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HCV Molecular Virology and Animal Models

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Contents

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Abstract Hepatitis C virus (HCV) infection was once considered a threat to life but is now curable. This miraculous achievement is the result of years of effort to understand basic HCV biology, which led to the development of HCV cell culture systems eventually enabling drug discovery. Initial studies focused on biochemical characterization of viral proteins and dissected their roles in the virus life cycle. Two of the viral proteins, NS3-4A protease and NS5B polymerase, were selected early on as potential drug targets, and subsequent collaborative efforts of academia and industry led to the development of highly effective inhibitors against these enzymes. Another HCV protein, NS5A that has no known enzymatic activity, was more recently identified as an unexpected target of a highly potent class of anti-HCV inhibitors. Various combinations of these protease, polymerase, and NS5A inhibitors now constitute the current anti-HCV regimens with cure rates of above 95%. This chapter is divided into two parts. The first part begins with a short introduction to HCV and its life cycle and reviews insights into biochemical and functional characteristics of HCV RNA elements and proteins. The second part discusses the HCV animal models and how their use yielded important insights into the viral life cycle, immunity, and disease pathogenesis.

Keywords HCV proteins, HCV replication, HCV RNA, Human liver chimeric mice, Non-primate hepaciviruses, Rodent hepaciviruses

1 Introduction

Identification of HCV in 1989 as the major cause of non-A, non-B chronic hepatitis elicited intense research. Initially, lack of suitable cell culture systems and small animal models slowed progress, but still great strides were made toward biochemical and functional characterization of various HCV RNA elements and proteins. These studies mainly relied on in vitro biochemical assays and/or expression of individual proteins in cell culture, and the results were at times validated in chimpanzees, the only HCV animal model available. Remarkable knowledge gained over these years set the stage for development of the first HCV in vitro replication system in 1999 [1]. This system uses truncated HCV genomes, called HCV subgenomic replicons that encode a subset of the viral nonstructural proteins and can autonomously replicate in cell culture. Although a great tool for studying various aspects of the HCV RNA replication, this system did not allow virus particle production. Eventually, success came in 2005 when the first efficient HCV cell culture (HCVcc) infection systems were reported [2, 3], finally allowing dissection of all the steps of the HCV life cycle. The HCVcc system has since been extensively used to expand our knowledge of HCV molecular biology.

2 HCV Genome Organization

HCV is a positive-sense, single-stranded RNA virus. Its genome is approximately 9.6 kb and comprises a long open reading frame (ORF) flanked at its 5′ and 3′ ends by untranslated regions (UTRs) [4–7] (Fig. 1). These UTRs contain important signals for replication and translation of the viral RNA. For example, the 5′ UTR has an internal ribosomal entry site (IRES) required for HCV RNA translation [9], and interaction between the 5′ and 3′ UTRs has been shown to be essential for genome replication [10–12]. Moreover, nucleotide sequences and RNA secondary structures in the ORF have also been implicated in translation and replication [13, 14].

The ORF encodes a polyprotein of just over 3,000 amino acids. This polyprotein then undergoes a series of cleavage events to generate three structural and seven nonstructural proteins. The structural proteins are produced at the N-terminus of the polyprotein and include the core protein and two viral glycoproteins, E1 and E2. The nonstructural proteins include the p7 viroporin, the NS2 protease, the NS3-4A protease/NTPase/RNA helicase, the NS4B membrane-remodeling protein, the NS5A phosphoprotein, and the NS5B RNA-dependent RNA polymerase (RdRp). The structural proteins and p7 are processed by two host proteases, signal peptidase

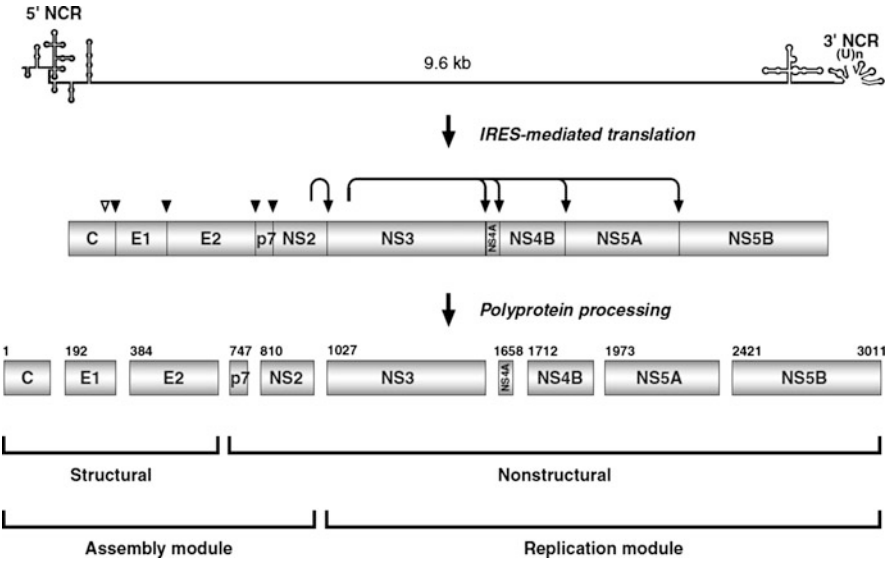

Fig. 1 HCV polyprotein processing. The HCV genome, shown on the top, is translated in an IRES-dependent manner to yield a polyprotein precursor that is processed by the host and viral proteases. Solid arrowheads indicate signal peptidase-mediated cleavages, while the open arrowhead indicates further C-terminal processing of the core protein by signal peptide peptidase. Arrows indicate cleavages by the HCV NS2 and NS3-4A proteases. Amino acid numbering above each protein refers to the HCV H strain (genotype 1a; GenBank accession number AF009606). Reproduced from "Hepatitis C Virus Proteins: From Structure to Function" [8], with permission from Springer

and signal peptide peptidase (SPP) [15], while maturation of the other proteins requires two viral proteases, NS2 and NS3-4A [16]. NS2 is an autoprotease that cleaves the polyprotein at the NS2/3 site, while all other cleavages are performed by NS3-4A.

3 Summary of the HCV Life Cycle

To enter a hepatocyte, HCV first interacts with glycans on the cell surface thereby docking onto the surface of the cell [17, 18]. The virus envelope proteins and virion-associated lipoproteins then interact with HCV receptors to facilitate virus internalization. Four key HCV receptors include scavenger receptor B1 (SR-B1), CD81, claudin-1 (CLDN1), and occludin (OCLN), which complete the viral entry process with the help of a number of host proteins, such as epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2), and Niemann-Pick C1-like1 (NPC1L1) (reviewed in [19]). HCV is a hepatotropic virus that only infects humans, and this specificity for human hepatocytes is at least partially defined by the entry step. For example, SR-B1, CLDN1, and NPC1L1 are highly expressed in hepatocytes and thought to be responsible for HCV hepatotropism, while CD81 and OCLN are critical for HCV human tropism – mouse cells refractory to HCV infection become susceptible when human CD81 and OCLN are expressed (reviewed in [20]).

Receptor-bound HCV is endocytosed in a clathrin-dependent process [21] and trafficked to early endosomes, where low pH-induced fusion between the viral and endosomal membranes releases the viral RNA into the cytoplasm [22, 23]. This RNA is then translated in an IRES-dependent manner to generate the ten major HCV proteins, which can be functionally classified into two modules: an assembly module (C-NS2, required for virion assembly) and a replication module (NS3-NS5B, components of the replicase complex) (Fig. 2). These modules do not operate in isolation though, as almost all replicase proteins have been shown to assist assembly (reviewed in [25]). The assembly module, however, is dispensable for HCV RNA replication.

HCV replicates its genome in a membrane-associated replication complex, which is composed of viral and host proteins [26]. Analogous to other positive-strand RNA viruses, HCV replication occurs in two steps, both of which are catalyzed by the viral NS5B polymerase. In the first step, HCV genomic RNA is used as a template to generate a complementary negative strand, which in turn serves as a template in the second step to produce molar excess of positive-strand progeny RNA. This progeny RNA has three possible fates: (1) it is translated to produce viral proteins, (2) it is used as a template to generate more negative-strand intermediates, or (3) it is trafficked to the assembly site where it is packaged into new virions.

Our understanding of how virions are assembled is incomplete. For assembly to occur, the HCV glycoproteins E1 and E2, the core protein, the newly generated viral RNA, and several viral and host proteins must come together. The current model posits that HCV core protein at the surface of lipid droplets (LD) recruits HCV

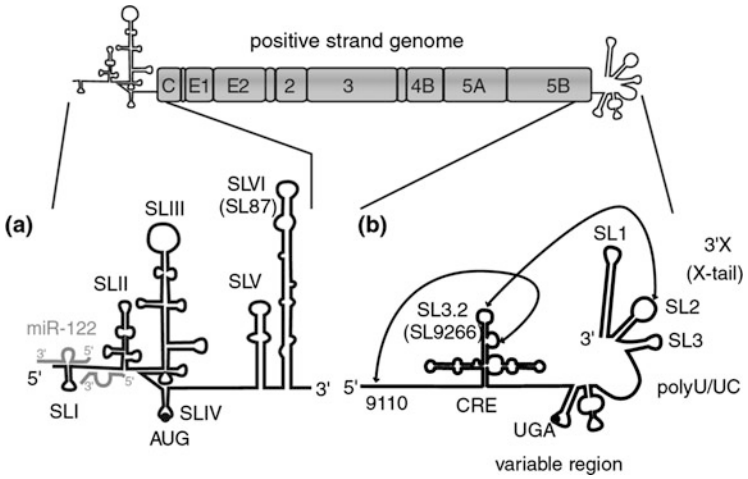

Fig. 2 Schematic representation of HCV genome. (**a**) 5′ UTR of the viral genome. Two copies of miR-122 bound to the genome are shown in gray. (**b**) 3′ UTR of the viral genome. Arrows indicate the long-range interactions of SL3.2 with sequences around 9,110 and with SL2 of the 3′ UTR. Reproduced from "Hepatitis C Virus RNA Replication" [24], with permission from Springer

replication complexes, primarily through its interaction with the NS5A protein [27]. This brings newly synthesized viral RNA in close juxtaposition to the assembly site and facilitates formation of nucleocapsids, which then acquire an HCV glycoprotein-studded envelope through budding into the ER lumen (reviewed in [28]). Once inside the ER lumen, the HCV particles appear to follow the canonical secretory pathway. Consistent with this, treating HCV infected cells with Brefeldin A, a potent inhibitor of ER-Golgi transport, blocks HCVcc egress and causes accumulation of infectious particles inside cells [29].

HCV particles further mature throughout the secretory pathway. This is reflected by differential biochemical properties of extracellular and intracellular particles. For example, the glycoproteins on the surface of extracellular virions contain complex glycans, whereas intracellular particles largely harbor simple, high-mannose glycans [30]. Since glycan maturation occurs in the Golgi, this suggests that HCV particles pass through Golgi en route to release at the cell membrane. Also, in contrast to the extracellular virions, intracellular HCV particles are highly sensitive to a low pH. It is believed that the ion channel activity of the HCV p7 protein blocks acidification of the secretory vesicles and protects the transiting virions from low pH [31]. Another difference is the comparatively lower buoyant density of the extracellular virions, implying that HCV undergoes post-synthesis lipidation, most likely in the post-ER compartment [29, 32].

Outside the cell, HCV exists as a lipoprotein-virus hybrid, sometimes called a lipo-viro-particle (LVP) that has an extremely low buoyant density (1.03–1.10 g/mL) [33, 34]. Sucrose gradient fractionation of HCVcc yields two virus populations: one with higher density but low specific infectivity and another of

low density and high infectivity [3, 35]. Interestingly, treatment with cholesterol, an essential component of the lipoproteins, improves the infectivity of the higher-density virus population [36]. Moreover, the lipid composition of highly purified infectious HCV particles closely resembles that of the lipoproteins [37]. However, despite compelling evidence that HCV is secreted from cells as LVP, it is unclear how and when during its assembly the virus associates with the lipoproteins or lipoprotein components. Future studies should shed light on this and several other aspects of the rather poorly understood steps of HCV assembly and egress.

4 Functional HCV RNA Elements

The HCV genomic RNA serves as a template for both translation and replication. Each of these processes requires *cis*-acting viral RNA elements and transacting cellular RNAs, such as micro-RNA-122 (miR-122). Since most of the functionally important *cis*-acting RNA elements are located in the HCV 5′ and 3′ UTRs, in this section, we will limit our discussion to these genomic regions.

4.1 The HCV 5′ UTR

The 5′ UTR is a highly structured 341 nucleotide region comprising four stem-loops (I–IV) (Fig. 2). The first two stem-loops (nucleotides 5–125) are essential for replication, as their deletion blocks RNA synthesis [38]. Although other regions of the 5′ UTR are not required for replication, their presence markedly enhances replication efficiency, suggesting that the signals for RNA replication are distributed throughout the UTR [38].

Stem-loops II–IV (nucleotides 40–341) plus a few nucleotides of the core-coding region form the HCV IRES, a class III IRES that requires only a subset of host translation initiation factors (eIFs) [39]. Indeed, the purified ribosomal 40S subunit can bind the HCV IRES in the absence of any eIFs or other proteins [40]. Inside infected cells, the 40S subunit directly binds to a pseudoknot structure in stem-loop III and positions itself such that the initiator AUG codon sits in the 40S P site [41]. Next, the eIF3 and the ternary complex (eIF2/GTP/Met-tRNA$_i^{Met}$) are recruited to form a 48S intermediate [40, 42]. GTP hydrolysis and subsequent release of initiation factors then allow 60S subunit joining and formation of a functional 80S ribosome complex [43, 44].

HCV requires a liver-specific and abundant micro-RNA, miR-122, for its replication [45, 46]. Two copies of miR-122 bind to two highly conserved target sites located between the first two stem-loops of the 5′ UTR (Fig. 2). Ablating these target sites or sequestrating miR-122 by antisense locked nucleic acid (LNA) inhibitors blocks HCV replication. Multiple mechanisms for miR-122 function have been proposed, including (1) it protects the HCV 5′ end from exonuclease

Xrn-mediated decay [47–49]; (2) it promotes IRES-mediated translation of the HCV genome [50, 51]; (3) it increases the fraction of HCV RNA available for replication, possibly by displacing the translation-associated proteins from the RNA template [52]; and (4) HCV RNA-mediated sequestration of miR-122 derepresses the normal, presumably proviral, host miR-122 targets and thereby promotes virus replication [53].

The absolute conservation of the miR-122 binding sites across HCV isolates and the indispensability of miR-122 for HCV replication made it an attractive target for antiviral therapy. Indeed, weekly administration of a miR-122 antagonist, SPC3649, decreased HCV titers in chronically infected chimpanzees by more than two orders of magnitude following 12 weeks of therapy. This first-in-class inhibitor (miravirsen/SPC3649) has shown promise in clinical trials [54], but was not developed as a first-line HCV therapy. It may however be considered as a salvage therapy in rare instances of treatment failure or as a component of one-shot injectable cure cocktail for difficult-to-treat PWID (people who inject drugs) populations.

4.2 The HCV 3′ UTR

The 3′ UTR has a tripartite structure comprising an about 40-nucleotide variable region immediately following the stop codon, a poly (U/UC) tract of varying length, and a highly conserved 98-nucleotide region, designated as the X-tail or 3′X [6, 55]. While the variable region and the X-tail are highly structured, with two and three stem-loops, respectively, the poly (U/UC) tract is unstructured (Fig. 2). The poorly conserved 40-nucleotide variable region is dispensable for RNA replication, although its presence enhances replication efficiency [56, 57]. Of interest, despite high-sequence diversity, the variable region contains an invariable sequence motif – a binding site for miR-122 [45]. Although no functional role for this miR-122 binding site has been demonstrated in cell culture, such high conservation suggests that it might have an as yet undefined function(s) in vivo.

The poly (U/UC) tract highly varies in length. It comprises a homouridine core (U core) flanked by 5′ and 3′ arms that contain stretches of uridines with interspersed cytidines. The poly (U/UC) tract is essential for replication [56, 58]. Also, its base composition appears to be under strong selection pressure, as replacement of the U core with any other nucleotide homopolymers is not tolerated [56, 58]. Additionally, shortening the U core beyond a certain length inhibits HCV; however, such mutants can extend their U core in cell culture and revert to the parental replication levels [58]. The fact that these revertants always acquire uridines, and not other nucleotides, further suggests that this region may have sequence-specific roles, such as recruitment of important viral or cellular proteins. This is in line with the observation that the HCV NS3 helicase, NS5A, and NS5B preferentially bind to uridine homopolymers [59–61].

The X-tail spans the last 98 nucleotides of the HCV genome and is the most highly conserved region in the 3′ UTR [6]. This region has three stem-loops

(SL1–3), and all of them are essential for replication [56, 57, 62, 63]. Importantly, both the structures and sequences of these stem-loops are critical, as even mutations that maintain the structures are barely tolerated [57, 63]. This could be due to the multiple interactions each nucleotide in this region is engaged in. For example, nucleotides in the SL2 base pair with a stem-loop in the NS5B-coding region to form a kissing-loop interaction (Fig. 2), which is essential for virus replication [13]. Alternatively, these sequences could be important for recruiting essential viral and/or host proteins.

The 3′ UTR also appears to communicate with the 5′ UTR and enhance RNA translation [10–12]. This communication is possibly a way to ensure that only complete, non-degraded HCV RNAs are translated and replicated. Exactly how HCV UTRs communicate with each other is unknown, but it seems to involve multiple regions in the 3′ UTR. A few host proteins, such as the NFAR complex and IMP-1, have also been implicated in this process [64, 65]. Our understanding of how 3′ UTR enhances HCV RNA translation is incomplete, but evidence suggests that it involves translation termination and ribosome recycling.

5 HCV Proteins

The HCV genome encodes ten proteins. The first three proteins (core, E1, and E2) are the structural components of the virus particles, while the remaining seven proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) function in genome replication and virion biogenesis without being incorporated into mature virions. In this section, we provide a brief overview of the contemporary knowledge of these proteins. For detailed insights, the readers are referred to reviews of individual proteins.

5.1 Core

The core protein constitutes the virion capsid and is synthesized at the most N-terminal portion of the HCV polyprotein (Fig. 1). During polyprotein biogenesis, an internal signal sequence between core and the E1 glycoprotein docks the nascent polypeptide on the ER membrane and induces translocation of E1 into the ER lumen [66]. The mature form of core is then released by two catalytic events: first, an ER-resident signal peptidase cleaves between E1 and the signal peptide, leaving behind an immature membrane-bound core of 191-aa. The signal peptide is further processed by an intramembrane SPP, releasing mature 177-aa core ($core^{177}$) from the membrane [67–69] (Fig. 3). This protein is then free for trafficking to LD, where it drives virion assembly [70]. A recent mass spec analysis of highly purified HCV particles identified $core^{177}$ as the protein form that is incorporated into mature virions [71].

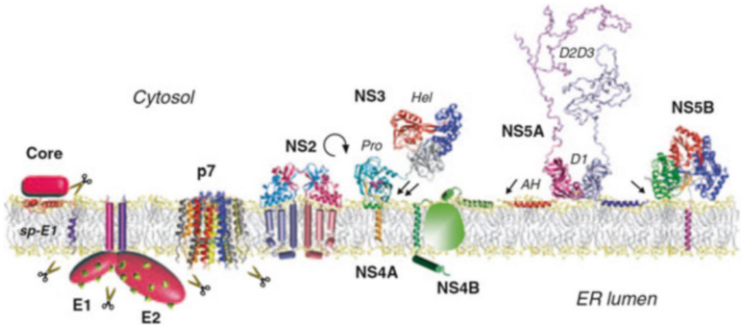

Fig. 3 Structure and membrane association of HCV proteins. Scissors on the ER luminal side indicate cleavages by the signal peptidases, while the one on the cytosolic side indicates the additional cleavage of core by signal peptide peptidase. The cyclic arrow shows NS2-mediated cleavage, while the black arrows denote NS3-4A-mediated cleavages. Reproduced from "Hepatitis C Virus Proteins: From Structure to Function" [8], with permission from Springer

The three-dimensional structure of the core protein is yet to be solved. The hydropathy profile suggests that the mature core comprises two domains: an N-terminal hydrophilic domain of ~122-aa (domain 1) and a C-terminal hydrophobic domain (domain 2) [72]. Domain 1 is highly enriched in basic residues, has RNA binding and homo-oligomerization activities, and is a key player in virion assembly. Domain 2 contains two amphipathic α-helices and a central hydrophobic loop and anchors the core protein to the membrane [73]. An important step during nucleocapsid formation is the packaging and compaction of the viral RNA, a process that requires substantial structural remodeling of the RNA. This is typically achieved by the RNA chaperone activity of the viral capsid proteins, and in the case of HCV, this function appears to reside in the domain 1 of the core protein [74].

How core and the viral RNA come together to assemble nucleocapsids, and exactly where in the cell this assembly occurs, is unknown. However, it is widely accepted that assembly occurs on the surface of LDs where the functional core protein resides. Interaction between core and NS5A appears to be important for the delivery of the viral RNA to the assembly site. It is unclear, however, if NS5A alone carries the viral RNA from an HCV replication complex to the assembly site or if the whole replication complex is moved to the LD. Once bound to the viral RNA, core is assumed to oligomerize and drive nucleocapsid formation (reviewed in [28]).

Additional core species, most likely produced by internal translation initiation, have also been reported [75]. Originally identified in cell culture systems, these 6–14-kDa minicores have recently been detected in patient sera as well [76]; however, their biological relevance is unclear. Evidence of an alternate reading frame (ARF) overlapping the core-coding region also exists. Although ARF proteins appear to elicit antibodies in patients, their function in viral persistence and pathogenesis is also unknown (reviewed in [77]).

5.2 HCV Glycoproteins

The HCV genome encodes two envelope glycoproteins, E1 and E2, that are released from the polyprotein by signal peptidase cleavage. As a general feature of the viral envelope proteins, HCV glycoproteins exhibit marked conformational plasticity, allowing them to play distinct roles at different stages of the virus life cycle, such as virion production and maturation, receptor binding, and fusion between the viral and cellular membranes.

HCV glycoproteins are type I transmembrane proteins with a large N-terminal ER-luminal ectodomain and a C-terminal hydrophobic transmembrane domain (TMD) (Fig. 3). A unique feature of the E1 and E2 TMDs is their dynamic topology provided by their special architecture – each TMD comprises a short stretch of hydrophilic residues connecting two hydrophobic segments, with the second segment serving as a signal sequence for the downstream protein. Before signal peptide cleavage, the TMDs have a hairpin structure with both ends of the hairpin oriented toward the ER lumen, but following cleavage, the second hydrophobic segment is reoriented toward the cytosol, resulting in a single membrane-spanning domain [78].

The TMDs contribute to several of the HCV glycoprotein properties, such as membrane association, ER retention, and E1E2 non-covalent heterodimer formation (reviewed in [79]). Like many ER-resident membrane proteins, the hydrophilic residues in the middle of the TMD appear to mediate the ER retention of E1 and E2. Replacing these residues with alanine abolishes ER retention. Similarly, deleting the E2 TMD or replacing it by the anchor signal from another protein disrupts E1 and E2 heterodimer formation, indicating that TMDs play critical roles in protein heterodimerization. Interestingly, although E1 and E2 makes non-covalent heterodimers at the ER membrane, they appear to be covalently bound on secreted virus particles [30].

It is unclear how and when E1E2 dimers acquire intermolecular disulfide linkages during HCV assembly. Although E1 and E2 ectodomains have numerous highly conserved cysteine residues, as first predicted and later confirmed by structural data, these cysteines are engaged in intramolecular disulfide associations and are therefore unavailable for intermolecular linkages [80–82]. However, as several regions of the HCV glycoproteins appear to possess remarkable conformational flexibility, it is possible that structural rearrangements at some point during virion assembly expose the cysteines and allow formation of intermolecular disulfide bonds.

In contrast to the flavivirus envelope (E) protein, HCV glycoproteins are heavily glycosylated, a feature required for proper protein folding, transport through the secretory pathway, and escape from the host immune surveillance. E1 and E2 have 4–6 and 9–11 N-linked glycosylation sites, respectively, which are decorated by high-mannose-type glycans as the proteins translocate into the ER lumen. These glycans then interact with an ER chaperone, calnexin, and assist in protein folding [83, 84]. As the assembled HCV particles pass through the Golgi, several of the high-mannose glycans are converted into complex glycans [30] and presumably protect the secreted virions from the humoral immune response [85].

HCV E1E2 heterodimers interact with cell surface receptors to initiate virus entry, which is a highly coordinated multistep process leading to virus uptake into endosomes. Low pH in the early endosomes then induces fusion between the viral and endosomal membrane, facilitating escape of the viral genome into the cytoplasm. Our knowledge of how fusion occurs is incomplete. In the case of flaviviruses, the E protein has a fusion peptide that is buried at the dimer interface at neutral pH. Upon exposure to low endosomal pH, the E protein undergoes structural rearrangement exposing the fusion peptide, which then inserts into the endosomal membrane and brings it closer to the viral membrane for fusion (reviewed in [86]). However, for HCV, which of the two glycoproteins mediates fusion is unclear.

For years, E2 was proposed to be a fusion protein; however, recent structures of the E2 ectodomain appear to disprove this notion. For example, type II fusion proteins, such as that of flaviviruses, have an elongated, three-dimensional architecture of over 140 Å in length, whereas HCV E2 ectodomain is a compact, globular protein of only 32 Å. Also, the predicted fusion peptide is buried inside the hydrophobic core of the E2 ectodomain where it is part of the secondary structural elements, seemingly ruling out a role in fusion [87]. Moreover, in contrast to typical fusion proteins, HCV E2 does not undergo major structural and oligomeric changes upon exposure to lower pH [80]. Is then E1 the fusion protein?

The crystal structure of the E1 N-terminal portion (aa 1–79) was recently solved. This so-called nE1 is 59 Å in length, and although it exists as a dimer in solution, its structure has no resemblance to other viral fusion proteins [88]. This raises the possibility that HCV fusion occurs by a completely different mechanism. Alternatively, the fusion peptide could be located in the regions of the HCV glycoproteins that were not part of the crystal structures, or fusion could be mediated by the E1E2 heterodimer. Also, the possibility of virion-associated lipoproteins or cellular receptors and cofactors mediating HCV fusion cannot be ruled out.

In addition to mediating cell entry, the HCV glycoproteins elicit neutralizing antibodies in patients. Consequently, the genes encoding glycoproteins are highly variable, and E2 further contains two hypervariable regions (HVR), differing by up to 80% among HCV isolates. HVR1 spans the first 27 amino acids of E2 and is the immunodominant region containing several epitopes that elicit type-specific antibodies [89]. HVR2 comprises amino acid 76–101 and shows massive sequence diversity. However, the significance of such a high diversity is unknown, as this region appears to play no roles in humoral immune responses [90].

5.3 P7

P7 is a small 63-aa protein located at the junction of the structural and nonstructural region in the HCV polyprotein and is processed by the signal peptidase cleavage. It is an integral membrane protein comprising two antiparallel transmembrane segments separated by a hydrophilic cytosolic loop [91] (Fig. 3). When reconstituted in

artificial membranes, p7 assembles into hexamers or heptamers and exhibits cation channel activity that is abolished by classical ion channel inhibitors, suggesting that p7 is a viroporin [92, 93].

To date, no high-resolution 3D structure of p7 has been solved. NMR studies of p7 monomers embedded in artificial membranes have revealed the following structural features [94]: (1) an N-terminal transmembrane segment comprising two α-helices connected by a turn, (2) a cytosolic loop containing a highly conserved dibasic motif, (3) a C-terminal transmembrane segment comprising an α-helix, and (4) an unstructured 7-aa C-terminal segment. P7 monomers oligomerize to make ion channels, but what these channels look like and how monomers are packed within these channels are unclear. Two studies, one based on a single-particle EM analysis and the other on structure simulation, have provided contrasting pictures: while the former revealed a conical-shaped channel, the latter showed a cylindrical, upright architecture [93, 95]. This discrepancy is likely due to the difference in the thickness of the artificial membranes the two groups used to reconstitute p7. In fact, the ability to adapt to the membrane thickness is a general feature of viroporins.

Several lines of evidence indicate that p7 is dispensable for HCV RNA replication in vitro. It is however essential for HCV infectivity in chimpanzees and for production of infectious particles in cell culture, suggesting that p7 is required at later stages of the virus life cycle (reviewed in [96]). To support this, an HCV mutant lacking the p7 viroporin activity failed to produce infectious particles, and this defect could be partially rescued by the influenza virus M2 viroporin. However, M2 failed to rescue infectivity of an HCV mutant lacking the whole p7 protein, suggesting p7 has additional viroporin-independent roles [31]. The p7 viroporin activity is believed to protect intracellular viruses from acidification, whereas its non-channel activity involves its interactions with other viral, and possibly host, proteins (reviewed in [96]).

5.4 NS2

NS2 is the first nonstructural protein encoded by the HCV genome and is processed by the signal peptidase at its N-terminus and by an intrinsic *cis*-protease activity at its C-terminus [97]. For efficient enzymatic activity, NS2 requires the protease domain, but not the protease activity, of the downstream NS3 protein [98] and is therefore described as NS2-3 protease. The protease-mediated NS2-NS3 cleavage is essential for HCV RNA replication [99]; however, following cleavage, NS2 loses its enzymatic activity and is no longer required for replication in cell culture. Consistent with this, HCV subgenomic replicons devoid of NS2 can efficiently replicate in cell culture. NS2 however is important for the production of infectious virions [1].

NS2-3 protease has no sequence or structural similarity with any known eukaryotic proteases. However, the finding that classical cysteine protease inhibitors such as iodoacetamide inhibit NS2-3 led to the suggestion that it is a cysteine protease [100]. Later, crystallization of the NS2 protease domain confirmed this assumption

and revealed a catalytic triad of His, Cys, and Glu, which is conserved across all HCV genotypes [101]. Replacing catalytic His and Cys with alanine abolishes NS2-3 enzymatic activity, whereas substitution of Glu is largely tolerated [97, 102]. NS2 forms a homodimer with two composite active sites (Fig. 3), each of which contains catalytic His and Glu residues contributed by one monomer together with a nucleophilic Cys contributed by the other [101].

The NS2-3 autoprotease activity leads to its inactivation, presumably because after cleavage, NS2 C-terminal residues occupy the active site [101]. The liberated NS2 is a 23-kDa protein localized at the ER membranes through its three N-terminal transmembrane segments (TMS), while the protease domain is cytoplasmically oriented [100, 103]. A number of mutations in the TMS and protease domain have been found to influence infectious particle production, suggesting an important role for NS2 [104–109]. Exactly how NS2 contributes to virus production is poorly understood but may involve a complex network of interactions with structural and other nonstructural viral proteins [104, 107, 110, 111].

5.5 NS3-4A Complex

NS3 is a multifunctional protein that makes a complex with and uses NS4A as a cofactor. The N-terminal one-third of NS3 is a chymotrypsin-like serine protease, while the C-terminal two-thirds has NTPase/RNA helicase activity. Both of these activities are essential for HCV replication (reviewed in [112]).

NS3-4A protease NS3-4A is responsible for all downstream cleavages that occur in the HCV polyprotein [113]. The cleavage at the NS3/4A site occurs in *cis*, while the rest require *trans*-cleavage activity of NS3-4A. There seems to be a preferential order in which NS3-4A processes the polyprotein: first NS5A/5B site is cleaved to produce the NS4A-5A intermediate, followed by cleavage at the NS4A/4B site to produce a relatively stable NS4B-5A precursor, which is the final intermediate to be processed [114]. The significance of this cleavage order is unknown, but the intermediates generated may have regulatory roles in the viral life cycle [115].

NS3-4A is an integral membrane protein complex, anchored on the ER membrane by an NS3 N-terminal amphipathic α-helix and an NS4A N-terminal transmembrane segment [116] (Fig. 3). The protease region of NS3 has a typical chymotrypsin-like fold and is composed of two ß-barrel domains flanked by two short α-helices. At the interface of these domains lies the active site, with a catalytic triad of His, Asp, and Ser; His and Asp are contributed by one domain, while the nucleophilic Ser by the other. Three Cys residues and a water molecule coordinate a Zn^{2+} ion required for enzyme stabilization (reviewed in [117]). The NS3 protease requires an obligate cofactor, NS4A, which is a 54-aa polypeptide comprising three regions: the N-terminal hydrophobic region that forms a transmembrane α-helix, the

central ß-strand that is incorporated into the N-terminal ß-barrel of NS3, and the C-terminal acidic region that forms a negatively charged α-helix. The C-terminal helix appears to play multiple roles in HCV RNA replication and virus particle production, most likely through binding with NS3 helicase and other replicase components [118, 119].

Aside from HCV polyprotein processing, NS3-4A cleaves several host proteins (reviewed in [120]). Its most well-defined target is MAVS, an adapter protein in the RIG-I branch of the interferon (IFN) production pathway. By cleaving MAVS, NS3-4A blocks RIG-I signaling and thereby antagonizes the major cellular antiviral immune pathway. Another immune component that NS3-4A has been shown to cleave is TRIF, an adaptor protein in the TLR branch of the IFN production pathway. Several other proteins, such as T cell protein tyrosine phosphatase (TC-PTP), UV-damaged DNA binding protein 1 (DDB1), and a membrane-associated peroxidase GPx8, are also targeted by NS3-4A, but the functional significance of these cleavages is poorly understood. This however shows that NS3-4A protease is a multifunctional protein, required on one hand for the maturation of viral proteins and, on the other hand, to disrupt cellular antiviral defenses.

Inspired by the clinical success of human immunodeficiency virus (HIV) protease inhibitors, and given the fact that NS3-4A protease has multiple essential roles in the HCV life cycle, intense efforts were started early to develop NS3-4A protease inhibitors. These efforts culminated in the development of highly effective HCV protease inhibitors that are now in clinical use. The details of these inhibitors are provided in the later sections of this book.

NS3 helicase The C-terminus of NS3 is a superfamily 2, 3′-5′ RNA helicase, which is thought to separate viral RNA strands, disrupt RNA secondary structures, and/or remove nucleic acid-associated proteins during HCV RNA replication (reviewed in [117]). An inherent NTPase activity of NS3 hydrolyzes ATP and thereby provides fuel for the translocation and RNA unwinding functions of the protein ([121] and references therein). Although NS3 NTPase/helicase is an independent enzyme, the presence of the NS3 protease domain and NS4A appears to modulate at least some of its activities. Ablating NS3 helicase function blocks HCV replication in vivo and in vitro, indicative of an essential enzymatic role (reviewed in [25]). However, exactly how NS3 helicase unwinds HCV RNA and its precise role in the virus life cycle remains elusive. Interestingly, in vitro assays have shown that NS3 also has a DNA helicase activity [122, 123], raising the formal possibility that it could interact with cellular DNA.

Crystal structures of the NS3 helicase domain as well as of the full-length NS3 protein in complex with its NS4A cofactor have been solved (reviewed in [117]). These structures revealed a Y-shaped helicase, where the N-terminal domain (D1) and the middle domain (D2) make arms, while the C-terminal domain (D3) makes stem of the "Y" (Fig. 3). D1 and D2 have similar topologies and contain all of the conserved helicase sequence motifs, including the signature DExH/D-box motif required for the binding and hydrolysis of NTP and coordination of a metal ion cofactor (Mg^{2+} or Mn^{2+}). Crystallization of NS3 helicase with bound nucleic acids

revealed that the substrate binding pocket of the enzyme resides in the cleft separating domain 3 from the other two domains [124].

Although HCV NTPase/helicase is not as extensively developed as a drug target as HCV protease and polymerase, it undoubtedly represents a potential target for antiviral therapy. Progress, however, has been slowed by a number of factors, including poor grasp of the NS3 helicase mechanism, a paucity of high-throughput assays for compound screening, and striking similarities between NS3 and cellular RNA helicases impeding identification of HCV-specific, nontoxic inhibitors. Improved understanding of the NS3 helicase could help overcome some of these barriers.

5.6 NS4B

Like all positive-sense RNA viruses, HCV replication occurs on highly remodeled cellular membranes, and although several viral and cellular proteins play roles in the biogenesis of these membranes, the NS4B protein is believed to be a major player [125, 126]. This 27-kDa, highly hydrophobic protein strongly associates with ER membranes and induces specific membranous structures designated the membranous web, which is the site of HCV RNA replication [127, 128]. In addition, NS4B has been reported to have RNA binding [129], NTP hydrolysis [130], and adenylate kinase [131] activities.

NS4B is an integral membrane protein comprising two amphipathic α-helices at its N-terminus, four transmembrane segments in the middle of the protein, and two α-helices at the C-terminus (reviewed in [132]). In addition, two palmitoylation sites in the NS4B C-terminus also appear to contribute to the membrane association [133]. How NS4B induces the formation of the membranous web is unknown; however, one way that membrane proteins induce membrane curvature and vesicle formation is by making oligomeric complexes. Multiple lines of evidence suggest that NS4B forms oligomers, involving a number of homo- and heterotypic interactions [125, 133, 134]. Mutations disrupting the NS4B membrane association and/or oligomerization severely impair the size and morphology of membrane vesicles with concomitant effects on virus replication [125, 134].

Different NS4B functions, including RNA binding, oligomerization, and enzymatic activities (NTP hydrolase and adenylate kinase), represent potential drug targets. It is therefore no surprise that NS4B has been pursued as a target for antiviral therapy, although as yet with little clinical success (reviewed in [135]).

5.7 NS5A

NS5A, the only HCV nonstructural protein without any apparent enzymatic activity, is perhaps the most enigmatic HCV protein and also the target of the most potent

clinically available drugs. This multifunctional, RNA-binding phosphoprotein plays essential roles in HCV RNA replication, virion assembly, and disease pathogenesis through its interactions with viral and cellular proteins (reviewed in [136]).

NS5A is a ~450-aa protein that is anchored on the ER membrane through an N-terminal amphipathic α-helix (Fig. 3). It comprises three domains (DI, II, and III) separated by two trypsin-sensitive low complexity sequences (LCS I and II) [137]. DI contains zinc-binding and RNA-binding motifs, is well conserved across HCV genotypes, plays essential roles in HCV RNA replication, and is the only domain crystallized so far [138]. The intrinsically unfolded DII and DIII are relatively less conserved than DI [139, 140]. Approximately, two-third of DII can be deleted without significant effects on HCV replication and virus particle production in culture [141]. DIII on the other hand is not needed for replication, as it can be deleted [141] or engineered to accommodate heterologous proteins such as GFP [142, 143], with no significant effects on viral RNA synthesis. However, this domain is essential for virion assembly, presumably through its interaction with the HCV core protein at the assembly site [141].

NS5A exists in the HCV infected cells in two major phospho-isoforms: p56 or basal phosphorylation form and p58 or hyperphosphorylation form. The p58 is converted from p56 and requires polyprotein processing [144]. Interestingly, the p56:p58 ratio appears to function as a molecular switch between virus replication and assembly, with p56 favoring RNA replication and p58 mediating a transition to virion assembly. This model stems from the observations that a high p56:p58 ratio achieved through genetic or chemical inhibition of NS5A hyperphosphorylation enhances HCV RNA replication in cell culture [145–147], while it inhibits HCV propagation in chimpanzees [148] and impairs virion assembly in the HCVcc-based experiments [149]. It should however be noted that some HCV isolates have yielded results which do not agree with the above model. One example is of the HCV JFH1 isolate for which high p56:p58 ratio has been shown to inhibit rather than enhance RNA replication [150].

NS5A is an integral part of the HCV replication complexes (RC), and several NS5A inhibitors have been shown to disrupt RC formation [151]. However, it is unclear if NS5A is directly recruited to RC through its N-terminal membrane anchor or if other viral or host proteins are involved. At least some evidence suggests that the NS4B protein may contribute to NS5A membrane localization [152]. Similarly, at least two host membrane-anchor proteins, VAPA [153] and FBL2 [154], bind with NS5A and are presumed to play a role in NS5A membrane localization. Inside the RC, NS5A binds with other components of the viral replicase machinery and seems to recruit host proteins essential for HCV replication. For example, NS5A-mediated recruitment of two host enzymes, PI4KIIIα [155] and cyclophilin A (CYPA) [156], is essential for the formation of functional RC. Several other proteins have been shown to bind NS5A, but the significance of these interactions is poorly understood.

NS5A plays important roles in virus particle production by channeling the progeny viral RNA to LDs, the assembly site. This function requires NS5A interaction with the core protein located on the surface of LDs and appears to be mediated

through NS5A DIII, as deletion of this domain blocks core-mediated NS5A trafficking to LDs and inhibits virion production. In contrast, this deletion has no effect on NS5A localization to the RC or HCV RNA replication (reviewed in [28]).

Due to the lack of a known enzymatic activity, NS5A was once considered a non-druggable protein. However, some of its important and extensively validated catalytic binding partners such as CYPA and PI4KIIIα, both of which are essential for HCV replication, have been long pursued as antiviral drug targets. An unexpected discovery came in 2010, when a small molecule, BMS-790052 (Daclatasvir), was found to directly target NS5A [157]. This compound inhibits HCV with half-maximal inhibitory concentration (IC_{50}) in the low picomolar range, and compounds in this class are now part of the HCV standard-of-care treatment. How daclatasvir hinders NS5A activity is an active area of investigation, but it seems to interfere with multiple functions, including NS5A hyperphosphorylation, dimerization, and NS5A protein-protein interactions, partly explaining the unusually high potency of this compound (reviewed in [158]).

5.8 NS5B

HCV NS5B polymerase is the catalytic subunit of the viral replicase complex responsible for two RNA polymerization steps. In the first step, it uses HCV genomic RNA as a template to generate the complementary negative-strand RNA intermediate, which, in the second step, serves as a template for the synthesis of the positive-strand progeny RNA. NS5B has been extensively characterized and contains all the sequence and structural motifs conserved among known viral RNA-dependent RNA polymerases (RdRp) [61, 159, 160]. The N-terminal 530 amino acids of this 591-aa protein constitute the catalytic domain that is connected to the C-terminal 21-aa membrane-anchor domain through a 40-aa linker (Fig. 3). The membrane association of NS5B is essential, as deletion of the C-terminal domain abrogates HCV RNA replication in cell culture [161]; however, this domain contributes minimally to nucleotide polymerization [162].

As a general feature of RdRp, the NS5B catalytic domain has discernable finger, thumb, and palm subdomains in reference to the catalytic domain's likeness to a cupped right hand. The purified catalytic domain is capable of performing de novo and primer-dependent RNA synthesis, requiring only divalent metals such as Mg^{2+} or Mn^{2+} as cofactors [163]; however, HCV RNA synthesis in vivo likely occurs by de novo initiation. The catalytic nucleotidyl transferase pocket is located in the palm subdomain and contains the signature sequences of $D^{220}xxxxD^{225}$ and $G^{317}D^{318}D^{319}$ ("x" represent any residue). The D^{220} and D^{318} chelate two catalytic metal ions that are crucial for the polymerase function. The template RNA enters the active site through a hydrophobic groove between the fingers and thumb subdomains, while NTPs access the active site via a specific tunnel beginning at the backside periphery of the palm subdomain.

Structural studies have revealed two unusual features of the NS5B polymerase [164–167]. First, the fingers and thumb subdomains heavily interact with each other resulting in a completely encircled active site and low intra-domain and possibly inter-domain flexibility, which may restrict structural rearrangements generally required during nucleotide binding and polymerization. Second, a thumb subdomain ß-hairpin loop of 12 amino acids protrudes into the active site, presumably contributing to the positioning of the HCV 3′ terminus for correct initiation [168]. Also, the ß loop imposes steric hindrance against docking the dsRNA, thus ensuring the preferential use of the HCV 3′ terminus as a template for de novo initiation of RNA synthesis. Consistently, deleting the ß loop promotes primer-dependent RNA synthesis, which requires binding of several base pairs of dsRNA [168].

The de novo RNA synthesis is a complex process, and a recent study that captured snapshots of the ternary assemblies (NS5B, template RNA, and NTPs) at different stages of HCV RNA replication suggests the following sequence of events [167] (Fig. 4): (1) in the NS5B apo enzyme, the active site is partially occupied by the ß loop and the C-terminus linker; (2) once the 3′ end of the HCV RNA and an initiating nucleotide enter the active site, the ß loop and the C-terminus linker are slightly retracted (~5 Å) to create space for the formation of a dinucleotide primer; (3) the first nucleotidyl transferase reaction then generates a dinucleotide primer; (4) extension of this primer by one to three nucleotides completely displaces the ß loop and the C-terminus linker, opening the RNA duplex exit channel; and (5) once the "path" is cleared of the ß loop and the C-terminus linker, the polymerase transitions from the dinucleotide-primed state to a rapid, processive elongation state.

NS5B does not replicate the viral genome in isolation, rather as a part of the replicase complex anchored on the ER membrane. The replicase complex is thought to comprise the host and viral proteins that might affect HCV replication activity. For example, protein kinase C-related kinase, PRK2, has been proposed to phosphorylate NS5B and thereby regulate HCV replication [169]. Similarly, NS5B binds to NS3, and the NS3 interacts with NS4A and possibility NS4B and NS5B [170]. NS5B has also been shown to directly interact with NS5A, which can possibly modulate NS5B functions [171]. How this complex array of interactions affects and/or regulates NS5B-catalyzed RNA synthesis is unclear.

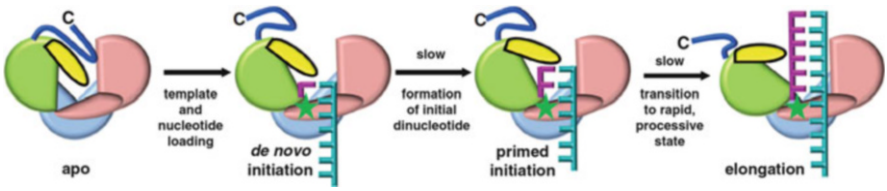

Fig. 4 Model of HCV replication by NS5B. NS5B C-terminus and the ß loop that occlude the RNA binding groove of the apo form of the polymerase are shown in blue and yellow color, respectively. See text for details of each step. Reproduced from Appleby et al. [167] with permission from the American Association for the Advancement of Science (AAAS)

Along with the NS3 protease, NS5B has long been extensively pursued as the most druggable HCV protein (reviewed in [172]). Although both nucleoside and non-nucleoside inhibitors of NS5B have been reported, only nucleoside inhibitors have demonstrated broad genotype coverage and high resistance barrier both in the laboratory and in human clinical studies. For these reasons, the nucleotide inhibitor sofosbuvir is now a backbone for several of the current interferon-free anti-HCV regimens containing HCV protease and NS5A inhibitors, with cure rates approaching almost 100%. The details of these inhibitors and their mechanisms of action are provided in subsequent chapters.

6 Summary

Thanks to the years of elegant research, we now have extensive knowledge of HCV proteins and how they contribute to the virus life cycle and pathogenesis. However, still much remains to be learnt. For example, high-resolution structures of most of the viral full-length membrane proteins are yet to be solved. Although X-ray crystallography of membrane proteins remains challenging, continuously improving methodologies and increasingly popular alternative systems such as cryo-EM should facilitate progress on this front. The ultimate success would be to solve the structure of the entire HCV replicase complex that will yield insights into the stoichiometric ratios and functions of individual replicase components. These structural and the associated functional studies will deepen our understanding of the replication mechanisms of HCV and potentially other positive-strand RNA viruses.

7 HCV Animal Studies

While the study of HCV molecular biology and the development of antivirals have greatly benefited from the availability of cell culture models, the key contributions of animal studies toward discovery of HCV and establishment of in vitro culture systems cannot be overstated. As the efforts to develop prophylactic HCV vaccines intensify, the use of animal models to test vaccine candidates and concepts will become even more important. In the next section, we provide an overview of HCV animal models, past and present, and their importance for studying HCV virology, immunology, and pathogenesis.

7.1 Animal Models

It took a decade from the discovery of HCV as the etiologic agent of non-A, non-B hepatitis (NANBH) in 1989 [173] to the development of HCV replicon system and

another 5 years to establish an HCV infectious cell culture system that would allow the study of the entire viral life cycle in vitro [1, 2, 174]. These experimental systems were milestones in HCV research that paved the way for the development of HCV DAAs that achieve a cure from chronic HCV infection [175]. On the organismal level, analyses of samples from HCV-infected patients have provided valuable insights into the natural history of infection, host immune responses, and pathogenesis [176]. Clinical research on HCV, however, is limited by the heterogeneity of human study cohorts and restricted access to human liver tissue.

HCV has a narrow host tropism limited only to humans and chimpanzees [177]. Studies in chimpanzees led to the discovery of HCV and were essential for many other breakthroughs in HCV research [178]. However, studies in these nonhuman primates were hampered by limited availability, high costs, and ethical concerns. The latter eventually led to a moratorium on government-funded chimpanzee research in 2015. Given these limitations, significant effort has been put into the development of alternative animal models for HCV.

Various species, such as woodchucks and numerous Old and New World monkeys, have been challenged with HCV and shown to be resistant [179–181]. Interestingly, the tree shrew (*Tupaia belangeri*) is susceptible to HCV infection [182]. However, the utility of this outbred species as a small animal model of HCV infection has been limited.

Numerous approaches have been taken to develop laboratory mouse models of HCV infection, including transgenic expression of viral proteins, xenotransplantation, genetic humanization, and viral adaptation [183]. Finally, HCV-related animal hepaciviruses provide the possibility to develop surrogate models of HCV infection [184].

In this section of the chapter, we discuss the chimpanzee model and selected HCV mouse models as well as models based on novel animal hepaciviruses in the context of HCV drug and vaccine development (see Table 1).

7.2 Chimpanzees

Chimpanzees are the only species besides humans that is readily susceptible to HCV infection. This first became apparent in the 1970s when NANBH virus was discovered as a new hepatitis-causing agent in human blood products and the only animal susceptible to the mysterious virus was a chimpanzee [185–187]. Ever since the chimpanzee model has played an essential role in HCV research.

In 1989, HCV was discovered as the etiological agent of NANBH by using the serum of an experimentally infected chimpanzee [173]. In the following decade, experiments in which in vitro transcribed viral RNA was directly injected into the liver of chimpanzees were essential to define the sequence of a full-length infectious clone [148, 188]. This was a prerequisite for the development of HCV replicon system, the first cell culture model of HCV [1, 174]. However, until the first HCV infectious cell culture system was developed in 2005, the chimpanzee remained the

Table 1 Summary of animal models for HCV and related hepaciviruses

Model	Infection	Immune response	Drug and antibody testing	Vaccine testing
Hepatitis C virus				
Chimpanzees	Acute and chronic	Yes, fully immune-competent	Yes	Yes
Liver chimeric mice	Chronic	No, highly immune-deficient	Yes	No
HCV entry factor transgenic nice	Prolonged low level viremia	No, immune-deficient	Yes	No
HCV-related hepaciviruses				
Monkeys (GBV-B)	Acute and chronic	Yes, fully immune-competent	n.d.	n.d.
Horses (NPHV)	Acute and chronic	Yes, fully immune-competent	n.d.	n.d.
Rats (RHV/NrHV)	Chronic	Yes, fully immune-competent	Yes	n.d.
Mice (RHV/NrHV)	Acute, chronic after transient immunosuppression	Yes, fully immune-competent	n.d.	n.d.

n.d. not determined

only experimental model recapitulating the entire HCV life cycle. Chimpanzees are susceptible to all six HCV genotypes, and infection can be initiated by intravenous injection of clinical isolates or HCVcc or by intrahepatic injection of in vitro transcribed RNA of infectious HCV cDNA clones [2, 185, 187–189].

The course of HCV infection in chimpanzees has been well characterized and differs from that in humans [177]. In humans, around 70% of infected individuals develop chronic infection, which frequently progresses to liver fibrosis and hepatocellular carcinoma (HCC). In contrast, chimpanzees mostly clear HCV in the acute phase of infection, and the animals that progress to chronicity do not develop fibrosis and HCC [178].

Nevertheless, the chimpanzee model has been of critical importance for defining antiviral immune responses and the nature of protective immunity following reinfection. Importantly, results from these immunological studies are in line with observations from HCV infection in humans. HCV infection in chimpanzees induces significant innate and adaptive immune responses in peripheral blood and liver and is associated with elevated ALT levels and hepatitis [190–193]. Acute clearance is associated with a strong hepatic innate immune response involving upregulation of hundreds of interferon-stimulated genes (ISGs) in hepatocytes and the expansion of HCV-specific CD4+ and CD8+ T cells [192–194]. The chronic phase of disease is

characterized by persistent viremia, sustained expression of intrahepatic ISGs, emergence of viral escape mutations in T cell epitopes, and mild hepatitis [192, 195, 196].

Experiments in chimpanzees further showed that clearance of a primary infection does not provide complete protective immunity against challenges with homologous or heterologous viruses [197, 198]. However, HCV viremia was often significantly shorter following reinfection and the likelihood of chronic infection reduced. Rapid HCV clearance post reinfection was associated with the induction of an HCV-specific memory T cell response. Antibody-mediated depletion of CD4+ or CD8+ T cells prolonged HCV viremia after re-challenge, thus directly demonstrating the critical role of both lymphocyte subsets in the clearance of HCV infection [199, 200]. Chimpanzees were also used to show that neutralizing antibodies may confer partial protection in primary HCV infection and upon reinfection [201, 202].

Chimpanzees have also been used to test numerous HCV therapeutic strategies, such as proof-of-concept that an interferon-free cure of HCV infection is possible with combinations of DAAs [203]. Further, it was shown that blockade of the liver-specific HCV host factor miR-122 by an antisense oligonucleotide prevents viral replication in vivo and leads to a slow but consistent decline of HCV viremia [204].

Chimpanzee studies have provided important insights into HCV vaccine design, and the animals have been used to assess the preclinical efficacy of vaccine candidates [205]. Immunization with an antibody vaccine comprising recombinant envelope E1/E2 glycoproteins or with a T cell vaccine consisting of adenovirus or plasmid DNA expressing HCV nonstructural genes has not led to induction of sterilizing immunity, but has prevented chronicity following experimental infection in chimpanzees [206, 207].

Finally the chimpanzee studies provided first insights into the functionality of the antiviral immune response post DAA cure. One animal cured of chronic infection with DAAs showed expansion of HCV-specific T cells post re-challenge but eventually progressed to a second chronic infection indicating that a DAA cure might not result in protective immunity [208]. These preliminary results may have further implications for vaccine design.

Overall chimpanzees have been of great importance for HCV research, and now historical studies in this model remain the gold standard for all other animal models.

7.3 HCV Mouse Models

Laboratory mice are the favorite model organism of biomedical research with numerous genetic variants and tools available to perform in-depth mechanistic studies. Consequently, significant effort has been put into the development of mouse models of HCV infection [209]. Since mice are resistant to natural HCV infection, sophisticated approaches were necessary to render them susceptible to

infection. Here we outline three strategies: (1) xenotransplantation, (2) genetic humanization, and (3) viral adaptation.

7.3.1 Xenotransplantation

Humanization of the mouse liver by transplantation of HCV-permissive human cells, such as hepatoma cell lines or primary hepatocytes, has been evolved as the most widely used strategy to study HCV infection in rodents. Xenotransplantation models have contributed to our understanding of many aspects of HCV biology, such as viral entry and mechanisms of neutralizing antibodies. However, a prerequisite of xenotransplantation is immunodeficiency of the host to prevent xenograft rejection. Thus, these models are not suitable to study antiviral immune responses. Here we describe distinct approaches of liver xenotransplantation and highlight strategies to overcome the lack of immune responses in these models.

Hepatoma Cell Transplantation Models

The human Huh7 hepatoma cell line containing an HCV replicon can be transplanted into severe combined immunodeficient (SCID) mice. In this model, HCV RNA replication was detectable in vivo, and responses to antiviral treatment such as interferon-α (IFN-α) could be analyzed [210]. A recent study from 2017 further developed the replicon cell transplantation mouse model to overcome its immune deficiency [211]. In this model, instead of human hepatoma cells, an H-2b positive mouse hepatoma cell line previously shown to support the HCV replicon [212] was transplanted into syngeneic immune-competent H-2b-restricted mice (C57BL/6). This approach led to HCV replication in vivo and the induction of HCV NS3- and NS4-specific T cell responses [211]. This model may become useful for HCV vaccine development. However, one has to keep in mind that immune responses induced against a replicon-containing hepatoma cell line are likely to be different from immune responses elicited during a natural infection.

Human Liver Chimeric Mice

In human liver chimeric mouse models, the liver of mice is xeno-engrafted with primary human hepatocytes. This can be achieved by intrasplenic injections of human cells into immunodeficient mice carrying a genetically engineered liver injury. The liver has a remarkable regenerative capacity upon injury, and the induction of murine liver injury before xenotransplantation gives human hepatocytes a growth and engraftment advantage. Two different liver injury mouse models are commonly used to generate liver chimeric mice: (1) urokinase plasminogen activator (alb-uPA) transgenic mice in which transgenic overexpression of uPA under the albumin promoter leads to liver injury [213, 214] and (2) fumaryl acetoacetate

hydrolase ($FAH^{-/-}$)-deficient mice in which defects in the tyrosine breakdown pathway lead to accumulation of hepatotoxic metabolites [215, 216]. When uPA mice are backcrossed on the immunodeficient strain SCID and $FAH^{-/-}$ mice on the $RAG2^{-/-}$ IL-$2R\gamma^{null}$ strain, respectively, the resulting uPA-SCID and FRG mouse models can achieve high levels of human liver cell chimerism and susceptibility to HCV infection with high titer viremia [214, 217–219].

Both the uPA-SCID and the FRG model have been widely used in HCV research [220]. Studies in these mice have contributed to our current understanding of basic aspects of HCV virology, the viral life cycle, and HCV particle composition [221]. Further, it has been shown that broadly neutralizing antibodies targeting viral envelope proteins as well as antibodies specific for HCV entry factors can protect against infection or abrogate a chronic infection in vivo in human liver chimeric mice [222–226]. Finally, these models have been proven useful for the preclinical evaluation of HCV DAAs [227, 228].

Significant effort has also been put into overcoming the immune deficiency in liver chimeric mice to broaden their application to immunological studies. A strategy to achieve this is to combine the xenotransplantation of human hepatocytes with that of human hematopoietic stem cells (HSC) into one immunodeficient host [183, 229]. In fact, approaches to engraft mice with human immune cells by transplantation of human fetal liver or cord blood-derived HSC have been developed for decades and applied in many research fields [230]. Only recently, however, the successful double engraftment of human hepatocytes and immune cells into mice was reported [231–233].

Despite significant progress, these complicated models still suffer from a variety of shortcomings in the functionality of the transplanted human immune system, e.g., caused by mismatches in mouse and human hematopoietic cytokines or MHC molecules [230]. These problems currently limit their utility to study HCV-specific immune responses or vaccine strategies. Efforts to improve these models will hopefully solve these caveats in the coming years.

7.3.2 Genetic Humanization of Mice

Genetic manipulation of the host, such as expression of human host factors or inactivation of inhibitory murine molecules, has been explored as another strategy to develop an HCV mouse model. For this approach, a detailed knowledge of factors that define HCV host tropism is necessary. Most progress in this regard has been made at the level of viral entry into hepatocytes. It has been shown that CD81, SR-B1, CLDN1, and OCLN represent the minimal set of entry factors required for HCV uptake into mouse hepatocytes and that CD81 and OCLN need to be of human origin [234]. Based on this knowledge, a genetically humanized C57BL/6 mouse model expressing all four human entry factors was constructed and shown to support HCV entry into the mouse liver in vivo [235, 236]. This model was then used to evaluate the efficacy of HCV envelope-specific neutralizing antibodies [237]. In addition, when innate immune signaling pathways (e.g., STAT-1 signaling) were blunted in

these mice, low levels of viral replication were detected suggesting that the entire HCV life cycle can be completed in mouse hepatocytes [235]. Using a similar approach, another study showed low-level HCV infection without immunosuppression in outbred ICR mice transgenic for CD81 and OCLN [238].

7.3.3 Viral Adaptation

A complementary approach to mouse humanization is the attempt to adapt HCV to entry and replication in mouse hepatocytes without the need of human factors. In fact, one adaptation strategy resulted in the selection of adaptive mutations in HCV E1 and E2 that enabled a so-called murine-tropic HCV (mtHCV) to enter mouse hepatocytes in vitro [239]. A recent follow-up study from 2016 further showed that mtHCV could infect the mouse liver in vivo. Viral replication, however, was not detected even when the mice were highly immune-compromised [240]. In conclusion, additional studies are needed to evaluate whether mice humanization and HCV mouse adaptation approaches can eventually lead to robust HCV replication in mice.

8 HCV-Related Hepaciviruses

For decades, the hepacivirus genus of the family *Flaviviridae* consisted of only two known members: HCV and GB virus B. In recent years, however, the phylogenetic diversity of hepaciviruses has expanded significantly [184, 241]. In 2011, a non-primate hepacivirus (NPHV) was discovered first in dogs and later in horses. Shortly thereafter more animal hepaciviruses where found in rodents, bats, cows, colobus monkeys, and even sharks. The discovery of these viruses reveals a so far unappreciated genetic diversity and host tropism of hepaciviruses and might help to provide new insight into HCV evolution and origin [242]. HCV-related hepaciviruses could also serve as a basis for the development of immune-competent surrogate HCV animal models. Here we discuss the potential of GB virus B, NPHV, and rodent hepaciviruses to serve as such models.

8.1 GB Virus B

GB virus B (GBV-B) was first described in 1967 following the experimental inoculation of marmosets with serum from a surgeon (GB: George Baker) suffering from acute hepatitis. In 1995 two viral genomes, GBV-A and GBV-B, were isolated from serum and liver of experimentally infected marmosets; however, only GBV-B caused hepatitis [243]. It has since been shown that a variety of other New World monkeys, such as tamarins and owl monkeys, are susceptible to GBV-B infection [244]. While GBV-B infection in monkeys is mostly acute and self-limiting, some

cases of chronic infection following intrahepatic inoculation of viral RNA have been reported [245]. Although GBV-B was supposedly isolated from a human sample, GBV-B infections in human populations have not been observed, and a potential viral reservoir in monkeys has also not been identified. As such the natural host of GBV-B remains unknown [184].

While HCV and GBV-B are related viruses with similar genome organization, there is significant divergence on the nucleotide and amino acid level. This limits the utility of GBV-B for HCV drug and vaccine testing.

8.2 Non-primate Hepaciviruses

In 2011, using high-throughput sequencing, a novel hepacivirus was identified in the respiratory tract of dogs during a respiratory illness outbreak in these animals [246]. The isolated virus was initially name canine hepacivirus (CHV). However, a follow-up screen performed in multiple species to further define the distribution and host tropism of this new virus revealed that CHV is actually most prevalent in horses and not in dogs [247, 248]. Following this discovery, the virus was renamed to non-primate hepacivirus (NPHV) [247]. NPHV is the closest known viral homolog to HCV with a nucleotide sequence convergence of approximately 50%. Multiple recent studies have characterized the natural course of infection and tissue tropism of NPHV in horses [249–251]. Collectively, these data show that NPHV causes a hepatotropic infection that can either be acute and self-limiting or progress to chronicity. Infection can be initiated by inoculation of horses with NPHV-positive horse plasma or intrahepatic injection of in vitro transcribed RNA derived from a NPHV cDNA clone [250].

Acute infection of NPHV is associated with mild hepatitis and liver damage. Interestingly, infection of immunodeficient foals led to significantly less pronounced liver disease as compared to immune-competent foals suggesting immune-mediated viral hepatitis [251]. Further, NPHV-infected horses showed activation of immune cells and delayed seroconversion. After clearance of primary infection, the horses were protected against reinfection [252].

In conclusion, NPHV infection in horses shows important similarities to HCV infection in humans. Thus this infection system might become a useful surrogate model to evaluate HCV vaccine strategies in the future. However, horses are large animals that are not genetically amenable or tractable, and there are limited equine-specific research tools available.

8.3 Rodent Hepaciviruses

In 2013, two studies independently reported the discovery of multiple rodent hepaciviruses (RHV) after screening over 5,000 individual rodents from 45 different

species [253, 254]. RHVs were isolated from species such as deer mice, desert wood rats, or bank voles. Further, in 2014 another report described the isolation of an RHV in rats from New York City, named Norway rat hepacivirus (NrHV) [255]. Phylogenetic comparison of RHVs with other hepaciviruses revealed that RHVs are more divergent from HCV than NPHV, but closer to HCV than the primate virus GBV-B [184].

The identification of hepaciviruses in wild rodents opened the possibility for the development of small laboratory animal models of hepacivirus infection. In fact, a recent study provided a detailed characterization of NrHV infection in laboratory rats, thus establishing an immune-competent rat surrogate model for HCV [256]. Rats developed a hepatotropic and miR-122-dependent chronic infection upon challenge with NrHV-positive serum or intrahepatic inoculation with in vitro transcribed RNA from an infectious clone. While spontaneous clearance was not observed, rats could be cured by treatment with the HCV DAA sofosbuvir. Similar to HCV infection in humans, chronic NrHV infection in rats was characterized by sustained intrahepatic immune activation, including persistent upregulation of type 1 interferon pathways [256].

Rodent hepaciviruses were not discovered in wild house mice [257]. However, it was recently shown that NrHV infection is not restricted to rats and that the virus can efficiently replicate in laboratory mouse strains [258]. Immunodeficient mouse strains developed a chronic infection while fully immune-competent C57BL/6 and Balb/c mice cleared NrHV within 3–5 weeks. Similar to rats, NrHV infection in mice was hepatotropic and miR-122-dependent. Acute clearance in mice was associated with the induction of a strong intrahepatic immune response and acute hepatitis. Transient depletion of CD4+ T cells resulted in viral chronicity in immune-competent mice indicating a major role of T cells in clearance of NrHV infection [258]. This is consistent with the T cell depletion studies performed in HCV-infected chimpanzees. Similar to HCV reinfection in chimpanzees and humans, mice were also not completely protected against secondary NrHV infection.

Both, the NrHV/RHV rat and mouse models provide fully immune-competent rodent HCV surrogate models that share important similarities with HCV infection in chimpanzees and humans. Both models provide for the first time a platform to mechanistically study basic aspects of hepacivirus infection and immunity in the liver. The utility of these models for HCV vaccine development still needs to be determined. Given the significant genetic divergence between HCV and RHVs, it remains to be determined if RHV models will be useful for the preclinical testing of HCV vaccine strategies.

9 Concluding Remarks

From the discovery of HCV until today, the chimpanzees have remained the only immune-competent animal model of HCV infection. As such studies in these large apes have been instrumental for achieving the medical milestone of HCV cure.

Nevertheless numerous other animal models of HCV infection have also provided valuable insight into HCV biology and served as platforms to test drug and vaccine candidates. A remaining challenge for HCV research is the development of a protective vaccine. Novel immune-competent HCV models that allow the mechanistic studies of immune responses and potentially the prioritization of vaccine candidates might help to achieve this goal.

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Compliance with Ethical Standards

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