

From Structure to Function: New Insights into Hepatitis C Virus RNA Replication

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Introduction

With an estimated number of about 180 million affected people worldwide and a limited therapy option, infection with the hepatitis C virus (HCV) is an important medical problem. Initial limitations in propagating HCV in cell culture have been overcome step-by-step in the past few years and culminated in the recent development of a system allowing efficient propagation of infectious HCV in tissue culture. Nevertheless, structural and biochemical studies of viral proteins as well as molecular analysis of viral RNA replication are still major challenges. The recent resolution of the three-dimensional structures of some HCV proteins or domains along with elegant reverse genetic and cell biological studies provided first insights into how HCV amplifies its RNA, exploits the cellular machinery and eventually overcomes innate immune responses.

Organization of the viral genome

HCV is a positive strand RNA virus that has been classified as the genus Hepacivirus in the *Flaviviridae* family. The HCV genome is an uncapped, linear molecule with a length of

~9600 nucleotides (nts; Fig. 1A). It carries a long open reading frame that is flanked at the 5' and 3' ends by short highly structured non-translated regions (NTRs). The 5' NTR has a length of about 340 nts and contains an internal ribosome entry site (IRES) required for translation of the HCV genome (1). This RNA element binds the 40S ribosomal subunit in the absence of other translation initiation factors in a way that the initiation codon is placed in the immediate vicinity of the P site (2). Part of the IRES (domain II) overlaps with RNA signals essential for viral replication (Fig. 1A) arguing for a possible role of domain II in regulating a translation-RNA replication switch. The 3' NTR has a tripartite structure composed of an ~40 nts long variable region downstream of the HCV coding sequence, a poly(U/UC) tract of heterogeneous length and a highly conserved 98-nts long sequence designated X-tail (Fig. 1A). Genetic studies have shown that a poly(U/UC) tract of at least ~25 nts as well as the complete X-tail are required for RNA replication in cell culture and for infectivity of the viral genome *in vivo*. An additional *cis*-acting RNA element (CRE) has been identified in the 3' terminal coding region of NS5B (Fig. 1A). This CRE (designated 5BSL3.2) (3) forms a long-distance RNA-RNA interaction

with SL2 in the X-tail (4), which is indispensable for RNA replication.

Expression of the viral proteins from the monocistronic genome is primarily achieved by production of a polyprotein that is proteolytically cleaved into the structural proteins (core, envelope proteins E1 and E2), the hydrophobic peptide p7 and the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig. 1A) (1, 5). Processing of the core to p7 region is mediated by host cell signalases, and in case of the core protein in addition by signal peptide peptidase. All remaining cleavages are carried out by two viral proteases: the NS2/3 protease mediating cleavage between NS2 and NS3 and the NS3 serine-type protease that is responsible for processing at all other sites in the NS polyprotein region. Alternative forms of the core protein generated by internal initiation of translation of an alternative reading frame or by ribosomal frameshifting have been reported (6). These proteins designated ARF-P or F-proteins (for 'alternative reading frame' or 'frame shift') are dispensable for RNA replication, at least in cell culture, and will therefore not be discussed in this review.

Components of the HCV replication complex

Our current understanding of the HCV life cycle and the molecular composition of viral particles is still mainly hypothetical. However, some details begin to emerge due to the availability of efficient *in vitro* systems, most notably the HCV replicon system (7, 8), HCV pseudoparticles (9, 10) and very recently, the first system for efficient production of infectious HCV in cell culture (11-13). Since the minimum components required for HCV RNA translation and replication are the NTRs and the NS3 to NS5B coding region, in the following we will only refer to these viral elements.

The non-translated regions

Apart from the genetic studies of the NTRs described above, detailed structural analyses have been performed only with parts of the 5' NTR, in particular the IRES and fragments thereof (2). By using cryo-electron microscopy Spahn and coworkers established a ~20 Å resolution map of the IRES bound to the 40S ribosomal subunit (14). This study revealed an important role of domain II in inducing conformational changes in the 40S subunit that

appears to be essential for assembly of the 80S ribosome. Very recently, cryo-electron microscopy reconstruction was also used to determine the structure of human translation factor eIF3 complexed to the HCV IRES and to model an HCV IRES-eIF3-40S ribosomal subunit complex (15). This model provides an explanation how eIF3 prevents premature joining of 40S and 60S ribosomal subunits.

The NS3 serine-type protease and its cofactor NS4A

NS3 is a bifunctional molecule that carries in the N-terminal ~180 amino acids a serine-type protease. This enzyme has a typical chymotrypsin-like fold and is composed of two β -barrel domains displaying on their interface the classical active site residues (H, D, S) that are a hallmark of serine-type proteases (Fig. 1B) (1, 5, 16, 17). Although NS3 possesses intrinsic proteolytic activity, polyprotein cleavage is dramatically enhanced by the NS4A cofactor. This short polypeptide of 54 amino acid residues serves multiple purposes. First, it anchors the protease to intracellular membranes *via* an N-terminal transmembrane segment present in NS4A; second, it contributes one β -strand to the N-terminal protease domain and thereby allows its complete folding (Fig. 1B); third, it stabilizes the protease against proteolytic degradation; fourth, it activates protease activity by changing the geometry of the catalytic triad. The 3D structure of the holoenzyme revealed in addition a tetrahedrally coordinated Zinc-ion required for proper folding of the C-terminal NS3 protease domain (Fig. 1B). The enzyme has an unusually shallow substrate binding pocket and therefore requires rather long interaction surfaces with the substrate (usually decapeptides). This made the design of efficient NS3-specific compounds with a sufficient therapeutic window challenging, but on the other side may explain why the enzyme can recognize several cellular substrates. In fact, it was found that NS3 can cleave two components of the dsRNA signalling pathway. One is the Toll-like receptor 3 (TLR-3) adaptor TRIF (also called TICAM-1) (18), the other is Cardif (also called IPS-1, MAVS or VISA) (19). Both adaptor molecules relay their signal from the dsRNA sensor [TLR-3 or retinoic acid inducible gene I (RIG-I)] to the kinase complex that is responsible for the phosphorylation of IFN regulatory factor-3 (IRF-3). Phosphorylated IRF-3 in turn, induces transcription of IFN- β and other antiviral genes. In this respect, NS3 is not only required for

polyprotein processing, but also for counteracting cellular antiviral defense pathways.

The NS3 helicase

The C-terminal ~400 amino acids of NS3 form a superfamily 2 DExH/D-box RNA helicase that is capable of unwinding RNA-RNA duplexes in an ATP-dependent manner (20). The protein binds to homopolymeric RNAs with a preference for poly(U). Moreover, some specificity of helicase binding to the terminal stem-loop in the X-tail was observed (21) arguing that a preferred binding to the 3' NTR also occurs *in vivo*. Earlier studies often came to the conclusion that NS3 protease and helicase function are largely independent. This view is difficult to envisage given the tight interaction of both domains observed in the crystal structure of full length NS3 (Fig. 1B) (5) and by the finding that an isolated NS3 helicase fragment showed a slower kinetic of duplex RNA unwinding as compared to the full length NS3 protein (22). Moreover, NS4A appears to act as an RNA loading factor that greatly enhances productive RNA binding of a full length NS3/4A complex (23) arguing for a cross-talk between protease and helicase domains.

Mutational and biochemical studies suggest that NS3 forms multimers, and in particular dimers. Although monomeric NS3 was found to bind RNA with high affinity, it was convincingly shown that RNA unwinding requires an NS3 dimer (24). The molecular mechanism underlying RNA unwinding is not yet clear. However, recent kinetic analyses have shown that the enzyme undergoes highly coordinated cycles of fast dsRNA unwinding with high processivity for about 20 nts (23-25). Thereafter, the enzyme 'pauses' and may fall off the template or start a new cycle of duplex unwinding.

The crystal structure of the HCV helicase revealed a Y-shaped molecule composed of 3 nearly equally sized subdomains (Fig. 1B) (26, 27). The cleft between domains I and II forms the nucleotide binding site whereas the interfaces between the 3 domains are predominantly involved in nucleic acid recognition and binding. The helicase domain is connected to the serine-protease domain *via* a flexible linker that may allow a rotation of both domains against each other and in this way a fine regulation of their respective enzymatic activities (Fig. 1B) (22, 28). It will

therefore be interesting to determine whether inhibitors of the protease also affect NS3 helicase activity.

The role of the NS3 helicase in the HCV life cycle remains to be unraveled. The enzyme may be involved in initiation of RNA replication by unwinding stable stem-loop structures at the termini of positive and negative strand RNA. NS3 helicase may also contribute to processivity of the replicase complex (RC) by removing stable RNA secondary structures in the template strand and by displacing bound proteins that may otherwise interfere with RNA synthesis. Finally, the helicase may be required for dissociation of the replicative form into positive and negative strand HCV RNA.

NS4B: inducer of a membrane scaffold for the viral RC?

NS4B is a highly hydrophobic protein predicted to contain 4 transmembrane domains while its N-terminal region may contribute to targeting of NS4B to intracellular membranes (29). Overexpression of NS4B in cell culture is sufficient to induce membrane alterations arguing that the main function of this protein is the formation of membranous structures serving as the scaffold of the HCV RC (30). However, the structure of NS4B-induced membrane alterations appears to be morphologically distinct from the membranous web suggesting that one or several viral and cellular components contribute to the formation of the viral RC. A nucleotide binding motif of the sequence GX₄GK was identified in NS4B and it was shown that a GFP-NS4B fusion protein can bind GTP upon expression in cell culture (31). Moreover, recombinant NS4B appears to possess GTPase activity. However, the results described by Einav and coworkers (31) are at variance to the reports by several other groups showing that some mutational alterations affecting the NTP binding site do not reduce but rather enhance RNA replication (1). A clarification of this issue and the mechanism by which NS4B induces membrane alterations awaits an appropriate biochemical *in vitro* assay. Given the hydrophobic nature of NS4B this is clearly a challenging task.

NS5A – an RNA binding protein and a key regulator of the HCV RC?

NS5A is composed of an N-terminal amphipathic α -helix serving as a membrane anchor (Fig. 1B) (32, 33) and 3 distinct domains which are separated by the low-complexity sequences (LCS) I and II (34). The N-terminal α -helix is an in-plane membrane anchor that carries a tryptophan-rich

hydrophobic side embedded in the cytosolic leaflet of the phospholipid bilayer and a fully conserved polar side that may serve as a platform for the recruitment of proteins involved in RNA replication (32, 33). Domain I (amino acid 36-213 of NS5A) appears to be involved in RNA binding (see below). Domain II may be involved in inhibition of the IFN-induced dsRNA activated protein kinase PKR (35). Domain III is only poorly conserved between different genotypes, can in part be deleted with only moderate reductions of RNA replication in cell culture and tolerates the insertion of rather large heterologous sequences (36, 37). In fact, functional RCs could be generated by using subgenomic replicons in which genes encoding for fluorochromes have been inserted into domain III of NS5A.

Recently, the X-ray crystal structure of domain I was solved at 2.5 Å resolution (Fig. 1B) (38). It is composed of a basic N-terminal subdomain IA and a predominantly acidic C-terminal subdomain IB. In subdomain IA a zinc ion is coordinated by a unique motif of 4 fully conserved cysteine residues which are absolutely essential for RNA replication (34, 38). In subdomain IB an unusual disulphide bond linking 2 cysteine residues near the C-terminal subdomain border was found. Since this modification is rather rare for cytosolic proteins a functional role can be envisaged. However, mutational analysis showed that it is not essential for HCV RNA replication (34). It remains to be determined whether the disulphide bond also forms *in vivo* and whether it plays a role in some steps of the viral life cycle.

Domain I forms homodimers *via* contacts near the N-terminal ends of the molecules resulting in a clamp or claw-like shape (Fig. 1B). The molecular surface involved in dimer formation is highly conserved indicating that homotypic NS5A interactions also occur *in vivo*. Intriguingly, domain I homodimers exhibit a strikingly asymmetric charge distribution generating a basic groove and a 'back'. The latter has been proposed to interact with the negatively charged phospholipid head groups presumably aided by the N-terminal α -helix (Fig. 1B) (38). The basic groove in the inner side of the claw is positioned away from the membrane and could interact with RNA (38). It was found that bacterially expressed NS5A binds to the 3' end of HCV positive and negative strand RNA with a preference for the

poly(U/UC) tract in the 3'NTR of positive strand RNA (39). Conceivably, NS5A dimers might oligomerize into large 2-dimensional assemblies forming an extended groove. This 'basic channel' may serve as a railway system facilitating viral RNA transport between functionally different compartments for RNA translation, replication and assembly. In addition, intensive sequestration of HCV RNA by binding to NS5A would protect it from nucleolytic degradation and prevent the activation of intracellular dsRNA sensors such as RIG-I. Thus far, the impact of domains II and III on structure and function of domain I is not known. However, one can speculate that these domains shield domain I by steric blockage of the basic groove or by recruitment of host cell factors and this block might be relieved by phosphorylation (see below).

NS5A is expressed as a basally phosphorylated and a hyperphosphorylated form (p56 and p58, respectively) with phosphorylation being mediated by as yet unknown cellular kinase(s) at serine, and to a much lesser extent, threonine residues (1). Basal phosphorylation depends on sequences from the C-terminal region of LCS I up to the end of NS5A while serine residues required for hyperphosphorylation reside in LCS I (40). The major phospho acceptor sites of NS5A appear to be genotype- or isolate-specific and have been mapped biochemically only for genotypes 1b (S2194) and 1a (S2321) (41, 42). Also the degree and the requirements for hyperphosphorylation seem to vary between different HCV genotypes and isolates. Thus far the functional relevance of the different phospho forms is unknown. However, phosphorylation of NS5A is a conserved feature among hepaci- and pestiviruses, and also found with flavivirus NS5, arguing that phosphorylation plays an important role in the HCV life cycle (43). Moreover, it was found that mutations reducing NS5A hyperphosphorylation can lead to a dramatic enhancement of viral RNA replication (36, 44). By using transfection of the human hepatoma cell line Huh-7 with HCV replicons, it was found that these RNAs amplify only to low levels unless they acquire 'cell culture adaptive' mutations. Many of these mutations cluster amongst others, in LCS I of NS5A and they often reduce hyperphosphorylation arguing that this modification is of disadvantage for efficient RNA replication (8, 45). In support of this assumption it was found that treatment of cells carrying non-adapted replicons with an inhibitor of the cellular kinase(s) responsible for NS5A hyperphosphorylation leads to an increase of RNA replication (46). However, when cells carrying

adapted replicons were treated with the same kinase inhibitor, replication was reduced. These results provide strong evidence that the NS5A phosphorylation status is a key regulator for HCV RNA replication.

NS5B: the RNA dependent RNA polymerase forming the catalytic core of the HCV RC

NS5B is the RNA dependent RNA polymerase (RdRp) and therefore the catalytic center of the HCV RC. The enzyme can initiate RNA synthesis *de novo*, at least *in vitro*, and it is assumed that *de novo* initiation is also operating *in vivo* (1). RdRp activity appears to be modulated by interaction with cyclophilin B (see below) as well as by interaction with the viral factors NS3 and NS5A. NS5B binds to homopolymeric RNAs with a preference for poly(U). In addition, a specific binding of NS5B to stem loop 5BSL3.2 has been described (47). This binding may recruit the enzyme to the 3' end of positive strand RNA for which the 5BSL3.2 and SL2 RNA - RNA interaction may be required (Fig. 1A).

The crystal structure of the NS5B catalytic domain revealed a structural fold comparable to other polymerases with palm, finger and thumb subdomains (Fig. 1B) (48, 49). One structural peculiarity of the enzyme is the fully encircled active site which is due to multiple interactions between the finger and thumb subdomains creating a tunnel in which a single stranded RNA molecule is directly guided to the active site. NTPs enter the active site *via* another positively charged tunnel. Binding of the RNA template and initiation of RNA synthesis are supposed to be regulated by a highly flexible β -hairpin loop (Fig. 1B) located in the thumb domain and pointing towards the active site (49, 50). This β -hairpin generates a narrow gate that prevents the 3' terminus of the template from slipping through the active site and that ensures initiation of RNA synthesis from the 3' end of the template. Another interesting feature of NS5B is a low-affinity GTP binding site located at the interface of the thumb and finger subdomain and residing on the surface of the molecule (51). Binding of GTP to this site may induce a conformational change resulting in efficient initiation of RNA synthesis (52). Interestingly, an overall structural similarity of NS5B and the RdRp of bacteriophage ϕ 6 was found (53) and a common model for *de novo* RNA synthesis by these two enzymes has been proposed.

The catalytic domain of the NS5B protein is membrane associated *via* a C-terminal transmembrane domain which is essential for HCV RNA replication (54). The recent structural analysis of this transmembrane domain (F. Penin and D. Moradpour, in preparation) allowed the molecular modeling of membrane associated NS5B. This model suggests that the RNA binding groove is stacked to the membrane interface, thus likely preventing the binding of RNA template (Fig. 1B). The activation of this inactive RdRp form possibly requires a conformational change at the level of segment 545-562 located in the RNA template-binding cleft of the catalytic NS5B domain and which connects it to its transmembrane anchor. Such a conformational change would liberate the RNA-binding groove and move the NS5B catalytic domain away from the membrane, while the 545-562 connecting segment might be involved in binding of viral or cellular factors required for RC formation.

Host cell factors involved in HCV RNA replication

Apart from the viral proteins and CREs, several host cell factors play an important role for HCV RNA replication based on the criteria that RNAi-mediated knock-down of each of these cellular genes and/or overexpression of dominant negative mutants decreases viral replication (55-57). The first one is the human vesicle-associated-membrane-protein associated protein (VAP-A) that was initially identified as an interaction partner of NS5A and NS5B (58). VAP-A is a widely expressed, endoplasmic reticulum/Golgi-localized protein involved in intracellular vesicle trafficking. It is implicated in directing NS-proteins to cholesterol rich, detergent resistant membranes on which RNA replication is thought to occur (59). Interaction between VAP-A and NS5A is enhanced by cell culture adaptive mutations suggesting that hyperphosphorylation of NS5A negatively affects association with this host factor (44). More recently, VAP-B, an isoform of VAP-A, was found to interact with NS5A and NS5B, too (60). siRNA-mediated knock-down of VAP-B expression reduced NS5B and HCV RNA abundance whereas the amount of NS5A was not affected. This result suggests that VAP-B also plays an important role in HCV RNA replication, but the underlying mechanism remains to be determined.

The third host cell factor involved in HCV RNA replication is the geranylgeranylated protein FBL-2 that also interacts with NS5A (55). Inhibition of geranylgeranylation in cells by disrupting the function of 3-hydroxy-3-methylglutaryl CoA

reductase or by impairing the enzymatic activity of geranylgeranyl transferase I led to the disassembly of replicase complexes and abolished HCV RNA replication (61). In contrast, increasing the availability of saturated and monounsaturated fatty acids stimulates viral replication, whereas polyunsaturated fatty acids and inhibitors of fatty acid synthesis have a negative impact (62). These observations clearly show that the host cell lipid metabolism is crucially involved in HCV replication.

The fourth host cell factor involved in HCV RNA replication is cyclophilin B (56). Starting with the observation that cyclosporinA (CsA) potently suppresses replication of HCV in cell culture (63) Watashi and co-workers identified cyclophilin B as the target of CsA action (56). In a series of elegant experiments they could show that this protein interacts with the C-terminal region of NS5B and appears to stimulate its RNA binding activity. Cyclophilin B is a peptidyl-prolyl cis-trans isomerase that may alter the conformation of NS5B. However, whether the HCV polymerase is a substrate of this enzyme remains to be determined.

Implications for the viral replication cycle

Based on the currently available data described in part above, the following model for HCV RNA replication can be envisaged. Upon infection and liberation of the viral RNA into the cytoplasm, HCV proteins are generated *via* IRES-mediated translation of the genome. Translation takes place at the rough endoplasmic reticulum (ER) where host cell signal peptidase, signal peptide peptidase and most likely also the viral proteases catalyze

cleavage of the polyprotein. Formation of membranous vesicles that accumulate to form a “membranous web” is induced by NS4B perhaps in concert with viral (e.g. NS5A) and host cell factors (e.g. VAP or FBL2). NS5B presumably in cooperation with additional viral (e.g. NS3/4A, NS5A) and host cell factors assembles at the 3' end of positive strand RNA to initiate *de novo* synthesis. Newly generated negative strand RNA then serves as template for production of excess amounts of positive strand RNA. These RNAs are either used for translation, a new round of RNA replication or are packaged into virus particles. It is unclear how the decision between these options is made but NS5A may play an important role in this respect by acting as a molecular switch. Two major lines of evidence support this assumption. First, alterations of the NS5A phosphorylation status, which can be achieved by cell culture adaptive mutations or by inhibition of cellular kinases, very much affect RNA replication. Second, at least for one HCV isolate it was shown that RNA replication enhancing mutations in NS5A strongly reduce virus production (T. Pietschmann and R. Bartenschlager, in preparation). This result suggests that adaptive mutations alter the RC in a way that RNA replication may be favored at the expense of virus assembly. It is unclear how this is achieved but one speculation is that basal phosphorylated NS5A strongly interacts with VAP-A which in turn may affect provision of viral RNA to the assembly machinery. With the advent of new cell culture systems for HCV that allow the production of infectious virus particles, these and many other important questions relating to the HCV replication cycle can now be addressed at the molecular level. It is well possible that these studies unravel novel putative strategies for antiviral intervention.

LEGEND TO THE FIGURE

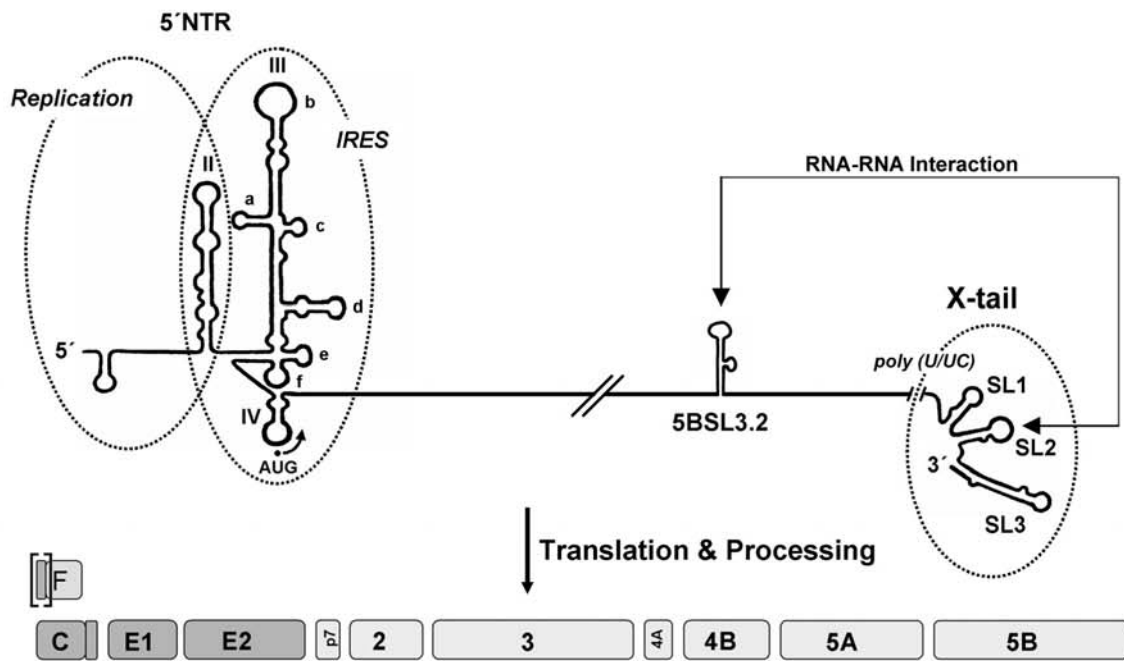
(A) A schematic of the HCV genome with the secondary structures of CREs is shown in the upper panel. The polyprotein with individual cleavage products is drawn below. Alternative forms of core proteins are given in the left. (B) Three dimensional structures of HCV proteins and molecular modeling of their membrane association. A ribbon diagram of the NS3 serine protease domain (cyan) associated with the central NS4A protease activation domain (yellow) and covalently complexed with a peptide-like inhibitor (stick structure in red) is shown in the upper left. Side-chain atoms of the catalytic-triad amino acids (H57, D81, S139) are represented as magenta spheres of the corresponding van der Waals radius. The structural zinc ion is shown as orange sphere. The ribbon diagram of the NS3 helicase domain in complex with a dU₈ oligonucleotide (stick structure in yellow) is shown in the upper right. Domains I, II and III are colored in silver, red and blue, respectively. The sulfate ion is shown as orange sphere. Its position is similar to that of the β -phosphate of ADP in the crystal structure of PcrA helicase. Conserved sequence motifs involved in the functioning of the helicase are colored in green. The small ribbon diagram of the crystal structure of full length NS3 complexed with the central NS4A protease activation domain is shown in the upper center. Protease and helicase subdomains and main structural features are colored as in the single structures. In this model, the position of the protease domain corresponds to a left rotation of 90° along the y-axis and a 180° left rotation along the z axis relative to the single structure in the left. The position of the helicase domain corresponds to a left rotation of 90° along the z-axis relative to the single structure in the right. The C-terminal segment of the helicase domain, which occupies the catalytic site of the protease subdomain, is represented as stick structure. A ribbon diagram of the NS5A domain I dimer and the N-terminal amphipathic α -helix (AH) in-plane membrane anchor in relative position to a phospholipid membrane is shown in the lower left [adapted from (38)]. Subdomains IA and IB are respectively colored in magenta and pink in one monomer, and cyan and iceblue in the other monomer. The corresponding N-terminal in-plane membrane helices colored in red and blue, respectively, are modeled to show the membrane association of domain I. The zinc atoms of the zinc-binding motif in subdomain IA are shown as orange spheres. The membrane is represented as a simulated model of POPC bilayer obtained from D.P. Tieleman (<http://moose.bio.ucalgary.ca/>). Polar heads and hydrophobic tails of phospholipids (stick structure) are colored in light yellow and tan, respectively. The positioning of the in-plane membrane helix at the interface between polar heads and hydrophobic tails of phospholipids was deduced from molecular dynamics simulations (N. Sapay and F. Penin, unpublished). The ribbon diagram of the NS5B RdRp catalytic domain (48, 49) associated with the membrane *via* its C-terminal transmembrane anchor is shown in the lower right. The fingers, palm, and thumb subdomains of the catalytic domain are colored in blue, red and green, respectively. In the presented conformation, the catalytic site lies within the center of the ectodomain and the RNA template-binding cleft is located vertically on the right along the thumb subdomain β -loop (colored orange) and the C-terminal part of the connecting segment 545-562 (colored silver) The transmembrane anchor domain is colored magenta. Structures shown in panel B are based on the following PDB accession codes: 1CU1 for NS3; 1DY9 for NS3 protease domain; 1A1V for NS3 helicase domain; 1GX6 for NS5B; 1R7E for NS5A N-terminal membrane anchor and 1ZH1 for domain I structure.

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