Ribosome–RNA interaction: a potential target for developing antiviral against hepatitis C virus*

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Hepatitis C virus (HCV), a member of Flaviviridae, encoding a positive-sense single-stranded RNA translates by cap-independent mechanism using the internal ribosome entry site (IRES) present in the 5′ UTR of the virus. The IRES has complex stem–loop structures and is capable of recruiting the 40S ribosomal subunit in a factor-independent fashion. As the IRES sequence is highly conserved throughout the HCV genotypes and the translation is the first obligatory step of the HCV life cycle, the IRES-mediated translation, or more specifically, the ribosome–HCV RNA interaction is an attractive target to design effective antivirals. This article will focus on the mechanism of the HCV IRES translation and the various ways in which the interaction of ribosome and IRES has been targeted.

Keywords: Antivirals, ribosomes, hepatitis C virus, replication and translation.

HEPATITIS C virus (HCV) is a positive-sense single-stranded RNA virus, belonging to the genus Hepacivirus of the family Flaviviridae. According to WHO estimates, around 3% of the total world population is infected with HCV and around 170 million of the patients are chronic carriers who have a high risk of developing hepatocellular carcinoma (HCC).

Interferon (IFN)-α is the drug of choice till date to treat chronic HCV infection, causing sustained viral clearance and leading to improvement in the liver condition. But IFN-α monotherapy has adverse side effects like flu-like symptoms, thrombocytopenia (low blood platelet count) and leukopenia. Only about 15% of the patients ultimately achieve sustained virological response (SVR) (sustained lack of detectable HCV RNA in the serum six months after completion of treatment)1,2.

IFN-α administration in combination with ribavirin (orally active guanosine analogue) indicates better effectiveness against the virus, showing up to around 40% SVR2. Current treatment of the HCV infection involves a combination of pegylated IFN-α and ribavirin, the ‘gold standard’ regimen against HCV, but the post-therapy relapse rate is quite high. No vaccine against HCV is currently available, and as the viral replication occurs through an RNA-dependent RNA polymerase that lacks proof-reading activity, viruses with new antigenic properties are constantly generated. Therefore, immune-mediated control of HCV infection may be difficult to achieve. Moreover, an important lesson learnt from the therapy of other viral infections is that multiple drug targets are required to prevent the emergence of drug-resistant varieties of the virus. Hence, it is the need of the hour to define new targets for controlling HCV infection. The development of inhibitors of the viral protease responsible for polyprotein processing is a step towards this objective3. However, any drug directed against viral proteins will still have the potential to generate drug-resistant escape mutants, due to the high rate of mutation of the viral genome. Therefore, multiple therapeutic strategies targeting various genomic processes of the virus, such as replication and translation need to be developed.

The positive-sense RNA genome of HCV is 96,000 nt in length. The viral RNA has a long open reading frame (ORF) which translates into the viral polyprotein consisting of 3000 amino acids4 (Figure 1). The open reading frame is flanked by a 5′-untranslated region (5′UTR) and a 3′-untranslated region (3′UTR). The 5′UTR harbours the internal ribosome entry site (IRES), where ribosomes can directly bind and initiate translation by a cap-independent mechanism (IRES-mediated translation). The 5′UTR is 341 nt long and is highly conserved. The 3′UTR varies between 200 and 235 nt in length. It includes a short variable region, a poly(U/UC) tract which is around 80 nt long and a conserved 3′X tail of about 98 nt5,6. This region is essential for RNA replication5,9.
The ORF of the HCV genome codes for two different classes of proteins: structural and non-structural proteins. The structural proteins include core, E1, E2 and p7, whereas NS2, NS3, NS4A, NS4B, NS5A and NS5B are the non-structural proteins. The life cycle of the virus begins with attachment and entry into host cells followed by uncoating of the viral RNA, translation, replication, assembly of new viruses and exit. Attachment of the virus to the hepatocyte surface followed by its entry is the first step of the life cycle. The viral entry, uncoating and release of the viral positive-stranded RNA into the cytoplasm is followed by viral RNA translation, which is mediated by the viral IRES.

**HCV IRES element**

The viral IRES encompasses elements essential for the translation of the viral RNA. The boundaries of the IRES have been observed to be between 25 and 46 nt of the 5’UTR, stretching up to about 30 nt within the coding region. The sequence upstream of the IRES (stem–loop I) is essential for RNA replication. Additionally, it has been reported that sequences inside the IRES are required to maintain a high degree of replication. Also, a micro RNA of liver origin (mir-122) binds to 5’UTR and enables enhanced RNA replication.

The highly conserved HCV IRES is composed of four highly ordered domains or stem–loop structures (SL I to SL IV). The first domain is not required for translation, but is important for replication. The remaining three along with the initial 24–40 nucleotides of the ORF constitute the IRES. SL II is comprised of a stem having several internal loops. SL III has many subdomains, such as SL IIIa–f. They form a four-way junction and a pseudoknot near the base of the stem–loop. SL IV is a small structure resembling a hairpin and contains the initiator AUG at position 342. The first 12–40 nucleotides after the initiator AUG, encompassing the beginning of the core protein-coding region are also important for IRES activity.
Internal initiation of translation of HCV RNA

Since the first round of IRES-mediated translation is obligatory for the HCV RNA to initiate its life cycle inside the cell, the IRES-mediated translation becomes an important target for developing therapeutic strategies against the virus.

Using elegantly designed experiments involving a reconstituted translation system, it had been determined that HCV IRES can capture the 40S subunit of ribosome independent of the initiation factors that are otherwise needed in the case of cap-dependent translation of eukaryotic mRNAs. In this regard the HCV RNA and 40S association can be said to mirror the prokaryotic system, in which the 30S ribosomal subunit binds directly to the mRNA via the Shine–Dargarno sequence. Additionally, the HCV translation initiation does not require scanning of the 5'UTR by the 40S ribosomal subunit, as it is directly recruited to the initiation codon. Presence of an additional initiation codon seven nucleotides upstream or eight nucleotides downstream of the actual initiator AUG leads to failure of recognition by the 40S ribosomal subunit, thereby indicating that the IRES has a specialized structure suitable to recruit the 40S only at the correct initiation codon. Further, it has been shown that an extensive interaction surface of the IRES RNA is required to facilitate the high-affinity interaction. This comprises the junctions among domains IIIabc, IIIId and IIIef. Domains II and IV also make contact with the 40S. This kind of large binding surface increases the stability of the interactions and is markedly different from the initiation mechanism in prokaryotes. Although the recruitment of the 40S subunit on the HCV IRES is initiation factor-independent, the localization of the initiator methionyl-tRNA on the 40S involves the eukaryotic initiation factor 2 (eIF2). eIF3 also enhances the formation of 40S RNA complex, although it is not an absolute necessity. Apart from domain III of the IRES, domains II and IV are also crucial for translation initiation. The domains II, IIIa and IIIabc junction facilitates the GTP-dependent formation of 80S ribosomal complex.

Interestingly, it has been shown that several domains of HCV IRES fold independently and some of them resemble structures present in the ribosomal RNA. The IIIabc junction has two sets of stacked helices resembling RNA structure in 50S subunit; IIIId has an asymmetric E-loop motif. It involves two S-turns similar to the sarcin–ricin loop of the ribosome. Sarcin–ricin loop is a site for binding of elongation factors. Domain II also has an E-loop motif like IIIId, but with a different orientation. These reports suggest that the HCV IRES RNA presents conserved interaction surfaces for recognition by ribosome-interacting factors.

The HCV IRES-mediated translation involves first the association of the 40S ribosomal subunit to IRES. The former interacts at multiple sites, including the stem–loop, pseudoknot and even the initiator AUG. This complex positions the initiator AUG directly at or near the P-site followed by the binding of eIF3 and also the eIF-2 : Met-tRNA : GTP ternary complex. The basal part of the HCV domain II is responsible for the 40S association. Whereas the 40S gets recruited to domains III, it also makes contact with domain II and IV.

The resulting 48S complex then gets associated with the 60S ribosomal subunit to form the 80S complex. This is the rate-limiting step and is dependent on GTP hydrolysis. Domain II of the IRES binds to the mRNA exit site, resulting in a conformational change in the 40S ribosomal subunit allowing the eIF5-mediated GTP hydrolysis. After the formation of the 80S complex, the translation can initiate by forming the first peptide bond at the initiator AUG. Apart from the canonical initiation factors, the IRES-mediated translation initiation is additionally facilitated by several other host factors or proteins called IRES trans acting factors (ITAFs), such as the human La protein, poly-C-binding protein, polyyrribimidine tract-binding protein (PTB), heterogeneous ribonucleoprotein L (hnRNP L), human D0, NS-1 associated protein 1 (NSAP 1), etc. The interaction of the viral proteins also modulates IRES activity.

Apart from the protein factors, there is increasing evidence of the role of naturally occurring microRNA (miRNA) on the functions of the HCV 5'UTR in a tissuespecific fashion. miR-122 is a liver-specific microRNA that modulates the HCV RNA function positively. Between SL I and SL II of the HCV IRES there are two partially complementary binding sites of miR-122. This regulates translation as well as replication of the HCV RNA. On the other hand, miR-199a has been shown to bind to SL II at a sequence present downstream of the second miR-122 binding site and reduce the efficacy of RNA replication.

Besides IRES, the 3'UTR also plays crucial role in the translation although its main role is to initiate replication. The 3'X region of the 3'UTR is the key element where PTB interacts. Because PTB interacts with IRES as well as 3'UTR and can also dimerize, it leads to circularization of RNA and might influence translation and/or replication.

Targeting the ribosome: HCV IRES interaction

In view of the above, translation initiation of HCV is highly regulated, and the IRES RNA and the protein-binding partners are interdependent, thus making each of them suitable for antiviral targeting provided it does not interfere with the host cell translation. Specifically, the ribosome binding that involves the three stem-loop structures of IRES, as well as various canonical and non-canonical factors that aid in ribosome loading are important targets to inhibit HCV translation.
HCV IRES is highly conserved amongst different viral genotypes and evidently plays a crucial role in the translation process. Owing to its conserved nature as well as the fact that it encompasses both the translation and replication elements, it is an attractive drug target. Interventions with the IRES functions have shown appreciable effect on the viral life cycle. Especially, SL III and SL IV of HCV IRES, that are most involved in the IRES-mediated translation are important targets against the HCV IRES functions.

HCV IRES can be targeted in cis and trans by either targeting the RNA itself or by targeting various host and viral factors involved in HCV IRES–ribosome interaction. The IRES has been targeted directly in several ways like anti-sense oligonucleotides, RNA interference (RNAi), oligonucleotide aptamers, ribozymes and DNAzymes, and small molecules such as RNA decoys or peptide. The following sections provide an overview of the various molecular strategies developed to target HCV IRES–ribosome interactions.

**Small RNAs: antisense, siRNA, aptamers and RNA decoys**

Antisense oligonucleotides have been extensively used to target viral translation. These may be DNA or RNA oligonucleotide and are 12–26 bp in length having complete sequence complementarity to the target RNA molecules. The binding of the antisense oligonucleotides to IRES leads to inhibition of translation. This translation inhibition might be due to steric hindrance or change in the structure of RNA or due to RNAase H-induced cleavage of the DNA–RNA duplex. Also, this results in the release of other antisense oligonucleotides which can now target another target RNA. Quite a number of antisense oligonucleotides against HCV 5’ UTR have entered the phase-II clinical trials. ISIS-14803 (Isis Pharmaceuticals, Carlsbad, CA, USA) and AVI-4065 (AVI-Biopharma, Bothell, WA, USA) are two such examples. A major problem in using antisense RNAs to target HCV IRES has been the instability of the antisense RNA, which has resulted in limited bioavailability of the RNA. Therefore, novel RNA modifications such as 2′-O-methyl RNA has been developed to impart greater stability to antisense RNAs. Also, peptide nucleic acids (PNAs), where the phosphodiester bonds in the RNA backbone are replaced by peptide bonds, enhancing the stability and efficacy of the antisense RNA have been developed. Alotte et al. have reported that when PNAs are used to target HCV SL IIIId and domain IV, they could inhibit HCV IRES-mediated translation efficiently. However, the efficacy of none of these molecules has been enough to go beyond the phase II trials. A recently developed strategy to utilize oligonucleotide-based inhibitory agents has been to use RNA interference (RNAi) against the HCV IRES using small interfering RNAs (siRNAs). The siRNA molecules are derived as a result of cleavage of double-stranded RNA (dsRNA) molecules by the dicer (double-stranded RNA-specific endonuclease) into small RNA fragments of about 22 nt length. The RNA-induced silencing complex (RISC) then loads these fragments on the target RNA, ultimately leading to degradation of the RNA. This mechanism of gene silencing is naturally occurring in eukaryotic cells but the concept has also been exploited to target specific RNAs. Chevalier et al. have shown that targeting domain III of HCV IRES using different siRNAs showed significant drop of HCV IRES translation. They could achieve almost 90% inhibition by targeting the region domain III if that forms a pseudoknot structure. On similar lines, stable overexpression of short hairpin RNAs, that lead to siRNAs in cells by intracellular processing has been used to inhibit the HCV RNA functions. However, as both antisense and siRNAs are sequence-based strategies of inhibition, they have high potential of giving rise to escape variants of the virus which fail to be inhibited due to mutations in the target sequences.

To avoid sequence-based inhibition strategies which give rise to escape variants, strategies based on RNA decoys or mimics, which target viral RNA–host protein interactions have been developed. Interaction of several IRES trans-acting factors (ITAFs) with the HCV IRES is crucial for HCV IRES-mediated translation, which when targeted might be deleterious for the virus. RNA decoys/mimics have been used to sequester ITAFs and prevent their interaction with IRES, aiming to inhibit the IRES-directed translation. A ~ 60 nt long RNA from yeast (IRNA) was found to inhibit HCV IRES translation, but not cellular cap-dependent translation. This inhibitory effect of IRNA was found to be due to its ability to bind with and sequester the La protein, a cellular ITAF required for HCV IRES–ribosome interaction.

In a further development of the RNA decoy strategy, a small RNA molecule which is a structural mimic of SL III e + f subdomain of the HCV IRES has been used to inhibit HCV IRES-mediated translation. It was demonstrated that the SL III e + f decoy RNA binds the ribosomal protein S5 protein of the 40S subunit and prevents the binding of the 40S subunit to IRES, thereby inhibiting translation initiation. The S5 protein has been shown to be crucial for the interaction of the 40S subunit with the HCV IRES RNA and for HCV IRES-mediated translation. However, the 40S subunit S5 protein has not been found to interact with eukaryotic mRNAs at any stage of translation. Thus, the SL III e + f RNA that interacts with the S5 protein inhibits the HCV IRES-mediated translation by reducing the 48S ribosomal complex formation, but does not affect normal cellular translation. Therefore, small RNAs that act as a decoy for ribosomes or other ITAFs are capable of inhibiting the IRES-mediated translation and are potential antiviral therapeutics.
Aptamer is another class of small oligonucleotide inhibitory agents. These can be DNA or RNA molecules that can fold into three-dimensional structures. These form binding pockets or structures specific for a particular target site. They are capable of binding to extensively structured RNAs as present in the HCV IRES, and thus prove to be a good candidate for translation inhibition. Also, the aptamers are highly specific and have been used to target various regions of HCV IRES. Kikuchi et al. have tested RNA aptamers against the IRES domains III and IV. They found one of the aptamers to be significantly inhibiting HCV IRES-mediated translation due to its interaction with the apical loop of domain IIId. They also demonstrated moderate inhibition by the aptamer against domain II. Interestingly, when they conjugated these two aptamers, they got an enhanced level of inhibition. These reports indicate that the aptamer strategy to target the HCV IRES–ribosome interaction and inhibit translation also has potential as antiviral therapy.

Ribozymes and DNAzymes

Ribozymes developed to target HCV cleave the HCV genomic RNA (Figure 2). The ribozymes are short RNA molecules containing an endoribonuclease activity which can carry out sequence-specific cleavage of a target RNA molecule. The flanking sequences of the ribozymes, which are complementary to the target sequence, account for its sequence specificity. There can be various ways to synthesize ribozymes, but the most common method is based on the hammerhead ribozyme. These are 50–100 nt long and are composed of a catalytic domain flanked by annealing arms directing the target complementary sequence. Heptazyme is a ribozyme that has been shown to have deleterious effect on the HCV RNA replication.

DNAzymes are similar to ribozymes, except that these are DNA molecules and are advantageous over ribozymes in having more stability. Moreover, the synthesis and delivery is easier. Recently, Roy et al. have reported DNAzymes against HCV SL IIIb and IIIId that inhibited the IRES-mediated translation significantly.

Inhibitory peptides

Peptides constitute another class of attractive anti-HCV agents targeting the proteins needed for the HCV IRES-mediated translation (Figure 3). These are derived from a cellular protein and function as competitive inhibitors for the latter. Ideally, the peptide sequence is derived from the RNA-binding site of the cellular protein, and it binds to the RNA and sequesters it away from the full-length cellular protein.

Figure 2. Ribozyme/DNAzyme-based inhibition. The ribozyme or DNAzyme molecule binds to the target RNA with help of the complementary sequence present on the flanking arms followed by cleavage of the target nucleic acid.

Figure 3. Inhibition of HCV RNA function by intervening with RNA–protein interaction. a. Schematic of RNA bound to a protein and the 40S ribosome. b. Peptide-based inhibition. A peptide derived from the RNA-binding region of the full-length protein acts as a competitive inhibitor by competing out the protein binding to the RNA. c. Small RNA-based inhibition. A small oligonucleotide derived from the sequence of RNA that interacts with the target protein is capable of sequestering the protein, thus inhibiting the RNA functions.
La protein has three major RNA recognition motifs (RRM1, which is also known as the La motif, RRM2 and RRM3). It has been shown that the RRM2 (101–208) of the La protein interacts with the HCV IRES in the SLIV region near the initiator AUG at the GCAC motif. Mutations at this region drastically affect the interaction of the La protein with HCV IRES and also inhibit the HCV IRES-mediated translation. It has been shown that LaR2C, a 24-mer peptide derived from the RNA recognition motif of human La protein interfered with La binding to the HCV IRES, resulting in inhibition of IRES-mediated translation. It was found that the 24-mer peptide has a 7-mer stretch that forms a turn-like structure. A 7-mer peptide (LaR2C-N7) comprising these amino acid residues retained the turn structure even in RNA-bound condition and could efficiently inhibit the HCV IRES-mediated translation and replication. This interference is due to the competition of the peptide with the full-length protein to bind to the IRES. As La binding to the IRES is necessary for efficient recruitment of the 40S subunit and its proper positioning at the initiator AUG, prevention of La binding leads to inhibition of IRES-mediated translation. Because of the relatively easy deliverability of the peptide into cells and the high stability of the peptide, this constitutes an attractive anti-viral strategy.

Small-molecule inhibitors

In addition to the above-mentioned methodologies, small molecules have also been utilized to target the IRES-ribosome interaction. For example, Seth et al. reported that a class of benzimidazole molecules that displays appreciable affinity for SL Iia of IRES could effectively inhibit the translation of the virus. This interaction was shown to result in a conformational change in IRES that hampered ribosome and RNA interaction.

All these therapeutic strategies appear to have potential to inhibit the HCV RNA function. But, the toxicity and stability of these agents need to be studied prior to their utilization as antiviral therapies. The other major problem has been the delivery of these agents to the target tissues, such as the liver. Recent trials with liposomes, polymers, nanoparticles and viral vectors have however shown improved delivery efficacy and tissue-specificity in some cases.

Concluding remarks

Yet another roadblock in the development of anti-HCV therapeutics has been the lack of a suitable small animal model for testing the efficacy and pharmacokinetics of any antiviral agent. Before the recent report of a humanized mice model, the only established animal model for studying HCV infection was the chimpanzee. However, it involved restricted usage, high cost and ethical issues. In few cases an alternative model, the chimera human liver uPA-SCID mouse, was utilized. However, as SCID mouse was used, the immune response studies could not be done utilizing this model. Recently, another potential model, the tree shrew, Tupia belangeri, has been introduced. Also, Dorner et al. have reported a genetically humanized mouse model for HCV infection. It had been previously shown by Ploss et al. that CD81 and occludin are the minimal human factors needed for infection by HCV in rodents. Dorner et al. have shown that an expression of these two receptors on mice liver could lead to efficient uptake of the virus by these mice that are otherwise immuno-competent. This might lead to a new era of testing therapeutic agents against HCV, that will have the potential to become frontline candidate drugs against the virus.

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