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A Minireview: Molecular Understanding of HCV Infection Mechanism

Animesh Sarker[1,*], Marufa Nasreen[1], Rafiad Islam[1], Tasnim Ahmed[2], Fahima Rahman[1]

[1]Department of Biotechnology and Genetic Engineering, Mawlana Bhashani Science and Technology University, Santosh, Tangail-1902, Bangladesh
[2]Department of Biotechnology and Genetic Engineering, East-West University, Dhaka-1200. Bangladesh

Abstract Hepatitis C (HCV) is a positive polarity single-stranded (ss) RNA virus belongs to *Flaviviridae* family. It infects about 2% people annually throughout the world (WHO, 2012) and causes both acute and chronic hepatitis consequences permanent liver damage, hepatocellular carcinoma (HCC) and eventually death. The absence of effective means of treatment makes HCV infection a global health hazard. Due to lack of pin point of molecular mechanism, precise drug target and efficient preventive measure is still unclear. Therefore, identifying and understanding mechanistic underpinnings of viral entry, replication, assembly, and budding are crucial in owing to the development of antiviral therapy. Current host-pathogen interactions data and the infection model suggest that RNA dependent RNA polymerase activity of NS5B, along with NS5A and NS3 play central role in HCV infection mechanisms. It has been shown in numerous studies that the interactions between 5′ and 3′ UTRs (Un-translated regions) and the interactions UTRs verses host proteins play fundamental role in regulating replication and translation processes as well as their successive switching.

Keywords Viral RNA, IRES element, PKR, Signal peptidase, Replicase complex

1. Introduction

Hepatitis C is one of the most common liver diseases that results from infection with the hepatitis C virus. It is transmitted through blood contact with an infected person. Currently WHO estimated that around 3–4 million people are infected with the hepatitis C virus annually. Approximately 150 million people are chronically infected with hepatitis C virus, and more than 350,000 people die every year from hepatitis C-related liver diseases. HCV infected patients, whether acute or chronic; often have no symptoms [1]. But Persistent infection with HCV often leads to cirrhosis and hepatocellular carcinoma (HCC) [2]. Protective vaccine and other therapeutic alternative are still limited against this virus [3].

The HCV is one of the members of Hepacivirus genus and belongs to Flaviviridae family. It‘s a small, enveloped, spherical, positive-sense RNA virus [4, 5]. So far six major genotypes of HCV (HCV 1-6) have been reported yet, each of them contains multiple subtypes. Genotype 3 is mostly prevalent in South-East Asia [6] and genotype 1 is in the US. Genotypes 1 and 4 are less responsive to treatment than other genotypes 2, 3, 5 and 6 [7].

* Corresponding author:
animesh_du_geb@yahoo.com (Animesh Sarker)
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The HCV infection solely depends on the interaction between virus and host cell. Further pathogenesis take place due to subsequent host-viral protein-protein (PPI) and protein-RNA (PRI) interactions. As our understanding of molecular interactions between host and the virus increases, more focused HCV infection mechanism being clear. This current review indentifies experimentally validated host-virus interaction partners and sequentially arranged them into a infection model. This conceptual model focuses on recent advances in molecular mechanism of HCV translation, replication as well as their regulatory switch.

2. HCV Lifecycle

HCV enters the host cell via receptor mediated endocytosis and following pH mediated membrane fusion of the HCV nucleocapsid escapes into the cytoplasm [8]. The positive sense RNA genome is transported to the endoplasmic reticulum (ER), where it is translated to produce viral structural and non-structural proteins. These viral proteins further engage in subsequent rounds of RNA synthesis. While minus (-) strand HCV RNA is synthesized from the parental (+) strand, serves as a template for further replication. These newly generated positive-sense RNA molecules further undergo for second round translation, generating more structural proteins required for RNA packaging and virus assembly [9]. This coordinated process yields virions which becomes maturated in the golgi

apparatus and finally secreted via the host secretory pathway [10].

3. HCV Genome and Proteome

The HCV genome comprises of 9.6 kb and encodes a poly-protein around 3000 aa in length [12]. It carries single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs), which are essential for viral translation, replication and infection [13]. The 5′ conserved secondary structure IRES (internal ribosome entry site) directs translation and other 3′ structural elements facilitates newly RNA synthesis [13, 14, 15]. The genomic RNA serves as template mRNA for poly-protein bio-synthesis which is then co and post-translationally processed into four structural protein capsid (C), envelope1 (E1), envelope2 (E2) and (p7) and six non-structural proteins (e.g. NS2, NS3, NS4A, NS4B, NS5A and NS5B) (**Figure 2A)** [15].

5′ UTR: There are four conserved stem-loop structure in 5′ end of HCV RNA which are designated as motif I to IV [16]. Among them motif II and III serve as internal ribosome entry site (IRES), which directs viral translation in a cap-independed manner [17, 18]. This IRES element spans 340 nucleotides (nt) of 5′ UTR and includes a short stretch of the 5′ core coding sequence [19]. Under intracellular condition, motif III forms six distinct stem-loop structures (designated IIIa-f) that provide sites for interaction of numerous host and virus proteins [20]. Most vital motif of IRES element exists in IIId stem-loop structure spanning nucleotides from 253 to 279. This highly conserved IIId region again composed of two short helix separated by a loop E and capped by an apical loop folded into a U-turn motif (**Figure 1B**) [20, 21].

3′ UTR: It is evolutionarily conserved and consists of three principal structured elements spanning around 200 nucleotides from 3′ viral RNA [19, 22]. These elements are highly variable region (VR), a poly U/UC tract and the 3′ X tail (**Figure 1C**). The third element, 3′ X tail again composed of three stem-loops termed 3′ SLI, 3′ SLII, 3′ SLIII [22]. These elements are conserved and numerous experiments have showed their indispensable association in viral translation and replication [18-22].

Core (C): Viral structural core is an RNA-binding protein that is supposed to form the viral nucleocapsid. It is removed from the poly-protein by a host signal peptidase yielding the immature form of the protein [23]. Subsequent modification turns it in matured dimeric alpha-helical protein, which behaves as membrane like protein [24].

Envelope (E1 and E2): The envelope proteins are glycosylated and form a non-covalent complex around viral RNA packed core protein during virus morphogenesis [25]. The complex maturation and folding process of E1 and E2 glycoprotein depends on disulphide bond formation which is carried out by ER chaperone machinery [25, 26].

p7: It is a small, 63 amino acid containing protein often incompletely cleaved from envelope protein E2, positioned at the junction of the structural and non- structural protein [4, 25]. It possesses ion channel activity and belongs to the viroporin family [27].

NS2: NS2 is an integral membrane protein and assists NS3 protease activity during cleavage at the NS2-NS3 junction [26, 28]. The function of solitary matured NS2 protein is still ambiguous.

NS3–4A complex: NS3 undoubtedly a serine protease and also a member of the DExH/D-box helicases superfamily [4,23]. One third at the N-terminal takes protease activity and two thirds from the C-terminal take RNA helicase/NTPase activity [29]. As a cofactor polypeptide NS4A associates with NS3 to ensure finest proteolysis [25].

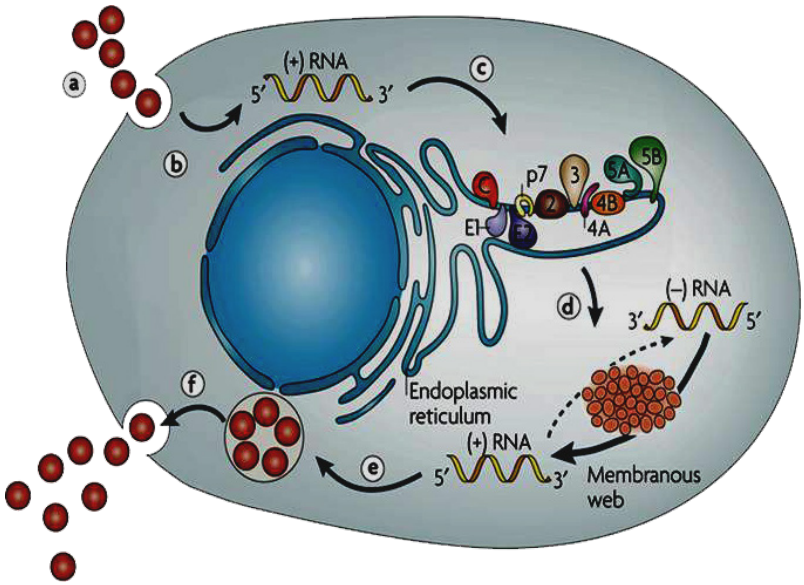

Figure 1. Overview of molecular mechanism of HCV infection [11]. (a) Virus binding and internalization; (b) cytoplasmic release and uncoating; (c) IRES-mediated translation and polyprotein processing; (d) RNA replication; (e) packaging and assembly; (f) virion maturation and release

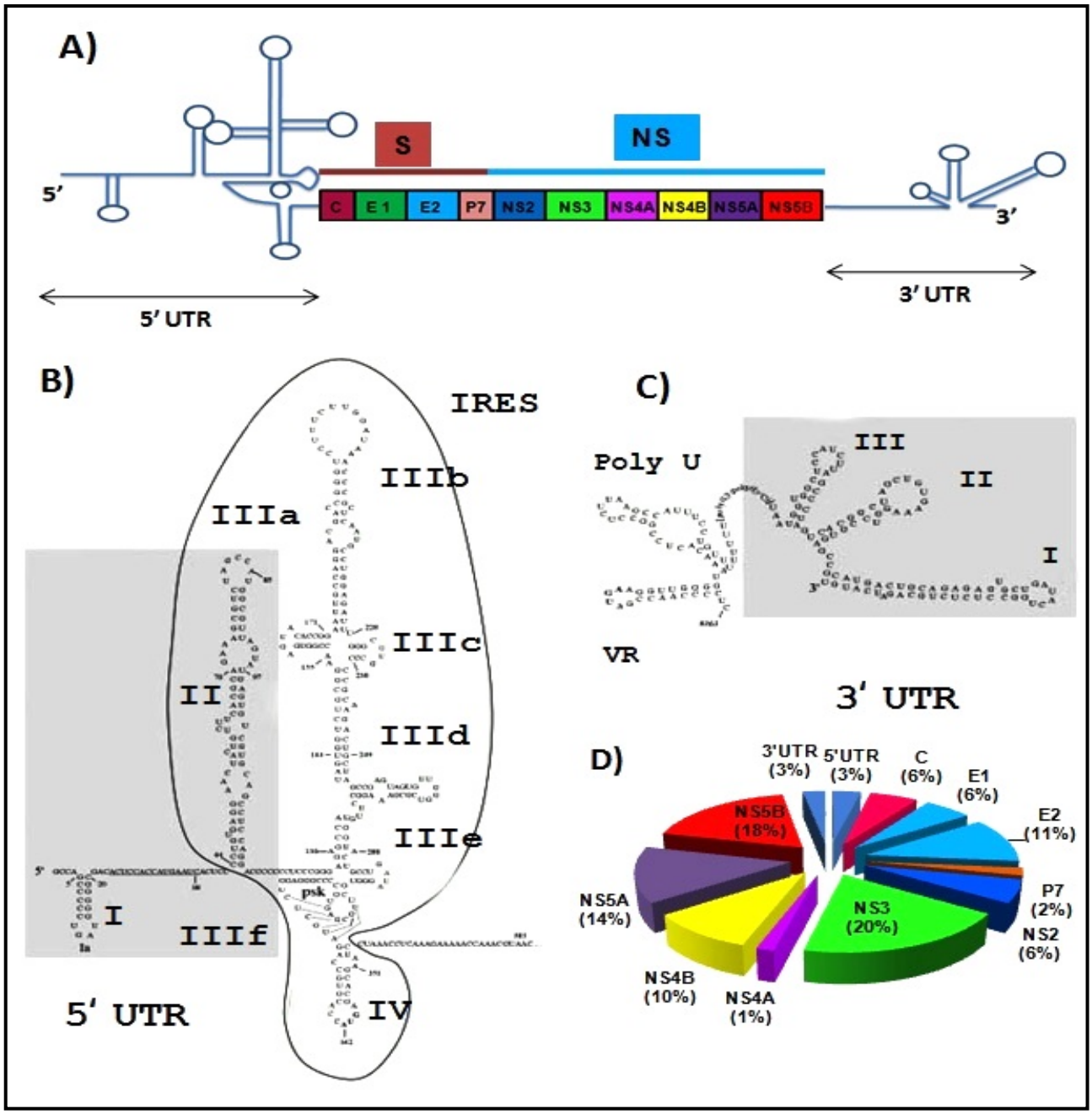

Figure 2. The Genomic Organization of HCV A) HCV genome composed of four structural protein and six non-structural protein flanked by 5′ and 3′ non-coding sequences; B) Stem-loop structure of HCV 5′ UTR which composed of four conserved motif designated (I to IV); C) Stem-loop structure of HCV 3′ UTR which composed of three conserved element known as highly variable region (VR), a poly U/UC tract and the 3′ X tail and D) Percent Distribution of HCV genomic content. NS5B comprises the highest percentage (18%) and NS4A the lowest percentage (1%) of HCV genome. Around 6% genome of HCV comprises 5′ and 3′ UTR which remain un-translated during translation

NS4B: The non-structural protein 4B (NS4B) is a highly hydrophobic ER-membrane integral protein [30]. It has an amphipathic helix both in N-terminal and C-terminal domains which both are involved membrane remodelling [31]. In the ER membrane NS4B oligomerizes and induces membrane curvature where viral replication takes place [32].

NS5A: NS5A has an amphipathic alpha-helix at N terminus and anchored to the ER membrane [33]. It has domain for both zinc (Zn^{2+}) binding and RNA-binding and also form complexes with NS5B [4]. Thus it could hold the replication complex with the ER membrane and regulate NS5B mediated polymerization reaction [4, 32].

NS5B: The most vital HCV protein is NS5B which is also anchored to the ER-derived “membranous webs" via its C-terminal 21 amino-acid residues [32, 34]. It acts as RNA dependent RNA polymerase (RdRp) and synthesizes complementary negative-strand RNA taking the positive strand RNA as template. Subsequently, negative- strand RNA replicates to yield massive positive strand RNA [35].

Table 1. Viral proteins and their role in HCV life cycle

Protein	Length(nt)	Mol. Mass	Function
C	573	21kDa	Nucleocapsid assembly
E1	576	31-35 kDa	Virus morphogenesis. Cell entry.
E2	1089	70 kDa	Building blocks of viral envelope.
P7	189	7 kDa	Virus assembly, export and infectivity
NS2	551	21 kDa	Polyprotein processing. Viral assembly.
NS3	1893	69 kDa	Serine protease activity, Helicase activity, HCV genome replication
NS4A	162	6 kDa	Co-factor of NS4B and NS5A
NS4B	945	27 kDa	Formation of membranous web structures
NS5A	1344	56-58 kDa	Part of the replication complex
NS5B	1773	68 kDa	RNA-dependent RNA polymerase

4. Molecular Mechanism of HCV Infection

Virus Entry: The primary targets of HCV are hepatocyte cells of human and chimpanzee. B cells, dendritic cells and other cell types have also been reported as its alternative target. CD81 a tetraspanin protein, LDL receptor (LDLR), scavenger receptor class B type I (SR-BI) and claudin-1 are most common receptor for HCV endocytosis [36,37].

The LDLR is an attractive candidate due to the association of HCV with LDL and VLDL [38]. Together with glycosaminoglycans GAG, the LDLR and other cell surface proteins serve as primary collectors of HCV particles for further targeting to CD81 and additional receptor components (**Figure 3**). HCV E2 also binds to L-SIGN (liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin) which is a calcium-dependent lectin expressed on liver sinusoidal endothelial cells that facilitates the infection process by trapping the virus for subsequent interaction with hepatocytes [11]. Another tight junction component claudin-1 (CLDN1) was recently identified as an HCV co-receptor was found to be essential for HCV entry into hepatic cells. CLDN1 acts at the late stage of the entry process, after virus binding and interaction with CD81 [36]. Finally, HCV enters by clathrin-mediated endocytosis [39] with transit through an endosomal, low pH compartment [40] and presumably endosomal membrane fusion [41].

Shut off host cell protein synthesis: After penetration, viral endosome undergoes an uncoating process to expose its genome to host cell machinery. As a primary response, partially double stranded viral RNA and interferons α/β activates PKR through its dimerization and mutual phosphorylation [42]. Activated PKR then successively phosphorylates α-subunit of eIF2, preventing the GDP-to-GTP exchange factor eIF2B from recycling GTP on eIF2α, thereby globally inhibiting host cell regular protein synthesis [43].

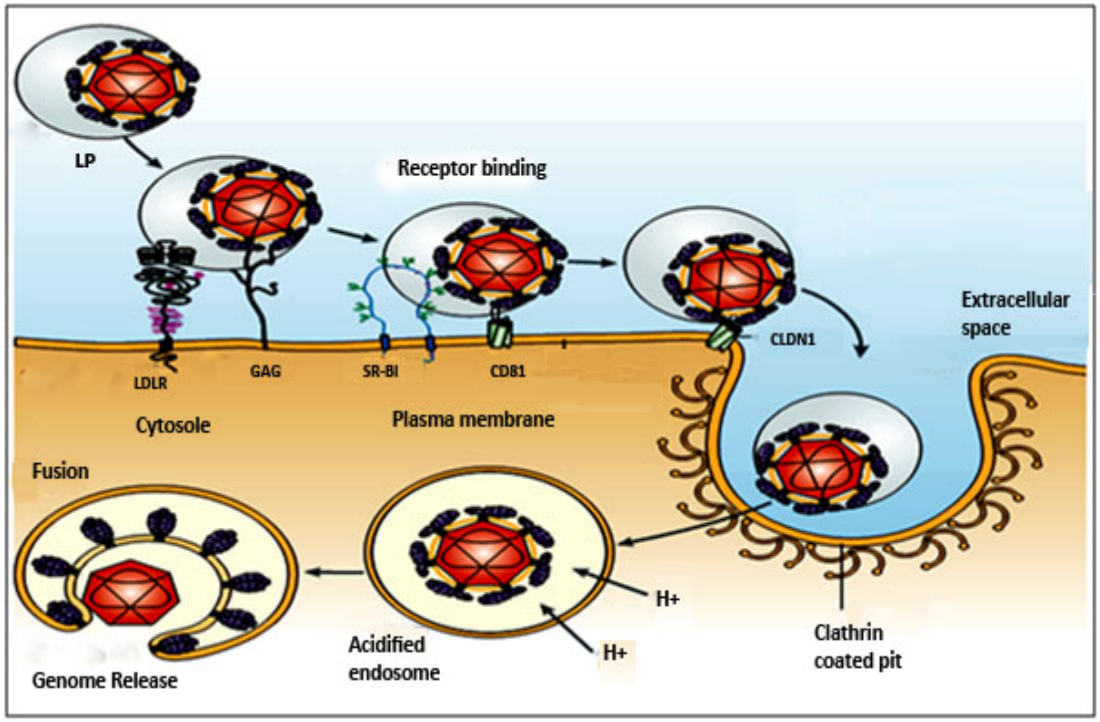

Figure 3. Hepatitis C virus entry process: HCV particles associate with low- and very-low-density lipoproteins (LP). Low density lipoprotein receptor (LDLR) initially interact virus particle on cell surface. Glycosaminoglycans (GAG), scavenger receptor class B type I (SR-BI), the tetraspanin protein CD81 and claudin-1 (CLDN1) all are involved in the entry of virus particle. Clathrin coated pit mediates endocytosis of virus particle. Acidification of the endosome induces HCV glycoprotein membrane fusion and genome release into the cytosol

Initiation of alternative translation: At the adverse situation of silencing regular protein synthesis, a number of viral RNAs can efficiently translated seizing alternative mechanism. This is exactly the case for HCV RNA, whose translation is reported to be refractory to reduced eIF2–GTP–Met-tRNAi ternary complex availability [45]. Recent evidences have showed that, in addition to the conventional eIF2-dependent initiation complexes formation, the HCV IRES can directly be assembled into 80S initiation complexes without eIF2 participation. This alternative pathway requires only eIF3 and eIF5B as initiation factors and does not require GTP hydrolysis. The 80S complexes thus formed are fully competent for translation elongation [46].

Reviving regular translation: Along with IRES mediated translation, HCV have evolved novel strategies for antagonizing PKR activation. Thus, it fails to inactivate eIF2–GTP recycling and revive regular translation process. HCV envelope protein E2 shares 12-aa phosphorylated domain with both the PKR regulatory site and eIF2α, biochemical studies have showed that HCV E2 domain interact with PKR and inactivate it [47]. HCV NS5A has also been reported as an inhibitor of PKR that mainly resist α/β interferon response and allows infected cell protein synthesis continuously [48]. Moreover, HCV protease NS3/4A, non-structural protein NS5A and core protein (C) have also cited as the blocker of JAK‑STAT pathway as well as interferon signalling [42, 49]. These entire event acts in collaboration to inhibit PKR and interferon signalling and rescue regular protein synthesis in an infected cell (**Figure 4**).

HCV translation: Generally, eukaryotic cell accomplish its protein synthesis in canonical cap dependent manner. Here, initiation factor eIF2 is crucial for the delivery of Met-tRNAi^{Met} to small ribosomal complexe which is mandatory for protein synthesis initiation except few exotic cases [52]. Viral infection as well as other stresses could activate PKR or interferon signals that phosporylate eIF2α and suppress initial tRNA delivery [43]. During this situation HCV translation is reported to switch from conventional canonical eIF2 dependent pathway to IRES mediated eIF2 independent pathway [53].

The probability of using such an alternative pathway might be explained by the unique features of HCV 5′ IRES element. In vitro reconstitution of initiation complexes on these IRESs have revealed that apart from the 40S subunit, IRES directly binds to eIF3 and holds Met-tRNAi at the P-site of ribosomal complex, that actually mimic with the formation of regular 48S initation complex [54]. During this non-canonical process, a stable mRNA–40S complex is formed directly, where the initiation codon properly positioned in ribosomal P site [55].

One of their foremost protein is poly T tail binding PTB that undoubtedly binds with poly U/C stretch of 3′ UTR and several others eg, hnRNP L, p210, RACK1 are identified to interact with 5′ IRES element. Paradoxically, hnRNP L and p210 are also found to be connected with PTB and 3′ poly U/C stretch that provide evidences of their role in genome circularization. as like as eukaryotic translation initiation factor eIF4G. Circular genome actually forms partially dsRNA which activates IFNα/β and PKR pathways. Finally, this double stranded RNA activated protein kinase (PKR) phosphorylates translation initiation factor IF2α and turned off regular cap dependent translation.

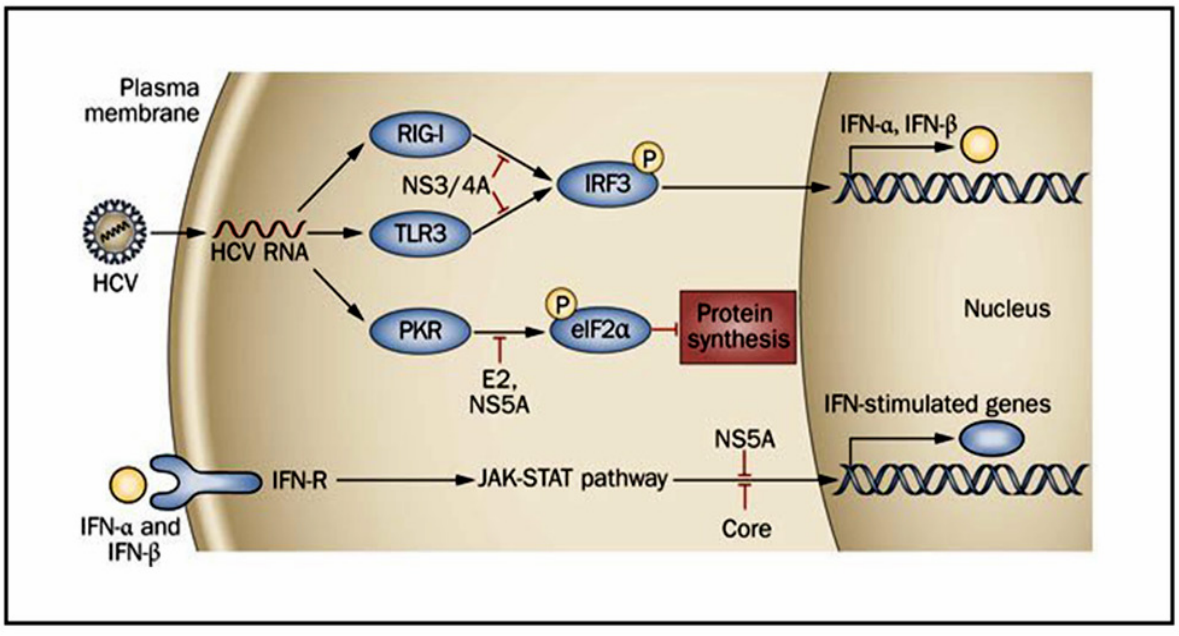

Figure 4. HCV interferes with IFN signalling, and antiviral effectors. HCV protease NS3/4A blocks downstream signalling of TLR3 and RIG‑I pathogen‑recognition receptors. HCV protein NS5A and Core also block IFN signalling via the JAK‑STAT pathway. E2 and NS5A inhibit PKR, an antiviral effector that leads to inhibition of protein synthesis in infected cells

Several studies have shown that, the IRES mediated cap independed translation solely dependent on viral RNA specially its 5´and 3´ UTR structure. Despite, some controversy PTB has unambiguously shown to unite specifically to the HCV 3´-UTR (Poly U/C stretch) and stimulates translation [56]. On the other hand, hnRNP L has also reported to interact with both PTB and 5´ IRES element, confirmed by yeast-two-hybrid screening and RNA gel shift assay respectively. Their synergistic interaction of PTB, hnRNP L and HCV UTRs suggests their association of long range 5´- 3´ interaction [57,58].

Another interaction partner of IRES is 210 kDa protein also found to be connected with poly U/C tract of 3´-UTR [58]. The computational study of this protein finds a functional and structural similarity to eukaryotic initiation factor 4G (eIF4G). Like 4G (eIF4G) in cap dependent translation this novel protein perhaps stabilizes the formation of closed-loop by bridging the 5´- and 3´-UTR.

Several other host factors (e.g. RACK1, NF90, NF45, RHA, NF100, La, PCB1/2, Unr, GADPH, hnRNPK, p87, p130, Rps25, Nucleolin and α-actinin) have also been reported to interact with viral RNA and have their functional role in viral translation process. Some are found to be interacted with 5′ UTR and some are 3′ UTR [**Table 1**]. According to their interaction pattern and functional co-relation it can be predict that they either participate in genome circularization or control translation process [58, 59, 60, 61, 62].

Post translational protein processing: HCV translation take place at the membrane of rough endoplasmic reticulum, where the synthesized poly-protein co and post-translationally processed by cellular and viral proteases (**Figure 5**) [63]. The structural proteins and the p7 polypeptide are liberated by cellular signal peptidase whereas NS2 and NS3 junction is cleaved by NS2 protease. Then, non-structural protein NS3 get free and form a complex with NS4A that could act as serine protease. Rest of the non-structural protein processing is exploited by this protease [64].

Induction of membrane curvature: While translation process increases the local concentration of viral proteins, they assemble into replication complex (RC) with the viral RNA at the membrane of rough endoplasmic reticulum [66]. Initially, RC is formed with the proliferation and invaginations of the ER membranes [67]. Presumably, this process is induced by NS4B and NS3, however other cellular and viral proteins are also identified with this complex. It is thought that NS4B induces curvature by inserting its amphipathic helix into ER membranes [68]. Oligomerization might then lead to increase the complex that force the membrane to remain curved [69]. These curved structures give rise to membranous vesicles or vesicle packets **(Figure 6)** that have been described as the site for viral replication. The interior of the vesicles are connected with the surrounding cytosol via a neck- like structure that allows constant supply of nucleotides for RNA synthesis [32, 68].

Viral Replication: According to in vitro studies 3′ UTR of HCV RNA fold back intra-molecularly and hybridize with 5′ UTR, generating a free 3′ OH end at the 5′ terminal that utilizes as primer of RNA dependent RNA polymerase (RdRp). Here, RdRp along with other host factors initiates replication by "copy-back" mechanism producing a single product about twice the length of the initial template RNA [70, 71]. However, in the presence of higher GTP or ATP concentration, RdRp could initiate de novo RNA synthesis. Concerning the template specificity, NS5B seems to bind preferentially to a sequence in its own 3′ coding region. Alternatively, the template specificity may be accomplished by the higher local concentration of both NS5B and the viral RNA within the replicase complex [72]. Efficient RNA replication also varies with other viral and host factors [58].

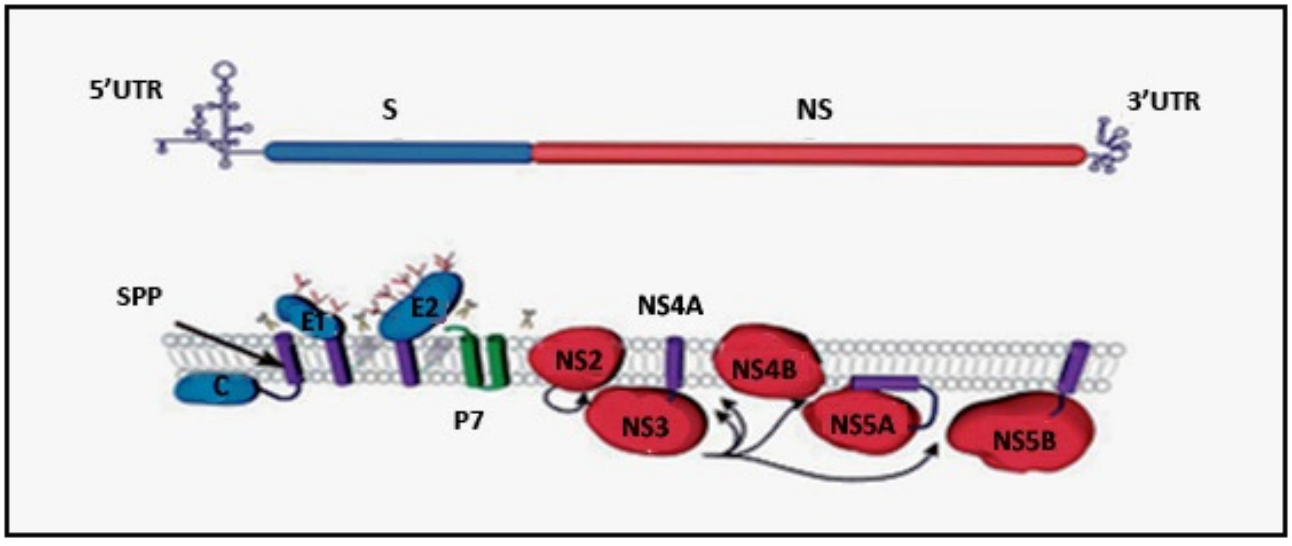

Figure 5. HCV poly-protein processing and its membrane topology. HCV encodes a single polyprotein with the structural proteins (S) and the non-structural proteins (NS). Scissors indicate cleavages by a host signal peptidase. Arrows indicate NS2-3 and NS3-4A cleavages. The intramembrane arrow indicates cleavage by a host signal peptide peptidase (SPP)

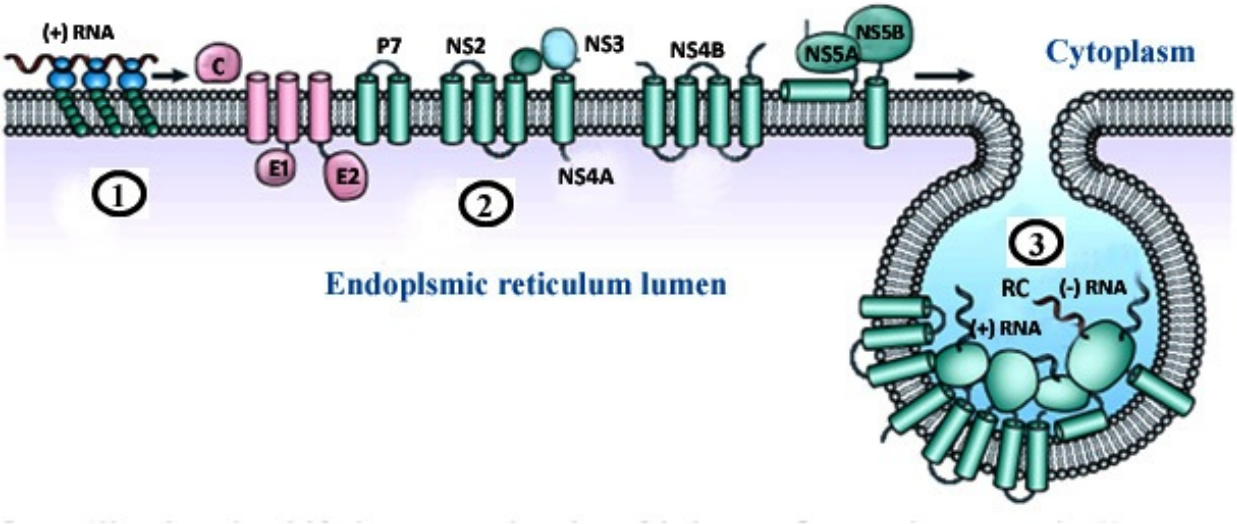

Figure 6. Topology of the HCV replication complex. (1) The viral genome is translated into a poly-protein (2) Poly-protein processed into structural (pink) and non-structural (green) proteins; (2) (3) viral non-structural protein NS4B induces the formation of membrane alterations which serve as a scaffold for the viral replication complex (RC) assembly

HCV RNA replication starts with the synthesis of a negative strand RNA genome which in turn serves as template for multiple rounds of nascent, positive-strand RNA synthesis, leading to an asymmetric accumulation of nearly 10 plus (+) strand RNA for every single minus-strand RNA [73].

Virion assembly and release: Since HCV RNA replication occurs at specialized sites on ER membrane as its replicated genomes needs to contact with core protein for virion assembly. Because of core is located on the cytosolic side of the ER membrane, assembly probably initiates in the cytosol before further maturation, and release occurs by transfer of nascent particles across the ER membrane to enable access to the secretory pathways in hepatocytes [74]. Several studies have identified cytosolic storage organelles, termed LDs, and the VLDL assembly pathway that occurs in the ER lumen as major contributors from the host cell to virion assembly which form virion capsid [75]. Little is known about the late steps of the viral lifecycle, as these have only recently become amenable to systematic study. NS2 and possibly other non-structural proteins, as well as yet to be defined RNA structure, are involved in these processes [11].

5. Proposed Mechanistic Model of HCV Infection

HCV carries only a positive sense RNA during invasion of host cell. It generally enters into the host cell via receptor mediated endocytosis and releases only its genetic material in to the host cell cytoplasm. Primarily, a number of cytoplasmic host proteins unite with viral genome and escort it to endoplasmic reticulum ER. A number of host proteins specially interact with conserved 5′ and 3′ UTR of viral RNA and assists long-range RNA-RNA interaction ensuring its full length genome circularization. Circular genome actually forms partially dsRNA which activates IFNα/β and PKR pathways. dsRNA activated protein kinase R (PKR) phosphorylates translation initiation factor eIF2α and turned off regular protein synthesis. Though, phosphorylated eIF2α could turn back by GADD34-pptase1 de-phosphorylation pathway and revive regular host cell protein synthesis.

However, the viral genome is so much tricky and between these phenomenons it can continue its translation through IRES mediated eIF2 independent pathways. In this case, translation initiation factor eIF3 and 40s ribosomal subunit directly bind 5′ IRES and form RNA-40s complex in which initiation codon is placed at the P site of ribosomal complex. Along with other host factor especially eIF5B and 60s ribosome 5′ viral genome form a functional translation initiation complex [46]. Ribosomal complex then carry out complete protein synthesis by eIF5B-GTP and charged tRNA recycling process. Viral genome synthesize as poly-protein which then undergoes for two stride proteolytic cleavage. The first stride structural proteins are processed by host single peptidase and the non-structural proteins are cleaved by viral NS3-NS4 and NS2 proteases.

The processed non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) are assembled in to replication complex (RC) to synthesize complementary RNA molecule yielding viral genome a double stranded replicative form (RF). The minus strand of RF then utilized by replication complex (RC) to synthesize manifold positive (+) strand RNA. The newly synthesized (+) strand RNA then goes either for second round translation or virion assembly. Structural protein and (+) strand RNA assemble at specialized sites on ER membrane. Further maturation and packaging occurs by transfer of nascent particles across the ER membrane to golgi bodies. Finally, complete packaged virus particle released out by host cell secretary pathway.

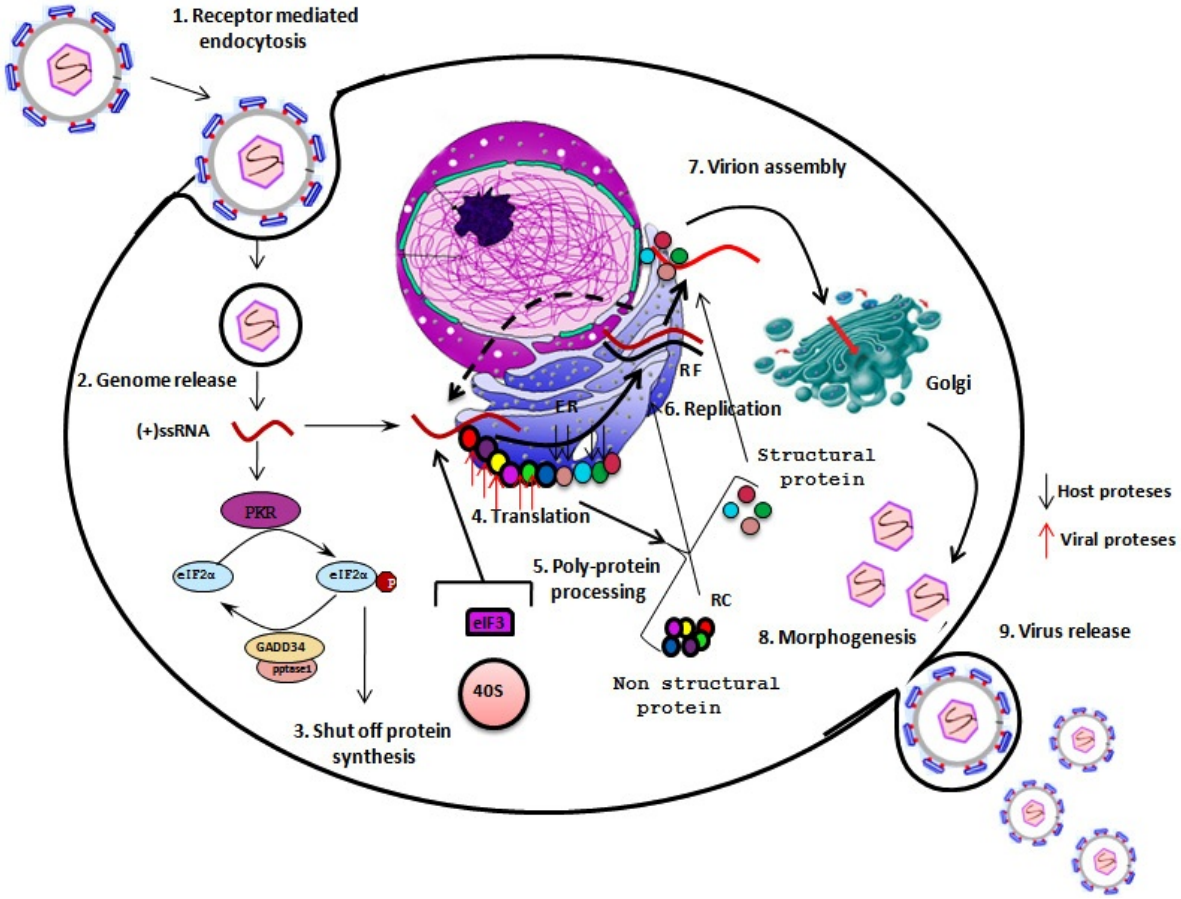

Figure 7. Hepatitis C virus infection mechanism. 1) HCV enters into host cell via receptor mediated endocytosis. 2) Viral positive strand RNA genome release into in host cell cytoplasm. 3) PKR phosphorylates eIF2 and shut off regular protein synthesis. GADD34-pptase further de phosphorylates eIF2 4) eIF3 and 40s ribosomal subunit directly bind 5′ IRES and along with other factors initiate viral translation in cap independent pathway 5) Viral poly-protein processed by host cell and viral proteses 6) HCV non-structural proteins are assemble into replication complex (RC) that carried out viral replication 7) Structural protein and (+) strand RNA assemble at specialized sites on ER membrane 8) Morphogenesis of complete packaged viral particle 9) Virus particle released out by host cell secretory pathway

6. Conclusions

The establishment of HCV infection requires temporal, spatial and mechanistic coordination of the molecular processes of viral replication. Sequences and secondary structures within the HCV genome dynamically interact with viral and host proteins to regulate translation and replication. While we have detailed knowledge of the cis and trans elements required for HCV protein and RNA synthesis, many questions about the coordination of these processes remain to be answered. Continuous development of molecular, biochemical and cell biological tools will facilitate elucidation of the precise mechanisms of first- and second-round translation and minus- and plus-strand replication while advances in proteomics and genomics are likely to provide information about the role of host factors in these processes. The HCV field is at an exciting point where new tools and approaches are advancing our understanding of the intricacies of HCV translation and replication.

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