Cellular and molecular biology of HCV infection and hepatitis

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ABSTRACT

HCV (hepatitis C virus) infects nearly 3% of the population worldwide and has emerged as a major causative agent of liver disease, resulting in acute and chronic infections that can lead to fibrosis, cirrhosis and hepatocellular carcinoma. Hepatitis C represents the leading cause of liver transplantation in the United States and Europe. A positive-strand RNA virus of the Flaviviridae family, HCV contains a single-stranded RNA genome of approx. 9600 nucleotides. The genome RNA serves as both mRNA for translation of viral proteins and the template for RNA replication. Cis-acting RNA elements within the genome regulate RNA replication by forming secondary structures that interact with each other and trans-acting factors. Although structural proteins are clearly dispensable for RNA replication, recent evidence points to an important role of several non-structural proteins in particle assembly and release, turning their designation on its head. HCV enters host cells through receptor-mediated endocytosis, and the process requires the co-ordination of multiple cellular receptors and co-receptors. RNA replication takes place at specialized intracellular membrane structures called 'membranous webs' or 'membrane-associated foci', whereas viral assembly probably occurs on lipid droplets and endoplasmic reticulum. Liver inflammation plays a central role in the liver damage seen in hepatitis C, but many HCV proteins also directly contribute to HCV pathogenesis. In the present review, the molecular and cellular aspects of the HCV life cycle and the role of viral proteins in pathological liver conditions caused by HCV infection are described.

HCV (HEPATITIS C VIRUS) GENOME AND PROTEINS

HCV research: a brief review of history

Originally termed NANBH (non-A, non-B hepatitis), hepatitis C was first discovered in post-transfusion hepatitis patients in 1975 [1]. The identification of the aetiological agent of NANBH took more than a decade, mainly because of the inability of the virus to propagate efficiently in cell culture. The cloning and characterization of the HCV genome by Michael Houghton’s group [2] revealed that HCV is a member of the Flaviviridae family and a hepacivirus genus and permitted molecular characterizations of HCV. The identification of the additional 3′-X sequence [3] and the construction of a cDNA clone that is infectious in chimpanzees [4,5]

Key words: hepatitis C virus (HCV), liver disease, pathogenesis, RNA replication, virus life cycle.

Abbreviations: Apo, apolipoprotein; ARFP, alternate reading frame protein; CLDN, claudin; CRE, cis-acting regulatory element; CyP, cyclophilin; eIF, eukaryotic initiation factor; ER, endoplasmic reticulum; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVcc, HCV cell culture; HCVpp, HCV pseudoparticle; IFN, interferon; IRES, internal ribosome entry site; LDL, low-density lipoprotein; LDLR, LDL receptor; miRNA, microRNA; NANBH, non-A, non-B hepatitis; NS, non-structural protein; ORF, open reading frame; PK, proteinase K; pRb, retinoblastoma-susceptibility protein; RC, replication complex; CRC, crude RC; RdRp, RNA-dependent RNA polymerase; RIG-I, retinoic-acid-inducible gene-1; RNS, reactive nitrogen species; ROS, reactive oxidative species; siRNA, small interfering RNA; SL, stem–loop; SR-B1, scavenger receptor class B type 1; TM, transmembrane; UTR, untranslated region; VAP, human vesicle-associated membrane protein-associated protein.

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confirmed the in vivo infectivity of the HCV genome. The resolution of the crystal structures of the NS3 (non-structural protein 3) protease [6,7] and NS5B polymerase [8–10] facilitated the drug-development effort to find HCV-specific inhibitors. Studies of HCV replication received a major boost with the development the subgenomic replicon in 1999 [11], another decade after the cloning of the HCV cDNA. Because of a selectable antibiotic marker gene incorporated into the viral genome, the replicon RNAs, either full-length or subgenomic, can maintain long-term RNA replication in culture cells without producing viral particles. The subgenomic replicon system was optimized further and deposited into the NIH (National Institutes of Health) AIDS Research & Reference Reagent Program (http://www.aidsreagent.org) [12] for public distribution. Accordingly, tremendous progress in both basic research and drug development of HCV infection has been made in the past decade. Most recently, with the advent of a robust cell culture infection system [JFH-1/HCVcc (HCV cell culture)] based on a unique isolate from a Japanese fulminant hepatitis patient in 2005, the complete life cycle of HCV infection could be studied in cell culture for the first time [13–16]. Subsequent development of cell-culture-infectious clones of additional genotypes and chimaeric genomes [17–20] should facilitate the study of genotype-specific differences in the viral life cycle and pathogenesis.

**Genome**

HCV is genetically heterogeneous and the six major genotypes have distinct regional distribution patterns [21]. The HCV genome consists of a positive-sense single-stranded RNA approx. 9600 nucleotides in length. It encodes a single ORF (open reading frame) that is flanked by 5′- and 3′-UTRs (untranslated regions) (Figure 1A). The 5′-UTR contains six secondary structure domains termed SLs (stem–loops) I–VI (Figure 1B). SLII, III and IV form an IRES (internal ribosome entry site) that facilitates the translation of the capless HCV RNA [22]. The 5′-UTR also contains essential replication signals for the negative-strand RNA [23], which serves as the replicative intermediate. A liver-specific miRNA (microRNA), miR-122, was identified to bind to two tandem sites just downstream of SLI [24–26]. The interaction between miR-122 and HCV 5′-UTR regulates HCV replication and translation, and ectopic expression of this miRNA increases HCV replication in non-hepatic cells [27]. Although a more recent study found no correlation between miR-122 expression level and viral load in vivo, an inverse correlation between the level of miR-122 pretreatment and the sensitivity to IFN (interferon) therapy was identified, suggesting a potential application of this miRNA as a biomarker for IFN therapy [28].

The 3′-UTR has a tripartite structure and is essential for HCV replication [29–32]. It starts with a short
variable region, followed by a poly(U/UC) tract and a highly conserved 3′-X tail [3]. Different genotypes differ in the sequence of the variable region, but in general isolates of the same genotype it is fairly well conserved. The length of the poly(U/UC) tract varies significantly and accounts for the length variation observed in the 3′-UTR. The poly(U/UC) tract, along with the 5′ triphosphate of the genomic RNA, constitutes the major HCV-associated molecular pattern that is recognized by the innate immunity receptor RIG-I (retinoic-acid-inducible gene-I) [33]. The 3′-X tail is highly conserved and contains three SL structures totalling 98 nucleotides in length. In addition to directing the synthesis of the negative-strand RNA, the 3′-UTR can also increase HCV IRES-mediated translation in the proper genomic context (see [34] and references within).

CREs (cis-acting regulatory elements) are also found in the protein-coding region of the genome. Three SLs have been identified in the NS5B coding region [35–37]. One of these, 5BSL3.2, forms base pairs with the 3′ SLII from the 3′-X tail to form a kissing-loop interaction that is critical for replication [38]. In addition, 5BSL3.2 can interact with an upstream CRE in the NS5B coding region to produce a pseudoknot structure [39]. Finally, mutagenesis studies of the core coding region suggest the existence of two more SLs that are important for efficient replication in vitro [40] (Figure 1B).

Proteins

The HCV polyprotein encoded by the single ORF is approx. 3000 amino acids long and is co-translationally and post-translationally processed by cellular and viral proteases to form three structural proteins (core, E1 and E2), an ion channel protein (p7) and six NSs (NS2, NS3A, NS4A, NS4B, NS5A and NS5B). The structural proteins are located at the N-terminus, whereas the non-structural proteins are situated at the C-terminus (Figure 1C).

The structure proteins are released from the polyprotein by host signal peptidases that cleave signal peptides located between core/E1, E1/E2, E2/p7 and p7/NS2 [41,42]. Core is processed further by a signal peptide peptidase into a mature protein of approx. 21 kDa. This cleavage/maturation process promotes the transport of core from the ER (endoplasmic reticulum) membrane to the surface of the lipid droplets [43], the site of HCV particle assembly [44]. Accordingly, signal peptide peptidase cleavage of core is important for the production of both virus-like particles and infectious virions [45,46]. The N-terminus of core is highly basic and has been implicated in virus-like particles and infectious virions [45,46]. The N-terminus is highly basic and has been implicated in RNA binding and homo-oligomerization, both important properties of a nucleocapsid protein. An alternative ORF, produced by ribosome shifting, overlaps with the core gene and encodes a family of core-related proteins called the ARFPs (alternate reading frame proteins) (reviewed in [47]). Although both reactive sera and a positive T-cell response against ARFP/F-protein have been reported [48,49], the function of the short-lived ARFPs in the HCV life cycle remains unclear [50].

E1 and E2 are envelope glycoproteins on the viral surface that are responsible for receptor binding and entry of HCV into target cells. Inside the cells, the C-terminal TM (transmembrane) domains of these proteins direct them to the ER membrane, the presumed site where HCV capsid particles pick up their envelopes before leaving the cell. E1 (192 amino acids) and E2 (363 amino acids) are both heavily glycosylated and form a non-covalent heterodimer. Although the correct folding of these two proteins appears to depend on each other [51–54], E2 is the major ligand that binds to CD81 and SR-B1 (scavenger receptor class B type I), two of the several HCV receptors identified so far [55,56].

The p7 protein is a hydrophobic peptide of 63 amino acids. Both N- and C-termini of p7 face the ER lumen, indicating that it contains two TM domains [57]. HCV p7 oligomerizes on the ER membrane and forms hydrophobic pores with ion channel activity [58,59]. Although dispensable for RNA replication, p7 is essential for the production of infectious viruses both in vivo and in vitro, indicating a potential role of this protein in viral assembly and release [60–62].

NS2 is a TM protein of approx. 21–23 kDa. Its N-terminal portion contains TM helices that anchor the protein to the ER membrane [63]. The C-terminal half of NS2 contains a protease domain that, together with the N-terminal protease domain of the NS3, forms a catalytically active protease that cleaves the NS2/NS3 junction [64,65]. As with p7, NS2 is not required for RNA replication, but is vital for the production of infectious viruses in vivo [18,61,66]. Interestingly, the C-terminal protease domain, but not the catalytic activity, of the NS2–NS3 protease is required for this function [61,66]. The crystal structure of the C-terminal protease domain of NS2 has been resolved and reveals a dimer structure and composite active sites consisting of residues contributed by each monomer [67].

The 69 kDa NS3 contains two distinct enzymatic activities. The N-terminal third of NS3 is a serine protease that cleaves all other downstream NS junctions (NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B) with the assistance of a non-covalently attached cofactor, NS4A. Its C-terminal two-thirds contain an RNA helicase/NTPase. Both the protease and the helicase activities are important for HCV replication [68,69]. In addition, the protease activity of NS3–NS4A plays an important role in the ability of HCV to blunt RIG-I-mediated innate immunity [70] by cleaving MAVS (mitochondrial antiviral signalling protein; also known as IPS-1 (IFN-β promoter stimulator-1), VISA (virus-induced signalling adaptor) and CARDIF (CARD (caspase recruitment domain) adaptor inducing IFN-β) off the mitochondrial membrane (reviewed in [71]). The NS3–NS4A protease can also cleave the Toll-like receptor 3 adaptor protein TRIF (Toll/interleukin-1...
receptor domain-containing adaptor protein inducing IFN-β) [72], diminishing further the ability of the host cell to mount an antiviral response against HCV infection. Both its essential role in the HCV life cycle and its ability to counteract host innate immunity make the NS3–NS4A protease an attractive target for STAT-C (specifically targeted antiviral therapy for HCV), and the available crystal structure and cell culture systems greatly facilitated these efforts. Several HCV protease inhibitors are currently being tested in human clinical trials [73].

The C-terminal helicase domain of NS3 folds independently and is connected to the protease domain through a linker peptide [74,75]. There is also evidence of functional cross-talk between the helicase and the protease [76–78]. The NS3 helicase is a member of the superfamily 2 DExH/D-box helicase that unwinds RNA–RNA substrates in an ATP-dependent manner. In addition to its potential role in resolving secondary structures during RNA replication, the NS3 helicase domain has been reported recently to be involved in an early step of viral assembly [79]. The development of NS3 helicase inhibitors as HCV therapeutics lags behind that of protease or polymerase inhibitors, mainly because the mechanism of action of the helicase in the replication and assembly process is not well understood [80,81].

NS4A is a 54-amino-acid polypeptide that serves as an essential co-factor for the NS3 protease. A TM helix in the hydrophobic N-terminus anchors the NS3–NS4A complex to intracellular membranes, where proteolytic processing and RNA replication take place [82]. The central region (amino acids 21–34) of NS4A is hydrophobic and required for the activation of NS3 [83–85]. The acidic C-terminus plays an important role in HCV replication by regulating NS5A phosphorylation [86,87].

NS4B is a 27 kDa integral membrane protein containing both hydrophobic TM and amphipathic helices [88,89]. Its best-known function is the induction of a specialized membrane compartment called the 'membranous web', which is the presumed site of RNA replication [90]. Recently, NS4B has been shown to bind HCV RNA, and a chemical compound that blocks RNA binding inhibits HCV replication [91]. Studies using 1b/1a chimaeric replicon constructs suggest the polymorphism at the NS4B gene affects replication efficiency, and a genetic interaction between NS3 and NS4B exists [92,93]. Whether this interaction is related to either the membrane-altering or the RNA-binding function of NS4B is currently unknown. The C-terminus of NS4B contains a GTP-binding site [94] and is also palmitoylated at two cysteine residues [95], but the mechanism by which NTP binding or palmitoylation facilitates HCV replication remains unclear. In addition, a cellular protein localized in the early endosome Rab5 was found to be associated with NS4B in replicon cells and may play a role in the formation of the RC (replication complex) [96].

NS5A does not contain any TM domains; instead, it is tethered to the intracellular membranes by an N-terminal helix that inserts itself horizontally into the lipid bilayer [97,98]. In this setting, the length of the helix runs perpendicular to the tails of the phospholipids and the helix is only in contact with the cytosolic leaflet of the membrane [99]. The amphipathic nature and the in-plane membrane association of the N-terminal helix are conserved features of NS5As encoded by several related members of Flaviviridae [100]. Both bioinformatics and proteolysis data suggest that NS5A consists of three domains following the N-terminal amphipathic α-helix [101]. The crystal structure of the largest and most conserved domain, domain I, revealed a dimeric structure that contains a putative RNA-binding groove [102], consistent with ability of NS5A to bind RNA in vitro [103]. Domain II and domain III are smaller and exhibit more sequence diversity among the different genotypes. Although domain III is important for virus production, it is dispensable for RNA replication [104], and replicon constructs with large insertions into this domain are replication competent [105]. Such modified replicons have provided insights into the dynamics of the RC in living cells [106] and are a convenient flow-cytometry-based tool to monitoring replication in vitro [107].

NS5A is phosphorylated and exists in two forms: a basally phosphorylated form of 56 kDa and a hyperphosphorylated form of 58 kDa [108]. Multiple lines of evidence point to an important role of the phosphorylation status of NS5A in viral replication. Adaptive mutations in NS5A and other NSs that enhance replication abolish NS5A hyperphosphorylation [109,110], and selective inhibitors of NS5A phosphorylation stimulated the replication of wild-type HCV RNA [111]. NS5A is important for the assembly of infectious viral particles in cell culture as well. Deletion of the C-terminal domain III or the substitution of a single serine residue with an alanine residue in domain III abolished the production of infectious viruses in the HCVcc system [112,113]. Interestingly, the phosphorylation state of domain III is also involved in this regulation [112]. A recent report of an NS5A inhibitor that suppresses HCV replication at concentrations between 9 and 50 pmol/l highlights the critical requirement for this protein in the viral life cycle [114].

NS5A also interacts with many cellular proteins that are implicated in HCV replication. The interaction between NS5A and hVAP-A (human vesicle-associated membrane protein-associated protein A) is regulated by the phosphorylation status of NS5A and may be involved in the formation of the viral RC [109,115]. A number of other NS5A-associated proteins, including the geranylgeranylated F-box protein FBL2 (F-box and leucine-rich repeat protein 2), VAP-B, FKBP8 (FK506-binding protein 8), the Rab GAP (GTPase-activating protein) TBC (Tre-2/Bub2/Cdc16) domain protein
TBC1D20 and FBP [FUSE (far upstream element of the c-myc proto-oncogene)-binding protein], are also shown to be required for HCV replication [116–119].

The 68 kDa NS5B is the RdRp (RNA-dependent RNA polymerase) responsible for synthesizing the complementary negative strand of the genome as the replication intermediate and then genomic RNA using the negative strand as the template. The HCV RdRp can initiate RNA synthesis in vitro by two different mechanisms: de novo initiation from the 3′-end of RNA template [120,121] and primer-dependent initiation [122]. The de novo synthesis is probably used in vivo because no primers corresponding to either end of the genome are found inside the cells. The crystal structure of NS5B reveals a right-handed structure made up of fingers, palm and thumb domains typically seen in all RdRps [8–10]. The active site of NS5B is fully encircled by the fingers and thumb domains, which closely interact with each other. The characterization of allosteric non-nucleoside inhibitors revealed that there is extensive interactions between the different domains (reviewed in [123]). Furthermore, a point mutation in the thumb domain was able to rescue the otherwise lethal phenotype of C-terminal mutation in the genotype 1b replicon [124]. In addition to the essential RdRp enzymatic activity, the membrane association of NS5B, which is mediated by a C-terminal TM domain, is also critical for HCV replication [125]. Other important features of the HCV NS5B include a β-hairpin loop [126], a GTP-binding site [127,128] and the ability to homo-oligomerize [129,130].

A functional interaction between NS5A and NS5B has been reported [131], and the proposed function of NS5A is to serve as a cofactor for NS5B [132]. NS5B also binds to several cellular proteins that are implicated in HCV replication. PRK2 (protein kinase C-related kinase 2) binds to the fingers domain and may regulate the phosphorylation of NS5B [133]. The glycine/arginine-rich domain of nucleolin mediates its interaction with NS5B, and siRNA (small interfering RNA) directed at nucleolin mRNA suppressed HCV replication in vitro [134]. NS5B also interacts with the NS5A-binding protein hVAP-B, which may be a component of the RC [117]. CyPA (cyclophilin A) and CyPB, the intracellular targets of cyclosporine A, which potently inhibits HCV replication, also interact with NS5B in vitro and in vivo [135,136]. Despite similar NS5B-binding abilities in vitro, the two cyclophilins differ greatly in their expression levels in vivo; the most abundant form, CyPA, comprises approx. 90% of the total CyP pool in liver cells and tissue [135,137]. Consequently, silencing of CyPA, but not of CyPB, significantly suppressed HCV replication and infection in vitro [124,135]. Finally, NS5B binds to pRb (retinoblastoma-susceptibility protein) through a site overlapping its polymerase motif, although this interaction presumably contributes to the oncogenic property of HCV rather than the replicase function of NS5B [138].

**HCV LIFE CYCLE**

HCV mainly infects liver in vivo, and this tropism is recapitulated in vitro, as both HCVcc and HIV particles pseudotyped with HCV envelopes [HCVpp (HCV pseudoparticles)] mostly infect cell lines of liver origin (reviewed in [139]). The virus uses specific attachment and entry factors to cross the plasma membrane of hepatocytes and release its genomic RNA. Once released, the RNA serves as a template for replication and translation of the polyprotein. The polyprotein must then be directed to specific cellular locations where it is co-translationally and post-translationally modified. Matured viral proteins are then able to assemble into new virions which leave the host cell using the secretory pathway (Figure 2).

**Entry and receptors**

The first step of HCV infection is the attachment of the virus to the host cell surface, where attachment molecules such as glycosaminoglycans (heparin sulfate) [140,141] and LDLR [LDL (low-density lipoprotein) receptor] [142–145] may mediate this initial interaction. Subsequently, specific binding between viral envelope glycoproteins and entry receptors induces receptor-mediated endocytosis and the ingress of HCV particles across the cell plasma membrane.

CD81, a member of the tetraspanin protein family, is probably the best-characterized receptor molecule for HCV entry. It was first identified as a soluble E2-binding protein [55], and its critical role in HCV entry has since been confirmed in virtually all model systems (reviewed in [146]). Furthermore, a recent in vivo study showed that pretreatment with anti-CD81 antibodies completely blocked infection of human liver uPA (urokinase plasminogen activator)/SCID (severe combined immunodeficiency) mice by HCV of multiple genotypes, suggesting agents that disrupt HCV–CD81 interaction could have prophylactic value [147]. On the other hand, a CD81-independent cell-to-cell transfer route of infection in vitro has also been reported recently [148]. CD81− HepG2 cells, which are non-permissive to cell-free virus, became infected after being co-cultured with HCVcc-infected Huh-7.5 cells, even in the presence of neutralizing anti-CD81 antibodies [148]. In addition, HCVcc bearing a mutant E2 protein defective in CD81 binding or cell entry was still capable of transmitting through cell-to-cell contact [149]. The relevance of CD81-independent infection in vivo awaits further characterization.

The E2-binding site on CD81 has been mapped to a large extracellular loop of 89 amino acids. Interestingly, an N-terminal truncated product (EWI-2wint) of a previously identified CD81 partner, EWI-2 [150], blocks the E2–CD81 interaction and inhibits HCV entry. EWI-2wint was shown to be absent from HCV-permissive cells, suggesting a potential contribution of this ‘natural’ entry inhibitor to HCV tropism [151].
Entry into target cells initiates HCV infection

HCV first attaches itself to the host cell surface by means of weak interactions with glycosaminoglycans (GAG) and/or the LDLR. Once bound and concentrated on the cell surface, virions are able to interact with entry receptors such as CD81 and SR-BI with high affinity. The virus–receptor complex then translocates to the tight junctions where CLDN and occludin (OCLN) act as cofactors and induce receptor-mediated endocytosis. The virus-containing endosome is acidified in the cytoplasm, a process that triggers the envelope glycoproteins to initiate fusion and release the nucleocapsid. HCV genomic RNA is then released and translated into polyprotein. The polyprotein is co- and post-translationally modified to produce mature viral proteins which can form RCs and assemble into new virions. These progeny virions bud into the lumen of the ER and leave the host cell through the secretory pathway, completing the viral life cycle. An animated version of this Figure is available at http://www.ClinSci.org/cs/117/0049/cs1170049add.htm.

Ectopically expressed CD81 is necessary, but not sufficient, to allow entry of HCV into non-permissive cells, indicating the presence of additional entry factors. Accumulating evidence supports a critical role for human SR-BI in the HCV entry process. Both anti-(SR-BI) antibodies and siRNAs directed at SR-BI mRNA could inhibit infection of HCVpp and HCVcc [152–155]. As with that of CD81, the expression level of SR-BI in Huh-7-derived cell lines can regulate cell permissiveness or HCV infectivity [156–158]. Because SR-BI interacts with both LDL/HDL (high-density lipoprotein) and soluble E2 [56,159], whether the interaction between SR-BI and HCV is direct or mediated by HCV-associated lipoproteins remains unclear.

Recognizing that even the simultaneous expression of CD81 and SR-BI on the cell surface of many cell lines was insufficient to support HCVpp entry, Evans et al. [160] set out to identify additional entry cofactors using an expression cloning approach. Their effort successfully identified human CLDN-1 (claudin-1), a tight-junction protein, a yet another protein essential for HCV entry. Subsequently, two more related CLDN family members, CLDN-6 and CLDN-9, have also been shown to be able to mediate HCV entry [161,162]. Interestingly, an interaction between CD81 and CLDN-1 has been observed, suggesting a co-receptor complex formation [163,164]. In addition, inhibition of PKA (protein kinase A) activity, which interfered with formation of the complex between CD81 and CLDN-1, inhibited HCV entry and reduced infectivity in cell culture [165].

More recently, another tight-junction protein, occludin, has been shown to be required for HCVcc entry in vitro. Occludin could interact with E2 expressed in HCVcc-infected cells, and suppression of occludin with siRNA inhibits HCV entry [166]. Of note, the expression of occludin was down-regulated upon HCVcc infection, potentially contributing to the exclusion of superinfection reported previously [167,168]. Occludin was also independently identified, again by expression cloning, as one of the human entry factors required for HCVpp entry into murine cells. Of the four entry factors identified so far, CD81 and occludin determine
the species specificity of HCV entry between mouse and human, as mouse CLDN-1 and SR-B1 functioned similarly as the human counterparts [169].

The list of cellular proteins involved in HCV entry will probably continue to grow [170]. On the other hand, very little is known about how these factors co-ordinate to facilitate the actual viral entry process. A current model predicts a multistep process that includes attachment and receptor binding, post-binding association with tight-junction proteins and then internalization by endocytosis, which is followed by a pH-dependent step that results in the fusion of membranes and the release of viral RNA into the cytoplasm of the host cells [171]. Elucidation of the details involved in these various processes will require a significant amount of future research.

**Translation**

Eukaryotic translation normally requires a 5′-7meG-cap structure that recruits eIF4F (eukaryotic initiation factor 4F) to the mRNA and allows the small (40S) subunit of the ribosome to bind to the 5′-end and scan to the initiation codon in an ATP-dependent manner. HCV RNA, however, does not contain a 5′-cap and uses an IRES-based cap-independent approach for protein translation [22]. Located within the 5′-UTR, the HCV IRES is made up of SLII, III and IV and the first few codons of the core ORF (Figure 1B). SLIII and IV form a pseudoknot structure that is confirmed by X-ray crystallography and NMR studies (reviewed in [172]). Single particle reconstructions of IRES bound to the 40S ribosome subunit and eIF3 showed that the IRES can induce conformational changes in the 40S subunit that allow the formation of the active 80S complex in the absence of a 5′-cap or ATP-dependent scanning [173–175]. In addition, direct assembly of the 80S ribosome complex on the HCV IRES without any eIFs can also be achieved in vitro at high Mg2+ concentrations [176].

**RNA replication**

Although HCV infection can be studied in animal models such as chimpanzees [4,5] and mice with chimeric human livers [177], the majority of research on HCV RNA replication is performed in cell culture models, such as the replicon or the HCVcc system, for obvious reasons of economy and accessibility. With the exception of the JFH-1 isolate [178], replicons require adaptive mutations for efficient RNA replication in cell culture [12,179–181]. One mechanism by which these mutations enhance replication appears to involve the suppression of hyperphosphorylation of NS5A [109,110], which negatively regulates the replication of wild-type replicon in vitro [111]. Paradoxically, the cell-culture-adaptive mutations that enable subgenomic replicon to propagate in vitro abolished the infectivity of the full-length viral genome in chimpanzees [182]. Several reasons could account for this discrepancy; among them are differences between cultured hepatoma cell lines and hepatocytes in liver tissue and/or the requirement of a delicate balance between RNA replication and later steps in the HCV life cycle including viral assembly.

As with all positive-strand RNA viruses, HCV replication occurs on intracellular membranes [183]. With all the NSs anchored to membranes, HCV forms an RC that is associated with altered cellular membranes [90,184], and CRCs (crude RCs) that maintain the replicase activity in vitro can be isolated by membrane sedimentation or flotation techniques [185–189]. Note that these CRCs can only use the endogenous template that is already in the complex, but not exogenously added templates for replication. The precise composition of the HCV RC is not known, but will certainly include the viral genome, NSs and cellular cofactors. Interestingly, the membrane association of the NSs is necessary but not sufficient for incorporation into the RC. In the replicon cells, only a small fraction of each NS is associated with the RC; the majority is not [190,191]. These two fractions could be distinguished by their sensitivity to PK (protease K) digestion after cell permeabilization by digitonin. The NSs incorporated into the RC are protected from PK digestion, whereas the rest is degraded by the protease. On the basis of these findings, a model of the relationship between the intracellular membrane and the HCV RC has been proposed [191]. In this model, invagination of the ER membrane forms vesicular membrane structures that house all of the active components of the functional RC (Figure 3A). Similar vesicular membrane-bound structures (spherules) have been observed in cells harbouring the RC of another positive-strand RNA virus, the brome mosaic virus [192]. Several factors may account for the production of the large amount of ‘excess’ NSs that are not part of the RC. They could be the by-products of polyprotein synthesis that generates large amounts of structural proteins; they could play active roles in processes such as counteracting innate immunity or cell cycle regulation; or they could participate in the formation of infectious virions.

Many cellular cofactors regulate HCV replication in vitro, including miRNAs [24,193], the various host proteins that interact with the NSs, and components of the RNA interference pathway [194]. In addition, the status of cellular physiology can have a profound effect on the efficiency of HCV replication. Cell confluence/nucleotide starvation, a temperature shift and ROS (reactive oxidative species) production have all been reported to suppress replication or infection in vitro [107,180,195–198]. The importance of the host cell environment in HCV replication is highlighted by the narrow range of cell lines permissive for replication, with the Huh-7-derived cells dominating cell-culture-based research.
Formation and release of viral particles

The previous reports of robust production of infectious HCV particles in tissue culture [13–19] is ushering in a new era of exciting research focused on the characterization of the physical properties and assembly pathway of HCV particles. HCV has long been suggested to complex with LDLs in chronically infected patients [199,200]. The in vivo observation is corroborated by buoyant density analysis of HCV particles produced in vitro. In the HCVcc system, higher infectivity is typically associated with lower density [201], and the buoyant density of the secreted particles is different from that of the intracellular virions, suggesting a lipoprotein-related maturation involved in the virion production process [202]. The role of lipoproteins in HCV assembly and release is supported by additional lines of evidence. Human Apos (apolipoproteins) are detected in HCV virions [203–205] and both genetic and pharmacological inhibition of ApoE significantly reduces HCV production [204]. HCV assembly appears to occur on lipid droplets, and the core protein clearly coats the surface of this organelle [44]. Lipids may also influence the infectivity of the mature virions; for example, cholesterol and sphingolipid that are associated with HCV virions have recently been shown to play a critical role in viral infectivity [153,206,207]. Finally, blockade of fatty acid synthase activity can down-regulate the expression of CLDN-1 and suppress HCV entry [208].

The contribution of NSs to virus production and infectivity was first suggested by the observation that
both intra- and inter-genotypic chimaeras require the JFH-1-derived NSs to produce infectious virus [14,18]. A detailed mapping study identified NS3, NS5B and the 3'-X tail of the JFH-1 isolate as important determinants of its unique ability to generate virions in cell culture [209]. More recently, mutations in NS5A, NS2 and NS3 that do not affect RNA replication have been shown to block the production of infectious viruses, suggesting a role of these NSs in the assembly and/or release of viral particles [61,66,79,112,113]. Composition studies of highly purified virions should soon provide a comprehensive picture of the NSs that are associated with or incorporated into the viral particles [210].

The area of HCV assembly and release is still in its early stages and rapidly progressing. Although the details are still lacking, a consensus view is emerging regarding the role of both the VLDL (very-LDL) pathway and NSs in this important process. A reasonable proposal is that NSs are divided into at least two pools in the infected cells; one participates in the replication process and is incorporated into the RC, whereas the other is involved in particle assembly. In addition, it is likely that the association with lipoproteins and lipids affects the buoyant density of the virions. A generalized model of HCV assembly integrating these views has recently been proposed (Figure 3B).

HCV PATHOGENESIS

Acute and chronic hepatitis C
A small percentage of patients (10–25%) spontaneously clear the virus during the acute phase (first 6 months after infection) of HCV infection. A strong HCV-specific CD8+ T-cell response, which is probably responsible for acute liver damage [211], is correlated with the control of viraemia during the acute phase [212]. The majority of HCV-infected individuals are unable to clear the virus during the acute phase and become chronically infected. Chronic hepatitis C is characterized by liver inflammation, fibrosis/cirrhosis and, in some patients, HCC (hepatocellular carcinoma) (Figure 4). The major contribution of cytotoxic T-cells to liver damage in chronic hepatitis C is well-documented and will not be covered here (for a recent review of this topic, the reader is referred to [211]). We will direct our discussion to the less-clear topic of the direct effects of HCV proteins on the progression of chronic hepatitis that can ultimately lead to HCC.

Oxidative stress
HCV infection is associated with increased oxidative stress, which is marked by an increase in oxidants and a decrease in antioxidant capacity of the cells [213]. In addition to the contribution by chronic inflammation caused by infection, a direct induction of ROS/RNS (reactive nitrogen species) and mitochondrial dysfunction by the virus is likely. In cell culture systems, HCV expression, replication and infection can induce oxidative stress [214–216]. Furthermore, individual viral proteins, such as core, NS3 and NS5A, have all been reported to increase ROS production and induce oxidative stress both in vitro and in vivo (reviewed in [213]). A role of ROS in liver fibrosis has been suggested by the ability of ROS to up-regulate the profibrogenic cytokine TGF-β (transforming growth factor-β) [217]. A recent proteomics study of liver biopsies from HCV-infected patients at different stages of fibrosis revealed a correlation between the down-regulation of antioxidant proteins and the later stages of liver fibrosis, consistent with a role of oxidative stress in the progression of liver fibrosis and cirrhosis [218]. Increased levels of ROS/RNS may also accelerate the development of HCC by inducing mutations and damaging cellular DNA [219,220], although the causes of HCV-induced HCC will certainly include many more different aspects [221].
Steatosis
Hepatic steatosis occurs in over half of chronic HCV patients and is associated with the development of HCC [222]. A role of the HCV core protein in insulin resistance and hepatic steatosis has been proposed. Core induces lipid droplet accumulation and redistribution in cell culture [223,224], and the higher lipid-binding affinity of the genotype 3 core protein is correlated with a higher prevalence and more severe grades of steatosis in genotype-3-infected patients [225].

HCC
Despite the strong epidemiological evidence linking HCV infection to HCC, exactly how this cytoplasmic RNA virus causes the cancerous transformation of hepatocytes is unknown. It is worth noting that HCC in the setting of HCV infection occurs mostly in the presence of cirrhosis, suggesting that the perturbation of signalling pathways by chronic inflammation and oxidative stress again plays a major role. On the other hand, the direct oncogenic properties of some HCV proteins have also been reported and may be involved in the process of hepatic oncogenesis.

Several HCV proteins have been reported to alter the regulation of cell proliferation and to be potentially oncogenic. The core protein has been reported to influence cell growth though diverse mechanisms, including the activation of transcription factors/proto-oncogenes, inhibition of apoptosis, stimulation of growth factors and their receptors, and modulation of a tumour-suppressor gene, p53 (reviewed in [226]). Although core has been shown to possess a nuclear localization signal [227], it is localized exclusively in the cytoplasm in HCVcc-infected cells, consistent with the cytoplasmic life cycle of HCV. Whether core can also localize to the nucleus of hepatocytes in the infected patients to exert some of the proposed functions remains unclear. In addition to core, both NS3 and NS5A have been shown to interact with p53 and to suppress p53-mediated transcription activation and apoptosis [228–231].

The second major tumour suppressor gene pRb is also a target of HCV proteins. Both core and NS5B have been reported to reduce the abundance of pRb in hepatoma cell lines [138,232]. In particular, NS5B directly interacts with pRb in both replicon and HCV-infected cells to recruit the E3 ubiquitin ligase E6AP (E6-associated protein), which leads to ubiquitination and subsequent degradation of pRb [233]. The pRb-binding site of NS5B partially overlaps with its polymerase active site and, as a result, recombinant pRb blocked the RdRp activity of NS5B in a dose-dependent manner [234].

The simultaneous interference with the p53 and pRb pathways by HCV parallels the one–two punch strategy employed by HPV (human papillomavirus) to induce oncogenesis. A highly oncogenic DNA virus, HPV blocks p53 and pRb function with two of its early gene products, E6 and E7 proteins, and efficiently transforms infected cells [235].

CONCLUDING REMARKS
The past decade has witnessed an explosion in both basic research and drug development activities in the HCV field thanks to the development of cell culture systems capable of measuring HCV replication and infection in vitro. Despite the tremendous progress summarized above, many important questions remain regarding the viral infection cycle. The precise composition and structure of the HCV virion is not yet known; a significant knowledge gap exists between the in vitro RdRp activity and in vivo replicase function because a biochemically tractable RC is not yet available; the virion assembly and release processes are just beginning to be understood, etc. On the clinical side, a specific anti–HCV drug has yet to be developed, and efforts to develop a prophylactic vaccine are in its early stages. Future directions of HCV research will focus on a deeper understanding of the complete life cycle of this human pathogen both in vivo and in vitro and the development of a wider range of drug targets and drug candidates. The elucidations of the virion structure and the host immune responses, both relevant to vaccine development, deserve equal if not more attention. It is our hope that now that considerably more advanced research tools are available and a large talent pool is pursuing HCV research, many of these ongoing efforts should come to fruition in the next decade.

ACKNOWLEDGEMENTS
We thank Dr Kunitada Shimotohno and Dr Chen Liu for providing images and diagrams, Dr Charlie Rice and Dr Tianyi Wang for sharing unpublished work, and Dr Anne B. Thistle and Ms Diana L. Lambert for proofreading the manuscript prior to submission. We would like to apologize to colleagues whose original work could not be cited here, as a result of page limits.

FUNDING
The authors’ work on HCV replication was supported by the James Esther King Biomedical Research Program of the Florida Department of Health [grant number 06NIR-10]; and the American Heart Association [grant number 0535154N].

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