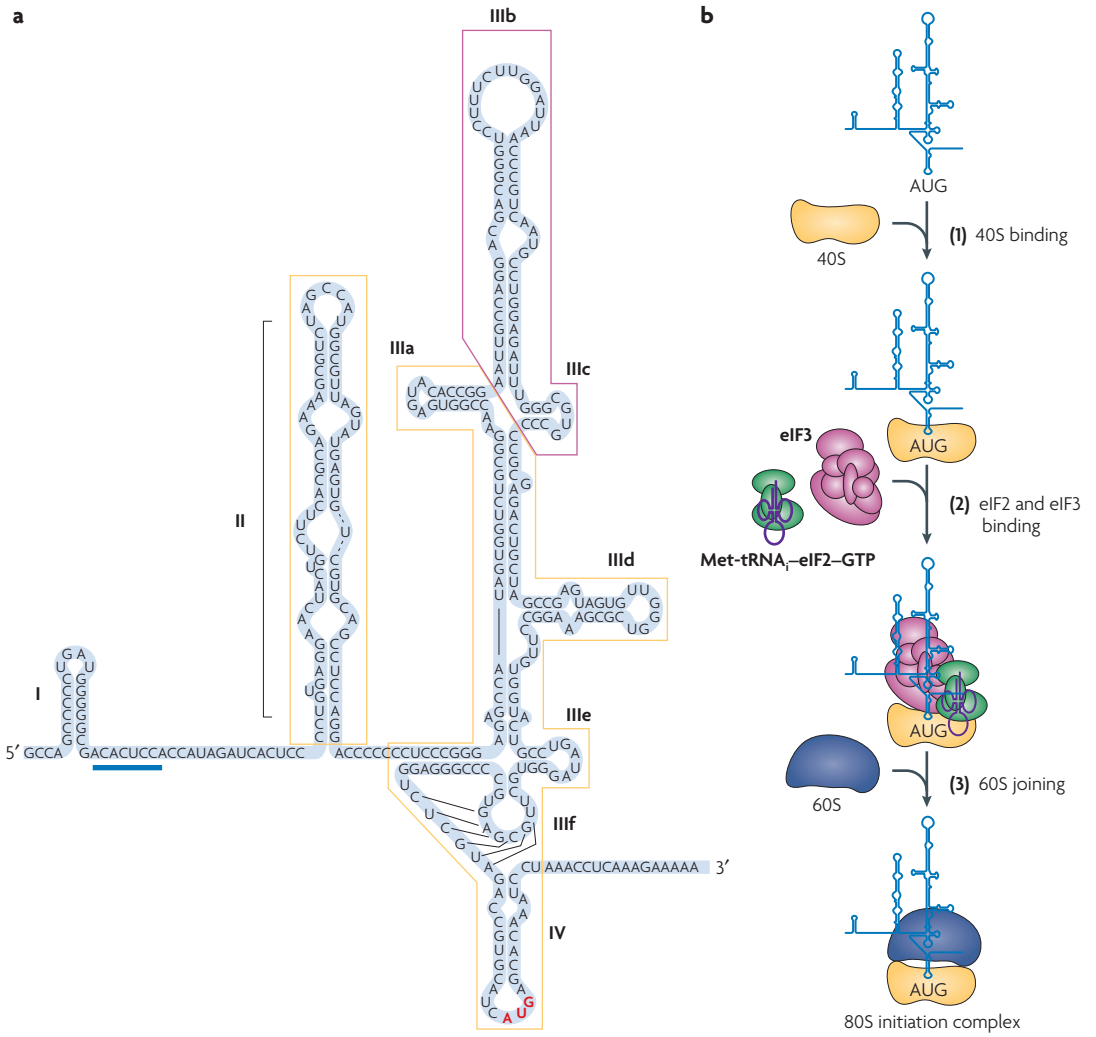


Figure 1 | **Secondary structure of the 5′ UTR of the HCV genome and model for the formation of the 80S initiation complex. a** | The secondary structure of the 5′ UTR of the HCV genome consists of four domains labelled I–IV. The HCV IRES includes three domains (II–IV), and the sub-domains (a–f) within domain III are also indicated. The domains that are important for 40S binding are boxed in yellow and the location of the binding site for the initiation factor eIF3 is boxed in magenta. The AUG codon is coloured red and the predicted microR-122 binding site is underlined in blue. **b** | Translation initiation on the HCV IRES begins with the recruitment of the 40S ribosomal subunit directly to the IRES in the absence of any initiation factors (1). Both eIF2–GTP (bound to Met-tRNA$_i$) and eIF3 are then recruited to the surface of the 40S ribosomal subunit (2), followed by a GTP hydrolysis step that promotes the joining of the 60S ribosomal subunit to form an 80S initiation complex (3). HCV, hepatitis C virus; IRES, internal ribosome entry site; UTR, untranslated region.

required for a high-affinity interaction[21,26–29] (FIG. 1). Although providing no measured additional affinity, domains II and IV also contact the 40S ribosomal subunit. The large binding surface that is required for stable interaction between the 40S ribosomal subunit and the HCV IRES contrasts with the relatively short (~2–10 nucleotides) Shine–Dalgarno sequence required for the interaction between the prokaryotic small ribosomal subunit and mRNA. Therefore, there is a significant difference in how the HCV IRES versus prokaryotic mRNA recruits the small ribosomal subunit.

As the 40S ribosomal subunit is directly recruited to the HCV IRES, it was important to determine which of the canonical initiation factors are necessary for efficient protein synthesis of the HCV mRNA. Given that no ribosome scanning is necessary, the factors needed for efficient scanning are not required for initiation codon recognition[21,30]. However, localization of the initiator methionyl-tRNA$_i$ (Met-tRNA$_i$) on the surface of the 40S ribosomal subunit by eIF2 is essential. Also, although not essential for correct placement of the initiator tRNA, the large multisubunit initiation factor eIF3 significantly enhances formation of the 40S ribosome initiation complex on the HCV IRES[21]. This enhancement is probably a result of the stabilizing effect of eIF3 on the Met-tRNA$_i$–eIF2 complex[31–33]. Furthermore, this stabilizing effect is essential for the formation of a translationally competent 80S ribosome on HCV mRNA[21]. The initiation factor eIF3

Sarcin–ricin loop
A highly conserved RNA loop in the rRNA from the large ribosomal subunit that forms a site for the binding of protein synthesis elongation factors. This association is inhibited by the ribotoxins α-sarcin and ricin.

Platform region
A large domain in the small ribosomal subunit above which is the mRNA and tRNA binding cleft.

has intrinsic RNA-binding activity, and therefore it was surprising to establish that eIF3 interacts with the HCV IRES in a specific manner[27,34,35]. The junction domain IIIabc, together with loops IIIa and IIIb, are essential for this interaction, which indicates that the junction domain IIIabc has a pivotal role in binding to both the 40S ribosomal subunit and eIF3. Therefore, domain III of the HCV IRES is essential for recruiting the 40S ribosomal subunit and eIF3 to the mRNA.

Early evidence indicated that domains II–IV of the HCV IRES are required for efficient translation *in vitro* and *in vivo*[14,15]. The high affinity of 40S ribosomal subunits for the HCV IRES structure is not the only requirement for efficient protein synthesis, as some IRES mutants that maintain efficient IRES–40S binary complex formation are nonetheless defective in translation initiation[11,14,7,36]. This indicates that the HCV IRES structure has additional roles beyond recruiting 40S ribosomal subunits.

Assays to measure intermediate thermodynamically stable complexes that are formed during the early events leading to 80S ribosome formation have indicated possible stages of initiation on HCV mRNA and the regions of the IRES that are required for each stage. The presence of the HCV IRES in distinct initiation complexes, identified using sucrose-density gradient centrifugation and RNA-based affinity purification, indicates that the HCV IRES might first bind to the 40S ribosomal subunit and then recruit eIF3 and eIF2–Met-tRNA$_i$[25,33,36] (FIG. 1b). Once the IRES associates with the 40S ribosomal subunit, domain IIIb regulates the recruitment of eIF3 and Met-tRNA$_i$–eIF2 (REF. 33). Although eIF3 and Met-tRNA$_i$–eIF2 can associate with an IRES–40S complex in the absence of the IIIb region, the stability of these initiation factors for such a complex is significantly reduced. This reduction in thermodynamic stability is reflected by the inefficiency of protein synthesis using an HCV IRES that lacks domain IIIb[36]. The IRES domains II, IIIa and the IIIabc junction influence the subsequent GTP-dependent formation of an 80S ribosomal complex[33,36]. Therefore, domains that contact and stabilize both the 40S ribosomal subunit and eIF3 are necessary for efficient 80S complex formation by the HCV IRES.

Whereas the likely dynamic flexibility of the entire HCV IRES complicates structure determination by X-ray crystallography and nuclear magnetic resonance (NMR), atomic-resolution structures have been determined for five individual domains and the four-way IIIabc junction of the IRES, providing details of how independent domains are arranged in the absence of their binding partners[37–42]. These studies reveal that the structures of the IRES domains fold independently and in some cases resemble RNA structures seen in ribosomal RNA. The domain IIIabc junction, which is required for 40S and eIF3 binding, forms two sets of stacked helices that resemble an RNA structure in the 50S ribosomal subunit[38], whereas domain IIId has an asymmetric E-loop motif, which is composed of two S-turns that have similarity to the sarcin–ricin loop of the ribosome[40]. Structure determination of domain II also identified an E-loop motif which is similar to that found in domain IIId[41], but differs in its orientation, resulting in distinct binding surfaces in these two domains that can be used for interaction with the 40S ribosomal subunit. Perhaps the similarities between the domain structures of the HCV IRES and those found in ribosomal RNA indicate conserved interaction surfaces that are used by RNA to recognize protein and RNA binding partners.

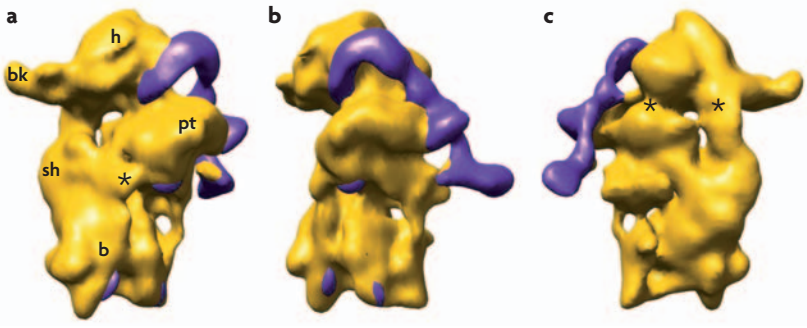


Figure 2 | **Cryo-electron microscopy (EM) reconstruction of the HCV IRES associated with the 40S ribosomal subunit.** The cryo-EM map corresponding to the association of the HCV IRES (purple) with the 40S ribosomal subunit (yellow) is shown in three different views at a resolution of ~20 Å (REF. 43). The views from the 60S (subunit interface) side (**a**), the platform side (**b**) and the solvent side (**c**) are shown. Most of the IRES, consisting of domain III, is positioned on the back of the platform region on the solvent side of the 40S ribosomal subunit. The HCV IRES also contacts the ribosome in a region of the head of the 40S ribosomal subunit. The contact made by domain II in this region partially occupies the exit site that is usually filled by a deacylated tRNA before it leaves the ribosome. Domain II also extends into the mRNA cleft close to the peptidyl site, in which Met-tRNA$_i$ is positioned to recognize the AUG start codon. Asterisks indicate conformational changes in the 40S ribosomal subunit induced by the association of the HCV IRES. Some landmarks of the 40S subunit are indicated as follows: b, body; bk, beak; h, head; pt, platform; sh, shoulder. HCV, hepatitis C virus; IRES, internal ribosome entry site. The figure is reproduced from REF. 43 © (2001) American Association for the Advancement of Science.

HCV IRES–40S ribosomal subunit interactions

The initial interaction between the HCV IRES and the 40S ribosomal subunit might occur in the absence of initiation factors[36]. Studies using single-particle cryo-electron microscopy (EM) provided the first look at the structure of the full-length HCV IRES and its interaction with the 40S ribosomal subunit during this important event[43]. In agreement with biochemical data, the reconstruction of the HCV IRES–40S ribosomal subunit complex at a resolution of ~20 Å showed that the IRES adopts a single conformation when bound to the 40S ribosomal subunit (FIG. 2). IRES–40S contacts occur mainly in the head and platform regions of the 40S subunit. Most of the IRES, consisting of domain III, is positioned on the back of the platform region on the solvent side of the 40S ribosomal subunit. As domain III is required for the high-affinity interaction with the 40S ribosomal subunit, it is of interest that this domain associates with the outer surface of the ribosomal platform. Interestingly, site-specific crosslinking has indicated that domain IIIe associates with ribosomal proteins rather than with ribosomal RNA[44], which agrees with earlier studies indicating that the interaction between the HCV IRES and the 40S ribosomal subunit

Exit (E) site
The site from which the deacylated tRNA molecule is ejected.

Difference mapping
This is used to determine conformational changes between closely related structures. The electron density map of one structure is subtracted from another structure revealing the difference in conformation between the structures.

is through contact with ribosomal proteins[45]. The lower degree of evolutionary conservation of the platform might explain why the HCV IRES cannot associate with plant ribosomes[21]. Another point of contact between the HCV IRES and the ribosome occurs in a region of the head of the 40S ribosomal subunit. Domain II contacts this region and as a result partially occupies the exit (E) site that is usually filled by a deacylated tRNA before it leaves the ribosome. Domain II also extends into the mRNA cleft close to the peptidyl (P) site where Met-tRNA$_i$ must be placed to recognize the AUG start codon. The structure of domain II observed by NMR agrees well with the density of domain II observed by cryo-EM, indicating that this domain is preformed in the absence of the 40S ribosomal subunit[41].

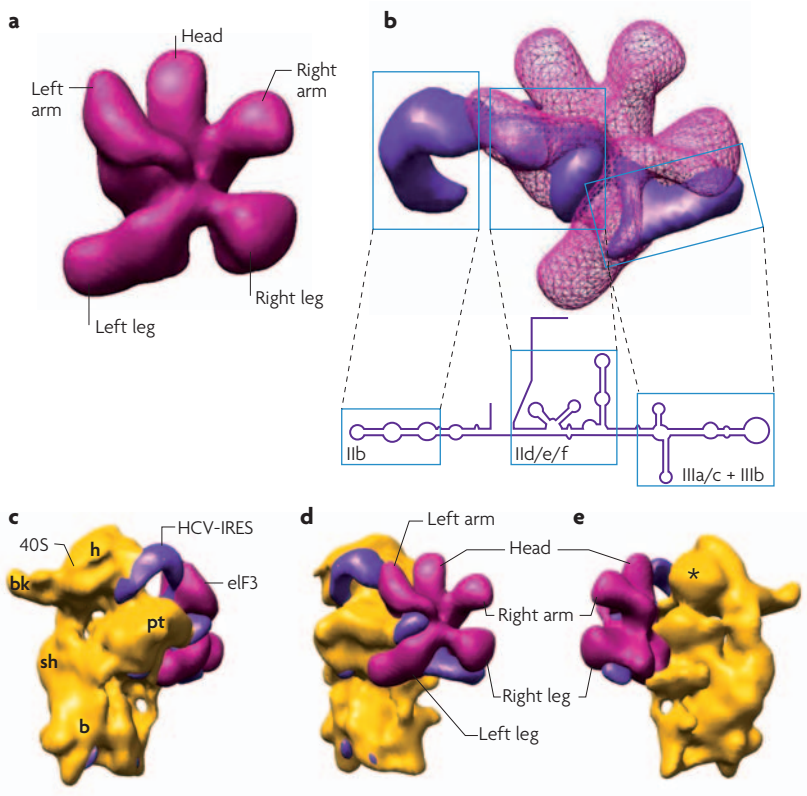


Figure 3 | **Cryo-electron microscopy (EM) reconstruction of the initiation factor eIF3, and a structural model of the IRES–40S–eIF3 complex. a** | The cryo-EM reconstruction of eIF3 at a resolution of ~30 Å is shown[48]. The domains of eIF3 are labelled according to body parts and range between 60 and 100 Å in length. **b** | The HCV IRES that is bound to eIF3 is shown, together with its corresponding secondary structure[48]. The secondary structure is arranged so that it matches the cryo-EM structure previously obtained[43], with domains II and III indicated. The IRES extends across eIF3 from the left arm to the right leg, apparently threaded through a cleft in eIF3. **c–e** | Shows three different views of a model of the ternary complex formed by the HCV IRES (purple), eIF3 (magenta) and the 40S ribosomal subunit (yellow), proposed on the basis of cryo-EM reconstructions of sub-complexes[43]. The views from the 60S (subunit interface) side (**c**), the platform side (**d**) and the solvent side (**e**) are shown. The location of the receptor for activated C-kinase (RACK1) is indicated by an asterisk — protein kinase C associates with RACK1 *in vitro* and *in vivo* and stimulates the formation of the 80S ribosome. Some landmarks of the 40S subunit are indicated as follows: b, body; bk, beak; h, head; pt, platform; sh, shoulder. HCV, hepatitis C virus; IRES, internal ribosome entry site. The figure is reproduced with permission from REF. 48 © (2005) American Association for the Advancement of Science.

A significant conformational change in the 40S ribosomal subunit occurs on association with the HCV IRES[43]. A rotation of the head relative to the body takes place that results in the movement of a latch structure that in turn opens the mRNA entry channel of the 40S ribosomal subunit. Once the mRNA is bound, this latch is thought to clamp around the incoming strand of mRNA to stabilize the mRNA on the 40S ribosomal subunit[46,47]. Interestingly, domain II is responsible for inducing this conformational change as no rotation of the head of the 40S ribosomal subunit is seen on association with an IRES construct that lacks domain II. It has yet to be determined whether this conformational change that is induced by the HCV IRES mimics that induced by canonical initiation factors on binding to 40S subunits on capped mRNAs. At present, it is not clear at which stage during protein-synthesis initiation the mRNA entry channel must close. It is not known if the entry channel must remain open during the scanning process on 5′-capped mRNAs or if it closes once the mRNA has associated with a 40S ribosomal subunit before cap-dependent scanning can take place. One possibility is that the channel only closes on AUG codon recognition.

Nonetheless, the combination of structural and biochemical data available so far provides a better understanding of how 40S subunit positioning at the initiation codon takes place in two distinct stages. One stage is determined by the high-affinity association of the 40S ribosomal subunit with the HCV IRES, whereas the second stage depends on the conformational changes that must occur to open the mRNA entry channel.

Interactions of the HCV IRES with eIF3

It has been proposed that initiation factors required for efficient protein synthesis associate with the ribosome following binding of the HCV IRES to the 40S ribosomal subunit[36]. The multisubunit initiation factor eIF3 has a central role in structurally organizing the surface of the 40S ribosomal subunit. Despite its importance in the initiation of both cellular and viral protein synthesis, the structural basis for its many functions has only recently begun to be elucidated. Single particle cryo-EM provided the first look at the structure of eIF3 and its interaction with the HCV IRES[48]. The reconstruction of eIF3 at ~30 Å revealed an architecture that consists of five distinct domains that are named according to body parts, including arms, legs and a head (FIG. 3a). Using cryo-EM and difference mapping, the binding site of the HCV IRES on eIF3 in the absence of the 40S ribosomal subunit was determined. Interestingly, the HCV IRES binds across the entire length of eIF3 with an intricate threading of the RNA into the initiation factor complex (FIG. 3b). The extended binding interface corresponds well with previous crosslinking data that identified numerous subunits of eIF3 that are close to the RNA[34]. Domain II of the HCV IRES protrudes from the left arm of eIF3, whereas domain IIIdef is located in between the left arm and left leg of eIF3. The stability of eIF3 on the IRES–40S complex requires the junction domain IIIabc

Modification interference
An RNA sequence is chemically modified so that a proportion of molecules cannot function correctly in a given assay. The RNA that cannot function is recovered and the site of modification is determined.

Footprinting technique
A technique that determines the site of a nucleic acid–protein interaction using the fact that a protein bound to a nucleic-acid region will protect it from enzymatic cleavage.

Translocation
The movement of an mRNA across the ribosome by one codon together with the movement of tRNAs between the aminoacyl, peptidyl and exit sites, catalysed by elongation factors and GTP hydrolysis.

and domain IIIb[33,36], which reside close to the right leg of eIF3. Identification of such an extended interaction surface on eIF3 indicates that modification interference and footprinting techniques, which identified domain IIIabc as the primary site of eIF3 binding, might have under-represented the weaker and potentially more dynamic interacting regions across the surface of eIF3 (REFS 11,34).

Structural model of the IRES–40S–eIF3 complex

The discovery that the structure of the HCV IRES, when associated with eIF3, is similar to that when it is associated with the 40S ribosomal subunit, allowed the superposition of the structure of eIF3 associated with the HCV IRES with a previous reconstruction of the IRES–40S complex[48] (FIG. 3c–e). The resulting model places eIF3 on the solvent-exposed platform region of the 40S ribosomal subunit, in agreement with a previous low-resolution reconstruction of the 40S–eIF3 complex[49]. The relatively flat face of eIF3 contacts the 40S ribosomal subunit with the left arm extended towards the tRNA E site and the left leg localized just below the platform region.

The position of the left leg of eIF3 in this model provides a possible explanation for its role in preventing the premature association of the large 60S ribosomal subunit (reviewed in REF. 50). Two crucial intersubunit bridging contacts are made between the two ribosomal subunits in bacteria and eukaryotic ribosomes. One of these bridge contacts (B4) involves binding of the 40S ribosomal protein rpS13 to helix 34 of the 60S ribosomal subunit, and another bridge contact (B2a) involves an interaction between helix 44 of the 40S ribosomal subunit and helix 69 of the 60S ribosomal subunit[51–53]. The left leg of eIF3 seems to bind over rpS13, probably preventing the B4 contact. Furthermore, it is possible that eIF3 stabilizes other initiation factors that occlude the B2a contact[54], therefore preventing the premature association of the 60S ribosomal subunit.

HCV IRES interactions with the 80S ribosome

The association of the 60S ribosomal subunit to form the 80S ribosome during the final stage of protein-synthesis initiation on the HCV mRNA has been investigated using cryo-EM[55]. This stage of initiation is dependent on GTP hydrolysis and requires domains II, IIIa and the IIIabc junction structure of the IRES to occur efficiently[33,36]. Human 80S ribosomes were assembled on the HCV IRES by using an *in vitro* translation extract and stalled prior to the elongation phase of protein synthesis using the drug cycloheximide. Affinity purification of the resulting 80S complex allowed structural analysis by cryo-EM to a resolution of ~20 Å (REF. 55; FIG. 4). At this resolution the structure of the HCV IRES is essentially the same for both the IRES–40S and IRES–80S complexes. However, comparisons of the mRNA-entry channel conformation in empty 40S ribosomal subunits with those observed in IRES–40S and IRES–80S complexes reveal significant differences. The mRNA-entry channel conformation is determined in part by the movement of the ribosome head relative to the body. The head has been identified as a rather mobile domain of the small ribosome subunit in both prokaryotes[56,57] and eukaryotes[53]. It is likely that head-domain movement relative to the body of the small ribosomal subunit is required for the efficient translocation of the peptidyl-tRNA and mRNA in the ribosome during protein synthesis. During the initiation of protein synthesis driven by the HCV IRES, a change in the orientation of the head relative to the body occurs in the 40S subunit, resulting in the opening of the mRNA entry channel on binding of the HCV IRES[53]. The orientation of the head changes again to close the mRNA entry channel on association of the 60S ribosomal subunit to form the 80S ribosome[55]. However, in both the 40S–IRES and the 80S–IRES structures the mRNA exit channel remains closed, perhaps as a result of domain II of the HCV IRES interacting with protein S5 in the head of the 40S ribosomal subunit[55]. Interestingly, the 80S–IRES structure indicates that the HCV IRES contacts the 60S ribosomal subunit only at the L1 protuberance, a dynamic domain that protrudes from the ribosome surface (FIG. 4e). Studies of prokaryotic ribosomes indicate that the L1 stalk removes the deacylated tRNA bound in the tRNA E site of the ribosome during elongation[58]. It is possible that the L1 stalk also aids the removal of domain II of the HCV IRES on the first translocation step.

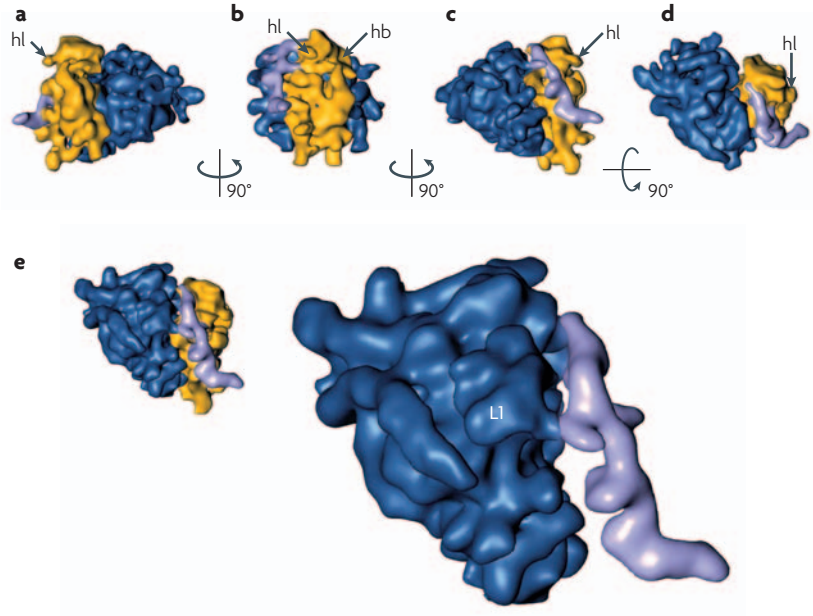


Figure 4 | **Cryo-electron microscopy (EM) reconstruction of the 80S–HCV IRES complex. a–d** | The cryo-EM reconstruction of the HCV IRES associated with an 80S ribosome at ~25 Å resolution is shown in four views[55]. The 40S ribosomal subunit is shown in yellow, the 60S ribosomal subunit in dark blue and the HCV IRES in purple. The HCV IRES interacts primarily with the 40S subunit. Arrows indicate the major structural differences observed between the IRES–80S complex and the 80S ribosome in the absence of the IRES. These are located in the head lobe (hl) and the head beak (hb) of the 40S ribosomal subunit. **e** | The only contact of the HCV IRES with the 60S ribosomal subunit is located at the L1 protuberance (L1). HCV, hepatitis C virus; IRES, internal ribosome entry site. The figure is modified with permission from REF. 55 © (2005) Cell Press.

Other viral IRES elements

Viral IRES elements can be classified into three groups that are structurally and functionally distinct from each other (FIG. 5). Picornaviruses (for example, encephalomyocarditis virus (EMCV)) use nearly all of the known initiation factors, including eIF4G and eIF4A; the *Flaviviridae* family of viruses (for example, HCV) directly associate with the 40S ribosomal subunit and do not need the initiation factors required for scanning, whereas the *Dicistroviridae* family (for example, cricket paralysis virus (CrPV)) also associate directly with the 40S ribosomal subunit and do not require any initiation factors for efficient protein synthesis. Interestingly, recent findings have identified several animal and avian picornaviruses that contain an IRES with a similar structure to that of the HCV IRES (reviewed in REF. 59). These IRES elements, similar to that of HCV, require only eIF2 and eIF3 to initiate protein synthesis and it is likely that the movement of IRES domains between virus families occurred through non-homologous recombination during co-infection. Recent work has identified an inhibitor that has been used to help distinguish between HCV-type IRES elements and those of the picornavirus. This inhibitor prevents the function of eIF4A, a DEX/HD helicase that is required for protein synthesis directed from picornavirus IRESs but not from HCV-type IRESs[60].

The CrPV IRES. The CrPV and Taura syndrome virus (TSV) of the *Dicistroviridae* family each contain an IRES that, similar to the HCV IRES, associates directly with 40S ribosomal subunits[61,62]. However, whereas efficient translation initiation of the HCV mRNA requires the recruitment of Met-tRNA$_i$ to the initiation (AUG) codon in the P site of the 40S ribosomal subunit, these IRES elements initiate protein synthesis by a mechanism that is independent of positioning Met-tRNA$_i$ in the P site, instead initiating from the aminoacyl (A) site of the 40S ribosomal subunit at a non-AUG codon. The well-characterized ~180 nucleotide CrPV IRES has a predicted secondary structure that is distinct from the HCV IRES and functions by occupying regions of the A, P and E sites of the 40S ribosomal subunit. This allows a 60S ribosomal subunit to associate, forming an 80S ribosome in the absence of any initiation factors. Once formed, the 80S ribosome and the associated CrPV IRES can initiate protein synthesis at the GCU codon placed in the ribosomal A site. The association of the CrPV IRES with the 40S ribosomal subunit and the formation of the 80S ribosome have been studied using cryo-electron microscopy[63]. In agreement with biochemical data, the CrPV IRES was found to form a tertiary fold that associates with regions of the A, P and E sites, which contrasts with the HCV IRES that only associates with the E site. However, a similar 40S conformational change is seen in both the HCV IRES–40S complex and the CrPV IRES–40S complex. The mRNA entry channel is opened in both cases, perhaps allowing the 3′ region of the mRNA to be inserted into the mRNA entry channel. It is possible that these changes occur as part of the general mechanism of initiation by eukaryotic ribosomes and might be regulated by initiation factors on 5′-capped mRNAs.

Aminoacyl (A) site
The site on the small ribosomal subunit that holds the incoming tRNA molecule that is charged with an amino acid.

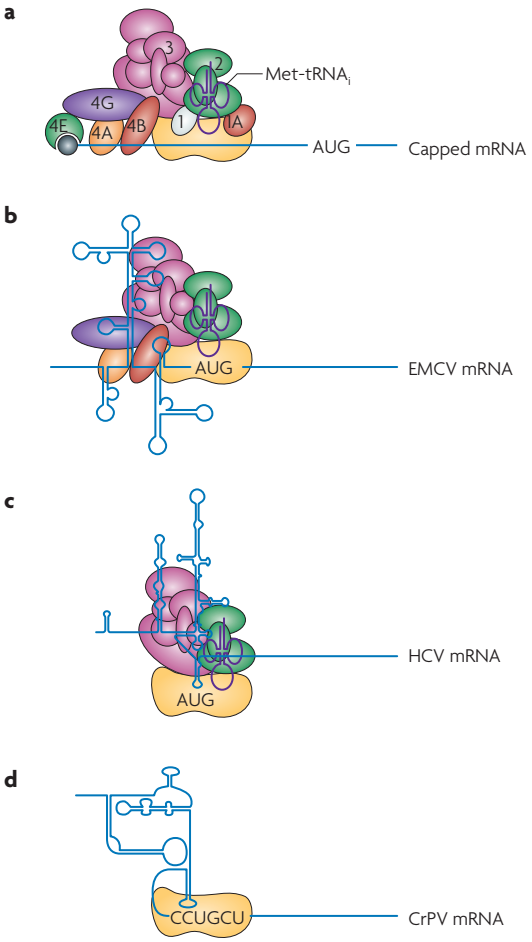


Figure 5 | **Capped mRNA and viral mRNA ribosome recruitment strategies.** Comparison of canonical eukaryotic cap-dependent translation initiation (**a**) and the internal initiation mechanism used by the picornavirus encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) (**b**), hepatitis C virus (HCV) IRES (**c**) and the cricket paralysis virus (CrPV) IRES (**d**). **a** | Cap-dependent translation requires recognition of a modified nucleotide at the 5′ terminus of the mRNA by the eIF4F complex (made up of eIF4E, eIF4A and eIF4G). The 43S particle (40S subunit–eIF3–Met-tRNA$_i$–eIF2–GTP–eIF1–eIF1A) together with eIF4B binds to the mRNA–eIF4F complex primarily through interactions between eIF3 and eIF4G. Once bound to the mRNA the 43S particle can migrate to the AUG codon. **b** | The EMCV mRNA bypasses the requirement for the cap-binding protein eIF4E, instead recruiting the 43S particle through direct interaction between the IRES and eIF4G. As no scanning occurs, both eIF1 and eIF1A are not required and eIF4B only increases the efficiency of the EMCV IRES. **c** | Translation of the flavivirus HCV mRNA begins with direct, specific recognition of the 40S subunit and eIF3 by the IRES RNA tertiary structure, eliminating the need for the cap-structure and cap-binding factors. In this case, the AUG codon is placed directly into the peptidyl (P) site of the 40S ribosomal subunit. **d** | The CrPV mRNA, like the HCV IRES, associates directly with the 40S ribosomal subunit but without the requirement for any initiation factors. This mRNA uses a GCU codon to initiate protein synthesis, which is placed directly in the aminoacyl site of the 40S ribosomal subunit.

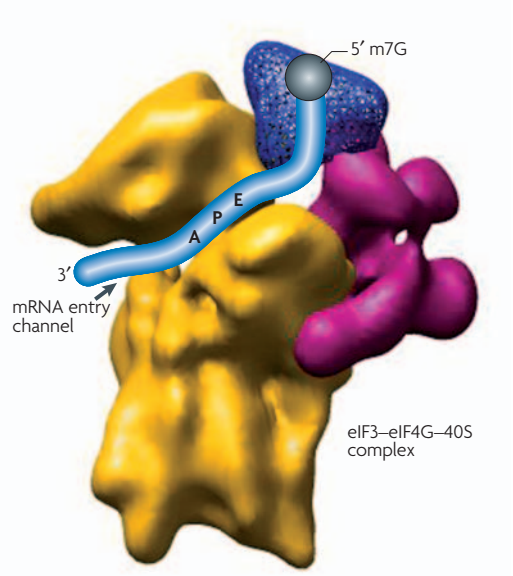


Figure 6 | **Model of cap-dependent mRNA loading onto the 40S ribosomal subunit.** Cryo-electron microscopy reconstructions of 40S–HCV IRES, eIF3–HCV IRES and eIF3–eIF4G complexes were used to propose the position of the cap-binding complex on the 40S ribosomal subunit[48]. In the resulting model for mRNA association with the 40S ribosomal subunit, the 5′ end of the mRNA (blue line) is located close to the eIF4G density (shown in purple), whereas the mRNA extends through the exit (E), peptidyl (P) and aminoacyl (A) sites, with the 3′ end of the mRNA extending past the mRNA entry channel as indicated. HCV, hepatitis C virus; IRES, internal ribosome entry site; 5′m7G, 5′-methylguanosine. The figure is modified with permission from REF. 48 © (2005) American Association for the Advancement of Science.

The picornaviral IRES elements. Picornaviruses contain 5′ UTRs that range in length from ~600 to 1,500 nucleotides and that are predicted to form complex secondary structures. These mRNAs are not capped and were the first family of mRNAs to be identified to initiate protein synthesis by a cap-independent and 5′-independent mechanism[64,65]. Indeed, the EMCV IRES can direct protein synthesis even in a circular mRNA[66]. So far, the poliovirus and EMCV IRES elements have been particularly well studied (reviewed in REFS 67–69). The region of RNA that defines the IRES of EMCV is ~450 nucleotides, making it larger than both the HCV and CrPV IRES elements. Although both the HCV and EMCV IRES elements allow for direct entry of the 40S ribosomal subunit at the initiation codon, the EMCV IRES cannot associate directly with the 40S ribosomal subunit. In contrast to the HCV IRES, the association of the 40S ribosomal subunit with the EMCV IRES is ATP dependent, although no scanning of the 40S ribosomal subunit is required for the correct positioning of the initiation codon in the P site. Instead, positioning of the 40S ribosomal subunit at the initiation codon requires two additional initiation factors that are not required for the efficient initiation of protein synthesis by the HCV IRES. Biochemical reconstitution experiments have shown that the large scaffold protein eIF4G is essential for the recruitment of the 40S ribosomal subunit by the EMCV IRES[70,71]. In addition, the presence of eIF4A is required, perhaps inducing local unwinding of RNA structure in the EMCV IRES that is likely to aid the association of the 40S ribosomal subunit with an unstructured region of the EMCV IRES[72]. Consequently, strengthening the secondary structure near the AUG codon strongly inhibits the HCV IRES, but the EMCV IRES is impervious to such manipulations, as the unwinding of RNA structure is possible by virtue of the recruitment of eIF4G and eIF4A by this IRES element.

Implications for cap-dependent translation

Mechanistic and structural studies of IRES-mediated translation initiation have indicated possible functions for cap-dependent translation factors in recruiting ribosomes to 5′-capped mRNAs. For example, cryo-EM reconstructions imply that the HCV IRES might have a similar role to that of eIF4G, a protein that forms part of the cap-binding complex (BOX 1). In separate cryo-EM reconstructions, the HCV IRES and eIF4G were each shown to bind to the same region of initiation factor eIF3[48]. In structural models of 40S–eIF3–IRES or 40S–eIF3–eIF4G ternary complexes based on cryo-EM of their components, the HCV IRES or eIF4G are each located near the site in which mRNA exits the 40S decoding cleft (FIG. 6). This position of eIF4G indicates how it anchors the 5′-capped end of an mRNA adjacent to the decoding cleft, and how a similar localization of viral mRNA is achieved by the HCV IRES.

The position of eIF3 or eIF4G is close to the location of the receptor for activated C-kinase (RACK1) on the head of the 40S ribosomal subunit[48,73]. Protein kinase C (PKC) associates with RACK1 *in vitro* and *in vivo* and stimulates the formation of the 80S ribosome[74]. Several subunits of eIF3 (REF. 75), eIF4E (REF. 76) and eIF4G (REF. 77) are phosphorylated *in vivo* in response to changes in nutrient levels (reviewed in REFS 78,79). It is possible that RACK1, and kinases that associate with it, might regulate the functions of these initiation factors during the initiation process (reviewed in REF. 80). Recently, another kinase, mammalian target of rapamycin (mTOR), has been identified to associate with eIF3 on the surface of the 40S ribosomal subunit and promote the association of eIF4G with eIF3 (REFS 81,82). Clearly, eIF3 has a central role in the assembly and regulation of the many initiation factors on the surface of the 40S ribosomal subunit.

Summary and concluding remarks

During the past decade the combination of structural and biochemical studies have greatly extended our understanding of how the HCV IRES can recruit ribosomes and translation factors from the host. Atomic-resolution structures of sub-domains are available but we are still limited by low-resolution structures and models for the larger complexes of the HCV IRES with its binding partners. A considerable challenge that remains is to understand at higher resolution how the HCV IRES can hijack the host translational machinery, possibly

enabling the structure-based discovery of HCV inhibitors. In addition, insights into the recruitment of ribosomes by the HCV IRES are likely to significantly enhance our understanding of how ribosomes are recruited by the standard scanning mechanism. Together with the recent development of a cell-culture system that produces fully viable HCV *in vivo*[83–86], it is hoped that significant advances in understanding the viral lifecycle and the development of successful treatments for hepatitis C will be possible.

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Competing interests statement
The authors declare no competing financial interests.

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