Role of Nonstructural Proteins in HCV Replication

Tetsuro Suzuki and Ryosuke Suzuki

Abstract The open reading frame of the HCV RNA genome is translated at the rough endoplasmic reticulum, yielding a polyprotein precursor that is co- and posttranslationally processed by cellular and viral proteases. The carboxy-terminal two-thirds of the polyprotein encodes various nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), which, it is thought, are not incorporated into virus particles but instead coordinate the intracellular aspects of viral replication. Together with host factors, NS3, NS4A, NS4B, NS5A, and NS5B form a membrane-associated replication complex. Cleavage of the precursor at the NS2/3 junction is accomplished by a cysteine protease encoded by NS2 and the N-terminal portion of NS3. The NS3-4A heterodimeric complex contains a serine protease domain and an RNA helicase/nucleoside triphosphatase domain. The protease activity is responsible for downstream cleavage events in the NS region. NS4B and NS5A have no enzymatic activity. NS4B is thought to serve as a scaffold for the viral replication complex and to induce substantial rearrangements of membrane vesicles. NS5A exists in hypo- and hyperphosphorylated forms and interacts with a large number of host proteins that are important for assembly and function of the replication complex. NS5B RNA-dependent RNA polymerase, which presumably mediates de novo RNA synthesis, is the catalytic core of the HCV replication machinery. Evidence clarifying the roles of several NS proteins is accumulating. Here, we present an overview of the mechanisms by which HCV NS proteins contribute to the viral replication cycle.

Keywords NS2 • NS3 • NS4A • NS4B • NS5A • NS5B • Replication

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1 Introduction

HCV open reading frame of the RNA genome is translated at the rough endoplasmic reticulum (ER), yielding a polyprotein precursor of about 3000 amino acids (aa) that is co- and post-translationally processed by cellular- and viral proteases. Nonstructural (NS) proteins are processed by two viral proteases: cleavage between NS2 and NS3 is a rapid intramolecular reaction mediated by NS2-3 protease, while the remaining four junctions are processed by NS3-4A protease. It is considered that the NS3-NS5B region is processed by sequential cleavage: NS3/4A→NS5A/5B→NS4A/4B→NS4B/5A (Tanji et al. 1994; Failla et al. 1995; Lin et al. 1994; Bartenschlager et al. 1994). NS3, NS4A, NS4B, NS5A and NS5B are the viral components of functional replicase complexes required for genome RNA replication. Understanding of molecular structures of individual NS proteins and their roles in the viral lifecycle has increased substantially over the last decades. Progress in the virus research as well as development of powerful model systems stimulated efforts for drug development. Recently, direct antivirals targeting NS3, NS5A and NS5B have been approved successively, as described in detail in *Volume II*.

2 NS2

The N-terminus of NS2 is cleaved by host signal peptidase from the p7 protein. NS2 is a 23-kDa transmembrane protein, with a highly hydrophobic 96-residue N-terminus. The N-terminal domain consists of three transmembrane helices that insert into the ER membrane (Jirasko et al. 2008; Phan et al. 2009). The C-terminal domain of NS2 resides in the cytoplasm, together with one-third of the N-terminal domain of NS3, enabling the NS2-3 protease activity that is necessary for cleavage of the NS2/3 junction. Although NS2 is not essential for the replication of subgenomic RNA replicons (Lohmann et al. 1999), NS2/3 cleavage is required for formation of the viral replication complex (RC), or replicase. Accumulating evidence from in vitro studies of HCV infection suggests that NS2 is required for virus assembly, independent of protease activity (Jones et al. 2007; Jirasko et al. 2008).

The NS2-3 protease was initially thought to be a metalloprotease because its enzymatic activity is stimulated by zinc and inhibited by EDTA (Hijikata et al. 1993). However, a later study revealed that the zinc-binding domain is located in the NS3 region, a domain that is important for NS2 protease activity, as discussed below (Schregel et al. 2009). NS2-3 is currently thought to function as a cysteine protease (Lackner et al. 2004). Mutational analyses showed that the highly conserved His-143, Glu-163, and Cys-184 residues are essential for NS2-3 protease activity (Grakoui et al. 1993; Welbourn et al. 2005; Hijikata et al. 1993). Analysis of the crystal structure of the C-terminal domain of NS2 revealed that the region can homodimerize, creating two composite active sites (Lorenz et al. 2006). The

catalytic His-143 and Glu-163 residues are contributed by one monomer, and Cys-184 is contributed by the other monomer, forming the catalytic triad (Lorenz et al. 2006). NS2/3 processing is critical for genome replication (Welbourn et al. 2005); however, some studies have demonstrated replication of the subgenomic replicon that encodes NS3-5B (Lohmann et al. 1999), indicating that NS2 is not essential for replication of the viral genome in vitro.

The involvement of NS2 in the production of infectious virus independent of its protease activity was initially demonstrated using a dicistronic expression system with the viral genome containing the internal ribosomal entry site from encephalomyocarditis virus between NS2 and NS3 (Jones et al. 2007; Jirasko et al. 2008; Dentzer et al. 2009). Several adaptive mutations in NS2 that enhance virus production have also been reported (Kato et al. 2008; Popescu et al. 2011; Russell et al. 2008; Yi et al. 2007; Jensen et al. 2008; Scheel et al. 2008). Furthermore, there is increasing evidence for genetic and biochemical interactions between NS2 and other HCV proteins, including E1, E2, p7, NS3-4A, and NS5A (Phan et al. 2009; Popescu et al. 2011; Ma et al. 2011; Jirasko et al. 2010; Stapleford and Lindenbach 2011; Yi et al. 2009; Selby et al. 1994; Kiiver et al. 2006; Counihan et al. 2011). Thus, it is now thought that NS2 functions as a scaffold, coordinating interactions between structural and NS proteins during viral assembly. Recently, signal peptidase complex subunit 1 (SPCS1), a component of the microsomal signal peptidase complex that is responsible for cleavage of the signal peptides of many secreted and membrane-associated proteins, was identified as an NS2-binding host factor (Suzuki et al. 2013). SPCS1 was shown to interact with both NS2 and E2, forming an E2-SPCS1-NS2 complex. SPCS1 thus plays a critical role in the assembly of infectious HCV particles (Suzuki et al. 2013), as interaction between E2 and NS2 is important for infectious viral particle assembly (Popescu et al. 2011; Stapleford and Lindenbach 2011; Jirasko et al. 2010).

Cyclosporin A (CsA) is known to inhibit replication of the HCV RNA (Watashi et al. 2003) as described below. Besides inhibiting the replication of the subgenomic replicon containing NS3 to NS5B genes, it has been shown that its inhibitory effect was remarkably enhanced in cultures of full-length virus (Ciesek et al. 2009). The CsA's inhibitory activity is dependent on NS2 as well as on the peptidyl-prolyl cis-trans isomerase (PPI) activity of cyclophilin (Cyp) A (Ciesek et al. 2009).

3 NS3-4A

NS3 is a 70-kDa protein containing a serine protease motif in the N-terminal domain and nucleoside triphosphatase (NTPase) and RNA helicase motifs in the C-terminal domain. NS4A is a 54-aa 8-kDa protein that acts as a cofactor for NS3 serine protease (Bartenschlager et al. 1994; Failla et al. 1994). The NS3-4A complex associates with the ER membrane through the transmembrane α-helix of

the NS4A N-terminus (Brass et al. 2008). The NS3-4A complex is essential for viral polyprotein processing and replication of the RNA genome.

Numerous studies utilizing various expression systems have revealed that NS3-4A cleaves at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites (Hijikata et al. 1993; Bartenschlager et al. 1993; Eckart et al. 1993; Tomei et al. 1993; D'Souza et al. 1994; Manabe et al. 1994; Shoji et al. 1995; Suzuki et al. 1995). The cleavage of NS3/4A is an autocatalytic and cotranslational event that is mediated in cis, whereas cleavage at the NS4A/4B, NS4B/5A, and NS5A/5B sites occurs in trans (Bartenschlager et al. 1994; Tanji et al. 1994; Lin et al. 1994; Failla et al. 1994). The catalytic triad of NS3-4A protease, which is a member of the trypsin/chymotrypsin superfamily of serine proteases, is formed by His-57, Asp-81, and Ser-139. Detailed mutagenesis experiments have indicated that the N-terminal domain of NS3 and the central domain of NS4A are important for stable interaction between NS3 and NS4A (Koch et al. 1996; Shimizu et al. 1996). Analyses of the NS3 serine protease crystal structure have revealed that NS3 has a trypsin-like fold and a structural zinc-binding site (Love et al. 1996). Zinc is tetrahedrally coordinated by Cys-97, Cys-99, Cys-145, and His-149. This domain is also important for cleavage of the NS2/3 junction by NS2 protease (Schregel et al. 2009). Direct interaction between NS3 and synthetic NS4A peptide has also been confirmed by X-ray crystallography (Kim et al. 1996). Sequence alignment of NS3-4A substrate among various HCV isolates indicates that a consensus sequence would include the Asp or Glu residue at the P6 position and Cys for Thr residue at the P1 position, and Ala or Ser residue at the P1′ position (D/E-X-X-X-X-C/T|A/S-X-X-X) (Bartenschlager et al. 1995).

RNA helicase and NTPase activities in the C-terminal domain of NS3 have also been demonstrated. The RNA helicase/NTPase domain is presumably responsible for unwinding replicative double-stranded (ds)RNA intermediates in order to remove RNA secondary structures or separate the genome from RNA-binding proteins during RNA replication. Recombinant NS3 expressed in *E. coli* has been shown to bind to the poly(U) sequence located at the 3′ end of the HCV RNA (Kanai et al. 1995). Furthermore, the NTPase activity is enhanced by adding poly (U) to the reaction mixture. NS3 helicase, a DExH/D-box helicase, potentially unwinds dsRNA in the 3–5′ direction (Tai et al. 1996). Crystal structure analyses revealed that NS3 helicase has distinct NTPase and RNA-binding domains. This structure supports a mechanism of helicase activity involving initial recognition of a 3′ single-stranded region on the nucleic acid by an Arg-rich region of the RNA-binding domain (Yao et al. 1997). These findings suggest that NS3 binds to the 3′ end of the HCV genome and regulates the replication of viral RNA.

Adenosine 5′-triphosphate (ATP), the major energy currency of cells, is involved in a variety of processes, including viral replication. ATP-dependent reactions essential for replication of viral genomes are catalyzed by various virus-encoded enzymes, including helicases and polymerases. High-energy phosphoryl groups are required for NS3 RNA helicase and NS5B RNA polymerase activities (see below). Previous research demonstrated that creatine kinase B (CKB), which is critical for the maintenance and regulation of cellular energy stores, accumulates in particular

subcellular sites where the viral genome potentially replicates in infected cells. It is likely that CKB is directed to the HCV replication machinery through interaction with NS4A and that the enzyme functions as a positive regulator of the viral replicase by providing ATP (Hara et al. 2009).

It is now apparent that HCV NS3 is also involved in viral morphogenesis (Ma et al. 2008; Han et al. 2009; Suzuki et al. 2012), although its precise role and the underlying molecular mechanism(s) have not fully been elucidated. Three cell-culture adaptive mutations involved in HCV assembly have been identified in the *NS3* gene. The Q221L mutation in helicase subdomain 1 results in approximately 30-fold higher production of HCV without affecting the NS3 enzymatic activity (Ma et al. 2008). The M260K adaptive mutation is also located in subdomain 1 of the NS3 helicase (Han et al. 2009). The N556D mutation, located in subdomain 3 of the helicase, enhances the assembly of infectious viral particles by increasing specific infectivity without affecting the efficiency of viral RNA replication (Suzuki et al. 2012). Considering the possibility that NS3 plays a role in linking the viral replicase and assembly sites (Jones et al. 2011), it is likely that the NS3 helicase mediates interactions with structural proteins.

4 NS4B

NS4B is a 27-kDa integral membrane protein consisting of a 69-residue N-terminal domain, a central transmembrane domain (aa residues 70–190), and a C-terminal domain (aa residues 191–261). NS4B is thought to play an essential role in viral replication through remodeling of the intracellular membrane by self-oligomerization, providing a platform for the viral RC (Egger et al. 2002; Lundin et al. 2003; Gouttenoire et al. 2010b; Paul et al. 2011). NS4B induces the formation of a complicated multivesiculated cytoplasmic structure known as the membranous web. This specialized membrane structure, derived at least in part from the ER, is rich in HCV NS proteins and viral RNA and likely contains single-, double-, and multi-membrane vesicles. It was recently reported that double-membrane vesicles are possible sites of viral genome replication (Romero-Brey et al. 2012; Ferraris et al. 2010; Gouttenoire et al. 2010a; Paul et al. 2013). The N-terminal domain of NS4B contains two amphipathic helices (designated amphiphathic α-helix [AH]1 and AH2) and reportedly has a dual-membrane topology (Lundin et al. 2003, 2006). Although the N-terminal domain is predicted to reside in the cytoplasmic face, NS4B AH2 was shown to translocate at least partially across the membrane into the ER lumen, presumably through a posttranslational mechanism (Gouttenoire et al. 2009; Lundin et al. 2006). Such a topology change might contribute to the induction of membranous vesicle formation. AH2 is also a major determinant of NS4B oligomerization (Gouttenoire et al. 2010b). The central domain of NS4B contains four predicted transmembrane segments. The C-terminal domain, which comprises two α-helices, is believed to be on the cytosolic side of the ER membrane and includes arginine residues that may be important in binding between RNA and

NS4B (Einav et al. 2008). Two conserved dimerization motifs (GXXXG and S/T clusters) have been identified within the transmembrane domains, and mutations in each of the putative motifs result in a reduction in viral replication (Han et al. 2011). In addition, a nucleotide-binding motif (Thompson et al. 2009) and two palmitoylation sites (Yu et al. 2006) were indicated in the region. Heterotypic interaction between the highly conserved cytoplasmic C-terminal domain and the NS4B N-terminus was shown to be important for induction of functional membrane vesicle formation (Paul et al. 2011). Specific charged residues in the N- and C-termini of NS4B play a role in formation of the viral RC (Blight 2011). Further studies on the impact of mutations in the C-terminal domain on the HCV infection cycle demonstrated that this NS4B domain is not only essential for viral genome replication but contributes to the assembly of infectious particles, possibly in the process of genome RNA encapsidation (Paul et al. 2011; Han et al. 2013).

5 NS5A

NS5A is an approximately 450-residue hydrophilic phosphoprotein that functions as a key regulator of viral genome replication and virion assembly. NS5A is predicted to contain three domains that are separated by repetitive low-complexity segments (LCS1 and 2). The N-terminal domain (D1; aa residues 28–213) immediately follows the membrane-anchoring α-helix (Brass et al. 2002; Penin et al. 2004; Tellinghuisen et al. 2004) and forms a dimeric structure with an unconventional zinc-coordinating motif (Tellinghuisen et al. 2005). Two different crystal structures for D1 have been reported. Although the dimeric D1 interface differs markedly in each structure, both models suggest that the dimeric structure associates with the lipid bilayer in membrane-bound HCV RCs (Love et al. 2009; Tellinghuisen et al. 2005). The model for dimer formation at the membrane surface led to the proposal that an RNA-binding groove is exposed to the cytosol (Tellinghuisen et al. 2005); binding of RNA by the protein has been demonstrated (Huang et al. 2005).

NS5A interacts with uridylate- and guanylate-rich RNAs as well as the 3′ ends of positive- and negative-stranded HCV RNAs (Huang et al. 2005). In the native state, domains II (D2; aa residues 250–342) and III (D3; residues aa 356-) are unfolded monomers (Hanoulle et al. 2009; Verdegem et al. 2011), but no other structural information has been reported for these domains. Such structural flexibility might enable this enigmatic protein to interact with a variety of host-derived proteins. D3 exhibits genotypic length polymorphisms. D1 and part of D2 are required for genome RNA replication. Most of D3 is unessential for this process; however, D3 is important for assembly of the infectious particles (Appel et al. 2008; Masaki et al. 2008; Hughes et al. 2009; Kim et al. 2011; Tellinghuisen et al. 2008).

Although NS5A has no known enzymatic activity, it has a variety of other functions, foremost as an essential component of the viral RC. A large number of cell culture-adaptive mutations mapped to NS5A gene have been shown to enhance

RNA replication of HCV replicons in human hepatoma HuH-7 cells (Blight et al. 2000; Krieger et al. 2001; Lohmann et al. 2001). Unlike the other NS proteins, NS5A can be trans-complemented in viral replicons containing mutated NS5A that does not support viral RNA replication (Appel et al. 2005a). Association of HCV NS proteins with the membrane is a key feature of RC formation in the specialized membrane compartment. Mapping and structural studies have shown that the N-terminal amphipathic α-helix of NS5A, which is well conserved among HCV isolates, serves as a membrane anchor, allowing association with ER-derived membrane, in which NS5A embeds in the plane of the cytosolic leaflet of the membrane bilayer (Penin et al. 2004). It has been proposed that the N-terminal domain of NS5A forms a platform that is involved in specific protein-protein interactions essential for the assembly of the RC (Penin et al. 2004).

Interaction between NS5A and RNA and other NS proteins is thought to be a crucial aspect of this protein's function in RNA replication. NS5A also interacts with a variety of cellular proteins that are potentially involved in viral replication. Cyps and FK506-binding proteins (FKBPs) are classified as immunophilins, proteins capable of binding to the immunosuppressants cyclosporine and FK506, respectively. Cyps exhibit PPI activity, catalyzing the cis-trans isomerization of peptide bonds preceding proline residues. Cyps also mediate de novo protein folding and isomerization of native proteins. CypA is a major host factor required for HCV replication (Kaul et al. 2009), and its PPI activity is thought to play a role in viral replication (Liu et al. 2009; Kaul et al. 2009). Increasing evidence supports a direct interaction between NS5A and CypA; D2 and LCS2 of NS5A contain sites shown to be important for binding to CypA (Hanoulle et al. 2009; Grise et al. 2012; Fernandes et al. 2010; Ross-Thriepland et al. 2013). How the CypA-NS5A interaction influences HCV replication remains to be elucidated, but it can be speculated that CypA-catalyzed isomerization of prolines in NS5A is required for proper binding of NS5A to viral RNA and/or for high polymerase activity of NS5B molecules (see below) directly associated with NS5A.

The immunophilin FKBP8 also plays a role in HCV genome replication. FKBP8 interacts with D1 of NS5A and recruits the molecular chaperone heat shock protein 90 (Hsp90) to the HCV RC through interaction of the carboxylate clump structure of FKBP8 with the C-terminal MEEVD motif of Hsp90 (Okamoto et al. 2008, 2006). Research suggests that Hsp90 and cochaperones are involved in formation of the HCV RC through interaction with NS5A or other HCV proteins (Taguwa et al. 2008, 2009; Ujino et al. 2009; Nakagawa et al. 2007). Human butyrate-induced transcript 1, which has significant homology with cochaperone p23, is also thought to be involved in HCV replication through interactions with NS5A and Hsp90 (Taguwa et al. 2008).

Small-interfering RNA screening studies identified phosphatidylinositol-4-phosphate kinase III α (PI4KIIIα) as a critical factor for HCV replication. The lipid kinase PI4KIIIα belongs to the family of type III phosphatidylinositol-4 kinases, which catalyze the conversion of phosphatidylinositol to phosphatidylinositol-4-phosphate (PI4P). PI4P is the most abundant monophosphorylated inositol phospholipid in mammalian cells and is thought to play roles in intracellular signaling

and membrane trafficking pathways. PI4KIIIα was shown to interact with NS5A, leading to activation of lipid kinase activity and increased PI4P levels (Bianco et al. 2012; Tai and Salloum 2011; Berger et al. 2011; Reiss et al. 2011). Another study demonstrated that mutations within the PI4KIIIα-binding site in the C-terminal portion of NS5A D1 result in reduced HCV RNA replication and alterations in the morphology of possible replication sites; the phenotypes are the same as those observed in gene silencing or treatment with inhibitors of PI4KIIIα (Reiss et al. 2013). It is of interest that the level of basally phosphorylated NS5A increases with overexpression of PI4KIIIα, indicating that the phosphorylation status of NS5A is regulated by PI4KIIIα (Reiss et al. 2013).

Vesicle-associated membrane protein (VAMP)-associated protein (VAP) subtypes A (VAP-A) and B (VAP-B) also interact with NS5A. VAP-A and VAP-B localize primarily at the cytoplasmic face of the ER and Golgi apparatus and are involved in maintaining ER homeostasis and vesicular trafficking. In HCV-replicating cells, VAP-A can be detected in the detergent-resistant membrane fraction that contains the viral RC. Interaction between VAP-A and NS5A is required for the efficient replication of HCV genomic RNA (Gao et al. 2004). VAP-B may also be involved in viral replication through the formation of homodimers and/or heterodimers with VAP-A (Hamamoto et al. 2005). VAP-A and VAP-B form either hetero- or homodimers through their transmembrane regions and interact with NS5A and NS5B. A recent study showed that VAP-A and VAP-B are enriched in the cytoplasmic membrane compartment, the formation of which is induced by HCV RNA replication (Paul et al. 2013). VAP-C, a third VAP subtype that is an alternatively spliced isoform of VAP-B, has been shown to act as a negative regulator of HCV replication. It is likely that VAP-C is involved at least in part in the determination of virus tissue tropism (Kukihara et al. 2009). Other host-derived factors, such as F-box, leucine-rich repeat protein 2 (FBL2) (Kapadia and Chisari 2005; Wang et al. 2005), and tail-interacting protein 47 (TIP47) (Vogt et al. 2013) may play roles in viral replication via interaction with NS5A.

NS5A is phosphorylated on multiple serine and threonine residues and exists in basally phosphorylated (p56) and hyperphosphorylated (p58) forms (Reed et al. 1997; Tanji et al. 1995; Kaneko et al. 1994). Regions around the center and near the C-terminus of NS5A appear to be required for basal phosphorylation, whereas hyperphosphorylation primarily targets a number of serine residues located within LCS1 (Tanji et al. 1995; Blight et al. 2000; Appel et al. 2005b; Evans et al. 2004). Phosphorylation of NS5A occurs primarily on serine residues, with minor phosphorylation on threonine residues. No phosphorylation on tyrosine residues in NS5A has been shown. It is thought that phosphorylation of NS5A modulates its function. It has been suggested that HCV replication could be regulated by controlling the p58:p56 ratio. Some adaptive mutations that reduce the level of NS5A hyperphosphorylation enhance HCV replicon replication (Appel et al. 2005b; Blight et al. 2000). Similarly, suppression of NS5A hyperphosphorylation through either the use of kinase inhibitors or mutagenesis enhances RNA replication in non-culture-adapted replicons (Neddermann

et al. 2004; Appel et al. 2005b). In contrast, HCV RNA replication is inhibited in cells carrying adapted replicons after treatment with the same kinase inhibitor (Neddermann et al. 2004). Thus, viral RNA replication might be regulated through a delicate balance between basal- and hyperphosphorylation of NS5A.

The C-terminal region of NS5A D3 is essential for the production of infectious virus, presumably because this region mediates the interaction between NS5A and core proteins (Appel et al. 2008; Masaki et al. 2008; Hughes et al. 2009; Kim et al. 2011; Tellinghuisen et al. 2008). In infected cells, a proportion of the NS5A molecules present in the cell localizes with core in close proximity to lipid droplets, the site of virion assembly. Alanine substitutions in the serine cluster in D3 impair phosphorylation of NS5A, leading to a decrease in NS5A-core interaction, perturbation of the subcellular distribution of NS5A, and disruption of virion production (Masaki et al. 2008; Tellinghuisen et al. 2008; Appel et al. 2008). It has been proposed, therefore, that NS5A acts as a transport vehicle, carrying newly synthesized viral genomes from replication sites to encapsidation sites (Masaki et al. 2008). Other genetic evidence suggests that NS5A, in association with NS2, functions at a later step in virus production, following virion assembly but prior to virus release (Yi et al. 2009).

Many studies have attempted to identify the cellular protein kinases responsible for NS5A phosphorylation, and several serine/threonine protein kinases have been identified as potential candidates (Chen et al. 2010; Kim et al. 1999; Coito et al. 2004; Ide et al. 1997; Quintavalle et al. 2007; Tellinghuisen et al. 2008). The α isoform of casein kinase I (CKI-α) was shown to be involved in replication of the subgenomic replicon derived from genotype 1b, with moderate inhibition of viral RNA replication resulting from CKI-α knockdown (Quintavalle et al. 2006). A study utilizing inhibitors of NS5A hyperphosphorylation coupled with inhibitor affinity chromatography identified NS5A as a direct substrate of CKI-α (Quintavalle et al. 2007). Recently, a kinome-wide in vitro high-throughput binding screening combined with phosphorylation analysis identified CKI-α as a major NS5A-associated kinase that is critical for NS5A hyperphosphorylation and the production of infectious virus (Masaki et al. 2014). A phosphoproteomic analysis of NS5A with and without CKI-α depletion in HCV-replicating cells identified an LCS1-derived peptide fragment that contains eight highly conserved serine residues that are important for CKI-α–mediated hyperphosphorylation. Subsequent mutagenesis analyses demonstrated that the NS5A (genotype 2a) residues serine-225 and serine-232 might be important for NS5A hyperphosphorylation and hyperphosphorylation-dependent regulation of virion production (Masaki et al. 2014). Casein kinase II (CKII) is also known to be involved in NS5A phosphorylation. In vitro, CKII binds to and phosphorylates the C-terminal domain of NS5A (Kim et al. 1999). Mapping studies have suggested that a specific serine residue in a consensus CKII phosphorylation motif within NS5A D3 is important for regulation of infectious HCV production (Tellinghuisen et al. 2008). Polo-like kinase 1 (Plk1) was identified as a candidate for NS5A phosphorylation through siRNA screening. Plk1 interacts with NS5A and plays a role in viral replication through hyperphosphorylation of NS5A (Chen et al. 2010). Other serine/threonine kinases, including AKT, p70S6K, MEK1, and MKK6 have also been shown to

phosphorylate NS5A in vitro (Chen et al. 2010). Considering the complexity of NS5A phosphorylation, it is likely that the differential phosphorylation of NS5A by multiple kinases coordinates the specific protein-protein interactions that regulate NS5A activity during HCV replication.

6 NS5B

NS5B is a 65-kDa tail-anchored protein that has characteristics of viral RNA-dependent RNA polymerase (RdRp). NS5B is the catalytic core of the viral RC and plays a key role in the synthesis of complementary negative-strand RNA during viral replication, using the genome as a template and subsequently the genomic RNA from the negative-strand RNA template. The fidelity of NS5B RdRp is approximately one million times lower than that of typical prokaryotic and eukaryotic DNA polymerases, presumably because it exhibits no exonuclease or proofreading activities. A 21-residue C-terminal region forms an α-helical transmembrane domain that is dispensable for in vitro polymerase activity but is necessary for posttranslational targeting to the cytoplasmic side of the ER in cells (Moradpour et al. 2004; Schmidt-Mende et al. 2001).

The structure of NS5B RdRp resembles a typical "right hand" polymerase shape and contains finger, palm, and thumb subdomains, similar to other template-dependent polymerases (Ago et al. 1999; Bressanelli et al. 2002; Lesburg et al. 1999). The finger and thumb subdomains serve important functions as sites for interacting with the nucleic acid substrate. The palm domain contains conserved active-site motifs, such as the GDD motif, in which Asp residues coordinate divalent cations (Mg^{2+} and/or Mn^{2+}). It has been shown that Mn^{2+} ions stabilize the conformation of the active site (Ranjith-Kumar et al. 2002). An unusual feature of this RdRp is that the finger and thumb subdomains interact extensively, leading to the formation of a fully enclosed, or encircled, active site in which nucleotide binding is controlled and de novo RNA synthesis is initiated (Moradpour and Penin 2013). Because such a closed conformation appears insufficient to accommodate the partially duplexed RNA that must form during RNA synthesis, it is highly likely that the RdRp must transition to a more open conformation after initiation of RNA synthesis. It has been suggested that the closed conformation is almost suitable for de novo initiation, with enough room to bind a single strand of template RNA and priming nucleotides (Schmitt et al. 2011). Both partially open as well as closed forms have been identified by X-ray crystallography of genotype 2a-derived RdRp, indicating that the crucial difference between the two conformations is the relative orientation of the thumb domain in relation to the finger and palm domains (Biswal et al. 2005). HCV RdRp also has an unusual 12-residue hairpin loop that protrudes into the active site. The loop structure purportedly interferes with binding to dsRNA and helps position the 3′ end of the RNA template for proper initiation of RNA synthesis (Butcher et al. 2001; Hong et al. 2001).

It is highly likely that the initiation of HCV RNA synthesis at the 3′ end of the positive- and negative-strand RNAs in infected cells begins de novo with a

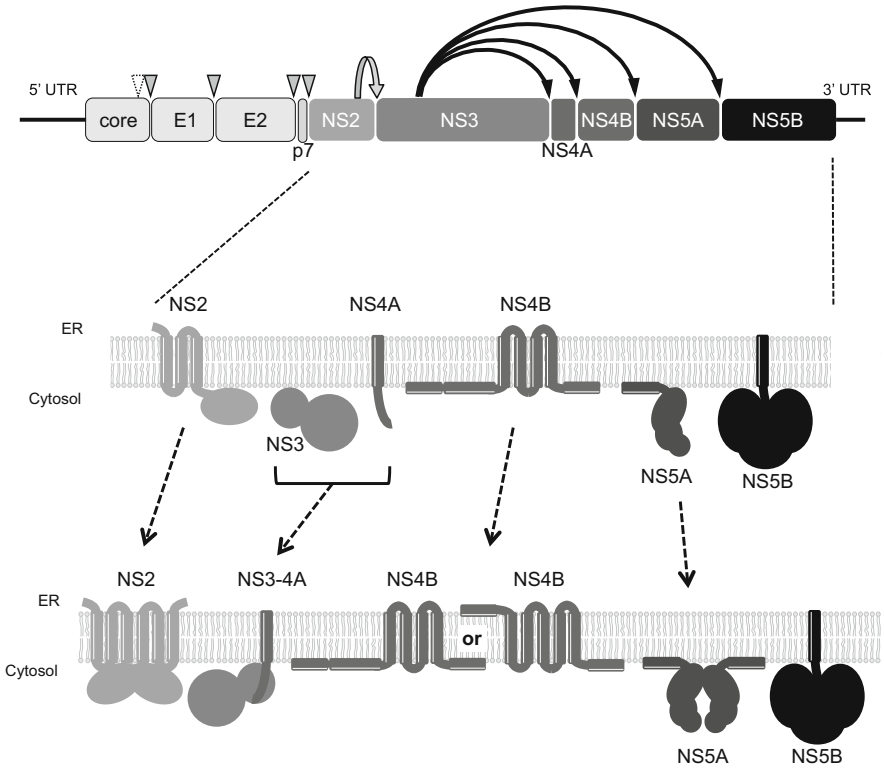

Fig. 1 Gene organization of HCV, processing of viral proteins and maturation of NS proteins (*top*). The HCV RNA genome contains a large open reading frame encoding a precursor polyprotein. Untranslated regions of 5′ and 3′ (5′ UTR, 3′ UTR) are shown on the left and the right side of the genome structure, respectively. Cleavages at the core/E1, E1/E2, E2/p7, and p7/NS2 junctions are mediated by host signal peptidases indicated by *gray triangles*. The *open triangle* indicates cleavage by host signal peptide peptidase. Processing at the NS2/NS3 junction mediated by the NS2–3 protease is indicated by a *gray arrow*. NS3-4A serine protease cleavage sites are indicated by *black arrows* (*middle*). Membrane topologies of HCV NS proteins (*bottom*). NS2 and NS5A are shown as a homodimer. NS3 associates ER membrane through interacting with NS4A. The two alternative membrane topologies of NS4B are shown

1-nucleotide primer, as copy-back or self-priming RNA synthesis cannot produce authentic viral RNA with precise 5′ and 3′ ends. HCV RdRp tends to selectively utilize purine nucleotides for initiation of RNA replication, and de novo initiation in vitro occurs most efficiently with guanine (Zhong et al. 2000; Luo et al. 2000). A study to determine the initiating nucleotides in HCV-replicating cells demonstrated that the replication of positive-strand RNA is preferentially initiated with purine nucleotides, whereas negative-strand RNA replication is invariably initiated with ATP (Cai et al. 2004). Coordination of the catalytic Asp residues with divalent metals may facilitate the formation of a phosphodiester bond between the initiating and second nucleotides (O'Farrell et al. 2003; Ferrari et al. 1999).

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