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# Hepatitis C viral life cycle<sup>☆</sup>

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## Abstract

Hepatitis C virus (HCV) has been recognized as a major cause of chronic liver diseases worldwide. Molecular studies of the virus became possible with the successful cloning of its genome in 1989. Although much work remains to be done regarding early and late stages of the HCV life cycle, significant progress has been made with respect to the molecular biology of HCV, especially the viral protein processing and the genome replication. This review summarizes our current understanding of genomic organization of HCV, features of the viral protein characteristics, and the viral life cycle.

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**Keywords:** Hepatitis C virus; Translation; Polyprotein processing; RNA replication; Viral assembly

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## 1. Introduction

Since its discovery in 1989, representing a turning-point in the search for infectious agents associated with post-transfusion non-A, non-B hepatitis, hepatitis C virus (HCV) has been recognized as a major cause of chronic liver disease and affects approximately 200 million people worldwide at the present time [1–3]. Persistent infection with HCV is associated with the development of chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma [3–8]. In general, people with chronic hepatitis C are relatively asymptomatic and have few, if any, clinical manifestations prior to the development of cirrhosis.

HCV is a small, enveloped RNA virus belonging to the *Hepacivirus* genus of the *Flaviviridae* family, which also includes several classical flaviviruses, including dengue virus and yellow fever virus, as well as pestiviruses, such as bovine viral diarrhea virus and the unassigned GB viruses [9,10]. This review summarizes our current understanding of genomic organization of HCV, as well as features of the viral protein characteristics, and the viral life cycle.

## 2. Genomic organization

The HCV genome consists of a single-stranded positive-sense RNA of approximately 9.6 kb, which contains an open reading frame (ORF) encoding a polyprotein precursor of approximately 3000 residues flanked by untranslated regions (UTRs) at both ends [11]. The precursor is cleaved into at least 10 different proteins: the structural proteins Core, E1, E2 and p7, as well as the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig. 1).

An important feature of the HCV genome is its high degree of genetic variability [12,13]. Mutation rates, however, vary in different regions. The E1 and E2 regions are the most variable, while the 5'UTR and terminal segment of the 3'UTR have the highest degree of sequence conservation among various isolates. The 5' UTR, which is ~341 nucleotide (nt) in length, contains an

internal ribosomal entry site (IRES), which is essential for cap-independent translation of viral RNA, from which four highly structured domains (domains I–IV) are produced (Fig. 1) [14–19]. These are largely conserved among HCV and related viruses [15,16]. As with other RNA viruses with IRES-mediated expression, the HCV 5'NTR is thought to contain determinants for translation, as well as cis-acting elements for RNA replication. It has been shown that (i) the sequence upstream of the IRES is essential for viral RNA replication, (ii) sequences within the IRES are required for high-level HCV replication, and (iii) the stem-loop domain II of the IRES is crucial for replication [20]. A recent study has revealed that the 5'UTR is capable of binding to a liver-specific microRNA, miR-122, resulting in enhanced HCV RNA replication [21]. (Fig. 2).

The 3'UTR varies between 200 and 235 nt in length, including a short variable region, a poly(U/UC) tract with an average length of 80 nt, and a virtually invariant 98-nt X-tail region [22–24]. The X region forms three stable stem-loop structures that are highly conserved among all genotypes and, as a result, the HCV genome likely ends with a double-strand stem structure. It appears that the 3'X region, as well as the 52 nt upstream of the poly(U/C) tract, are crucial for RNA replication, while the remainder of the 3'UTR plays a role in enhancement of replication [25,26].

To date, hepaciviruses are divided into six principal genotypes of HCV that differ in their nucleotide sequences by 31–34%, and in their amino acid sequences by ~30%. HCV, like many other RNA viruses, circulates in infected individuals as a population of diverse but closely related variants referred to as quasispecies [12]. HCV heterogeneity is primarily due to a high error rate of the RNA-dependent RNA polymerase encoded by the NS5B gene. The existence of different quasispecies of the HCV genome appears to contribute to viral persistence. It has been shown that patients with chronic hepatitis C have greater genetic complexity in terms of the population of quasispecies they possess than patients with spontaneous clearance [13]. During the course of chronic infection, random genetic drift steadily induces the development of quasispecies primarily due to changes in the

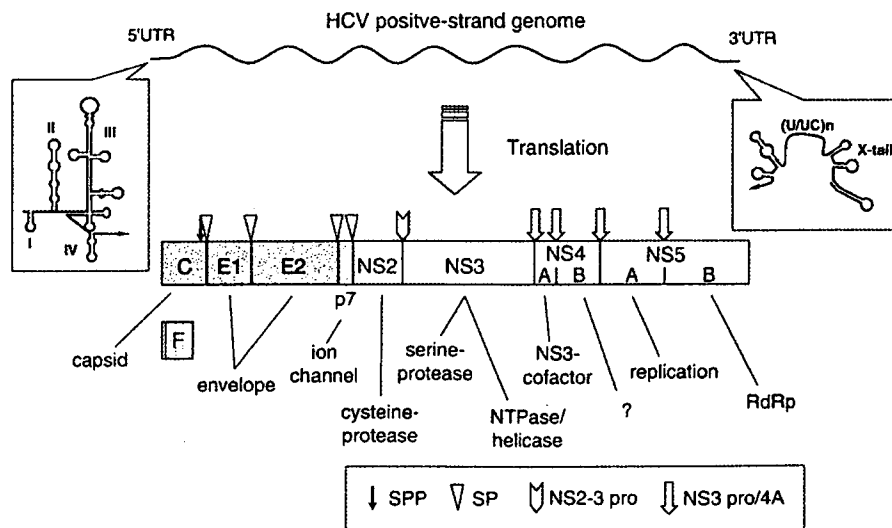


Fig. 1.

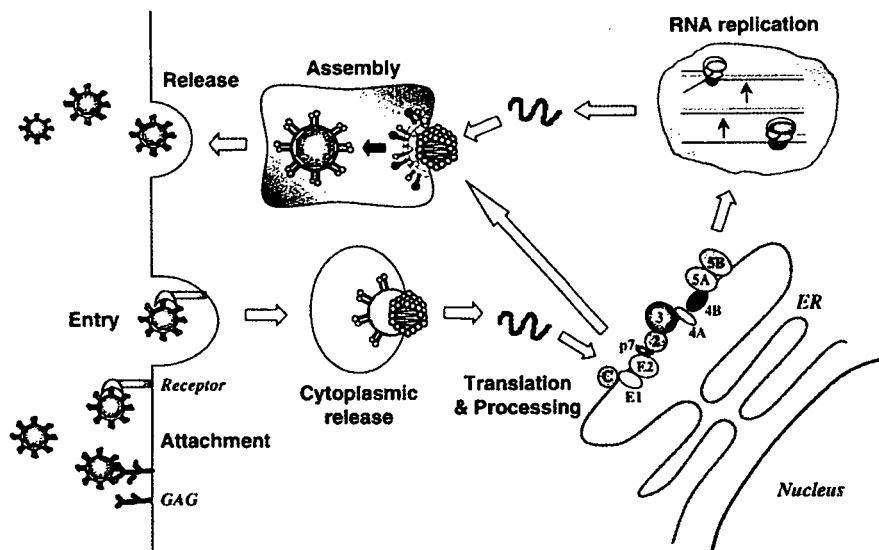


Fig. 2.

hypervariable region 1, involving the 27 N-terminal 27 residues of the E2 envelope protein.[27–29].

### 3. Features of the viral proteins

#### 3.1. Core protein

The HCV core protein, which is derived from the N-terminus of the polyprotein, most likely forms the viral nucleocapsid given similarities between its position and that of sequences encoding viral nucleocapsids in other flavivirus genomes. The amino acid sequence of the core protein is highly conserved among different HCV strains, compared with other HCV proteins. HCV core protein has been extensively used in a number of serologic assays since anti-core antibodies are highly prevalent among HCV-infected individuals. Although several core proteins of varying molecular weights have been identified [30–33], the core protein is released as a 191-residue precursor of 23 kDa and further processing yields the predominant form of 21 kDa. The N-terminal domain of the core protein is highly basic, while its C-terminus is hydrophobic. Several groups have reported a complex intracellular localization of the core protein [30,33–42]. The core protein is primarily detected in the cytoplasm, in association with the endoplasmic reticulum (ER), lipid droplets, and mitochondria. In some studies, a fraction of the core protein has also been found in the nucleus.

The ubiquitin–proteasome pathway, a major route by which selective protein degradation occurs in eukaryotic cells, is involved in post-translational modification of the core protein [32,43–45]. An initial report indicated that processing at the carboxyl-terminal hydrophobic domain of the core protein produced efficient polyubiquitylation and proteasomal degradation [32]. Recently, ubiquitin ligase E6AP has been identified as an HCV core-binding protein that enhances ubiquitylation and degradation of mature, as well as carboxyl-terminus truncated-core protein, and it has been suggested that E6AP-dependent degradation of the core protein is common to a

variety of HCV isolates and plays a critical role in the HCV life cycle [45].

The core protein is likely multifunctional and essential for viral replication, maturation, and pathogenesis. It is involved not only in formation of the HCV virion, but also has a number of regulatory functions, including modulation of signaling pathways, cellular and viral gene expression, cell transformation, apoptosis, and lipid metabolism [reviewed in 46].

#### 3.2. E1 and E2 envelope proteins

The E1 and E2 proteins are essential components of the virion envelope and are necessary for viral entry. These glycosylated proteins extend from aa 192–383 (E1) and from aa 384–746 (E2) of the polyprotein, and have molecular weights of 33–35 and 70–72 kDa, respectively [47]. Along the precursor polyprotein, it has been suggested that the C-terminal transmembrane domains of E1 and E2 form hairpin structures that pass through the membrane twice, thereby allowing processing by a signal peptide in the ER lumen [48]. Upon signal peptidase cleavage, the C-termini are thought to translocate into the cytoplasm in order to generate the type I membrane topology of mature E1 and E2. Mature E1 and E2 remain noncovalently associated, interacting in part through their C-terminal transmembrane domains, which also mediates retention of the E1–E2 complex in the ER. It has recently been demonstrated that, in addition to this conventional type I membrane topology, E1 protein also adopts a polytopic topology, in which the protein twice spans the ER membrane with an intervening cytoplasmic loop spanning aa 288–360 [49].

#### 3.3. p7 protein

The p7 protein is a small (63 aa) hydrophobic polypeptide that adopts a double membrane-spanning topology. This protein is essential for the production of infectious virions *in vivo* [50] and may belong to a small protein family of viroporins, which are known to enhance membrane permeability. It has been revealed

that p7 protein forms an ion channel in artificial lipid bilayers, suggesting it may function as a viroporin [51,52].

### 3.4. NS2 protein

The NS2 protein is a transmembrane protein of 21–23 kDa, with 96 highly hydrophobic N-terminal residues, forming either three or four transmembrane helices that insert into the ER membrane. The C-terminal part of NS2 presumably resides in the cytoplasm enabling zinc-stimulated NS2/3 autoprotease activity together with the N-terminal domain of NS3. Efficient cleavage at the NS2/3 site requires the 130 C-terminal residues and first 180 aa of the NS3. Site-directed mutagenesis has revealed that His-952, Glu-972, and Cys-993 may comprise the active site for proteolytic activity [53,54]. Deletion of NS2 from the nonstructural polyprotein has not been observed to abolish HCV RNA replication in cell cultures, indicating that NS2 is not essential for viral RNA replication [55,56]. However, the NS2 protein is essential for completion of the viral replication cycle in vitro and in vivo [57,58]. A recent report regarding the crystal structure of the C-terminus of NS2 suggests that the cytoplasmic domain of NS2 forms a dimeric cysteine protease with two composite active sites, in which His-952 and Glu-972 comprise the active site of one monomer, and Cys-993 contributes to the active site of the other [59].

### 3.5. NS3–4A complex

NS3–4A is a complex bifunctional molecule essential for viral polyprotein processing and RNA replication. NS3 is a fairly hydrophobic protein of 69 kDa with a serine protease encoded by its N-terminal one-third region that non-covalently binds the NS4A cofactor, which is a 54-aa polypeptide [reviewed in 60]. The catalytic triad is formed by residues His-1083, Asp-1107 and Ser-1165 of NS3. The central portion of NS4A is important for efficient processing of the nonstructural proteins by NS3. It has been suggested that the N-terminus of NS4A might form a transmembrane helix that anchors the NS3–4A complex to the cellular membrane [61]. Crystal structural analyses of the NS3/4A complex have demonstrated structural similarities between the NS3 serine protease and trypsin, with two large domains primarily composed of six-stranded beta barrels separated by a cleft containing the active site and substrate binding pocket [62–64]. Of note, NS4A forms an integral part of this structure and interacts with the extreme N-terminal residues of NS3 to form two additional anti-parallel beta-strands. The NS3–4A complex has a shallow substrate-binding pocket, thus requiring extended interaction sites with the substrate.

The final 442 aa of the C-terminal of NS3 comprise the helicase-NTPase domain, which is a member of the superfamily-2 DexH/D-box helicase, which unwind RNA-RNA substrates in a 3'-to -5' direction [reviewed in 65]. This is supported by crystal structure analysis indicating the presence of NTPase domains and RNA binding within the protein [66]. During RNA replication, the NS3 helicase is believed to translocate along the nucleic acid substrate by changing its protein conformation, utilizing the energy of NTP hydrolysis [67–69]. Its helicase activity is

positively modulated by the NS3 protease domain and NS4A [70].

### 3.6. NS4B protein

NS4B is an integral membrane protein of 27 kDa, which is predicted to contain at least four transmembrane domains and an N-terminal amphipathic helix that is responsible for membrane association [71]. NS4B has the ability to induce the formation of a specialized membrane compartment, a sort of membranous web where viral RNA replication may take place [72,73].

### 3.7. NS5A protein

NS5A is a membrane-anchored phosphoprotein that is observed in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms. Based on the results of a comparative sequence analysis following limited proteolysis of purified protein, NS5A is predicted to contain three domains: domain 1 (aa 1–213), domain 2 (aa 250–342) and domain 3 (aa 356–447) [74]. A recent structural study has demonstrated that domain 1 immediately follows the membrane-anchoring alpha-helix and forms a dimeric structure with an unconventional zinc-coordinating motif [75]. Thus, it may interact with viral and cellular proteins, as well as membranes and RNA.

While its function has not fully been elucidated, NS5A is believed to be important in viral replication. A large number of cell culture-adaptive mutations mapped to the NS5A have been shown to enhance RNA replication [76–78]. These adaptive mutations often affect hyperphosphorylation of NS5A, suggesting that the phosphorylation status of NS5A might influence replication efficiency. NS5A has been reported to interact with other HCV nonstructural proteins [79–81]. In addition, several cellular proteins interact with NS5A, resulting in assembly of the viral replication complex and/or regulation of RNA replication, as described below.

### 3.8. NS5B protein

NS5B is a 68-kDa protein with a conserved sequence motif characteristic of viral RNA-dependent RNA polymerase (RdRp), including a hallmark GDD motif that produces catalytic activity. NS5B is a tail-anchored protein and its C-terminal 21-aa region forms an alpha-helical transmembrane domain, which is dispensable for polymerase activity in vitro but is responsible for post-translational targeting to the cytoplasmic side of the ER [82,83]. Analysis of the crystal structure of NS5B has revealed that the HCV RdRp resembles a right hand and contains fingers, palm, and thumb subdomains, similar to other template-dependent polymerases [84–86]. Unlike the more open structures of other template-dependent DNA polymerases, such as the Klenow Fragment and the human immunodeficiency virus 1 reverse transcriptase, the HCV RdRp has a fully encircled active site through extensive interactions between the fingers and thumb subdomains, resulting in a protein that predominantly exists in a “closed” conformation. HCV RdRp also has an unusual hairpin loop that protrudes into the active site and helps position the 3'-

end of the RNA template for proper initiation of RNA synthesis and inhibits extension from a primed template [87].

#### 4. HCV life cycle

##### 4.1. Attachment and entry

Attachment of the virus to a cell followed by viral entry is the first step in the virus life cycle. In order to enter the host cell, the virus must first bind to a receptor on the cell surface. The specific interaction between a host cell receptor and viral attachment proteins on the surface of the virion determines tissue tropism and host range.

Low levels of HCV replication in cultured cells hindered study of the HCV life cycle. In order to overcome this, alternative models have been developed to study viral attachment and entry using recombinant HCV envelope proteins, including virus-like particles produced by baculovirus [88,89], vesicular stomatitis virus and retrovirus pseudotypes (HCVpp) [90–93], as well as infectious particles derived from a JFH-1 isolate (HCVcc) [94–96].

By using soluble E2 as a probe to identify cell-surface proteins potentially involved in HCV entry, CD81 was first identified as a putative HCV receptor [97]. CD81, a widely expressed 25-kDa cell surface protein, belongs to a family of tetraspanins and is involved in a number of activities, including cell adhesion, motility, metastasis, cell activation and signal transduction [98]. CD81 has a small and large extracellular loop, which mediate binding to recombinant E2 [97,99]. Studies with HCVpp and HCVcc confirm the involvement of CD81 in HCV entry. HCVpp shows a restricted tropism for human hepatic cell lines expressing CD81 [92,93,100–102]. Although necessary, CD81 expression alone is not sufficient for cell entry of HCVpp. Of note, HepG2, which does not express CD81 on its cell surface, is resistant to HCVpp infection, but over-expression of CD81 renders the HepG2 permissive to HCVpp infection [102–105]. Significant infection of CD81-negative cell lines with HCVpp has not been reported. However, as mentioned, not all CD81-positive cell lines can be infected [93,101,103]. Expression of CD81 in host cells is also required for infectivity of HCVcc. Recombinant CD81 and antibodies to CD81 have been observed to neutralize infection [94–96]. Thus, CD81 may function as a post-attachment entry co-receptor and may play a role after binding of the virion to another receptor.

The human scavenger receptor class B type I (SR-BI) has been identified as another putative receptor for HCV [106]. SR-BI is an 82-kDa glycoprotein with two C- and N-terminal cytoplasmic domains separated by a large extracellular domain involved in cellular lipometabolism. SR-BI is expressed in a wide variety of mammalian tissues and cell types [100,107,108], with particularly high levels of expression in the liver and steroidogenic tissue [107,109,110]. SR-BI recognition by soluble E2 requires the HVR1 of E2 [103,111]. A role of SR-BI in HCV cell entry has been confirmed using HCVpp in receptor competition assays using polyclonal anti-SR-BI serum, which has been observed to specifically inhibit HCVpp entry efficiently in a dose-dependent manner [103]. Recent reports have demonstrated that serum factors, especially high-density

lipoprotein (HDL), a ligand to SR-BI, enhance the infectivity of HCVpp [105,112–115]. These results suggest that SR-BI modulates HCV entry.

Several human cell lines co-expressing CD81 and SR-BI are non-permissive for HCVpp infection [100,102,103], suggesting that another cell surface molecule(s) may be required for HCV entry. C-type (calcium-dependent) lectins, such as L-SIGN, DC-SIGN, and the asialoglycoprotein receptor, have also been investigated as potential HCV receptors based on their affinity for recombinant HCV envelope proteins [116–119]. However, L-SIGN and DC-SIGN are not expressed on hepatocytes and therefore cannot be receptors for HCV entry. A possible role of L-SIGN and DC-SIGN involves the capture and transfer of HCV to hepatocytes [120,121]. The LDL receptor is another candidate receptor based on the finding that HCV particles associate with lipoproteins in serum and their infectivity correlates with lipoprotein association. The LDL receptor has been shown to mediate HCV internalization by binding to virion-associated LDL particles [122]. However, a role for the LDL receptor in virus entry has not been confirmed using HCVpp [93], likely since the binding is mediated by lipoproteins rather than viral components.

Recently, a tight junction component claudin-1 has been identified as a co-receptor of HCV [123]. Claudin-1 appears to be critical for HCV entry into hepatic cells and is thought to act during the late stages of viral entry.

##### 4.2. Translation

As opposed to cellular capped mRNA molecules which are translated via a cap-dependent scanning mechanism, the naturally uncapped RNA molecules of viruses such as flaviviruses and picornaviruses are translated via a cap-independent IRES-mediated process, in which viral protein expression is regulated by direct recruitment of each ribosome to the start site of translation [18,19]. The first 40 nt of the 5'UTR, which include a single stem-loop (domain I), are not essential for translation. Of note, the 5' border of the IRES was mapped between nt 38 and 46 [17,124,125]. Other domains in the 5'UTR are more complex: domain II consisting of a stem with several internal loops, domain III consisting of a pseudoknot connected to a four-helix junction, as well as stem-loop IIIId and domain IV, a small hairpin containing the AUG start codon at nt 342. It has been suggested that the first 12 to 40 nt downstream of the start codon are also important for IRES activity [126–128].

Structural analysis of the HCV IRES indicates that all of the RNA elements adopt tertiary structures capable of binding to the translation initiation complex with high affinity [129]. IRES-mediated translation of HCV RNA is initiated by direct binding of a vacant 40S ribosomal subunit to the IRES. The 40S subunit appears to interact with the viral RNA at multiple sites including stems, loops, pseudoknots, as well as the start codon. This binary complex then binds to eukaryotic initiation factor (eIF) 3, as well as the ternary complex eIF-2: Met-tRNA<sub>i</sub>: GTP to form a 48S-like complex dependent upon both the basal domain III and the start codon. Subsequent formation of the 80S complex, which is the rate-limiting step, is dependent upon GTP



hydrolysis and attachment of the 60S subunit, after which the first peptide bond is formed [130].

In addition to the requirements described above, additional factors modulate IRES activity. Cellular factors such as the La autoantigen [131–133], heterogeneous ribonucleoprotein L [134], poly-C binding protein [135], and pyrimidine tract-binding protein (PTB)[136], have also been shown to bind to the IRES element and modulate HCV translation. HCV translation is also regulated through various interactions with viral proteins and the IRES.

We have found that HCV core protein expression inhibits HCV translation, possibly through binding to domain III<sub>d</sub>, particularly a GGG triplet within the hairpin loop structure of the domain [137–139]. We therefore propose a model in which competitive binding of the core protein for the IRES and 40S subunit regulates HCV translation. Although there is an increasing body of evidence to suggest involvement of the core protein in translational regulation, there is conflicting data regarding the mechanism by which this occurs. In contrast to studies describing modulation of initiation of HCV translation by expression of the core protein [137,139–141], another study suggest that the core protein sequence, and not the core protein itself, modulates HCV IRES function through a long-range RNA-RNA interaction [142].

In addition to the 10 known viral proteins (Fig. 1), the core-coding region of HCV has also been observed to express low levels of a 16–17 kDa protein [143–145]. This protein, which has been named the F protein, is thought to be produced by a +1 translational frameshift by ribosomes initiating translation at the start codon during synthesis of the HCV polyprotein, which shifts the reading frame between codons 9 and 11 of the polyprotein.

#### 4.3. Polyprotein processing

The main translation product of the HCV genome is a large precursor polyprotein that is subsequently processed by cellular and viral proteases into mature structural and nonstructural proteins (Fig. 1). As deduced from the hydrophobicity profile and dependence on microsomal membranes, junctions at core/E1, E1/E2, E2/p7, p7/NS2 are processed by host signal peptidases. Secondary structure analysis of the core protein has revealed that all major alpha helices are located in the C-terminal half of the protein. A predicted alpha helix encoded by aa 174–191 is extremely hydrophobic and resembles typical signal peptide sequences. Further post-translational cleavage close to the C terminus of the core protein takes place, removing the E1 signal sequence by a signal peptide peptidase [146–149]. This peptidase has been identified as a presenilin-type aspartic protease [150] and shown to exhibit protease activity within cellular membranes, resulting in cleavage of peptide bonds in the plane of lipid bilayers.

As described above, HCV nonstructural proteins are processed by two viral proteases: cleavage between NS2 and NS3 is a rapid intramolecular reaction mediated by a NS2-3 protease spanning NS2 and the N-terminal domain of NS3, whereas the remaining four junctions are processed by a serine protease located within the 180 N-terminal residues of the NS3 protein. The NS3-NS5B

region is presumably processed by sequential cleavage: NS3/4A → NS5A/5B → NS4A/4B → NS4B/5A [151–154]. Processing at the NS3/4A site is intramolecular, whereas cleavage at the other sites occurs intermolecularly.

#### 4.4. RNA replication

As with other positive-strand RNA viruses, HCV replication is assumed to start with synthesis of a complementary negative-strand RNA using the genome as a template, after which genomic positive-strand RNA is produced from a negative-strand RNA template, both steps of which are catalyzed by the NS5B RdRp. The positive-strand RNA progeny are transcribed at a level 5- to 10-fold that of negative-strand RNA. Recombinant NS5B protein demonstrates RdRp activity *in vitro*, however, appears to lack strict template specificity and fidelity, which are essential for viral RNA synthesis. Thus, other viral and/or host factors are believed to be responsible for RNA replication and formation of the replication complex (RC), together with NS5B, which is required for catalyzing HCV RNA synthesis during replication. Several research groups have demonstrated HCV RC-mediated replication *in vitro* in crude membrane fractions of cells harboring subgenomic replicons [155–158]. Studies of cell-free replication systems, which provide a useful source of viral RCs have revealed that RNA synthesis can be initiated in the absence of additional negative-strand template RNA, suggesting that pre-initiated template RNA co-purifies with viral RCs [156–159].

Co-precipitation and immunostaining studies have revealed that newly synthesized HCV RNA exists as distinct specks of material, while all of the viral nonstructural proteins coexist [160]. These distinct structures may be equivalent to a membranous web, as described above. Expression of all structural and nonstructural proteins in the context of the entire HCV polyprotein has been observed to induce similar membrane changes [72]. It is of interest that morphologically similar structures, termed sponge-like inclusions [161], have been identified by electron microscopy within the hepatocytes of HCV-infected chimpanzees. Thus, HCV RC may exist in the context of a membranous web in infected cells. Because all nonstructural proteins of HCV are associated with the ER membrane in cells harboring subgenomic replicon RNA molecules [162,163], and since the membrane web is frequently observed in close proximity with the ER membrane, it is likely that the membranous web in HCV-infected cells is derived from the ER membrane.

On the other hand, there is accumulating evidence to support an association between HCV RNA replication and detergent-insoluble membrane domains or lipid rafts, which are microdomains rich in cholesterol and sphingolipids. Membrane flotation analysis and replication assays have shown that viral RNA and proteins exist within detergent-resistant, lipid-raft membranes, and that RNA replication occurs even after treatment with detergent [155,164]. Inhibitors of *de novo* sphingolipid synthesis have been shown to inhibit HCV replication, presumably by disrupting the association of viral nonstructural proteins with lipid rafts [165,166]. It is now accepted that HCV nonstructural proteins synthesized at the ER localize to lipid raft membranes when they are actively engaged in RNA replication.

Membrane separation analysis has demonstrated that HCV nonstructural proteins exist both in the ER and the Golgi apparatus, but that viral RNA replication primarily occurs in the Golgi fraction [155]. Further studies to elucidate the cellular processes involved in HCV RC formation and replication of the HCV genome in infected cells are needed.

Studies of RNA replicons have demonstrated the greatest viral RNA levels during the growth phase of the cells, after which a significant drop is observed as the cells reach confluence, suggesting that HCV replication and/or translation is tightly linked to host cell metabolism [163]. Huh-7 cells, in which adapted replicons are cured by treatment with IFN, yield cell populations that are more permissive for the replicon tested. Thus, it is likely that some interplay between the cellular environment and specific adaptive mutations of viral RNA contributes to efficient RNA replication of HCV.

Several cellular proteins capable of interacting with NS5A, such as vesicle-associated membrane protein-associated protein (VAP) subtypes A and B (VAP-A and -B) [73,167], FKBP8 [168], FBL2 [169,170], growth factor receptor-bound protein 2 adaptor protein [171], SRCAP [172], and karyopherin b3 [173], as well as Raf-1 kinase [174], have been identified. VAP-A and -B and SNARE-like proteins are known to localize within the ER and Golgi apparatus and are essential for HCV replication by binding with both NS5A and NS5B. VAP-A interacts with VAP-B through its transmembrane domain. Thus, VAP-A and -B are thought to be involved in the formation of functional HCV RCs. FKBP8, a member of the FK506-binding protein family, and Hsp90 form a complex with NS5A, further contributing to viral RNA replication. Statins that decrease the production of mevalonate by inhibiting 3-hydroxy-3-methylglutaryl CoA reductase have been shown to inhibit HCV RNA replication [170,175], which can be reversed by adding geranylgeraniol, suggesting that viral replication requires geranylgeranylated proteins. A geranylgeranylated protein, FBL2, which contains an F-box motif and is therefore likely involved in protein degradation, has been identified as a NS5A-binding protein.

Host factors that interact with NS5B and might participate in HCV replication include cyclophilin B [176], p68 [177], nucleolin [178,179], and hnRNP A1 [180]. Cyclophilin B, a cellular peptidyl-prolyl cis-trans isomerase, interacts with the C-terminal region of NS5B to directly stimulate its RNA binding activity, and thereby contributes to efficient replication of HCV RNA. Redistribution of p68, an RNA helicase, from the nucleus to the cytoplasm occurs through its binding to NS5B, and the p68-NS5B interaction may further serve to mediate HCV replication. Nucleolin, a representative nucleolar marker, interacts with NS5B through two independent regions of NS5B and may be essential for HCV replication. hnRNP A1, a heterogeneous nuclear ribonucleoprotein, also interacts with septin 6, as well as the 5'-UTR and 3'-UTR of HCV RNA, and contains the cis-acting elements required for replication. Thus, hnRNP A1 and septin 6 play important roles in HCV replication through RNA-protein and protein-protein interactions. Other cellular components that bind to HCV RNA, such as PTB, may also be involved in viral replication. PTB has been observed to modulate HCV IRES

activity by binding to several sites within the viral genome [22,181–184]. Recent studies have shown that PTB also forms part of the HCV RC and participates in viral RNA synthesis [185].

#### 4.5. Viral assembly

Little is known about the assembly of HCV or its virion structure since efficient production of authentic HCV particles has only recently been achieved. As with related viruses, the mature HCV virion likely consists of a nucleocapsid and outer envelope composed of a lipid membrane and envelope proteins. Various forms of HCV have been reported to circulate in the sera of infected hosts, including (i) free mature virions, (ii) virions bound to low-density lipoproteins and very-low-density lipoproteins, (iii) virions bound to immunoglobulins, as well as (iv) non-enveloped nucleocapsids, which exhibit different physicochemical and antigenic properties [186–189].

Several expression systems have been used to investigate HCV capsid assembly using lysate from mammalian cells, insects, yeast, bacteria, and reticulocytes, as well as purified recombinant protein [88,89,190–195]. The results suggest that the immunogenic nucleocapsid-like particles of HCV are variable in size ranging from 30 to 80 nm in diameter. The N-terminal half of the core protein is important for nucleocapsid assembly [190,194,195]. HCV capsid formation occurs in the presence or absence of ER-derived membrane, which supports cleavage of the signal peptide at the C-terminus [195].

Nucleocapsid assembly generally involves oligomerization of the capsid protein and encapsidation of genomic RNA. This process is thought to occur upon interaction of the core protein with viral RNA, and the core-RNA interaction may be critical for switching from RNA replication to packaging. In fact, HCV core protein can bind to positive-strand HCV RNA through stem-loop domains I, III and nt 24–41 [138]. Two-hybrid systems have identified a potential homotypic interaction domain within the N-terminal region of the core protein (aa 1–115 or –122), with particular emphasis on the region encompassing aa 82–102 [196,197]. Using purified HCV core protein, a C-terminally truncated core protein (aa 1–124) and structured RNA have been implicated in nucleocapsid formation to produce homogenous spherical HCV particles. When core protein containing the C-terminus up to aa 174 is similarly examined, a heterogenous array of irregularly shaped particles is observed, suggesting that the C-terminus of the core protein influences self-assembly. Furthermore, Pro substitution within the C-terminal region has been observed to abolish core protein self-interaction [198]. Circular dichroism spectroscopy has further shown that a Trp-rich region spanning aa 76–113 is largely solvent-exposed and unlikely to play a role in multimerization. Recently, our group has demonstrated that self-oligomerization of the core protein is promoted by aa 72 to 91 of the core protein [49].

Once a HCV nucleocapsid is formed in the cytoplasm, it acquires an envelope as it buds through an intracellular membrane. Interactions between the core and E1/E2 proteins are thought to determine viral morphology. Expression of HCV structural proteins using recombinant virus vectors has succeeded in generating virus-like particles with similar ultrastructural

properties to HCV virions. Packaging of these HCV-like particles into intracellular vesicles as a result of budding from the ER has been noted [88,199,200]. Mapping studies to determine the nature of interaction between core and E1 proteins have demonstrated the importance of C-terminal regions in this interaction [201,202]. Since corresponding sequences are not well conserved among various HCV isolates, interactions between core and E1 proteins might depend more on hydrophobicity than specific sequences. In contrast, it has been shown that the interaction between self-oligomerized HCV core protein and the E1 glycoprotein is mediated through a cytoplasmic loop of the polytopic form of the E1 protein [49].

It is believed that HCV particles are released from the cell through the secretory pathway. HCV structural proteins have been observed both in the ER and Golgi apparatus [203]. In addition, complex N-linked glycans, which transit through the Golgi apparatus, have been detected on the surface of HCV particles isolated from patient sera [204].

## 5. Perspectives

Since the discovery of HCV, which is a major cause of liver disease worldwide, significant progress has been made regarding the molecular biology of this virus. However, details regarding early and late stages of the HCV life cycle, including cell entry, genome packaging, assembly and release, remain unclear. In addition, the role of some viral proteins and their importance to replication remains unclear, as well as the role of certain host factors in regulation of the HCV life cycle.

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## Review

# Molecular biology of hepatitis C virus

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Infection with hepatitis C virus (HCV), which is distributed worldwide, often becomes persistent, causing chronic hepatitis, cirrhosis, and hepatocellular carcinoma. For many years, the characterization of the HCV genome and its products has been done by heterologous expression systems because of the lack of a productive cell culture system. The development of the HCV replicon system is a highlight of HCV research and has allowed examination of the viral RNA replication in cell culture. Recently, a robust system for production of recombinant infectious HCV has been established, and classical virological techniques are now able to be applied to HCV. This development of reverse genetics-based experimental tools in HCV research can bring a greater understanding of the viral life cycle and pathogenesis of HCV-induced diseases. This review summarizes the current knowledge of cell culture systems for HCV research and recent advances in the investigation of the molecular virology of HCV.

**Key words:** hepatitis C virus, translation, polyprotein processing, RNA replication, viral assembly, ubiquitin

## Introduction

Hepatitis C virus (HCV), discovered in 1989, is a major etiologic agent of posttransfusion- and sporadic non-A, non-B hepatitis<sup>1</sup> and at present infects approximately 200 million people worldwide.<sup>2,3</sup> Persistent infection with HCV is associated with the development of chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma.<sup>3,4–8</sup> HCV is a small, enveloped RNA virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family.<sup>9,10</sup> Its genome consists of a single-strand of

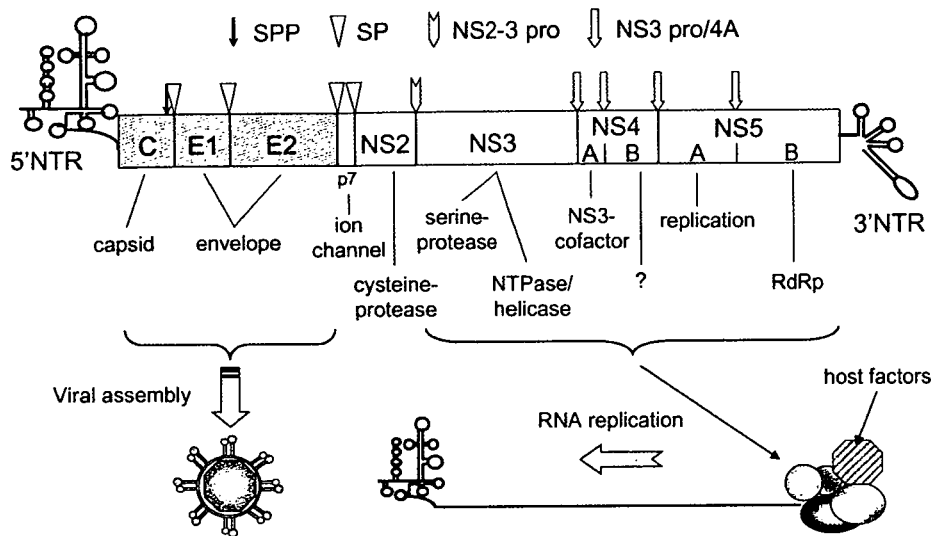
positive-sense RNA of approximately 9.6kb, which contains an open reading frame (ORF) coding for a polyprotein precursor of approximately 3000 residues.<sup>11</sup> The precursor is cleaved into at least ten different proteins: the structural proteins core, E1, E2, and p7, and the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Fig. 1).

To date, six major genotypes of HCV have been identified that differ by 31%–34% in their nucleotide sequence and by about 30% in their amino acid sequence. It has been shown that HCV, like many other RNA viruses, circulates in infected individuals as a population of diverse but closely related variants referred to as quasispecies.<sup>12</sup> This quasispecies model of mixed virus populations may confer a significant survival advantage, because the simultaneous presence of multiple variant genomes and the high rate of generation of new variants allows rapid selection of mutants better suited to new environmental conditions.<sup>13</sup>

Specific anti-HCV drugs that efficiently block virus production are not yet available. The current standard care is combination therapy with interferon (IFN)- $\alpha$  and the nucleoside analog ribavirin, which cures about 40% of hepatitis C patients infected by HCV genotype 1, the most prevalent genotype in industrialized countries, and about 80% of those infected by genotype 2 or 3.<sup>14,15</sup> Since many patients still do not benefit from the treatment and IFN therapy is associated with undesirable side effects such as headache, fever, severe depression, myalgia, arthralgia, and hemolytic anemia, development of innovative treatment alternatives for hepatitis C patients is immediately needed. Studies of HCV life cycle in cell cultures have been greatly facilitated by the development of genetically engineered viral genomes that are capable of self-amplifying to high levels (replicon system), and by recent establishment of a production system for recombinant infectious HCV. Such progress will aid in the development of significantly improved HCV antiviral agents.

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**Fig. 1.** Hepatitis C virus (HCV) genome organization and polyprotein processing. Posttranslational cleavages by signal peptide peptidase (*SPP*), signal peptidase (*SP*), NS2–NS3 protease (*NS2–3 pro*), and NS3 protease and NS4A complex (*NS3 pro/4A*) lead to the production of functional HCV proteins. *NTR*, non-translated region

### Cell culture systems for HCV research

Although substantial information on HCV protein structure and function has been obtained from the use of a variety of cell culture and *in vitro* expression systems, for many years, HCV research has been hampered by the restricted host range and the inefficiency of cell culture models for viral infection and propagation. The development of the HCV replicon system, therefore, is a milestone in HCV research and has allowed examination of viral RNA replication in cell culture.<sup>16</sup> Expression systems of heterologous virus genes based on RNA replicons have been established in a variety of positive-strand RNA viruses such as polio virus,<sup>17–20</sup> the alphavirus Semliki Forest virus,<sup>21</sup> Sindbis virus,<sup>22–25</sup> Kunjin virus,<sup>26</sup> human rhinovirus 14,<sup>27</sup> and bovine viral diarrhea virus.<sup>28</sup> In general, advantages of replicon systems are (1) a high level of gene expression and RNA replication, (2) easy construction of recombinants, and (3) a wide permissible host range.

The HCV replicons are typically composed of selectable, bicistronic RNA, with the first cistron containing the HCV 5' nontranslated region (NTR), which directs translation of the gene encoding the neomycin phosphotransferase, and the second cistron containing the internal ribosome entry site (IRES) of the encephalomyocarditis virus, which directs translation of HCV NS3 through NS5B region, and the 3' NTR. The prototype subgenomic replicon utilized a particular HCV genotype 1b clone termed Con1. Following transfection of RNA generated by *in vitro* transcription of the cloned replicon sequences into a human hepatoma cell line Huh-7, antibiotic G418-resistant cells could be obtained in which the subgenomic RNA replicated autonomously. RNA replication was first detected at relatively low frequency, followed by the identification of replicons harboring cell culture-adaptive mutations, which in-

creased the efficiency of replication initiation by several orders of magnitude.<sup>29–31</sup>

Adaptive mutations were found primarily at the N-terminus of the NS3 helicase, in NS4B, and in the center of NS5A, which is upstream of the region putatively involved in IFN sensitivity. Most of the mutations in NS5A are located at highly conserved serine residues and lead to change in the phosphorylation state of NS5A.<sup>32,33</sup> A combination of adaptive mutation in NS3 and NS5A resulted in the highest level of replication of a particular HCV genotype 1b isolate.<sup>31</sup> Later work, however, has indicated that adaptive mutations can arise in most of the viral nonstructural proteins.<sup>34,35</sup> The mechanisms by which adaptive mutations increase RNA replication efficiency are not well understood.

In the last 7 years, a variety of different replicons have been generated, including replicons with reporters or markers such as luciferase and green fluorescent protein, replicons from genotype 1a and 2a, and genome-length dicistronic HCV RNAs (genomic HCV replicons). HCV replicons with reporter genes allow us to execute fast and reproducible screening of large series of compounds for antivirals.<sup>36–38</sup> Huh-7 cells are the most permissive for HCV replicons. However, variability in the permissiveness for replicons has been observed for a given Huh-7 cell pool, and the cells that are able to support efficient replication of the viral genome are enriched during selection such as G418 treatment. A so-called “cured” cell clone, which can be prepared by removing the replicons by treatment with IFN, supports viral replication to a much higher level in many cases and is useful for introducing genome-length HCV RNAs.<sup>39,40</sup>

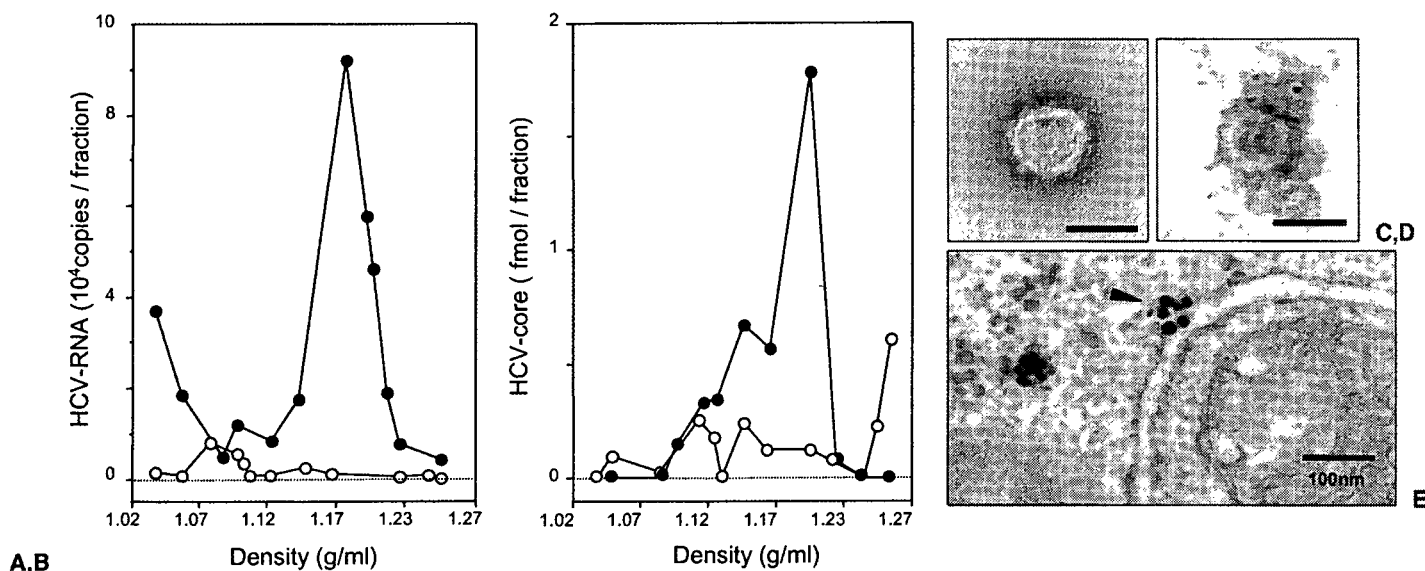
An HCV genotype 2a replicon with the JFH-1 strain, which was first isolated from the serum of a Japanese patient with fulminant hepatitis C by our group,<sup>41</sup> replicates efficiently in not only Huh-7 cells but also other

hepatocyte-derived cell lines, HepG2 and IMY-N9, and nonhepatocyte-derived cell lines, HeLa and 293.<sup>42-44</sup> Interestingly, the JFH-1 replicon does not require adaptive mutations for replicating in these cell lines, and enormously efficient RNA replication is detected by transient replication assay as well as by colony formation assay with G418 selection,<sup>42</sup> suggesting that the JFH-1 genome can replicate autonomously without the help of drug selection or the requirement of adaptive mutations. This observation laid the basis for a breakthrough in HCV research.

Transfection of the full-length JFH-1 genome into Huh-7 cells leads to the production of HCV particles that are infectious both for naïve cells and for animal models.<sup>45</sup> As a first attempt, an in vitro transcribed full-length JFH-1 RNA was introduced into naïve Huh-7 cells, which is the original cell line used for subgenomic replicon studies. Efficient RNA replication in the transfected cells was detectable by Northern blot analysis, and the viral-enveloped particles, which are spherical structures with an outer diameter of approximately 55 nm, were secreted to the culture medium.<sup>45</sup> Secreted virus was found to be infectious, although at low efficiency, for naïve Huh-7 cells, and its infectivity can be neutralized by anti-CD81 antibody and hepatitis C patients' sera.<sup>45</sup> Subsequently, to increase the infection efficiency, "cured" Huh-7 cell lines such as Huh7.5, Huh7.5.1, and Huh7-Lunet were used. Infectivity of these cured cell lines with JFH-1 became more intense

compared with standard Huh-7 cells, and the virus titers released from cells freshly transfected with the JFH-1 genome were markedly increased by continuous passage of the cells carrying persistent replicating viral RNA.<sup>46</sup> Further, chimeric constructs with the core to NS2 region of another genotype 2a clone, J6, improved the infectivity.<sup>47</sup> Thus, this recombinant infectious HCV cell culture system opens avenues of biochemical and genetic studies of the HCV life cycle.

Besides isolating functional molecular clones of HCV that replicate to high levels, to generate a cell culture model that mimics natural host cell environments may be advantageous for improving HCV production systems suitable for studying the virus-host interaction. It is likely that HCV morphogenesis occurs in a complex cellular environment in which host factors may either enhance or reduce the assembly and budding process. Generally, the interaction of viruses with polarized epithelia in the host is one of the key steps in the viral life cycle. A variety of enveloped viruses mature and bud from distinct membrane domains of the host cells.<sup>48-51</sup> We found that a dicistronic HCV genome of genotype 1b supports the production and secretion of infectious HCV particles in two independent three-dimensional (3D) culture systems, the radial-flow bioreactor (RFB) and the thermoreversible gelation polymer (TGP), but not in monolayer cultures, although its productivity is much lower than that observed in the JFH-1 system<sup>52</sup> (Fig. 2). The RFB system was initially aimed at the



**Fig. 2A-E.** Production of HCV particles in the three-dimensional, thermoreversible gelatin polymer (TGP) culture of the Huh-7 cell line (RCYM1) carrying genome-length dicistronic HCV RNA of genotype 1b. **A, B** Sucrose density gradient analysis of culture supernatants of RCYM1 cells. The culture supernatants were fractionated and then HCV RNA (**A**) and core protein (**B**) in each fraction were determined by enzyme-linked immunosorbent assay and real-time reverse transcription polymerase chain reaction, respectively. *Closed circles*, TGP culture; *open circles*, monolayer culture. **C, D** Electron microscopy of HCV particles in the supernatants of TGP-cultured RCYM1 cells. **C** Negative staining. **D** Immunogold labeling with an anti-E2 antibody. Gold particles, 5 nm; bars, 50 nm. **E** Silver-intensified immunogold staining with anti-E1 antibody. The *arrowhead* indicates virus-like particles reacting with anti-E1 antibody

development of artificial liver tissue, and the bioreactor column consists of a vertically extended cylindrical matrix through which liquid medium flows continuously from the periphery toward the center of the reactor.<sup>53</sup> In RFB culture, human hepatocyte-derived cells can grow spherically or cubically, and they retain liver functions such as albumin synthesis<sup>53-55</sup> and drug-metabolizing activity mediated by cytochrome P450 3A4.<sup>56</sup> TGP is a chemically synthesized biocompatible polymer which has a sol-gel transition temperature, thus enabling us to culture cells three-dimensionally in the gel phase at 37°C and to harvest them in the sol phase at 4°C, without enzyme digestion.<sup>57</sup> In contrast to other matrix gels made from conventional natural polymers, TGP has several advantages that allow us to investigate the functional characteristics of epithelial cells, their tissue-like morphology, and their potential clinical applications. For example, the use of 3D culture materials other than TGP requires treatment with appropriate digestive enzymes or heating to collect cells grown as spheroids from the culture media, and the matrices may damage the cultured cells to some extent. A 3D culture system based on RFB and TGP, in which human hepatoma cells can assemble into spheroids with potentially polarized morphology, is a valuable tool in studies of HCV morphogenesis.

### Translation

The approximately 341-nucleotide (nt)-long 5' NTR is one of the most conserved regions of the HCV genome, and the secondary structural model, which is also largely conserved, reveals four distinct RNA domains in the region, reflecting its importance in both viral translation and replication.<sup>58-61</sup> The 5' NTR forms four highly structured domains (domains I-IV), which may be conserved among HCV and related flaviviruses and pestiviruses,<sup>59,60</sup> and it is functionally characterized as an IRES to direct cap-independent translation of the genome.<sup>62,63</sup> To determine the minimal sequence required for HCV IRES-dependent translation, as in the earlier studies of picornaviruses, the bicistronic RNAs in which two reporter protein-coding sequences are separated by an IRES sequence were analyzed. Translation of the upstream reading frame occurs in a 5' end-dependent fashion, while translation of the downstream reading frame is driven by the IRES element. The IRES comprises nearly the entire 5' UTR of the genome. There is evidence to suggest that the first 12 to 30 nt of the coding sequence are also important for IRES activity.<sup>64-66</sup> The first 40 nt of the 5' NTR, which includes a single stem-loop (domain I), is not essential for the translation; the 5' border of the IRES was mapped between nt 38 and 46.<sup>61,67,68</sup> Domains II and III are relatively more complex

and contain multiple stems and loops.<sup>60,69</sup> Domain IV consists of a small stem-loop containing the polyprotein start codon at nt 342 and forms a pseudoknot via base-pairing with a loop in domain III.

Recruitment of the 43S ribosomal complex, containing a small 40S ribosomal subunit, eukaryotic initiation factor (eIF) 3, and a tRNA-eIF2-GTP ternary complex, to mRNA molecules is critical for initiation of eukaryotic protein synthesis. The 40S subunit and eIF3 can bind independently to the HCV IRES.<sup>64,70-72</sup> However, it appears that interaction between IRES RNA and the 40S subunit drives formation of an IRES-40S subunit-eIF3 complex, since HCV IRES RNA demonstrates similar affinity to both the 40S subunit and the 40S-eIF complex.<sup>71</sup> Other cellular factors such as La autoantigen,<sup>73-75</sup> heterogeneous ribonucleoprotein L,<sup>76</sup> poly-C binding protein,<sup>77,78</sup> and pyrimidine tract-binding protein,<sup>79,80</sup> also bind to the IRES element and modulate translation.

Regulation of IRES-dependent translation of HCV is also likely to involve viral factors. We found that the core protein specifically inhibits HCV translation, possibly by binding to a stem-loop IIIId domain, particularly a GGG triplet within the hairpin loop structure of the domain, within the IRES (Fig. 3).<sup>79-81</sup> Although a conflicting report has suggested that inhibition of HCV translation is due to an RNA-RNA interaction, rather than to an interaction between RNA and the core protein,<sup>82</sup> later studies support the role of a core protein sequence spanning amino acids (aa) 34-44 in inhibition of viral translation through its interaction with the IRES.<sup>83</sup> Furthermore, the N-terminal 20 residues of the core protein have been shown to selectively inhibit translation mediated by HCV IRES in a cell type-specific manner.<sup>84</sup> We propose a model in which competitive binding of the core protein to the IRES and 40S ribosomal subunit regulates HCV translation.

By analogy with other RNA viruses with IRES-mediated expression, the HCV 5' NTR has been expected to contain not only determinants for translation but also *cis*-acting elements for RNA replication. Recent studies demonstrated that (1) the sequence upstream of the IRES is essential for viral RNA replication, (2) sequences within the IRES are required for high-level HCV replication, and (3) the stem-loop domain II of the IRES is crucial for the replication.<sup>85</sup>

### Polyprotein processing

IRES-mediated translation of the HCV ORF yields a polyprotein precursor that is subsequently processed by cellular and viral proteases into mature structural and nonstructural proteins (Fig. 1). As deduced from the hydrophobicity profile and the dependence on micro-