Foundations for Understanding Antiviral Therapies in HCV

1 HCV Replication

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Introduction

Hepatitis C virus (HCV) is classified in the *Hepacivirus* genus within the family *Flaviviridae* and is the leading cause of chronic hepatitis and liver disease related morbidity worldwide. With an estimated 170 million people infected worldwide and the ability of the virus to establish a chronic infection in approximately 70% of cases, it is not surprising that HCV represents a major cause of global suffering and morbidity and a burden to many public health systems. Chronic HCV infection is often associated with development of serious liver disease, including cirrhosis, liver failure, and hepatocellular carcinoma. Accordingly, a thorough understanding of the life cycle and molecular biology of HCV and its interaction with the host are essential in the development of treatment and vaccine strategies. Although these studies have been hampered by the lack of a small-animal model and, until recently, a lack of a tissue culture system that accurately reflects the life cycle of HCV, significant progress has been made in the understanding of HCV molecular biology and pathogenesis. In this chapter we discuss recent advances in models to study HCV replication and the HCV life cycle.

The HCV Genome

HCV possesses a 9.6 kb single-stranded, positive-sense RNA genome composed of a 5′ UTR (untranslated region), a long open-reading frame (ORF) encoding a polyprotein of approximately 3000 amino acids, and a 3′ UTR (Figure 1.1). The polyprotein can be divided into three segments based on the functional aspects of the proteins: the NH2 terminal region comprises the structural proteins (core, E1, and E2); a central region consists of two proteins (p7 and NS2) that are not involved in HCV replication or are structural components of the virus, but probably play a role in virion morphogenesis; and the COOH-terminal proteins (NS3, NS4A, NS4B, NS5A, and NS5B) that are required for HCV replication (Figure 1.1). A detailed description and function of the HCV proteins can be found in an excellent review from Moradpour and colleagues [1]. After release of the HCV genome into the cytoplasm the genome is exposed to the host cellular machinery for translation of the viral polyprotein. The 5′ and 3′ UTRs are highly conserved and critical to viral genome replication and translation of the viral polyprotein. The 5′ UTR is approximately 341 nucleotides long and contains a highly structured RNA element known as the internal ribosome entry site (IRES) that is recognized by the cellular 40S ribosomal subunit to initiate translation of the RNA genome in a cap-independent manner. The importance of the secondary and tertiary structure of the IRES domain for initiation of translation has been demonstrated by mutational analysis. However, the primary sequence, particularly in stem-loop IIId and IIIe, is also critical for efficient HCV IRES activity [2,3]. Recently, the structural nature of HCV IRES interactions with the 40S ribosomal subunit and the eIF3 complex has been revealed by cryo-electron microscopy [4,5]. Preceding the IRES at the extreme 5′ end are elements required for viral replication that overlap partially with the IRES region (domain II), leading to speculation that this region is involved in regulation of a viral translation to replication switch [6]. Consistent with this speculation is the recent observation that a short highly conserved RNA segment at the 5′ end of the HCV genome binds a liver-specific
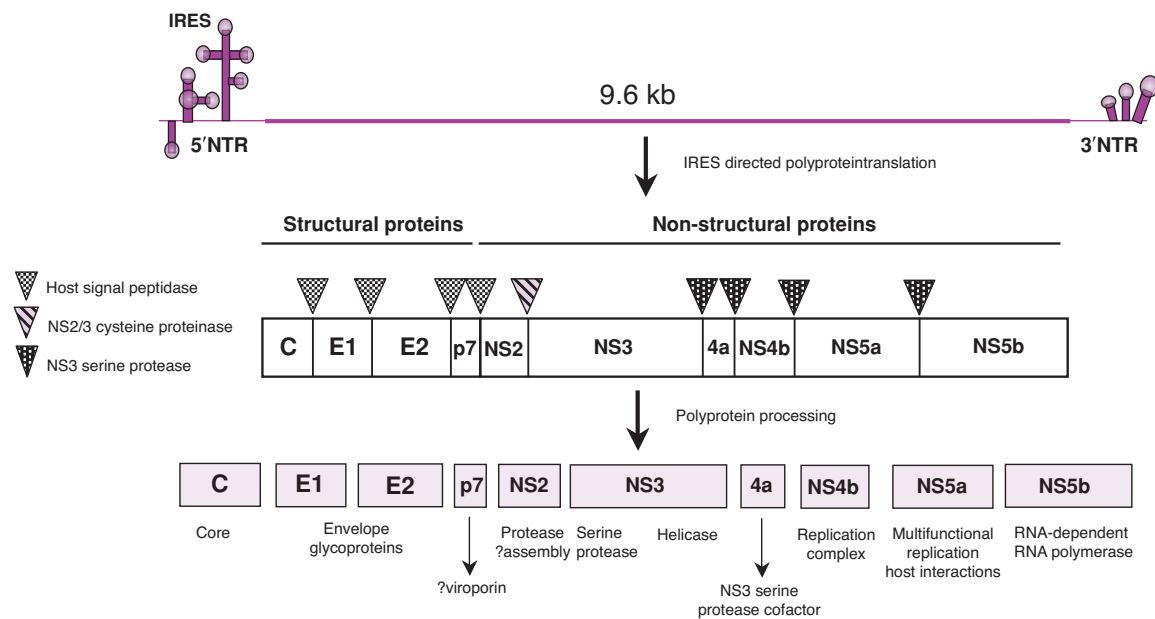
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Figure 1.1 Genomic organization and polyprotein processing of the HCV genome. The HCV genome consists of a positive-stranded RNA genome that is flanked by 5′ and 3′ UTRs of highly ordered secondary structure. The polyprotein is cleaved by either host- or viral-encoded proteases (depicted by triangles) to liberate the mature structural and non-structural proteins.

microRNA, miR-122, that is required for efficient HCV replication in cultured hepatoma cells [7]. However, in the HCV-infected liver no correlation was noted between miR-122 abundance and levels of HCV RNA in the liver or serum, which suggests that the impact of miR-122 may be less prominent *in vivo* than *in vitro* [8]. miR-122 may impact HCV replication indirectly through stimulation of translation of the viral polyprotein by enhancing association of ribosomes with the IRES [9]. Although more work is required to determine its role in the HCV life cycle and pathogenesis, modulation of miR-122 expression and activity presents as an attractive target for future therapy.

Like the 5′ UTR, the 3′ UTR of the HCV genome contains a high degree of secondary structure. This region is 200–300 nucleotides in length and is comprised of three major elements involved in replication: (i) a variable region (30–50 nucleotides), which directly follows the NS5B stop codon; (ii) a polyuridine (U/C) tract (20–200 nucleotides); and (iii) a highly conserved region (98 nucleotides), known as the 3′ X region, which forms a three stem-loop structure [10–12]. Mutational analysis has revealed that the poly-U/C tract and the 3′ X region play a more important role than the variable region in the synthesis of negative-strand RNA [13].

Models to Study HCV Replication

HCV Replicons

The development of the subgenomic HCV replicon system, first reported in 1999, significantly enabled the study of HCV replication in cultured cells for the first time [14]. Replicons represent autonomously replicating HCV RNAs, and typically contain an in-frame insertion of a selectable antibiotic marker (e.g., neomycin phosphotransferase: G418) within the amino terminal HCV core sequence, followed downstream by a heterologous IRES from encephalomyocarditis virus (EMCV), a picornavirus, to drive internal translation of the downstream HCV open reading frame (NS2 to NS5B) (Figure 1.2). The minimal requirements for a viable HCV replicon are HCV-derived 5′ and 3′ termini and the non-structural proteins (NS3 to NS5B) that form the replication complex, however, replication-competent HCV replicons encoding the complete HCV polyprotein are viable [15,16]. Transfection of Huh-7 (hepatoma-derived) cells with synthetically derived transcripts followed by selection with G418 results in the establishment of cell lines that harbor autonomous
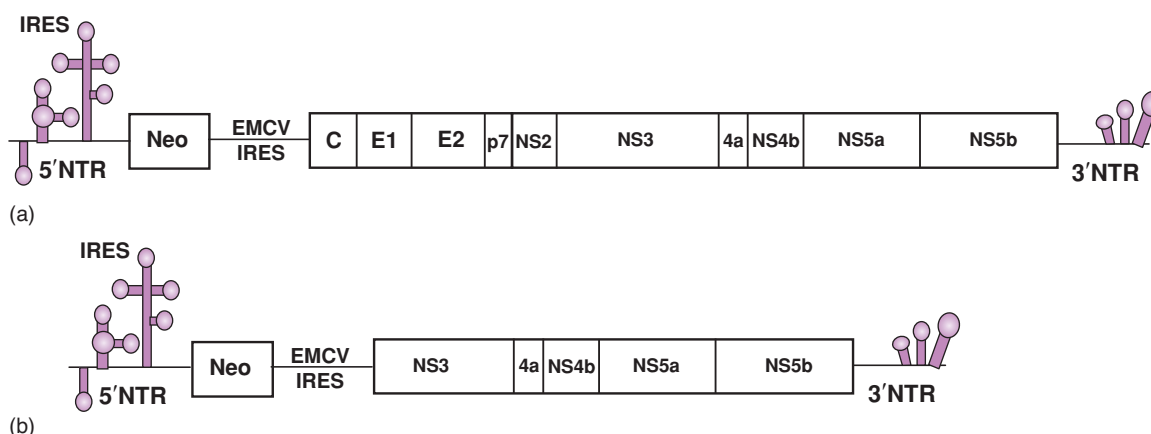
Figure 1.2 Schematic diagram of the organization of the (a) genomic and (b) subgenomic HCV replicons. In both cases the HCV IRES drives expression of the neomycin phosphotransferase gene while the EMCV IRES drives expression of the HCV proteins.

replication of the virus. HCV RNA isolated from cell lines under antibiotic selection often contains cell-culture adapted mutations that greatly enhance replication, although the molecular basis for this increased replication is unclear [15,17,18]. These adaptive mutations often map to the NS5A protein and may potentially influence phosphorylation, resulting in a hypophosphorylated state and increased replication. Adaptive mutations have also been mapped to NS3, NS4A, NS4B, and NS5B. Interestingly, these replication-adaptive cell-culture mutations have been shown to reduce *in vivo* infectivity in chimpanzees, highlighting the adaptive nature of these viruses derived from cell culture [19]. HCV replicons are not restricted to Huh-7 cells, and other cell lines such as HeLa and cells of murine origin have also yielded selected clones of replicating HCV, highlighting that HCV replication is not restricted to liver-derived cells of human origin [20,21].

The antibiotic selection process not only selects for HCV genomes with high replication capacity but also clones of Huh-7 cells that are highly permissive for HCV infection. One such cell line, Huh-7.5, has been "cured" of HCV by treatment with low doses of interferon-α, is hyperpermissive for HCV replication [22], and is clearly enriched for factors that promote replication and/or defects in innate viral sensing pathways. For example, Huh-7.5 cells have a spontaneous knockout of the dsRNA cellular sensing protein RIG-1 and do not mount a robust antiviral response to viral infection that allows for HCV replication. This highlights the importance of innate immune sensing in HCV infection and is con-

sistent with the ability of the HCV NS3/4A protein to cleave IPS-1, which is integral to the innate immune RIG-I pathway [23].

HCV replicons have been valuable tools for studying numerous aspects of the HCV life cycle and interaction with the host cell. However, their major limitation has been inability to produce infectious virus particles even when the complete complement of HCV proteins is expressed, for reasons that are not entirely clear [15,16,22]. The original replicon concept has undergone evolution and replicons are now available that contain various markers (e.g., GFP, luciferase) that allow quantitative assessment of HCV replication and have been useful in high-throughput screening of antiviral compounds.

Productive Viral Infection in Cell Culture

The recent identification in 2005 of a cloned HCV genome (genotype 2a), known as JFH-1, that is capable of initiating high-level replication in cell culture *and* production of infectious virus particles represents a major breakthrough in the pursuit of a cell-culture model for HCV [24,25]. In contrast to HCV replicon systems, transfection of Huh-7 cells with RNA synthesized *in vitro* from the cloned JFH-1 cDNA genome and a related genotype 2a chimera, FL-J6/JFH replicate efficiently in Huh-7 cells without the need for cell-culture adaptive mutations. Moreover, virus particles produced by these cells are infectious in chimpanzees and can be serially passaged *in vivo* [25] while the FL-J6/JFH virus can infect mice containing human liver grafts [24]. Interestingly, virus produced *in vivo* has a lower buoyant density than virus produced in cell

culture, suggesting association with low-density lipids [26]. This system represents a major advance in the study of virus-host interactions and the virus life cycle, all in the context of replicating virus. Similarly, the highly adapted genotype 1a HCV isolate known as H77-S (derived from the H77 isolate) [27] is also capable of instigating HCV RNA replication and production of infectious virus particles [28]. This represents another breakthrough in the generation of tools to study the HCV life cycle, particularly because this genotype is more prevalent worldwide and is associated with more significant liver disease. Intragenotypic and intergenotypic chimeras of HCV that contain the non-structural protein-encoding regions of JFH-1 and the structural protein-encoding regions of other HCV genomes may help define regions of structural proteins that influence the efficiency of virus particle synthesis and secretion [29]. This relatively new cell-culture model system will be invaluable in the study of many aspects of virus-host interaction, including viral entry, and assembly and release, which were previously inaccessible to manipulation.

The HCV Virion and Entry

The relatively low levels of HCV in plasma samples have hampered visualization of viral particles; however, virus-like particles have been identified by electron microscopy, which has indicated that infectious HCV virions are roughly spherical particles of diameter 55–65 nm with fine projections of approximately 6 nm ([25] and references therein). The major protein constituents of the host-derived lipid bilayer envelope are the highly glycosylated HCV envelope glycoproteins E1 and E2 that surround the viral nucleocapsid, composed of many copies of the HCV core protein and the genomic HCV RNA (Figure 1.3). HCV from serum and plasma fractionates with a wide range of buoyant densities that can be attributed to association of the virus with lipoproteins, in particular apolipoprotein-B (Apo-B) and apolipoprotein-E (Apo-E), which are components of host low density lipoprotein (LDL, Apo-B) and very low density lipoprotein (VLDL, Apo-B, Apo-E) and suggest a close association with circulating LDL/VLDL [26]. The physiological association of HCV with LDL/VLDL remains unexplained mechanistically; however, it could be involved in viral uptake (see below), or alternatively the association of Apo-B with HCV virions may indicate a role for the hepatic LDL/VLDL secretory pathway in release of the virus.
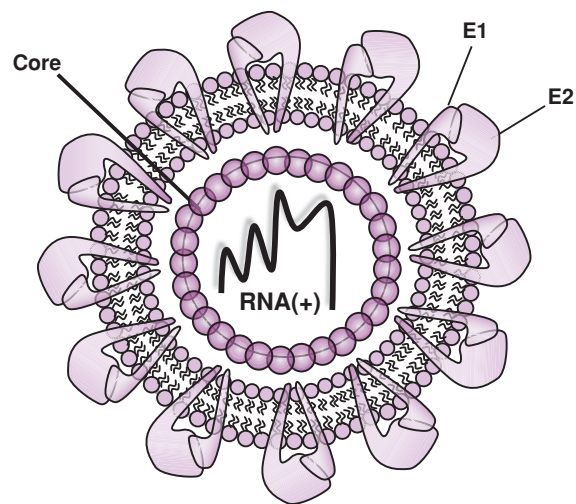

Figure 1.3 The HCV particle. The RNA genome is encapsidated by the icosahedral nucleocapsid consisting of the core protein. The nucleocapsid is enveloped by a host-derived spherical lipid bilayer that is enriched with heterodimers of the envelope glycoproteins E1 and E2.

Hepatocytes are the main target for infection with HCV; however, identification of the cellular receptors responsible for HCV entry has proven difficult due to the lack of appropriate model systems. However, using a combination of HCV pseudotyped particles (HCVpp) [30,31] and cell-culture-derived HCV (HCVcc) [25], the complement of HCV receptors now seems complete.

The 25 kDa tetraspanin molecule CD81 and the human scavenger receptor class B type I (SR-BI) both bind HCV E2 and are necessary but not sufficient for HCV entry [32]. For example, CD81 ectopic expression in hepatocyte-derived cell lines that are negative for CD81 confers susceptibility to HCVpp and HCVcc; however, expression of both factors in non-hepatocyte-derived cell lines does not concur infectivity [24,30]. Clearly additional hepatocyte factors are required for HCV entry. Using an interactive cloning and expression approach, the tight junction protein claudin-1 (CLDN1) was recently identified as an HCV co-receptor [33]. CLDN1 was found to be essential for entry into hepatic cells and rendered non-hepatic cells susceptible to infection. However, despite the identification of CD81, SR-B1, and CLDN1 as essential HCV entry co-factors, a number of human cell lines and those of non-primate origin remained resistant to HCV infection, suggesting an additional entry factor. Using a cyclic lentivirus-based screen of a cDNA library derived from a highly HCV-permissive hepatocarcinoma cell line (Huh-7.5) for

genes that render the non-permissive CD81+, SR-BI+ 293T cell line infectable with HCVpp, the remaining crucial factor was recently identified as occludin (OCLN), also a tight junction protein [34]. Although expression of all four entry factors (CD81, SR-B1, CLDN1, OCLN) renders mouse cell lines susceptible to HCVpp infection, these cells could not support HCVcc infection. This is not surprising given past reports of inefficient replication of HCV RNA in mouse cell lines, and suggests that specific hepatocyte factors are crucial for efficient HCV replication. The identification of CD81 and OCLN as the minimal human-specific entry factors (HCV can bind to murine SR-B1 and CLDN1) not only significantly advances our understanding of the molecular mechanisms of HCV entry but also provides important steps for the development of a mouse model of HCV infection and provides an attractive target for the development of novel antiviral strategies.

Other molecules have been suggested to be involved in HCV entry. The association of HCV virions in serum with LDL and VLDL suggests that the LDL receptor (LDLR) may be an attractive candidate receptor. However, its precise role remains to be determined [26,35]. LDLR is not sufficient itself for entry and it does not bind directly to HCV E2 [30]. Together with the glycosaminoglycans, the LDLR in concert with other cell-surface proteins may serve to collect HCV at the cell surface and facilitate binding with receptors crucial for HCV entry. Consistent with this, a role has been proposed for L-SIGN and DC-SIGN in HCV attachment although they do not seem to mediate cell entry of HCV and their role is unclear [36].

The precise molecular events underlying HCV binding and entry are not well understood. However, HCV binding to the cell surface is thought to occur in a stepwise process by binding to several receptors followed by transfer to the tight junction proteins CLDN1 and OCLN that may facilitate cellular uptake (Figure 1.4). Similar to other flaviviruses, HCV entry is thought to be mediated by clathrin-mediated endocytosis with delivery of the nucleocapsid from the endosome in a pH-dependent manner [37–39]. Furthermore, the E1 and E2 proteins are class II fusion proteins that result from the production of a fusion pore in the endosome membrane that facilitates genome release to the cytoplasm [40].

HCV Replication

The HCV replication process is summarized in Figure 1.5. After translation of the HCV proteins from the positive-sense RNA genome by direct interaction of the host 40S ribosomal subunit with the IRES within the 5′ UTR of the genome, HCV replication begins. This IRES-directed translation is cap-independent and enables virus translation/replication to continue even after host cell cap-dependent translation has been shut down in response to viral infection.

Similar to other positive-stranded viruses, HCV is believed to replicate in association with intracellular membranes in a complex called the membranous web, although the exact details of this association are not well understood. It is thought that the association predominantly with endoplasmic reticulum (ER) membranes may provide support for the organization of the replication complex, compartmentalization of the viral products, concentration of lipid constituents important for replication, and protection of the viral RNA from host-mediated innate immune defenses. This membranous web was first noticed in cultured cells harboring HCV replicons and contains detectable concentrations of the non-structural proteins NS3, NS4A, NS4B, NS5A, and NS5B, and is very similar to sponge-like inclusions noted in liver tissue from HCV-infected chimpanzees [41–44]. Expression of NS4B alone induces the formation of the membranous web, and recent work has shown that membrane association is facilitated by amino acids 40 to 69 of the N-terminal region of NS4B [45].

The phosphorylation status of NS5A appears to be a determinant of HCV RNA replication with mutations that reduce hyperphosphorylation of NS5A dramatically enhancing HCV RNA replication [46,47]. In this manner, hyperphosphorylation of NS5A may induce a switch from genome replication to viral protein translation. NS5A also interacts with several host proteins that may be important in HCV replication through formation of the replication complex or facilitating assembly. NS5A interacts with the SNARE-like vesicle-associated membrane host proteins, VAP-A and VAP-B [48]. NS5A also interacts with geranylgeranylated F-box protein, FBL2, which is essential for replication and seems to be part of the replication complex [49]. How this interaction contributes to replication is unclear but it may help anchor the replicase complex to membranes. Its involvement in the replication process highlights the close interaction between HCV replication and the host cholesterol biosynthetic pathway [50]. Another host factor, cyclophilin B, has also been implicated in HCV replication through interaction with NS5B and stabilization of RNA binding, and was originally discovered through the ability of the powerful immunosuppressive drug cyclosporin A (CsA) to inhibit HCV replication [51]. However, more recent work suggests that
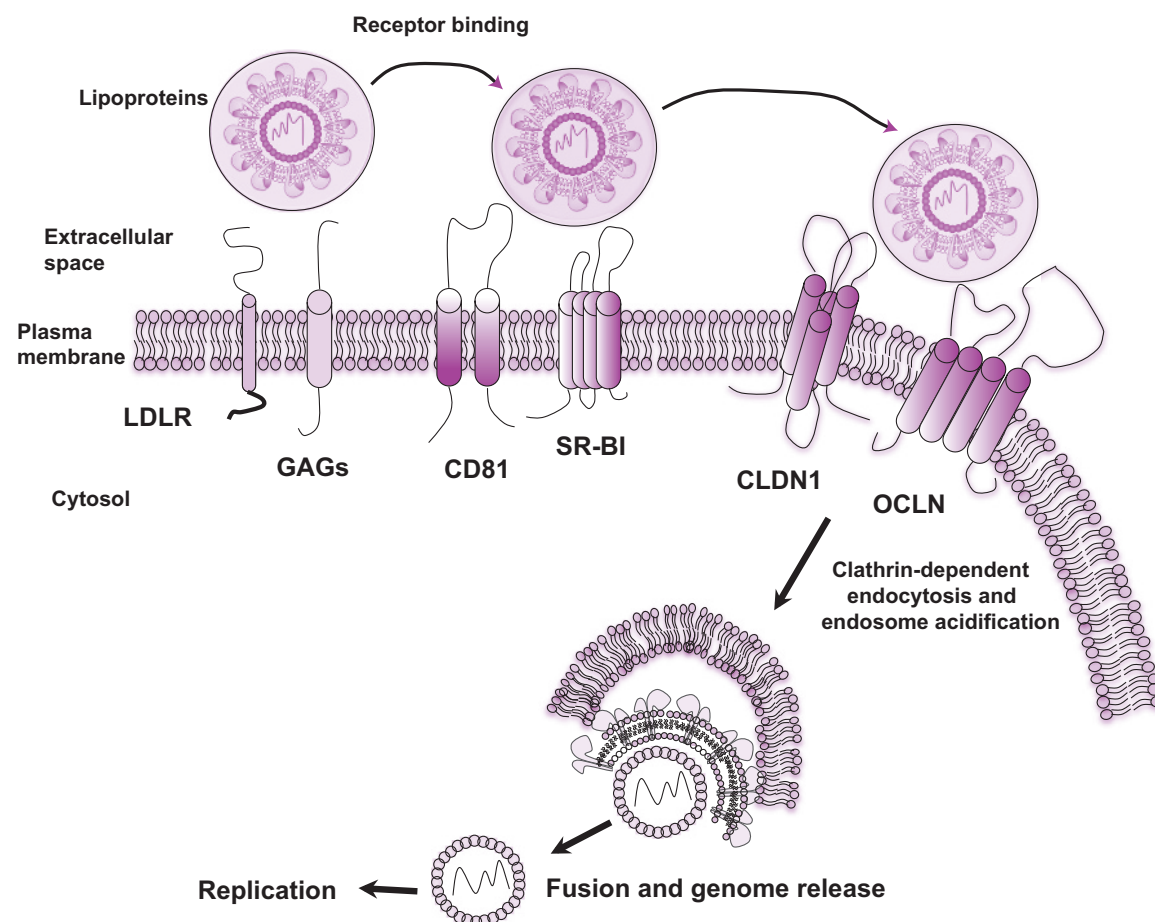
Figure 1.4 Model for HCV entry. HCV particles associated with LDL and VLDL are thought to be tethered to the hepatocyte surface by the LDL-R and GAGs and subsequent stepwise interactions with CD81 and SR-B1. HCV is transferred to the tight junction proteins OCLN and CLDN-1 from where virus enters the cell by endocytosis. Release of the HCV core containing the RNA is mediated by fusion of the E1 and E2 proteins with the endosome. The relative roles and the spatial distributions of each of the HCV receptors remain to be determined.

cyclophilin A plays a critical role in cleavage of NS5A/5B and assembly of the replication complex [52,53]. CsA analogs are currently being developed as antivirals against HCV [54].

The precise details of the HCV RNA replication process are still unclear but comparison with other flaviviruses suggests that the positive-stranded genome serves as a template for the synthesis of negative-strand RNA. Components of the membrane-bound replication complex associate with the 3′ end of the positive strand of the genome, with NS5B at the catalytic core, and initiate *de novo* synthesis of negative-strand RNA. These two strands remain base-paired, which results in the formation of a double-stranded RNA molecule that is copied multiple times by semiconservative replication by the RNA-dependent RNA polymerase (RdRp) NS5B to generate multiple progeny, positive-strand viral RNA genomes. Importantly, the NS5B RdRp has no proofreading capacity and as such is error prone. This lack of proofreading ability results in the generation of many different but closely related genomes, often referred to as quasispecies. This genetic diversity is ideally suited to escape of immune control and is a significant factor in the generation of antiviral resistance to select antiviral agents. While a proportion of new positive-strand genomes serve as templates for viral protein translation, others associate with
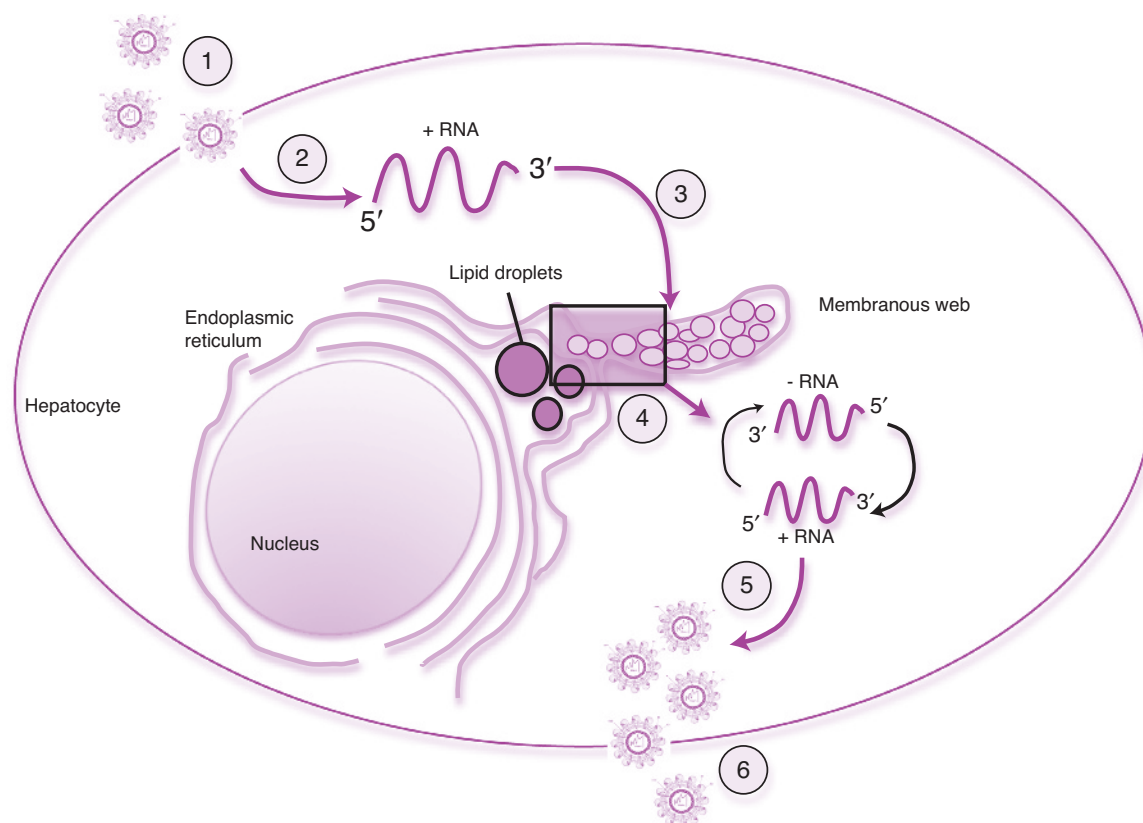
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Figure 1.5 Lifecycle of HCV. 1, Virus binding and internalization; 2, release of HCV RNA and translation of viral proteins; 3, association of HCV proteins in ER and formation of the replication complex in association with lipid droplets, replication of HCV RNA; 5, virion maturation and packaging of RNA; 6, release of virions.

the core protein and form dimers within core-protein-enriched nucleocapsids. The association of the core protein with cytoplasmic lipid droplets has emerged as a critical determinant of nucleocapsid and infectious viral particle assembly. It is thought that the core uses this platform to recruit replication complexes and associated new genomes from closely associated ER-derived "lipid-droplet-associated membranes" in the assembly process [55,56]. Core particles may then become enveloped via budding through the ER where viral glycoproteins (E1/E2 heterodimers) become embedded. Little is known about the process of viral particle egress except that particles change in their biophysical properties (increased density) upon exit. Recent studies have indicated that the processes of HCV particle assembly, maturation, and secretion are dependent upon the machinery involved in the assembly and secretion of VLDL by hepatocytes [57].

The development and use of *in vitro* cell-culture model systems described above has been and continues to be fundamental in dissecting the stages of HCV replication and identification of viral-host interactions at the molecular level (Figure 1.5). While these studies are important for our understanding of HCV biology, they also provide specific targets for the development of novel therapeutics designed to completely eradicate HCV infection across all genotypes. Current therapies for HCV focus on modulation of the host immune response. However, with a greater understanding of HCV replication and host interactions, we are currently in a phase of developing therapeutics that directly target various stages of the HCV life cycle. Drugs targeting HCV entry and fusion, viral helicase, and polymerase or protease function are all under clinical investigation, with some showing exceptional promise. These targeted therapies when used in combination with the

current therapeutic regime of Peg-IFN alfa-2 and ribavirin will provide the foundation for systemic eradication of HCV in infected persons. Furthermore, defining novel host factors essential for HCV replication and a greater understanding of the immunological correlates of immunity to HCV will provide the cornerstone for further development of novel therapeutics to combat HCV infection.

References

1. Moradpour D, Penin F, Rice CM. Replication of hepatitis C virus. *Nat Rev Microbiol* 2007;5(6):453–63.

2. Kieft JS, Zhou K, Jubin R, *et al.* The hepatitis C virus internal ribosome entry site adopts an ion-dependent tertiary fold. *J Mol Biol* 1999;292(3):513–29.

3. Psaridi L, Georgopoulou U, Varaklioti A, Mavromara P. Mutational analysis of a conserved tetraloop in the 5′ untranslated region of hepatitis C virus identifies a novel RNA element essential for the internal ribosome entry site function. *FEBS Lett* 1999;453(1–2):49–53.

4. Siridechadilok B, Fraser CS, Hall RJ, *et al.* Structural roles for human translation factor eIF3 in initiation of protein synthesis. *Science* 2005;310(5753):1513–15.

5. Spahn CM, Kieft JS, Grassucci RA, *et al.* Hepatitis C virus IRES RNA-induced changes in the conformation of the 40s ribosomal subunit. *Science* 2001;291(5510):1959–62.

6. Appel N, Schaller T, Penin F, Bartenschlager R. From structure to function: new insights into hepatitis C virus RNA replication. *J Biol Chem* 2006;281(15):9833–6.

7. Jopling CL, Yi M, Lancaster AM, *et al.* Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 2005;309(5740):1577–81.

8. Sarasin-Filipowicz M, Krol J, Markiewicz I, *et al.* Decreased levels of microRNA miR-122 in individuals with hepatitis C responding poorly to interferon therapy. *Nat Med* 2009;15(1):31–3.

9. Henke JI, Goergen D, Zheng J, *et al.* MicroRNA-122 stimulates translation of hepatitis C virus RNA. *EMBO J* 2008;27(24):3300–10.

10. Blight KJ, Rice CM. Secondary structure determination of the conserved 98-base sequence at the 3′ terminus of hepatitis C virus genome RNA. *J Virol* 1997;71(10): 7345–52.

11. Kolykhalov AA, Feinstone SM, Rice CM. Identification of a highly conserved sequence element at the 3′ terminus of hepatitis C virus genome RNA. *J Virol* 1996;70(6):3363–71.

12. Tanaka T, Kato N, Cho MJ, *et al.* Structure of the 3′ terminus of the hepatitis C virus genome. *J Virol* 1996;70(5): 3307–12.

13. Yi M, Lemon SM. 3′ nontranslated RNA signals required for replication of hepatitis C virus RNA. *J Virol* 2003;77(6): 3557–68.

14. Lohmann V, Korner F, Koch J, *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999;285(5424):110–13.

15. Ikeda M, Yi M, Li K, Lemon SM. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. *J Virol* 2002;76(6):2997–3006.

16. Pietschmann T, Lohmann V, Kaul A, *et al.* Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J Virol* 2002;76(8):4008–21.

17. Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. *Science* 2000;290(5498):1972–4.

18. Lohmann V, Korner F, Dobierzewska A, Bartenschlager R. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J Virol* 2001;75(3):1437–49.

19. Bukh J, Pietschmann T, Lohmann V, *et al.* Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. *Proc Natl Acad Sci U S A* 2002;99(22):14416–21.

20. Kato T, Date T, Miyamoto M, *et al.* Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon. *J Virol* 2005;79(1):592–6.

21. Zhu Q, Guo JT, Seeger C. Replication of hepatitis C virus subgenomes in nonhepatic epithelial and mouse hepatoma cells. *J Virol* 2003;77(17):9204–10.

22. Blight KJ, McKeating JA, Rice CM. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* 2002;76(24):13001–14.

23. Foy E, Li K, Wang C, *et al.* Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* 2003;300(5622):1145–8.

24. Lindenbach BD, Evans MJ, Syder AJ, *et al.* Complete replication of hepatitis C virus in cell culture. *Science* 2005;309(5734):623–6.

25. Wakita T, Pietschmann T, Kato T, *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005;11(7):791–6.

26. Nielsen SU, Bassendine MF, Burt AD, *et al.* Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. *J Virol* 2006;80(5):2418–28.

27. Kolykhalov AA, Agapov EV, Blight KJ, *et al.* Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* 1997;277(5325):570–74.

28. Yi M, Villanueva RA, Thomas DL, *et al.* Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc Natl Acad Sci U S A* 2006;103(7):2310–15.

29. Pietschmann T, Kaul A, Koutsoudakis G, *et al.* Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci U S A* 2006;103(19):7408–13.

30. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J Exp Med* 2003;197(5):633–42.

31. Drummer HE, Maerz A, Poumbourios P. Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins. *FEBS Lett* 2003;546(2–3):385–90.

32. Pileri P, Uematsu Y, Campagnoli S, *et al*. Binding of hepatitis C virus to CD81. *Science* 1998;282(5390):938–41.

33. Evans MJ, von Hahn T, Tscherne DM, *et al*. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 2007;446(7137):801–5.

34. Ploss A, Evans MJ, Gaysinskaya VA, *et al*. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 2009;457(7231):882–6.

35. Agnello V, Abel G, Elfahal M, *et al*. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc Natl Acad Sci U S A* 1999;96(22):12766–71.

36. Lozach PY, Amara A, Bartosch B, *et al*. C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. *J Biol Chem* 2004;279(31):32035–45.

37. Blanchard E, Belouzard S, Goueslain L, *et al*. Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J Virol* 2006;80(14):6964–72.

38. Koutsoudakis G, Kaul A, Steinmann E, *et al*. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *J Virol* 2006;80(11):5308–20.

39. Tscherne DM, Jones CT, Evans MJ, *et al*. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. *J Virol* 2006;80(4):1734–41.

40. Drummer HE, Poumbourios P. Hepatitis C virus glycoprotein E2 contains a membrane-proximal heptad repeat sequence that is essential for E1E2 glycoprotein heterodimerization and viral entry. *J Biol Chem* 2004;279(29):30066–72.

41. Egger D, Wolk B, Gosert R, *et al*. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* 2002;76(12):5974–84.

42. Gosert R, Egger D, Lohmann V, *et al*. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. *J Virol* 2003;77(9):5487–92.

43. Moradpour D, Brass V, Bieck E, *et al*. Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication. *J Virol* 2004;78(23):13278–84.

44. Moradpour D, Gosert R, Egger D, *et al*. Membrane association of hepatitis C virus nonstructural proteins and identification of the membrane alteration that harbors the viral replication complex. *Antivir Res* 2003;60(2):103–9.

45. Gouttenoire J, Montserret R, Kennel A, *et al*. An amphipathic {alpha}-helix at the C terminus of hepatitis C virus nonstructural protein 4B mediates membrane association. *J Virol* 2009;83(21):11378–84.

46. Appel N, Pietschmann T, Bartenschlager R. Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. *J Virol* 2005;79(5):3187–94.

47. Evans MJ, Rice CM, Goff SP. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc Natl Acad Sci U S A* 2004;101(35):13038–43.

48. Hamamoto I, Nishimura Y, Okamoto T, *et al*. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. *J Virol* 2005;79(21): 13473–82.

49. Wang C, Gale M, Jr., Keller BC, *et al*. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Mol Cell* 2005;18(4): 425–34.

50. Ye J, Wang C, Sumpter R, Jr., Brown MS, *et al*. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. *Proc Natl Acad Sci U S A* 2003;100(26):15865–70.

51. Watashi K, Ishii N, Hijikata M, *et al*. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* 2005;19(1):111–22.

52. Kaul A, Stauffer S, Berger C, *et al*. Essential role of cyclophilin A for hepatitis C virus replication and virus production and possible link to polyprotein cleavage kinetics. *PLoS Pathog* 2009;5(8):e1000546.

53. Liu Z, Yang F, Robotham JM, Tang H. Critical role of cyclophilin A and its prolyl-peptidyl isomerase activity in the structure and function of the hepatitis C virus replication complex. *J Virol* 2009;83(13):6554–65.

54. Flisiak R, Feinman SV, Jablkowski M, *et al*. The cyclophilin inhibitor Debio 025 combined with PEG IFNalpha2a significantly reduces viral load in treatment-naive hepatitis C patients. *Hepatology* 2009;49(5):1460–68.

55. Miyanari Y, Atsuzawa K, Usuda N, *et al*. The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* 2007;9(9):1089–97.

56. Shavinskaya A, Boulant S, Penin F, *et al*. The lipid droplet binding domain of hepatitis C virus core protein is a major determinant for efficient virus assembly. *J Biol Chem* 2007;282(51):37158–69.

57. Gastaminza P, Cheng G, Wieland S, *et al*. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. *J Virol* 2008;82(5):2120–29.