Hepatitis C Virus RNA Replication

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Abstract Genome replication is a crucial step in the life cycle of any virus. HCV is a positive strand RNA virus and requires a set of nonstructural proteins (NS3, 4A, 4B, 5A, and 5B) as well as cis-acting replication elements at the genome termini for amplification of the viral RNA. All nonstructural proteins are tightly associated with membranes derived from the endoplasmic reticulum and induce vesicular membrane alterations designated the membranous web, harboring the viral replication sites. The viral RNA-dependent RNA polymerase NS5B is the key enzyme of RNA synthesis. Structural, biochemical, and reverse genetic studies have revealed important insights into the mode of action of NS5B and the mechanism governing RNA replication. Although a comprehensive understanding of the regulation of RNA synthesis is still missing, a number of important viral and host determinants have been defined. This chapter summarizes our current knowledge on the role of viral and host cell proteins as well as cis-acting replication elements involved in the biogenesis of the membranous web and in viral RNA synthesis.

Abbreviations

HCV	Hepatitis C virus
IRES	Internal ribosome entry site
nts	Nucleotides
NTR	Nontranslated region
CRC	Crude replication complex
CRE	Cis-acting replication element
MW	Membranous web
DMV	Double membrane vesicle
MMV	Multi membrane vesicle
RdRp	RNA-dependent RNA polymerase
Ago2	Argonaute protein 2

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Contents

1 Introduction

Hepatitis C virus (HCV) is an enveloped positive-strand RNA virus belonging to the genus *Hepacivirus* in the family *Flaviviridae* (van Regenmortel et al. 2000). The genome of HCV encompasses a single ~9,600 nts long RNA molecule containing one large open reading frame (ORF) that is flanked by nontranslated regions (NTRs), important for viral RNA translation, and replication. The 5′NTR contains an internal ribosome entry site (IRES), enabling viral RNA translation in the absence of a cap structure. HCV proteins generated from the polyprotein precursor are cleaved by cellular and viral proteases into at least 10 different products (for further details see chapter "Hepatitis C Virus Proteins: From Structure to Function", this volume): core, envelope glycoproteins E1 and E2, p7, and the nonstructural proteins (NS) NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Core to NS2 is primarily involved in the formation of infectious virus (see chapter "Virion Assembly and Release", this volume), whereas the nonstructural proteins NS3 to NS5B are primarily involved in viral RNA replication, forming the viral replication complex (Bartenschlager et al. 2010), which will be the focus of this chapter. NS3 is a multifunctional protein, consisting of an aminoterminal protease domain required for processing of the NS3 to NS5B region (Bartenschlager et al. 1993) and a carboxyterminal helicase/nucleoside triphosphatase domain (Suzich et al. 1993; Kim et al. 1995). NS4A is a cofactor that activates the NS3 protease function by forming a heterodimer (Bartenschlager et al. 1995). The hydrophobic protein NS4B induces vesicular membrane alterations involved in RNA replication (reviewed in Gouttenoire et al. 2010a). NS5A is a phosphoprotein capable of RNA binding (Huang et al. 2005; Foster et al. 2010), which seems to play an important role in regulating viral replication and assembly (Appel et al. 2008; Tellinghuisen et al. 2008; Masaki et al. 2008). It exists in two phosphorylation variants (Tanji et al. 1995; Kaneko et al. 1994); a basally (hypo-)phosphorylated form (p56), which is supposed to be important for RNA replication (Appel et al. 2005b; Blight et al. 2000) and a hyperphosphorylated variant (p58), probably involved in assembly.

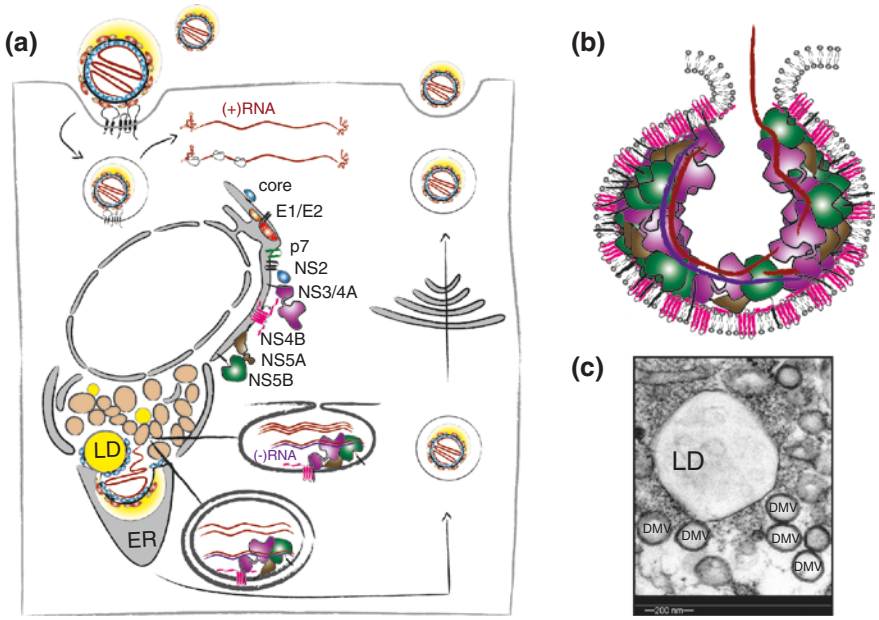

Fig. 1 **a** Schematic representation of the HCV replication cycle. Different potential substructures harboring the replication sites based on biochemical evidence as shown in (**b**) and DMVs recently detected by EM (**c**) are indicated. **b** Model of the HCV replication complex based on biochemical evidence (Quinkert et al. 2005). Multiple copies of the nonstructural proteins serve as structural components of a vesicular structure, containing probably only one replication intermediate and several progeny positive strands. Only a minor subfraction of the nonstructural proteins is supposed to have a function in RNA synthesis. A pore should allow the access of nucleotides and the exit of RNA. **c** Electron micrograph of DMVs in HCV-infected cells 16 h after infection (provided by Inès Romero-Brey unpublished). *LD* lipid droplet, *DMV* double membrane vesicle

NS5B is the RNA-dependent RNA polymerase of HCV, the key enzyme of viral RNA synthesis (Behrens et al. 1996; Lohmann et al. 1997).

After RNA translation and polyprotein processing NS3 to NS5B induce distinct membrane alterations, harboring the sites of viral RNA replication (Gosert et al. 2003; Fig. 1a), which is a typical feature of all positive-strand RNA viruses [reviewed in (Miller and Krijnse-Locker 2008)]. The minimal genetic unit necessary and sufficient for RNA replication has been defined by subgenomic replicons and encompasses the NTRs as well as the NS3 to 5B coding region (Lohmann et al. 1999a). The first step of RNA synthesis generates a negative-strand genome, which serves as template for progeny positive-strand RNA that is produced in 5- to 10-fold excess. It is generally assumed that RNA synthesis of complementary strands initiates at the very 3′terminus of the template strand by de novo initiation of RNA synthesis and that RNA replication involves a double-stranded RNA intermediate, although clear experimental proof for both assumptions is missing. The newly synthesized positive-strand RNA is either re-entering a new translation/replication cycle or is packaged into virions. This chapter summarizes

our current knowledge on HCV-induced membrane alterations, the role of the nonstructural proteins and cis-acting elements in distinct steps of RNA synthesis and provides a selective review of some important host factors involved in these processes.

2 Ultrastructure of the HCV Replication Sites

2.1 Model of the HCV Replication Complex

The establishment of robust cell culture models for HCV (see chapter "Cell Culture Systems for Hepatitis C Virus", this volume) provided the first opportunity to analyze structure and functions of the viral replication sites in detail. Early pioneering EM-studies of liver tissue from infected patients and chimpanzees indicated that HCV, like other positive-strand RNA viruses, induced membrane alterations in infected hepatocytes (Jackson et al. 1979; Shimizu et al. 1990; Shimizu 1992). Later it was shown that the expression of viral nonstructural proteins, particularly NS4B, indeed resulted in the induction of vesicle accumulations, which were designated the membranous web (Egger et al. 2002). These data were confirmed using cell lines harboring persistent subgenomic replicons (Gosert et al. 2003). Immunofluorescence analysis of the localization of viral nonstructural proteins revealed an ER-like distribution with distinct dot-like structures in these replicon cells also co-localizing with newly synthesized viral RNA (Gosert et al. 2003). At the ultrastructural level, these dot-like structures correspond to accumulations of vesicles, which stained positive for viral nonstructural proteins (Gosert et al. 2003). It seems likely that individual vesicles within the membranous web represent the sites of viral RNA replication. Based on analogy with related viruses, it is furthermore assumed that the vesicles are invaginations from the ER membrane, which are connected with the cytoplasm by a small pore allowing the exchange of small membrane-impermeable molecules like nucleotides for RNA synthesis [Fig. 1b; (Welsch et al. 2009)].

This model was further supported by biochemical studies of membrane preparations from replicon cells, so-called crude replication complexes (CRCs) and by selective permeabilization of replicon cells (Ali et al. 2002; Hardy et al. 2003b; Aizaki et al. 2004; El Hage and Luo 2003; Lai et al. 2003; Shi et al. 2003; Miyanari et al. 2003). CRCs and permeabilized replicon cells are capable of RNA synthesis in vitro and this process was shown to be resistant to treatment with proteases and nucleases (Quinkert et al. 2005; Miyanari et al. 2003; Targett-Adams et al. 2008), as well as to Triton X-100 treatment at 4°C (Aizaki et al. 2004; Shi et al. 2003). These results suggested that viral replication complexes were protected by detergent resistant membranes, most likely resembling those vesicular structures indicated by the EM analysis. To allow access of nucleotides to the sites of RNA synthesis, these vesicles should contain an opening allowing access for nucleotides, but small enough to protect the replication sites from nucleases and proteases

(Fig. 1b; Quinkert et al. 2005; Miyanari et al. 2003). Protease and nuclease digests of CRCs (Quinkert et al. 2005) and in permeabilized replicon cells (Miyanari et al. 2003) furthermore demonstrated that all of the negative-strand RNA, ~50 % of the positive-strand RNA, but less than 5 % of the nonstructural proteins were protected, indicating that the majority of NS-protein copies seem not to be involved in the formation of replication sites. Based on these data, it was assumed that an active replication site contained only one copy of negative-strand RNA, which might be part of a double-stranded replication intermediate (Targett-Adams et al. 2008), several copies of positive-strand RNA and 500–2,000 copies of nonstructural proteins [Fig. 1b (Quinkert et al. 2005)].

2.2 DMVs and MMVs

This simplistic model of the HCV replication complex has been challenged in several ways by more recent studies. First, it was shown that the majority of membrane alterations found in HCV-infected cells were not single membrane vesicles, but more complex structures, shelled by two or more membranes and termed double-membrane vesicles (DMVs; Fig. 1c) and multi membrane vesicles (MMVs) (Ferraris et al. 2010; Reiss et al. 2011), similar to membrane alterations identified for coronaviruses (Knoops et al. 2008; Gosert et al. 2002) and picornaviruses (Belov et al. 2012). HCV double-stranded RNA and nonstructural proteins have been found inside DMVs and DMV abundance clearly correlates with viral RNA replication (Ferraris et al. 2010; Romero-Brey et al. 2012), arguing for a functional role of these structures. However, connections of the vesicles to the cytoplasm were rarely observed; therefore, it is not clear how nucleotides get into and newly synthesized RNA out of the DMVs. The simple "ER-invagination model" (Fig. 1b) that was convincingly shown for the related Dengue virus (Welsch et al. 2009) was furthermore challenged by three-dimensional reconstructions, demonstrating that DMVs originate from protrusions rather than invaginations of the ER, with the outer membrane connected by a neck to the ER membrane (Romero-Brey et al. 2012). How this topology of DMVs can be linked to previous models of HCV and flavivirus replication sites (Welsch et al. 2009) is an open question. In case of picornaviruses, RNA replication takes place preferentially at complex single membrane structures originating from the cis-Golgi, which are later transformed into DMVs by membrane wrapping processes (Belov et al. 2012) with yet to be defined function. In case of SARS coronavirus, it has been suggested that active RNA replication occurs in circular single membrane structures known as convoluted membranes (Knoops et al. 2008). DMVs probably originate from these replication sites and represent a final storage compartment to hide replication intermediates from recognition by the innate immune response (Knoops et al. 2008). Similar models might be true for HCV, since DMVs are the dominant species of membrane alterations only at time points later than 16 h after transfection or infection (Paul et al. 2011; Romero-Brey et al. 2012).

2.3 Biogenesis of the Membranous Web

2.3.1 Viral Determinants

The term membranous web was originally established to designate distinct tightly packed vesicle accumulations induced by expression of NS4B (Egger et al. 2002). However, meanwhile, this term is used to generally subsume distinct membrane alterations induced by the HCV nonstructural proteins, containing the sites of viral RNA synthesis (Gosert et al. 2003). The membranous web most likely originates from the ER, as indicated by ultrastructural studies, biochemical evidence and cellular marker proteins, partially co-localizing with the viral replication sites (Gosert et al. 2003; Egger et al. 2002; Miyanari et al. 2003; El Hage and Luo 2003; Romero-Brey et al. 2012). In addition, the early endosomal marker Rab5 has also been found to colocalize with viral NS proteins, suggesting that the formation of HCV replication sites engages several organelles (Stone et al. 2007). Interestingly, the morphology of the membranous web is not depending on RNA replication, but is solely driven by the nonstructural proteins NS3 to NS5B, presumably in concert with cellular factors, since no obvious differences have been found between ectopic protein expression models, cells harboring replicons or infected cells (Romero-Brey et al. 2012; Egger et al. 2002; Gosert et al. 2003; Reiss et al. 2011; Ferraris et al. 2010; Paul et al. 2011).

NS4B has been identified as the main driver of the biogenesis of the membranous web, because sole expression of NS4B induced structures most closely resembling expression of NS3 to 5B (Egger et al. 2002). NS4B is predicted to contain four central transmembrane segments flanked by N- and C-terminal regions attached to the membrane by amphipathic α-helices (Lundin et al. 2003; Gouttenoire et al. 2009b; Gouttenoire et al. 2009a) (see also chapter "Hepatitis C Virus Proteins: From Structure to Function", this volume). Recent studies have shown that NS4B can oligomerize, thereby probably forming the scaffold of membranous vesicles (Yu et al. 2006; Gouttenoire et al. 2010b; Paul et al. 2011). Oligomerization is mediated by homotypic and especially heterotypic interactions involving the N-terminal amphipathic helix 2 and the C-terminus of the protein (Paul et al. 2011; Gouttenoire et al. 2010b). Mutations residing in the NS4B C-terminal region that are impaired in NS4B self-interaction and expressed in the context of the NS3 to NS5B polyprotein indeed generate aberrant DMV structures, supporting the notion that DMVs play an essential role in RNA replication (Paul et al. 2011; Aligo et al. 2009). However, NS4B is not the only determinant of membranous web morphogenesis and more recent data show that also the sole expression of NS3/4A, NS5A, and even NS5B gives rise to distinct vesicular membrane rearrangements (Romero-Brey et al. 2012), suggesting that the role of other nonstructural proteins in this process has been underrated. Interestingly, NS3/4A, NS4B, and NS5B induce single membrane vesicles, which are different from the DMVs and MMVs observed upon expression of the entire replicase module NS3–5B (Romero-Brey et al. 2012). In contrast, only NS5A induced vesicles containing several lipid bilayers and occasionally vesicles containing a pair of membranes morphologically identical to DMVs (Romero-Brey et al. 2012). Although we currently do not understand the role of these DMVs in HCV replication, these novel

data clearly indicate that morphogenesis of the membranous web is complex and engages a concerted action of several nonstructural proteins. NS4B might therefore serve as the major scaffold in this process, modulated mainly by NS5A with the help of NS3/4A and NS5B. A critical role of NS4B-NS5A interactions in web formation is also indicated by genetic studies (Paul et al. 2011) and by the physical interaction between NS3/4A and the C-terminal region of NS4B (Aligo et al. 2009).

2.3.2 Host Factors

In addition to viral proteins also several host factors have been shown to contribute to membranous web formation. Recently, the lipid kinase phosphatidylinositol 4-kinase III alpha (PI4KIIIα, PIK4CA, PI4KA) has been identified by several siRNA screens as a cellular protein essential for HCV RNA replication (Vaillancourt et al. 2009; Borawski et al. 2009; Li et al. 2009; Tai et al. 2009; Berger et al. 2009; Reiss et al. 2011). NS5A and NS5B interact with PI4KIIIα and activate its lipid kinase activity, giving rise to elevated intracellular phosphatidylinositol 4-phosphate (PI4P) levels (Reiss et al. 2011; Berger et al. 2011). Silencing of PI4KIIIα results in reduced DMV size and absence of MMV formation, suggesting that this enzyme is critically involved in web morphology (Reiss et al. 2011), probably mediated by PI4P. More recent data, furthermore, suggest that PI4KIIIα modulates NS5A phosphorylation, by promoting p56 synthesis, which might regulate the structure of the HCV replication sites as well (Reiss and Lohmann unpublished data).

The HCV replication cycle is, furthermore, tightly linked to host cell lipids in various other ways, which are mostly not well understood (reviewed in Alvisi et al. 2011). HCV alters expression of genes involved in cellular lipid metabolism, resulting in accumulation of intracellular lipids (Diamond et al. 2010; Blackham et al. 2010; Su et al. 2002), which is critical for viral RNA replication (Kapadia and Chisari 2005). On the one hand, increased lipid levels might be required to generate the membrane proliferations necessary to form the HCV replication sites, on the other hand, they might be necessary for protein modifications. FBL2, for example, is a geranylgeranylated protein interacting with NS5A and geranylgeranylation was shown to be critical for HCV RNA replication (Wang et al. 2005). NS4B is supposed to be palmitoylated at two cysteine residues at the C-terminus and this modification seems to facilitate oligomerization (Yu et al. 2006).

Lipid droplets (LDs) are also often found in ultrastructural studies of the membranous web (LDs, Fig. 1a, c). LDs are cellular storage organelles for neutral lipids surrounded by a phospholipid monolayer (Martin and Parton 2006). HCV core (Barba et al. 1997; Moradpour et al. 1996; McLauchlan et al. 2002) and NS5A (Shi et al. 2002; Brass et al. 2002) are associated to LDs, because membrane attachment of both proteins is mediated by an amphipathic helix, capable of association with membrane mono- and bilayers. LDs are currently believed to play a central role in the coordination of viral RNA synthesis and virion morphogenesis by physically associating replication and assembly sites (Miyanari et al. 2007; reviewed in Bartenschlager et al. 2011; see also chapter "Virion Assembly and Release", this volume). An interaction of core and NS5A domain III, which is probably regulated

by phosphorylation, has been shown to be critical for assembly of infectious virus, by recruiting both proteins to the same LDs (Appel et al. 2008; Tellinghuisen et al. 2008; Masaki et al. 2008). Although core seems to be the main driver in recruiting the viral replication sites to LDs (Miyanari et al. 2007), viral double-stranded RNA has been found surrounding LDs also in the absence of core, suggesting an additional role of these organelles in RNA replication (Targett-Adams et al. 2008).

Autophagy has also been suggested to contribute to the biogenesis of the membranous web and a very recent study even proposed that HCV RNA replication mainly takes place on autophagosomal membranes (Sir et al. 2012). Indeed, DMVs induced by HCV share morphological similarities with autophagosomes and colocalize with autophagosomal markers in some studies (Ferraris et al. 2010; Guevin et al. 2010). However, the functional role of autophagy in the HCV life cycle is still controversially discussed (reviewed in Dreux and Chisari 2011). Silencing of autophagy components indeed impaired HCV replication, but only very early in infection and not in persistent replication (Dreux et al. 2009; Guevin et al. 2010), suggesting an important role in translation of the viral RNA (Dreux et al. 2009), rather than in membranous web biogenesis. Autophagy has, furthermore, been discussed to be involved in subversion of innate immune responses against HCV (Ke and Chen 2011) and in production of infectious virus (Tanida et al. 2009).

In summary, the biogenesis of the membranous web is a complex process involving not only NS4B but all HCV nonstructural proteins and several host factors. In the light of the complexity of these membrane alterations, including DMVs, MMVs, LDs, and autophagosomes, we are far from understanding their precise functions in viral RNA replication.

3 RNA Synthesis

3.1 Limitations of Current Model Systems

Most of our knowledge on the distinct contribution of viral proteins to viral RNA synthesis is based on biochemical studies and structural analyses of individually expressed and purified proteins, particularly NS5B. This has been complemented by reverse genetics using replicons or infectious virus, particularly to dissect the function of cis-acting elements (see also chapter "Hepatitis C Virus RNA Translation", this volume). However, requirements of RNA replication in cell culture are complex, involving polyprotein processing, membranous web induction, interaction of cis-acting RNA elements (CREs) with proteins, RNA synthesis, and so on. This entire process seems to involve several cis-functions mediated by proteins synthesized on their original template, indicated by the limited possibility to rescue lethal mutants by transcomplementation, which is currently only possible for distinct mutations in NS5A and NS4B (Appel et al. 2005a; Jones et al. 2009; Fridell et al. 2011). Therefore, most mutations interfering with any step in the process of RNA synthesis will finally result in an abrogation of RNA synthesis, without providing further

mechanistic insight. Some limited information into the complex interactions between nonstructural proteins has been gained by studies using intergenotypic chimeras (e.g. Binder et al. 2007) and by the selection of pseudoreversions, rescuing replication deficient mutants and thereby providing genetic evidence for functional interactions (e.g. Lindenbach et al. 2007; Paredes and Blight 2008). Still, there are some major discrepancies between biochemistry and cell culture such as the promiscuity of viral proteins regarding template choice in vitro compared to the strict requirement for almost invariant cis-acting elements to allow RNA replication in cell culture. Biochemical studies on CRCs purified from replicon cells failed to close this gap. CRCs predominantly synthesize a single, full-length RNA product of predominantly positive polarity (Hardy et al. 2003a) and probably also some negative-strand RNA (Ali et al. 2002). RNA synthesis in this system requires at least helicase and polymerase activity as shown by specific inhibitors (Hardy et al. 2003a), involves de novo initiation of RNA synthesis (Hardy et al. 2003a) and gives rise to single- and double-stranded RNA products (Lai et al. 2003). However, inhibition of NS5B is mainly achieved by chain terminating nucleotides in this system (Migliaccio et al. 2003; Lai et al. 2003), whereas several classes of allosteric nonnucleosidic inhibitors of NS5B and heparin failed to inhibit RNA synthesis (Ma et al. 2005), suggesting that CRCs contain stable complexes of the replicase bound to its RNA template. Therefore, it has not been possible to feed exogenous templates into CRCs, which strongly limits their use in mechanistic studies. Furthermore, this system is not accessible to reverse genetics, since active replication in cell culture is a prerequisite of CRC production, thereby precluding the study of mutations affecting defined steps of RNA replication. We are therefore currently lacking adequate in vitro models to address more specific interactions between HCV nonstructural proteins and their specific template, particularly regarding the complex initiation of positive- and negative-strand RNA synthesis.

3.2 Cis-Acting RNA Elements

CREs are mainly, but not exclusively, found in NTRs at the termini of the viral positive- and negative-strand RNA (Fig. 2). The RNA secondary structures and the functional roles of most cis-acting elements in the HCV genome have been mapped extensively in vitro and in cell culture, but the distinct mechanistic functions of individual stem loops are not known due to the lack of appropriate in vitro models.

3.2.1 The 3′end of the Positive-Strand RNA

The 3′NTR is essential for viral RNA replication (Friebe and Bartenschlager 2002), presumably for the initiation and regulation of negative-strand synthesis (Binder et al. 2007). It is composed of a variable region, a polyU/UC tract of variable length and a highly conserved 98-bases element designated X-tail or 3′X, encompassing the 3′end of the viral genome (Fig. 2b; Tanaka et al. 1995; Kolykhalov et al. 1996).

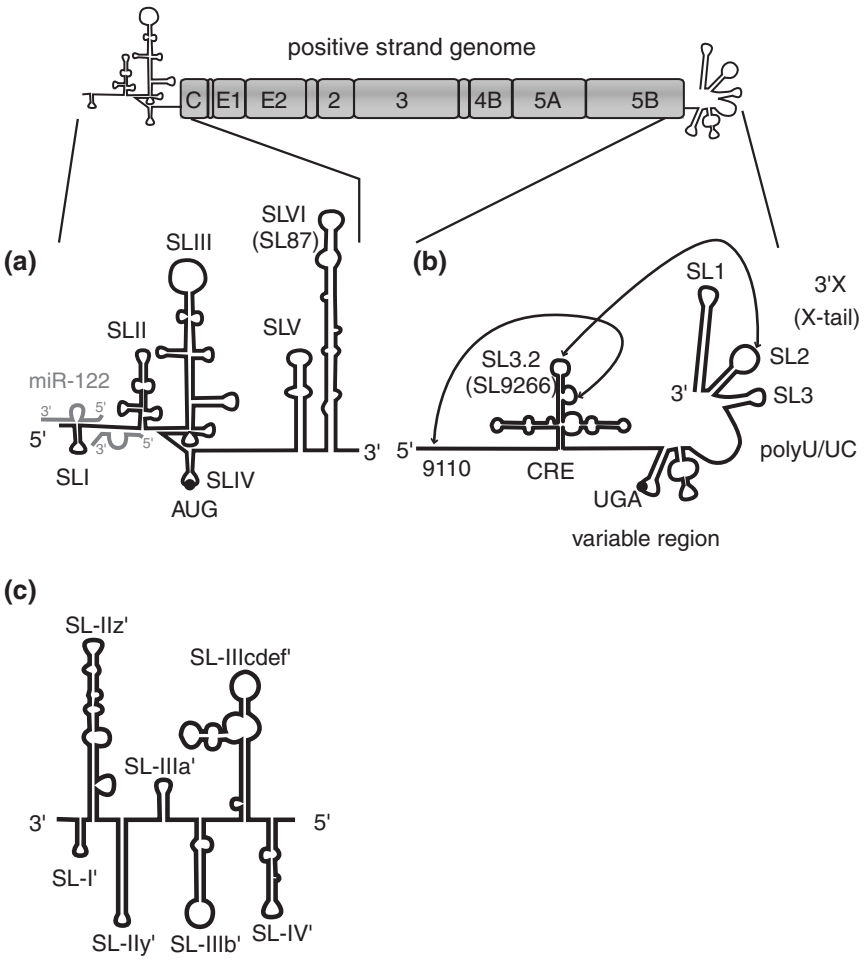

Fig. 2 Schematic representation of cis-acting replication elements. **a** 5′ end of the viral positive strand (Honda et al. 1996). Two copies of miR-122 binding to the 5′NTR are shown in *grey*. **b** 3′end of the viral positive strand (Blight and Rice 1997). Long range interactions of SL3.2 with sequences around 9,110 (Tuplin et al. 2012) and with the loop region of SL2 (Friebe et al. 2005) are indicated by arrows. **c** 3′ end of the viral negative strand (Smith et al. 2002; McMullan et al. 2007). Alternative nomenclatures of some structures are given in *brackets*

The variable region is predicted to form two stem-loop structures, which partly overlap with the very 3′-terminal region of the NS5B coding sequence. Deletion of the variable region results in replicons with significantly reduced replication efficiency, suggesting that this part of the 3′NTR is not essential, but contributes to efficient RNA replication (Friebe and Bartenschlager 2002; Yi and Lemon 2003a). The length of the polyU/UC tract varies between 30 and 90 nts among HCV isolates (Kolykhalov et al. 1996). It is composed of homopolymeric uridine stretches

interspersed by single cytosines. Uridine cannot be replaced by other homopolymeric nucleotides; however, a minimal length of 26–33 consecutive uridines is essential and sufficient for efficient RNA replication in cell culture (Friebe and Bartenschlager 2002; You and Rice 2008). Interruption of this minimal U homopolymer by C residues is deleterious for replication, but the position of homopolymeric U within the polyU/UC tract is flexible (You and Rice 2008; Yi and Lemon 2003a). This polyU stretch might provide a binding platform for viral and cellular proteins (Friebe and Bartenschlager 2002; You and Rice 2008), since NS3 helicase, NS5A, and NS5B have been shown to preferentially bind to polyU (Gwack et al. 1996; Huang et al. 2005; Lohmann et al. 1997). However, the distinct functional role of the polyU/UC region in viral RNA synthesis has not been clarified yet.

The 98-nt X-tail is almost invariant among HCV isolates and is supposed to contain the main regulatory elements required for negative-strand synthesis (Kolykhalov et al. 1996; Tanaka et al. 1995). It comprises three experimentally validated stem-loop structures (Blight and Rice 1997), which are all essential for viral replication (Friebe and Bartenschlager 2002; Yi and Lemon 2003b; Yi and Lemon 2003a) and barely tolerate mutations (Yi and Lemon 2003b; Yi and Lemon 2003a), indicating that not only the structures, but also the sequences are critical for RNA replication. The very 3′end of the HCV genome is a uridine residue in all HCV isolates analyzed so far and base paired in the very stable 3′-terminal SL1 (Fig. 2b). The terminal U can be replaced by C, in line with the requirements of the RdRp to initiate RNA synthesis with a purine base (G or A) (Cai et al. 2004). However, mutant replicons recovered from cell culture revealed reversions to U or even contained additions of U residues in all cases, suggesting a strong selective pressure for a terminal U (Cai et al. 2004; Yi and Lemon 2003b).

Another CRE, designated SL3.2 or SL9266, has been identified within the NS5B coding region. Stem-loop 3.2 is part of a larger predicted cruciform like secondary structure (Fig. 2b; You et al. 2004) and engaged in a kissing-loop interaction with SL2 in the X-tail. This interaction encompasses 7–8 complementary nts in the loop regions and is essential for RNA replication (Friebe et al. 2005). The position of SL3.2 can be moved into the 3′NTR and complementarity between the loop sequences of SL3.2 and SL2 was shown to be more important than the precise sequence (Friebe et al. 2005), arguing for a functional role of a pseudoknot structure at the 3′end of the genome. More recent studies suggest that the bulge region of SL3.2 can form an independent alternative pseudoknot structure with upstream sequences around nucleotide 9,110 (Diviney et al. 2008; Tuplin et al. 2012). Since both interactions of SL3.2 seem mutually exclusive, they might support a functional switch in the HCV replication cycle, e.g. from translation to replication (Tuplin et al. 2012).

3.2.2 The 5′NTR and the 3′end of the Negative-Strand RNA

The 5′NTR encompasses 341–342 nts and has a dual function in the HCV replication cycle; first in the positive strand by functioning as an IRES driving RNA translation, and thus polyprotein synthesis (see chapter “Hepatitis C Virus RNA Translation”, this

volume) and second in the negative strand, providing CREs proposed to direct progeny positive-strand synthesis. Interestingly, the 5′NTR and the complementary 3′end of the negative-strand RNA have been shown to adopt very different secondary structures, in line with their different functions in RNA translation and replication, respectively (Fig. 2a and c, respectively) (Honda et al. 1996 compared to Smith et al. 2002; Schuster et al. 2002). Genetic analyses mapped the minimal region required for RNA synthesis to the 3′-terminal 125 nts, comprising SL-I' and SL-IIz' (Fig. 2c; Friebe and Bartenschlager 2009; Friebe et al. 2001). SL-IIy' is important for efficient RNA replication, whereas the remaining stem loops only seem to have auxiliary functions (Friebe and Bartenschlager 2009). The distinct mechanistic roles of the stem-loop structures at 3′end of the negative strand are still ill defined. In case of SL-IIz', the structure of the stem rather than the discrete sequence seems to be the major determinant. Still it is interesting to note that two miR-122 seed sequences are located in the complementary region of SL-IIz' in the 5′NTR (Fig. 2a; Jopling et al. 2005), suggesting a role of miR-122 in the proper formation of RNA secondary structures. In case of SL-I' only the stem structure is essential for replication of genotype 1b, whereas genotype 2a is also sensitive to sequence alterations not affecting the stem structure (Friebe and Bartenschlager 2009; Luo et al. 2003). The 3′end of the HCV negative-strand genome encompasses a short single stranded region adjacent to SL-I' terminating mostly with C and sometimes with U in clinical isolates, again reflecting the need of the RdRp to initiate RNA synthesis with a purine base (Cai et al. 2004). However, after multiple rounds of replication in cell culture a terminal C was found to be replaced by U (Cai et al. 2004), suggesting that A is the preferred initiating nucleotide for positive- and negative-strand synthesis in cell culture.

Additional conserved stem-loop structures have been found in the core coding sequence. These sequences are not contained in subgenomic replicons, and therefore seem not to be essential for RNA synthesis (Lohmann et al. 1999a). However, disruption of SLVI/SL87 in full-length viral genomes caused severe replication defects in vivo and in cell culture, suggesting that this stem loop has important functions in the regulation of viral RNA synthesis (Fig. 2a; Vassilaki et al. 2008; McMullan et al. 2007).

3.3 The Viral RNA Polymerase NS5B

The viral RdRp NS5B is the driving force of RNA synthesis. The active enzyme can be expressed heterologously with recombinant baculovirus (Behrens et al. 1996) or in *E. coli* (Al et al. 1998) and a huge number of studies have shed light on the structure and biochemical properties of NS5B in vitro. The enzyme consists of a catalytic domain, followed by a linker sequence and a C-terminal membrane insertion sequence, which is essential for RNA replication in cell culture (Fig. 3a; Ivashkina et al. 2002; Moradpour et al. 2004), but seems not to significantly contribute to RNA synthesis in vitro (Lohmann et al. 1997; Yamashita et al. 1998). Removal of the membrane insertion sequence, comprising the C-terminal 21 amino acid residues, increases solubility

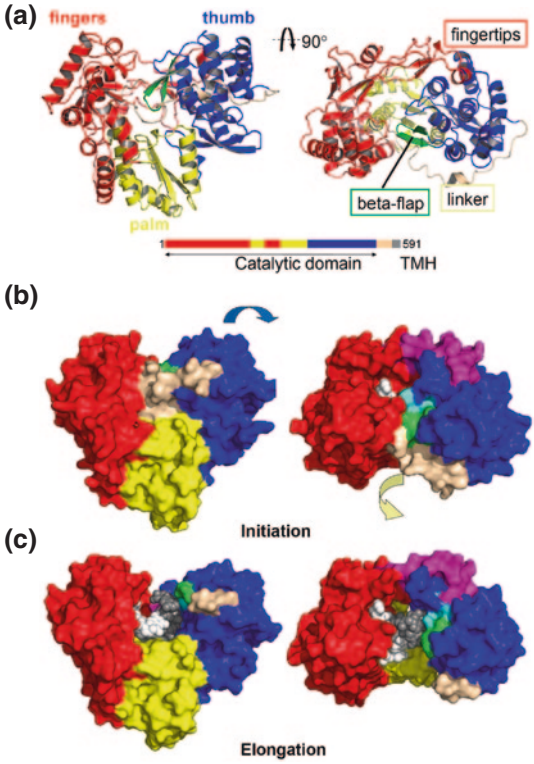

Fig. 3 Structure of the HCV polymerase in *front* views (*left panels*) and *top* views (*right panels*) (Bressanelli et al. 1999). **a** *Ribbon model* of HCV NS5B indicating the fingers (*red*), thumb (*blue*), palm (*yellow*) and linker (*wheat*) subdomains. Note the contact of fingertips and thumb, resulting in a closed structure. The beta flap in the thumb domain is indicated by *green color*. **b** Space filling model of the same structures as in (**a**) with template RNA (*light gray*) modeled. This structure represents the closed conformation, capable of binding the single-stranded template RNA and the two initiating nucleotides. Structural movements of the thumb and linker domains required for the transition to elongation are indicated by a *blue* and *wheat colored arrow*, respectively. **c** Crystal structure of the elongation mode of NS5B in a complex with a double-stranded replication intermediate consisting of the template RNA (*light gray*) and newly synthesized RNA (*dark gray*)

and facilitates purification of NS5B; therefore, most biochemical and all structural studies so far used the so-called NS5BΔC21 enzyme (Ferrari et al. 1999) or even C-terminal deletions encompassing up to 60 amino acid residues (Leveque et al. 2003).

Structural analysis of the catalytic domain revealed a so-called right hand shape, common to many single-subunit polymerases, with fingers, thumb, and palm subdomains [Fig. 3a (Ago et al. 1999; Lesburg et al. 1999; Bressanelli et al. 1999)]. All regular structures published until very recently reveal a closed conformation, encircled on one side by the "fingertips", which is a hallmark of viral RdRps, and on the other side by the linker and the so-called beta flap (or β-hairpin). The latter is specific to *Flaviviridae* RdRps [reviewed in (Lescar and Canard 2009)], but the linker or a variation thereof is common to de novo

initiating RdRps (Butcher et al. 2001). In HCV NS5B, its functional role has not been fully clarified yet. Deletion of the entire linker (so-called ΔC47 or ΔC60 constructs) strongly enhances polymerase activity in vitro and stimulates de novo initiation, suggesting a negative regulatory function (Leveque et al. 2003), whereas a recent study suggests that the linker plays an active role in the very first step of de novo initiation (Harrus et al. 2010). Anyhow, the closed conformation is supposed to represent the initiation state of the polymerase, since the catalytic core only provides space for a single-stranded RNA template and nucleotides for de novo initiation of RNA synthesis, but not for a double-stranded RNA (Fig. 3b; Simister et al. 2009). The closed conformation, therefore, seems to be actively inhibit primer-dependent RNA synthesis by forming a "locked" structure preventing access of primer-template complexes (Chinnaswamy et al. 2008; Ranjith-Kumar et al. 2003).

3.3.1 Template Requirements and Initiation Modes

Purified NS5B can initiate RNA synthesis by a primer-dependent mechanism or de novo (Behrens et al. 1996; Lohmann et al. 1997; Luo et al. 2000; Zhong et al. 2000; Sun et al. 2000). De novo initiation at the 3′end of the viral positive- and negative-strand RNA is likely to be the physiological mode of initiation of RNA synthesis in infected cells, although this has not been experimentally validated yet. De novo initiation in vitro requires a terminal purine, but is most efficient with a G (Zhong et al. 2000). It can take place even on homopolymeric polypyrimidine templates at high nucleotide concentrations (>50 μM; Luo et al. 2000), suggesting that no specific cis-acting elements are required. However, a stable secondary structure and a single-stranded sequence of at least three nucleotides has been shown to be optimal for de novo initiation on nonhomopolymeric templates (Kao et al. 2000), although a secondary structure is not absolutely required (Shim et al. 2002). The 3′end of the HCV negative-strand genome consists of a stem loop with a single-stranded overhang, thereby corroborating the ideal structure for de novo initiation (Fig. 2b). Interestingly, the 3′end of the HCV positive-strand genome is buried within a stable stem structure, which cannot bind to the closed initiation conformation of NS5B (Fig. 2a). In contrast, the positive-strand genome of the related pestiviruses terminates with 3–5 C residues adjacent to a stem loop, representing in theory an ideal template for de novo initiation by the polymerase (Yu et al. 1999). Indeed, it has been shown that the 3′end of the HCV negative strand is an excellent template for de novo initiation, whereas the 3′end of the positive-strand hardly gives rise to terminal de novo initiation (Reigadas et al. 2001; Binder et al. 2007), suggesting that auxiliary factors might be required to initiate negative-strand synthesis by NS5B, thereby probably allowing a tight regulation of this process.

However, de novo initiation by NS5B in vitro is not limited to the 3′end of the template, but can also take place internally (Binder et al. 2007; Shim et al. 2002) and on circular templates (Ranjith-Kumar and Kao 2006). This suggests that NS5B

in solution is in an equilibrium between the closed conformation observed with all crystal structures and an open conformation capable of binding to internal or circular initiation sites (Ranjith-Kumar and Kao 2006) and to primer templates (Lohmann et al. 1997; Behrens et al. 1996). Mn^{2+} ions seem to stabilize the closed conformation, thereby favoring terminal de novo initiation (Ranjith-Kumar et al. 2002).

3.3.2 Steps of RNA Synthesis

RNA synthesis by NS5B in vitro can be separated into distinct steps, namely RNA binding, initiation, elongation, and termination. NS5B binds to a plethora of heteropolymeric RNA templates with no clear specificity for virus-derived sequences, while the binding to homopolymers follows a distinct pattern (polyU > polyG > polyA > polyC) (Lohmann et al. 1997). RNA binding seems to be a very slow and inefficient process and accounts primarily for the low apparent specific activity of the enzyme in vitro (Liu et al. 2006). The enzymatic core of NS5B protects 8–10 nts from RNase digest (Kim et al. 2000) and binds to single-stranded RNAs of >7 nts with high affinity (Kim et al. 2005).

De novo initiation of RNA synthesis involves several steps, which have been characterized by biochemical studies, supported by structural evidence (Fig. 3b; Harrus et al. 2010). After binding of a single-stranded template and the first two nts matching to the 3′end, a dinucleotide primer is synthesized, requiring high concentrations of the priming nts (Ferrari et al. 2008). These dinucleotide primers are produced in great abundance and accumulate in vitro, suggesting that they dissociate rapidly from the NS5B-template complex, whereas progression to processive elongation seems to be inefficient and rate limiting (Harrus et al. 2010; Shim et al. 2002). This initial primer synthesis seems to be facilitated by a very closed conformation, since a very high de novo initiation efficiency observed for NS5B from isolate JFH-1 correlated with a particularly closed structure (Simister et al. 2009). It, furthermore, requires a "platform" to support the first nucleotide, which has to move out of the active center upon addition of the third base. This platform has not been clearly identified in the structure of the polymerase, but it might be provided by the C-terminal linker sequence (Harrus et al. 2010) or by the beta flap in coordination with a GTP bound close to the active site, as suggested for pestiviruses (Lescar and Canard 2009; Choi et al. 2004; D'Abramo et al. 2006). Such a role of coordinating GTP in stabilizing the initiation complex might also explain the strong stimulating effect of high GTP concentration on de novo initiation of NS5B in vitro (Lohmann et al. 1999b; Harrus et al. 2010; Ranjith-Kumar et al. 2003).

The switch from primer synthesis to processive elongation requires high concentrations of the third base to be incorporated (Ferrari et al. 2008), and is furthermore facilitated by high GTP concentrations (Harrus et al. 2010). This switch requires a major conformational change in the polymerase structure, resulting in a move of the "priming platform" and an opening of the entire enzymatic core. The C-terminal linker is removed to accommodate a double-stranded RNA, allowing egress of the template-primer duplex, and the fingertips shift and adjust their contacts with the

thumb (Fig. 3c). The existence of such an "open" conformation is supported by data from a genotype 2a NS5B (Biswal et al. 2005) and by a recent structure of NS5B in complex with a primer template, which was obtained after deletion of the beta flap (Mosley et al. 2012). In this structure, the C-terminal linker is disordered and no longer occludes the exit from the catalytic site and the thumb domain is moved relative to palm and fingers by 20°, generating a large cavity capable of binding double-stranded RNA (Mosley et al. 2012). Position 405 in the thumb domain seems to be a central switch in the transition from initiation to elongation, stabilizing a closed conformation for dinucleotide synthesis, on the one hand, and facilitating the transition to the open conformation, on the other hand, (Scrima et al. 2012) and this position is also critical for efficient RNA replication in cell culture (Schmitt et al. 2011). It should be noted that the C-terminal linker, supposed to take part in the major conformational switch to elongation, is directly connected to the transmembrane segment of NS5B (Ivashkina et al. 2002) and thereby will probably cause a repositioning of the entire enzyme relative to the membrane.

Once RNA synthesis is initiated, NS5B elongates the nascent RNA by about 100–400 nts per minute and is capable to processively copy an entire RNA genome in vitro (Oh et al. 1999; Lohmann et al. 1998; Simister et al. 2009), suggesting that no helicase is required to resolve secondary structures. In this stage, the polymerase is tightly bound to its template and elongation complexes can even be stalled and purified (Jin et al. 2012). Elongation requires only low nucleotide concentrations compared to the initiation reaction (Jin et al. 2012). Little is known about termination of RNA synthesis; however, the polymerase might just fall off after approaching the end of the template.

RNA synthesis by NS5B is error prone and provides the molecular basis of the high genetic variability of HCV isolates. A recent study revealed a high error rate of ca. 10^{-3} per site with a strong bias toward G:U/U:G mismatches for NS5B in vitro, which was corroborated by an observed 75-fold difference in transitions over transversions in vivo (Powdrill et al. 2011).

Although the polymerase is capable of de novo initiation and copying an entire genome in vitro without the help of other factors, there are still some open questions and discrepancies between the properties of NS5B in vitro and replication in cell culture. The overall slow and inefficient initiation reaction, requiring very high nucleotide concentrations in vitro, suggests that this process might be facilitated by auxiliary factors in vivo (Harrus et al. 2010). It is, furthermore, puzzling that the 3′end of the positive-strand RNA is not a bona fide template for de novo initiation, suggesting that initiation of negative-strand synthesis might be a tightly regulated process, requiring additional co-factors. The absence of a clear specificity for viral templates in vitro is in striking contrast to the importance of the CREs for replication in cell culture. Finally, NS5B shows a strong preference for G as initiating nucleotide in vitro, but constitutively initiates negative-strand synthesis with A in vivo and even tends to convert initiation of positive-strand synthesis from G to A in cell culture (Cai et al. 2004). Taken together, analysis of purified NS5B in vitro as well as structural studies provided a number of important insights into the mechanisms of HCV RNA synthesis, but did not reveal the complex regulation of this process in vivo.

3.4 Contribution of Other Viral Proteins to RNA Synthesis

Although it is clear from reverse genetics studies that NS3/4A and NS5A have essential functions in RNA replication, their distinct contribution to RNA synthesis is still unresolved. A study using intergenotypic replicon chimeras found that NS3 helicase, NS5A, and NS5B are required to recognize genotype-specific signals for positive-strand synthesis, suggesting that a complex of these proteins is engaged in initiation of RNA synthesis (Binder et al. 2007). However, deeper mechanistic insights are currently limited due to the lack of appropriate model systems. Since the individual functions of each nonstructural protein have been described in detail in the chapter "Hepatitis C Virus Proteins: From Structure to Function", this volume, the present chapter will focus on the knowledge about their interactions.

The role of the NS3 NTPase/helicase (NS3h) in RNA synthesis is particularly enigmatic. The helicase activity might be involved in initiation of RNA synthesis, e.g. by resolving strong stem-loop structures at the 3′end, thereby generating a template accessible to de novo initiation by NS5B. In addition, NS3h could support NS5B in the elongation phase by unwinding double-stranded replication intermediates. Finally, a ssRNA translocase activity might help to strip proteins off the RNA or deliver RNA for packaging into virions (Gu and Rice 2010). However, none of these functions could yet be validated in cell-based assays. Still, a number of cross-talks between NS3h and other nonstructural proteins have been demonstrated in vitro, which might point to important regulatory functions in vivo. On the one hand, the helicase activity of NS3 is regulated by the NS3 protease domain and by NS5B (Jennings et al. 2008; Zhang et al. 2005), on the other hand, NS3h stimulates the RdRp in vitro (Piccininni et al. 2002), suggesting that NS3 and NS5B indeed might function together.

An important regulatory role for the replicase has recently been assigned to NS4A, the cofactor of NS3 protease. Genetic evidence points to the C-terminal region functioning as an electrostatic switch, regulating NS3 protease function and NS5A phosphorylation (Lindenbach et al. 2007).

NS4B, besides its previously discussed role in organizing the membranous web, might also have more distinct roles in RNA synthesis. NS4B has been shown to inhibit NS5B in vitro (Piccininni et al. 2002). NS4B is furthermore capable of binding RNA (Einav et al. 2008; Einav et al. 2004) and has an NTPase activity; however, the role of the latter two functions has not been clarified yet. In addition, genetic evidence for an interaction of NS4B with NS3 has been reported (Paredes and Blight 2008), which might regulate RNA replication beyond the morphogenesis of the replication sites.

Essential functions of NS5A in viral RNA synthesis, particularly of the hypophosphorylated variant, are clearly suggested from genetic studies. Many cell culture adaptive mutations increasing RNA replication efficiency of genotype 1 isolates and reducing the level of hyperphosphorylated NS5A are found in NS5A (for further details see chapters "Cell Culture Systems for Hepatitis C Virus" and "Hepatitis C Virus Proteins: From Structure to Function", this volume). The analysis of the mechanistic role of NS5A is particularly hampered by the existence of these different

phospho-isoforms. The determinants involved in regulation of phosphorylation are ill defined as is the case for most of the phosphorylation sites and their role in viral RNA synthesis. However, the synthesis of p58 requires at least an NS3-5A polyprotein (Koch and Bartenschlager 1999); therefore, different purified phospho-isoforms have not been accessible for in vitro assays yet and it can be assumed that heterologously expressed NS5A might not be properly phosphorylated. Still, in vitro studies suggest that low doses of NS5A stimulate NS5B, whereas high doses are inhibitory to the polymerase (Shirota et al. 2002; Quezada and Kane 2009). An inhibitory function of NS5A was also found in a cell-based assay (Ranjith-Kumar et al. 2011), suggesting a regulatory role of NS5A for RNA synthesis. The most distinct biochemical property of NS5A that could be envisaged in RNA synthesis is its RNA-binding activity, which resides in domain 1 (Huang et al. 2005) and is modulated by domains 2 and 3 (Foster et al. 2010). One of the available crystal structures of NS5A domain 1, indeed, suggests that NS5A dimers form a basic cleft capable of accommodating RNA (Tellinghuisen et al. 2005), which might play a role in RNA transport, e.g. from replication to assembly sites (see also chapters "Hepatitis C Virus Proteins: From Structure to Function" and "Virion Assembly and Release", this volume). In addition, NS5A interacts with and recruits a plethora of host cell proteins, which might be directly or indirectly involved in RNA synthesis (see below).

3.5 Host Factors Involved in RNA synthesis

Several high content screening approaches, including recent siRNA screenings brought up a huge number of cellular proteins which are supposed to be involved in HCV RNA replication (e.g. Li et al. 2009; Tai et al. 2009). Host factors involved in the morphogenesis of the membranous web, like PI4KIIIα, have been described above; therefore, this part will focus on some of those which might be directly involved in RNA synthesis.

The human VAMP-associated protein A (hVAP-A) and its isoform hVAP-B were identified in yeast two-hybrid screens using NS5A as a bait and were shown to interact with NS5A and NS5B (Gao et al. 2004; Hamamoto et al. 2005). Because of their role in cellular vesicle transport hVAPs are discussed to be involved in the formation of viral membrane rearrangements (reviewed in Moriishi and Matsuura 2007). Interestingly, hVAP-A was found to bind only to hypophosphorylated, but not to hyperphosphorylated NS5A (Evans et al. 2004) and is still the only known host protein differentially interacting with the phospho-isoforms of NS5A. Therefore, hVAP-A was suggested to have a more direct role in RNA synthesis, e.g. regulation of viral replicase activity in a NS5A phosphorylation-dependent manner (Evans et al. 2004).

Cyclophilins are peptidyl-prolyl cis/trans isomerases and their essential role in HCV replication was identified by the inhibition of HCV replication upon cyclosporin A treatment of replicons (Watashi et al. 2003). Initially, cyclophilin B was found to interact with NS5B, regulating template binding of the polymerase (Watashi et al. 2005). More recent results rather suggest that cyclophilin A (CyPA)

is critical for viral RNA replication (Kaul et al. 2009; Liu et al. 2009) and resistance to cyclophilin inhibitors point to NS5A as the main target site of CyPA (Yang et al. 2010; Kaul et al. 2009). CyPA has, furthermore, been shown to directly convert proline residues in NS5A domains 2 and 3 (Coelmont et al. 2010; Verdegem et al. 2011), probably inducing conformational changes critically involved in the function of NS5A. Dependence on CypA can be overcome in part by reducing the cleavage kinetics at the NS5A/NS5B junction, arguing that a kinetically controlled folding step of NS5A plays an important role for viral replicase activity (Kaul et al. 2009).

The liver specific microRNA miR-122 is one of the most remarkable host factors of HCV, regulating RNA abundance in cell culture (Jopling et al. 2005) and in vivo (Lanford et al. 2010). MiR-122 is a critical factor restricting viral replication in cultured cells (Narbus et al. 2011) and might substantially contribute to the liver tropism of HCV. MiR-122 binds to two seed sequences in the 5′NTR, forming an unconventional micro-RNA/target complex, encompassing the 5′end of the viral genome, thereby probably preventing degradation by RNases and/or induction of innate immune responses (Fig. 2a; Machlin et al. 2011). Indeed, miR-122 has been found to stabilize the viral genome in an Ago2-dependent manner (Shimakami et al. 2012). In contrast to conventional microRNA functions on mRNA, miR-122 also stimulates translation of the viral RNA (see chapter "Hepatitis C Virus RNA Translation", this volume).

Cellular RNA-binding proteins are also supposed to serve important functions at different steps of HCV replication. However, 26 cellular proteins specifically binding to the IRES (Lu et al. 2004) and more than 70 interacting with the 3′NTR (Harris et al. 2006) have been identified in proteomic studies; therefore a detailed description of all of them is beyond the scope of this chapter, but several of them are discussed in the chapter "Hepatitis C Virus RNA Translation", this volume, with respect to their role in translation. Little is known about the role of cellular RNA-binding proteins in RNA replication, however, the NF/NFAR proteins have been shown to mediate interactions between the 5′ and 3′NTR of the viral positive-strand RNA and might be involved in the regulation of translation and replication of HCV and the related pestiviruses (Isken et al. 2007; Isken et al. 2003).

4 Intracellular Dynamics of RNA Synthesis

Little is known about the dynamics of HCV RNA replication in vivo and the HCV RNA copy number in infected hepatocytes. This is particularly due to the difficulties in detecting viral antigens and RNA in the liver, arguing for overall relatively low viral RNA and protein levels. However, mathematic modeling of viral decline after therapy revealed that about 10^{12} virions are produced per day in infected individuals (Neumann et al. 1998), suggesting also a relatively high dynamics of RNA replication in the infected liver. Previous studies in chimpanzees determined $10^3 - 3 \times 10^5$ genomes per μg of total liver RNA (equivalent

to ~10^4–10^5 cells), depending on the sample. Therefore, it was assumed that 0.1–30 % of hepatocytes are infected, assuming that an infected cell should contain at least 10 positive-strand RNA genomes to maintain persistent replication (Bigger et al. 2004). Recent studies using highly sensitive two-photon microscopy found that up to 20 % of human hepatocytes are infected, most of the antigen positive cells containing clearly detectable amounts of double-stranded RNA, arguing for much higher HCV RNA copy numbers than 10 per infected cell (Liang et al. 2009).

In cell culture, the dynamics of RNA synthesis is highly variable in transient replication models, depending on the viral isolate, the permissiveness of the host cells, host cell growth, and so on. (see chapter "Cell Culture Systems for Hepatitis C Virus", this volume). Detailed replication kinetics are only available for the most efficient system, replication of the genotype 2a isolate JFH-1 in Huh-7 cells, either upon transfection with subgenomic replicons (Binder et al. 2007) or after virus infection (Keum et al. 2012), revealing very similar results. First viral negative-strand RNAs are detectable 4 or 6 h after transfection or infection, respectively. After this time point, negative-strand RNA levels increase exponentially, reaching a plateau at 24–48 h and slightly declining later on. Most (>90 %) of the incoming positive-strand RNA is degraded within the first hours in both model systems reaching a minimum at the onset of negative-strand synthesis (Binder et al. 2007; Keum et al. 2012). At this time point, positive- to negative-strand ratios are ~1:1, then exponential positive-strand synthesis starts and parallels negative-strand synthesis, again reaching a plateau 24–48 h after transfection/infection, with a positive- or negative-strand ratio of ~10:1. This ratio stays constant in case of the replicon (Binder et al. 2007), but interestingly increases up to 100:1 in case of the infection model (Keum et al. 2012). These data overall suggest that only a minority of incoming positive strands are entering into a productive replication cycle, starting with a 1:1 conversion into probably double-stranded replication intermediates (Targett-Adams et al. 2008). The initial lag-phase of 4–6 h might represent the time required for polyprotein translation, generation of the membranous replication compartment and RNA synthesis (100–400 nts/min. in vitro) (Lohmann et al. 1998; Simister et al. 2009). Asymmetric RNA synthesis is established within a few hours later, rapidly reaching the 10:1 excess of positive-strand RNA typical for all positive-strand RNA viruses. The plateau of RNA synthesis reached within 24–48 h for JFH1 probably reflects restrictions by the host cell, e.g. due to limiting host factors involved in RNA synthesis (Lohmann et al. 2003). Less efficient genotype 1 replicons exhibit a much slower replication kinetics with no clear exponential phase and reach steady-state replication levels at much later time points (Binder et al. 2007; Krieger et al. 2001). The strong increase of positive-to-negtive-strand ratios observed late in infection, but not for replicons, is counterintuitive, since positive-strand genomes should rather be depleted due to the secretion of virions (Keum et al. 2012). However, negative-strand synthesis, in contrast to positive-strand synthesis, probably depends on the continuous generation of new replication sites by positive-strands entering into new translation/RNA replication cycles, which might be limited during the full viral life cycle because of the competition with virion production.

In steady-state cultures of HCV replicon cells, viral RNA and protein amounts have been thoroughly quantified and seem quite similar for different cell clones and viral isolates, arguing for a balance between cell growth and viral replication which is dictated by the selective pressure (see chapter "Cell Culture Systems for Hepatitis C Virus", this volume). Viral negative strands are the most limiting component in replicon cells with less than 100 copies per cell on average, which also represents by definition the maximal number of active replication sites per cell (Quinkert et al. 2005). In contrast, more than 1,000,000 copies of nonstructural proteins are found, indicating that not all vesicular structures seen in EM or antigen positive dots in IF studies can represent active replication complexes (Quinkert et al. 2005). Based on their resistance to proteases, only a subfraction of less than 5 % of these protein copies seem to be engaged in the formation of viral replication sites (Miyanari et al. 2003; Quinkert et al. 2005). Interestingly, roughly 1,000–5,000 positive-strand RNA molecules per cell were reported for transient and steady-state cell cultures and this number might represent a limit of Huh-7-based cell cultures (Quinkert et al. 2005; Keum et al. 2012; Blight et al. 2002).

Mathematic modeling revealed that the sequestration of viral RNA into membranous replication compartments might be an important factor in restraining viral RNA synthesis and defining these steady-state levels (Dahari et al. 2007). Since the half lives of viral NS-proteins and viral positive-strand RNA in replicon cells have been shown to be 11–16 h (Pietschmann et al. 2001; Pause et al. 2003), it can be estimated that only about 1,000 positive-strand RNA molecules are synthesized per day per cell by ca. 100 replicase complexes (Quinkert et al. 2005). In consequence, each newly synthesized positive strand has to be excessively translated to yield the ascertained surplus of proteins, whereas RNA synthesis is a rather rare event, which most likely is achieved only by a few nonstructural protein copies (Fig. 1b). However, it is currently unclear, which mechanisms render a few replicase copies active and the majority inactive.

5 Conclusions and Future Perspectives

Based on our still patchy understanding described above, it is tempting to summarize our current knowledge on viral RNA replication in a tentative succession of events. After release of the viral genome into the cytoplasm of the host cell, the positive-strand RNA is translated giving rise to numerous polyprotein copies at the ER-membrane, which are co- and post-translationally processed into structural and nonstructural proteins. A fraction of NS3/4A, NS4B, NS5A, and NS5B drives the formation of membrane alterations, mainly DMVs, supported by several host factors, e.g. PI4KIIIα. It seems likely that active replication sites are connected to the cytoplasm to allow access of nucleotides and release of newly synthesized RNA (Fig. 1a, b). Negative-strand synthesis is most likely initiated within such vesicular structures, resulting in a probably (partially?) double-stranded replication intermediate. Since most nonstructural proteins cannot be complemented in trans, it can

be assumed that RNA synthesis is initiated from a protein complex translated from the same RNA molecule. This protein complex might assemble at the polyU tract of the genome and probably contains the entire set of NS3 to 5B and some host factors. Initiation of negative-strand synthesis involves cis-acting elements within NS5B and the 3′NTR and might be the most tightly regulated step in the entire process of RNA replication. Since the 3′end of the positive strand is buried in a strong stem, it is not a template for de novo initiation of RNA synthesis by the polymerase; therefore, it seems likely that additional factors like the viral helicase might be essential at this step. NS5B initiates RNA replication by production of a dinucleotide primer, then undergoes a huge conformational change and processively copies the entire genome. Currently it is not clear, whether primer synthesis really takes place at the 3′end or at a different site of the genome, as in case of poliovirus (Paul et al. 2000), with a subsequent transfer of the polymerase/primer complex to the 3′end. Due to the low and limiting number of negative-strand RNA, it is furthermore tempting to speculate that negative-strand synthesis can be initiated only once from a positive strand by a cis-acting protein complex translated on the same RNA. In this scenario, initiation of negative-strand RNA would essentially require a preceding translation of the positive-strand RNA, resulting in the formation of a new replication vesicle and each replication site would indeed contain only one negative-strand RNA/replication intermediate (Fig. 1b). At least in vitro, synthesis of progeny positive strands can directly be initiated by the polymerase due to a favorable 3′ terminal structure of the negative strand, thereby probably allowing multiple initiation cycles, resulting in an overall surplus of positive-strand RNA. The progeny positive-strand RNA is released into the cytoplasm by a yet to be defined mechanism, which could involve NS5A and the translocase function of the NS3 helicase. Mechanisms terminating the lifespan of an active replication site are not known so far. However, the connection to the cytoplasm might simply be constricted after a certain time, sequestering the replication intermediates inside DMVs to prevent recognition by innate immunity, as suggested for arteriviruses (Knoops et al. 2011).

Many of the mechanisms in this model are hypothetical and require experimental validation. Particularly, enigmatic are the transition from translation to replication, the role of the NS3 helicase, the function of NS5A and the distinct roles of its phospho-isoforms, the interplay of CREs with viral and host proteins, the initiation and regulation of RNA synthesis, and the morphology of active replication sites, just to name a few. The complex linkage between translation/replication and cis-/trans-acting functions in the replication cycle of positive-strand RNA viruses severely impedes the analysis of individual steps of RNA synthesis in cell culture models. Therefore, further mechanistic insights will require more sophisticated in vitro models allowing further dissecting the complexities governing HCV RNA synthesis.

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