

Review

Molecular Virology of Hepatitis C Virus (HCV): 2006 UpdateVolker Brass¹, Darius Moradpour² and Hubert E. Blum¹

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Received: 2005.12.30; Accepted: 2006.03.10; Published: 2006.04.01

Fascinating progress in the understanding of the molecular biology of hepatitis C virus (HCV) was achieved recently. The replicon system revolutionized the investigation of HCV RNA replication and facilitated drug discovery. Novel systems for functional analyses of the HCV glycoproteins allowed the validation of HCV receptor candidates and the investigation of cell entry mechanisms. Most recently, recombinant infectious HCV could be produced in cell culture, rendering all steps of the viral life cycle, including entry and release of viral particles, amenable to systematic analysis. In this review, we summarize recent advances and discuss future research directions.

Key words: helicase, hepatitis C virus, protease, polymerase, replicon

1. Introduction

The hepatitis C virus (HCV) belongs to the *Flaviviridae* family and is the only member of the Hepacivirus genus. HCV infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) worldwide [1]. Therapeutic options are improving but are still limited and a protective vaccine is not available to date. In 50% to 80% acute HCV infection persists and 4% to 20% of patients with chronic hepatitis C will develop liver cirrhosis within 20 years. In patients with liver cirrhosis, the risk to develop HCC is 1-5% per year. Current standard therapy is the combination of pegylated interferon- α (PEG-IFN- α) and ribavirin. Depending on the HCV genotype and other factors, this strategy results in a sustained virologic response in 50-80% of patients [2-5]. However, many patients do not qualify for or do not tolerate standard therapy [6]. Therefore, more effective and better tolerated therapeutic strategies are urgently needed. The development of such strategies depends on a detailed understanding of the molecular virology of HCV infection.

The investigation of the HCV life cycle and pathogenesis has been complicated by the lack of efficient cell culture systems and small animal models. Nevertheless, significant progress could be made using heterologous expression systems, functional cDNA clones [7], the replicon system [8, 9], and functional HCV pseudoparticles [10, 11] (see refs. [12-14] for reviews). Most recently, a major milestone was the production of recombinant infectious HCV particles in cell culture [15-17]. The model systems, summarized in Table 1, are the current basis for the study of the HCV life cycle and the development of novel antiviral strategies.

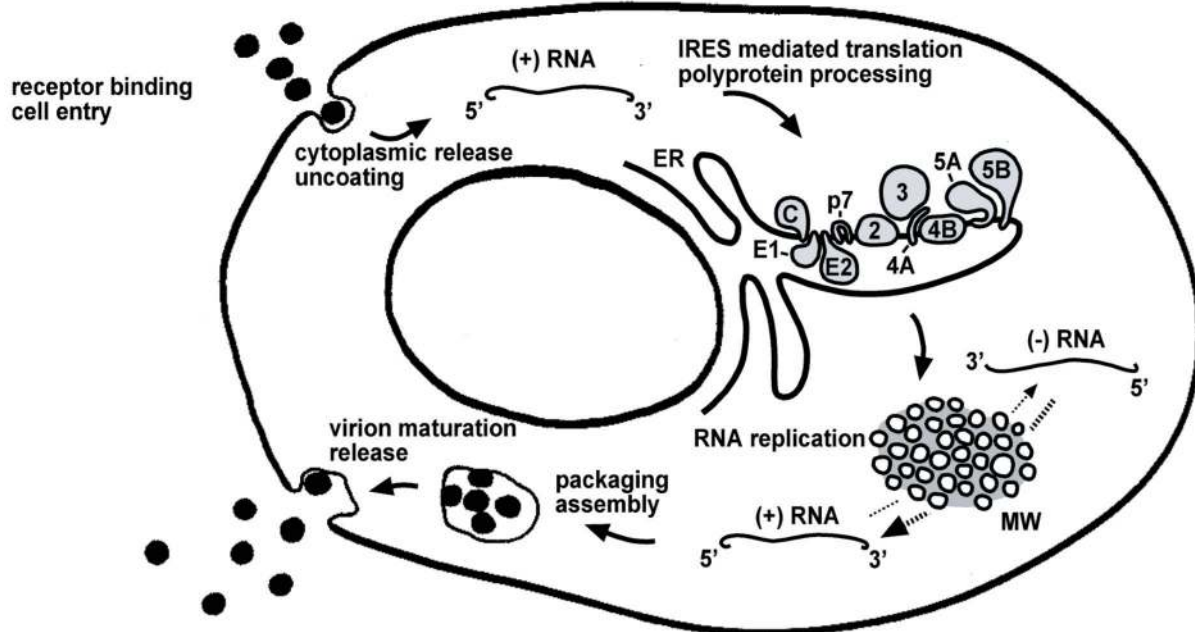
Table 1. *In vitro* and *in vivo* models to study HCV.*In vitro* models

- Transient expression systems
- Stably transfected cell lines (constitutive / inducible expression)
- Infection of primary hepatocytes and established cell lines
- Retroviral pseudoparticles displaying functional HCV glycoproteins
- Replicons (subgenomic / full-length; selectable / transient)
- Recombinant infectious cell culture system
- Chimeric viruses (e.g., poliovirus – HCV)
- Related viruses (e.g., GBV-B in tamarin hepatocytes)

In vivo models

- Transgenic mice
- Immunodeficient mice / hepatocellular reconstitution models
- Chimpanzee (*Pan troglodytes*)
- Tree shrew (*Tupaia belangeri chinensis*) ?
- Related viruses (e.g., GBV-B in tamarins)

Figure 1. Life cycle of HCV. The steps of the viral life cycle are depicted schematically. The topology of HCV structural and nonstructural proteins at the endoplasmic reticulum (ER) membrane is shown. HCV RNA replication occurs in a specific membrane alteration, the membranous web (MW). IRES-mediated translation and polyprotein processing as well as membranous web formation and RNA replication, illustrated here as separate steps for simplicity, may occur in a tightly coupled manner.



2. The replicon system

For many years, HCV research was hampered by the extremely restricted host range and the inefficiency of *in vitro* models based on the incubation of cells in culture with HCV particles. Therefore, the establishment of a replicon system for HCV was a milestone in HCV research [8, 18]. The prototype subgenomic replicon utilized a particular HCV genotype 1b clone termed Con1. In this system, the structural region was replaced by the sequence encoding the neomycin phosphotransferase gene resulting in G418 resistance. Expression of the nonstructural proteins is directed by a heterologous encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) downstream of the neomycin phosphotransferase gene. After transfection into the human HCC cell line Huh-7 G418 resistant colonies contain self-replicating HCV RNA. Critical for the usefulness of this system was the identification of specific amino acid substitutions, i.e., cell culture-adaptive changes, that increased the efficiency of replication initiation by several orders of magnitude [9]. With the replicon system it became possible, for the first time, to study efficient and genuine HCV RNA replication *in vitro* as well as structural aspects of the replication complex, basic replication processes, virus-host cell interactions, and antiviral agents.

In the last 6 years, a large panel of different replicon systems has been developed (Fig. 2). These include replicons from genotype 1a [19] and 2a [20], transient systems expressing easily quantifiable marker enzymes [21], replicons with green fluorescent protein (GFP) insertions in NS5A to track replication complexes in living cells [22], and full length replicons. In addition, the spectrum of permissive host cells has been expanded [23].

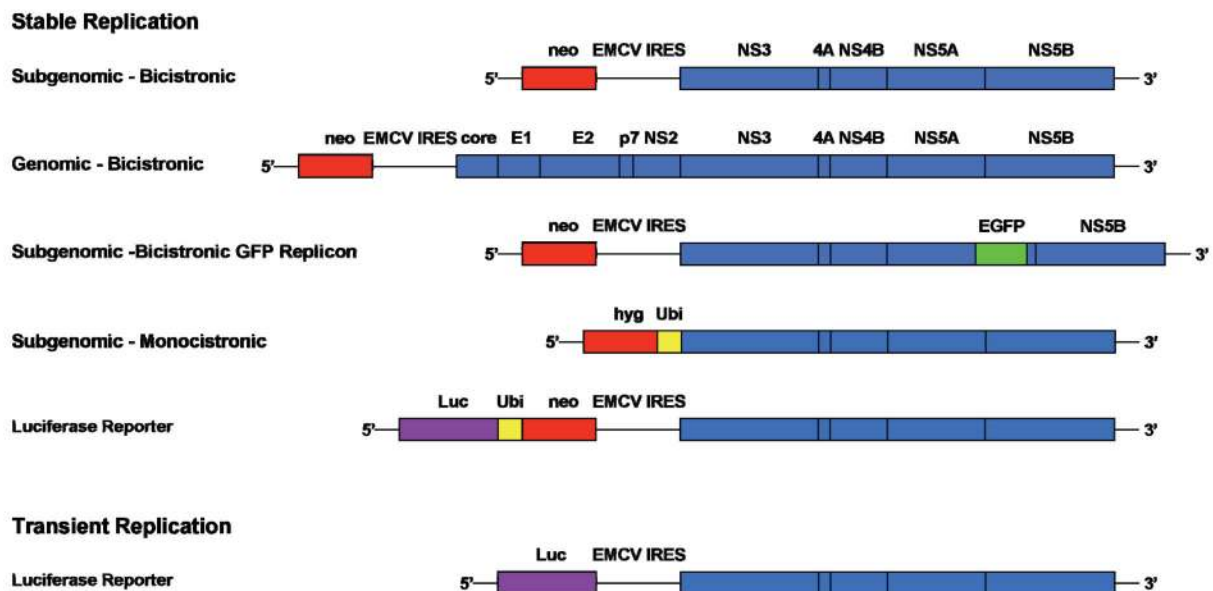
The replicon system revolutionized the research on basic replication processes. However, the step of infection and entry as well as the release of viral progeny could not be analysed to date. Wakita and colleagues, however, generated a genotype 2a replicon (JFH-1) that was isolated from the serum of a patient with fulminant hepatitis C [20]. This system turned out to replicate very efficiently in different cell types. Furthermore, the full-length JFH-1 sequence produced infectious viral particles that could be passaged in cell culture [15, 17]. Further, chimeric constructs with the structural region of the J6 genotype 2a clone improved the infectivity of this system significantly [16]. This recombinant infectious HCV cell culture system represents the last major milestone in the field and renders the complete viral life cycle accessible to detailed analysis *in vitro*.

3. Cell entry

Surrogate models for the study of the early steps of viral life cycle have been established, including infectious retroviral pseudotypes displaying functional HCV glycoproteins. These pseudotypes turned out to provide a robust model system for the study of viral entry [10, 11]. HCV pseudoparticle infectivity is pH-dependent and restricted primarily to human hepatocytes and hepatocyte-derived cell lines. Thus, HCV entry likely involves transit through an endosomal, low pH compartment and fusion with the endosomal membrane.

HCV E2 binds with high affinity to the large external loop of CD81, a tetraspanin found on the surface of many cell types including hepatocytes [24]. However, CD81 is not sufficient to mediate cell entry and several cofactors appear to be required. The low density lipoprotein receptor (LDLR) [25] and scavenger receptor class B type I (SR-BI) [26] have, among others, been proposed as components of a putative HCV receptor complex. The concept, that lipoproteins could play an important role for cell entry is supported by recent data from studies on HCV pseudotypes that demonstrate an enhancement of infectivity by certain components of human serum [27, 28]. In particular, association to high density lipoprotein (HDL) seems to enhance SR-BI guided cell entry and could protect viral particles from neutralizing antibodies [29].

Figure 2. Comparison of different replicon systems. A selection of different bicistronic and monocistronic replicon constructs including subgenomic and full-length HCV sequences is depicted schematically. Stably replicating systems are equipped among others with a neomycin phosphotransferase or a hygromycin phosphotransferase cassette that allow selection of cells with continuous RNA replication. Transient systems include a marker gene that allows quantification 48 h after transient transfection. In general, luciferase (Luc) is used as easily quantifiable enzyme in this context and values are normalized based on the amount of input RNA. Monocistronic replicon constructs avoid the translation of proteins from the heterologous encephalomyocarditis virus (EMCV) IRES. The resistance gene is released after cleavage *via* a ubiquitin sequence (Ubi).



4. Translation and polyprotein processing

The HCV genome consists of a 9.6-kb positive-strand RNA that comprises a long open reading frame flanked by 5' and 3' noncoding regions (NCR). The 5' NCR is highly conserved among different HCV isolates and contains an IRES. The IRES directly binds the 40S ribosomal subunit independently of the pre-initiation factors necessary for cap-dependent translation. The three-dimensional structure of the HCV IRES bound to the 40S ribosomal subunit illustrates the molecular basis of this cap-independent translation process [30]. High-resolution structural data on critical elements of the IRES and detailed characterization of the translation initiation complex further improves the view on the HCV translation initiation [31, 32].

Translation of the HCV genome leads to a polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases to produce the mature structural and nonstructural proteins. The N-terminal one-third of the polyprotein harbors the structural proteins core, E1 and E2 that form the viral particle. The structural region is followed by the p7 polypeptide which may be involved in ion channel formation (see below). The nonstructural proteins 2-5B coordinate viral replication by the formation of a membrane-bound replication complex. Processing of the polyprotein at the core/E1, E1/E2, E2/p7, and p7/NS2 sites by the host cell signal peptidase produces all structural proteins and p7. Two viral proteases are responsible for the maturation of the nonstructural proteins. The NS2-3 autoprotease cleaves at the NS2/NS3 junction while all downstream sites are processed by the NS3-4A serine protease.

5. Molecular aspects of viral proteins

5.1 Structural proteins

5.1.1 Core protein

The HCV core protein is a highly basic, RNA-binding protein which presumably forms the viral nucleocapsid. Of note, the core protein has been reported to interact with numerous cellular proteins and to affect host cell functions such as gene transcription, lipid metabolism, apoptosis and various signaling pathways [33]. Further, it has been associated with the induction of steatosis and HCC [34-36].

5.1.2 Envelope glycoproteins

The envelope glycoproteins E1 and E2 are type I transmembrane proteins with C-terminal hydrophobic anchors. The ectodomains translocate to the ER lumen where they are modified by extensive N-linked glycosylation. E1 and E2 form non-covalent heterodimers which are believed to represent the building blocks for the viral envelope. The processes of particle assembly and release are poorly understood and have only recently become amenable to systematic investigation. In this context, structural studies on recombinant HCV particles confirmed earlier electron microscopy observations [15].

5.2 ARFP/F protein

The synthesis of a protein encoded by an alternative reading frame within the core region was reported by several groups [37]. It was designated ARFP (alternative reading frame protein) or F (frameshift) protein and comprises up to 160 amino acids. The ARFP/F protein is dispensable for HCV RNA replication. Whether it is expressed during natural HCV infection has still to be clarified.

5.3 p7

p7 is a 63-amino acid polypeptide located at the junction between the structural and nonstructural region. It is unknown whether p7 is packaged into viral particles. It is composed of two transmembrane domains and has recently been reported to form hexamers with ion channel activity [38, 39]. It is believed that p7 could be important for viral assembly because the corresponding protein of the related bovine viral diarrhea virus (BVDV) is essential for the production of infectious progeny virus but not for RNA replication [40].

5.4 Nonstructural proteins

5.4.1 NS2-3 autoprotease

The NS2/3 junction is cleaved by a remarkable autoprotease consisting of NS2 and the N-terminal third of NS3. Although NS2-3 protease activity is required for the replication *in vivo*, it is dispensable for replication of subgenomic replicons *in vitro*. It is unclear whether NS2 fulfills any further functions after separation from NS3.

5.4.2 NS3-4A

NS3 is a multifunctional protein because it harbors a serine protease located in the N-terminal one-third that is responsible for the downstream cleavage in the nonstructural region and a NTPase/RNA helicase domain in the C-terminal two-thirds. NS4A, a 54-amino acid polypeptide, targets NS3 to intracellular membranes and is required as a cofactor for the NS3 serine protease. The crystal structure of the NS3-4A complex revealed that NS4A is an integral component of the enzyme core [41]. Surprisingly, the NS3 serine protease recently turned out to influence the innate cellular host defense by inhibition of RIG-I and TLR3 signalling [42, 43]. This observation renders the NS3 protease particularly attractive as an antiviral target [44]. Serine protease inhibitors have emerged as extremely efficient antiviral components in first 'proof-of-principle' studies in patients with chronic hepatitis C [45, 46].

The enzymatic activity of the NS3 NTPase/helicase activity is indispensable for RNA replication. Putative functions during replication could be to unwind replicative double strand RNA intermediates, to eliminate RNA secondary structures or to separate the genome from nucleic acid binding proteins. Recent advances in the understanding of the molecular mechanisms of this enzyme could enable a specific inhibition as a novel antiviral strategy [47, 48].

5.4.3 NS4B

Due to its very hydrophobic properties, NS4B belongs to the difficult-to-study HCV proteins that are poorly understood. So far, it is known that NS4B is a 27-kDa integral membrane protein that localizes to an ER-derived membranous compartment [49]. Interestingly, the expression of NS4B induces a specific membrane alteration, designated as membranous web, that serves as a scaffold for the formation of the viral replication complex [50, 51].

5.4.4 NS5A

NS5A is a phosphorylated zinc metalloprotein of unknown function. Numerous potential functions and a huge list of interaction partners have been described [33]. However, surprisingly little efforts has been devoted to a rigorous biochemical characterization of this protein and

its definitive role in viral replication remains elusive. NS5A has initially attracted considerable interest because of its potential role in modulating the IFN response (reviewed in ref. [52]). These findings are still controversial, however. A striking observation was the concentration of cell culture adaptive replicon mutations within the central part of NS5A [9, 53]. Considering the fact that NS5A phosphorylation has an impact on replication efficiency, these observations support the concept that NS5A plays an important role in the regulation of viral replication [54-56]. The membrane association of NS5A is mediated by a unique amphipathic alpha-helix which is localized at the N-terminus [57, 58]. Limited proteolysis experiments recently allowed the definition of three protein domains within the cytosolic domain [59]. More recently, the three-dimensional structure of the N-terminal domain I could be resolved by crystallography. After dimerization, it forms a basic groove facing the cytosol at the surface of the membrane [60]. This 'claw like' structure is believed to provide an RNA binding site that could be involved in regulated genome targeting within the replication complex.

5.4.5 NS5B

The key enzyme of the replicase that promotes synthesis of new RNA genomes is the NS5B RNA-dependent RNA polymerase (RdRp). NS5B is a tail-anchored protein, characterized by a transmembrane domain at the C-terminus of the protein responsible for posttranslational membrane targeting [61-63]. The structural organization of NS5B is a typical 'right hand' polymerase shape with finger, palm, and thumb subdomains surrounding a completely encircled active site [64]. Replication proceeds *via* synthesis of a complementary minus-strand RNA using the genome as a template and the subsequent synthesis of genomic plus-strand RNA from this minus-strand RNA intermediate. As central component of the HCV replicase, NS5B has emerged as a major target for antiviral intervention [44].

6. RNA replication

As in all positive-strand RNA viruses investigated thus far (reviewed in ref. [65]), HCV forms a membrane-associated replication complex, composed of viral proteins, replicating RNA, altered cellular membranes and additional host cell factors [50, 51]. A specific membrane alteration, referred to as the membranous web, was recently identified as the site of RNA replication in Huh-7 cells harboring subgenomic HCV replicons [51]. Thus, intracellular membranes play a crucial role in HCV replication. Recent data underline the importance of a specific lipid environment for HCV RNA replication [66, 67].

In addition to coordinated protein-protein and protein-membrane interaction, essential *cis*-acting replication elements (CRE) of the RNA genome were recently discovered. For instance, the sequence coding for the C-terminal domain of NS5B consists of an essential stem-loop, designated SL3.2, within a larger cruciform RNA element, designated SL3 [68]. Detailed characterization of the SL3.2 domain indicated a functionally important kissing loop interaction with the 3' NCR [69].

Several host cell factors, including hVAP-A, FBL2 or cyclophilin B that influence HCV RNA replication *via* interaction with different viral proteins have been

identified [54, 70, 71]. However, the regulation of replication, the switch to translation or assembly and the release of viral particles are still poorly understood.

7. Future research directions

The pace of research in the HCV field has increased enormously with the establishment of the replicon system. The infectious JFH-1 cell culture system promises exiting progress in the understanding of steps in the viral life cycle that have been difficult to study thus far. In particular, HCV entry, cytoplasmic release and uncoating, the initial steps of replication, virus assembly, the release of viral progeny, and the detailed virion structure will be characterized in the infectious cell culture system. Furthermore, the impact of viral proteins such as p7 and NS2 for viral particle formation and possibly of NS5A for the switch between replication and assembly can be explored in this context. New insights into the molecular virology of HCV should identify novel targets for antiviral strategies.

Conflict of interest

The authors have declared that no conflict of interest exists.

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