

The Hepatitis C Virus Replicase: Insights into RNA-dependent RNA Replication and Prospects for Rational Drug Design

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Abstract: The enzymes involved in the replication of the Hepatitis C Virus (HCV) have been some of the most intensely studied proteins in recent history because they are targets for rational drug design. HCV is an established and growing menace to human health that is without a current vaccine or a widely affordable and effective treatment. Traditional antiviral screening is difficult with HCV because of the lack of a convenient animal model or tissue culture system. Consequently, two viral replicative proteins have been intensely studied as drug targets: the NS3 protein, which possesses serine protease, ATPase, and helicase activities, and the NS5B RNA-dependent RNA polymerase. Structural and mechanistic studies of the HCV replicative proteins have not yet led to antiviral HCV drugs. However, new insights have been gained into the mechanisms of actions of the enzymes comprising the viral replicase. This review discusses recent advances in understanding the HCV NS5B RNA-dependent RNA polymerase and the NS3 helicase mechanisms and suggests how this new information could be exploited for the potential development of future antiviral agents.

INTRODUCTION

The Hepatitis C virus (HCV)¹ is the main agent responsible for contagious hepatitis not due to the "classical" hepatitis viruses A and B. There is presently no vaccine or widely effective treatment for the resulting illness, which was previously called non-A-non-B hepatitis. HCV infection has been recognized as an urgent health crisis primarily because approximately 2% of the world's population tests positive for HCV [1]. Because the virus causes few acute symptoms, most HCV patients are unaware of their infection. However, if left untreated, the majority of all HCV infections lead to chronic hepatitis, which can develop into cirrhosis and liver cancer. Currently in the United States alone, HCV is responsible for more than 3,700 deaths and 1,600 liver transplants annually. These numbers are predicted to quadruple in the next 10 to 20 years [2]. Because current HCV therapies are costly and produce debilitating side effects, new antiviral therapies are clearly needed.

HCV antiviral drug development has been difficult (see [3] for a recent review) because the virus cannot be conventionally cultivated in tissue culture or a convenient animal model. Consequently, the viral replicase has been intensely targeted for rational drug design. Like the majority of viruses, HCV does not use DNA to store its genetic material. Instead, the RNA genome of HCV is translated by host ribosomes directly into viral structural components and the machinery required for viral replication. This "replicase" is composed of several proteins that work together to produce new viral particles from the RNA genome. The two components that are best characterized are the NS5B RNA-dependent RNA polymerase and the NS3 protease/helicase. This review discusses recent progress in understanding the mechanism of action of these proteins.

HCV VIROLOGY

The lack of a robust culture system or animal model has prevented the use of classical virology to study HCV. Thus, almost all information regarding HCV biology has been garnered indirectly using recombinant DNA technology. The virus was discovered in 1988 by analyzing the RNA sequences of patients with symptoms of non-A-non-B hepatitis [4]. The RNA sequence shared by HCV patients was remarkably similar to viruses in the family *Flaviviridae*. At that time, *Flaviviridae* contained two virus genera, the *flaviviruses* and the *pestiviruses*. The prototype *flavivirus* is yellow fever virus, to which the family owes its name (Latin *flavus*, yellow). *Pestiviruses* are pathogenic animal viruses. *Flaviviridae* now contains a third genus, *hepacivirus*, to which all the various genotypes and quasispecies of HCV belong. The discovery that a virus-like RNA sequence could be found in patients with non-A-non-B hepatitis did not directly prove that this RNA actually caused the disease, however. Almost ten years passed until it was proven using infectious HCV clones that the HCV RNA was the agent responsible for non-A-non-B hepatitis [5, 6]. Infectious HCV clones can be used to study the development of the disease in chimpanzees, the only HCV animal model.

All members of *Flaviviridae* are positive sense single-stranded RNA ((+)ssRNA) viruses. This means that their ssRNA genomes directly encode the viral proteins by acting like a messenger RNA in a host cell. The genome organization of HCV is shown in Fig. (1). When the (+) ssRNA genome of HCV enters the host cell, HCV RNA is translated into a single polypeptide more than 3,000 amino acids long that is processed into 11 functional proteins. This processing has been inferred from analyzing the peptides produced when a vaccinia virus containing the HCV reading frame is transiently-expressed in various human hepatocyte cell lines [7, 8]. As determined by genetic knockouts of regions in the open reading frame, two HCV-encoded proteases process the polyprotein as shown in Fig. (1). The two non-structural (NS) proteins that cleave the polyprotein are the autocatalytic NS2/3 zinc-containing protease [9, 10]

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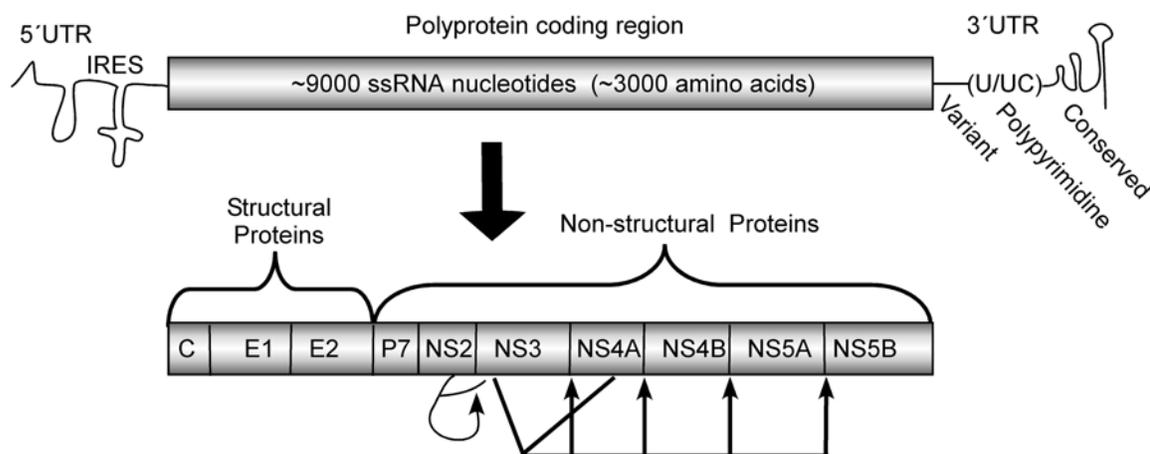


Fig. (1). The HCV genome. The viral RNA is translated in cell into a single polyprotein. Host and viral proteases process HCV polyprotein into 10 functional proteins. The NS2/3 protease cleaves the NS2/3 junction, and the protease domain of the NS3 protease/helicase, with its NS4A cofactor, cleaves the NS3-NS5B region. Arrows indicate cleavage sites.

and the NS3 serine protease [11, 12]. The NS4A protein also participates in polyprotein processing, albeit indirectly. The NS4A protein binds tightly to NS3 and stimulates its protease activity. The proteases that are responsible for the first few cleavages of the viral polyprotein are still unknown, but they are widely assumed to be host proteins.

The HCV replicase likely contains some, if not all, of the mature nonstructural (NS) proteins and possibly some host factors. These proteins act together to produce new HCV particles. The 3 mature structural proteins form the core and envelope of new virus, whereas the non-structural proteins are responsible for viral replication. The key component of the HCV replicase is the NS5B protein. NS5B is an RNA-dependent RNA polymerase, which synthesizes the viral genome from nucleoside triphosphates (NTPs) using viral RNA as a template. The second key protein is NS3. In addition to its protease activity mentioned above, the NS3 protein possesses a second functional region that unwinds duplex RNA, double stranded DNA, and DNA:RNA hybrids.

At either end of the HCV genome are located regions of RNA that are not translated into proteins (Fig. (1)). The 5 untranslated region (5 UTR, sometimes referred to as the 5 nontranslated region, 5 NTR) contains an internal ribosome entry site (IRES) from which the viral polyprotein is translated in a cap-independent manner [13, 14]. Very few cellular RNAs are translated in this manner and most are recruited to the ribosome by proteins that bind the m⁷GpppN capping nucleotide placed on the 5' ends of mRNAs. The 3 untranslated region, (3 UTR or 3 NTR) is comprised of three regions, a variable region, a polypyrimidine tract, and a conserved 98 nucleotide tail (sometimes called the X tail). Deletion of all or part of the 3 UTR disables infectious clones [15]. As discussed below, there is accumulating evidence that the 3 UTR helps dictate replicase assembly.

Recent Progress in Systems to Study HCV

All the human hepatitis viruses have been difficult to culture *in vitro*, and a robust tissue culture system for HCV remains an elusive goal. Substantial progress has been made in the last few years with the help of recombinant DNA technology and transgenic animals. Although HCV viruses

isolated from natural sources are not normally viable *in vitro*, self-replicating RNAs, called "replicons," have been created by splicing portions of the HCV genomes with selectable markers and portions of other viruses. The first widely used HCV replicons contained a gene encoding neomycin phosphotransferase in place of the structural proteins [16]. Cells expressing neomycin phosphotransferase are resistant to the drug G418. Thus, when HCV subgenomic replicon RNA is transfected in the human hepatoma cell line Huh-7, only cells expressing the HCV replicase will survive in the presence of G418. Both viral RNA and mature HCV proteins can be detected in this system. Similar replicons have also been constructed containing the HCV full-length genome [17]. Recently, several groups reported HCV replicons that have been adapted to replicate at higher levels in cell culture [18-21]. These adapted replicons carry several point mutations in the HCV polyprotein and make the study of HCV genetics and replication in cells finally possible. However, the resulting data will still need to be cautiously interpreted in light of recent revelations that infectious HCV clones containing mutations found in adaptive replicons are not infectious to chimpanzees [22]. In other replicons, HCV RNA is expressed under control of a bacteriophage T7 promoter. Introduction of the T7 RNA polymerase into cells containing T7/HCV replicons leads to autonomous RNA replication even in the absence of selective pressure [23-25]. The T7 binary replicon system has been used to study both HCV genotypes 1a [23] and 1b [25, 26]. In addition to this progress towards an *in vitro* system to cultivate HCV, small animal models for HCV are currently under intense development. For example, in a recently reported mouse system, portions of human livers are grafted onto transgenic immuno-compromised mice making the mice sensitive to HCV infection [27, 28].

Genotypes and Quasispecies

Like all RNA viruses, the genome of HCV evolves very rapidly. There are dozens of distinct HCV strains with highly divergent genetic sequences [29, 30]. HCV evolves so rapidly that in individual patients infected with certain genotypes, HCV exists as a heterogeneous population of quasispecies. Both viral genotype and quasispecies diversity

influence disease severity and treatment response (reviewed in [31, 32]). Patients infected with genotype 1 often do not respond to antiviral therapy, whereas patients with other genotypes respond more favorably [33]. Strains that more rapidly evolve into diverse quasispecies more readily evade the host immune system, leading to chronic hepatitis [34], and respond less well to therapeutic intervention [35]. Exactly how HCV genetic variation leads to these clinically important viral phenotypes is still largely a mystery.

HCV genetic diversity can also make interpretation of HCV biochemical studies quite difficult. Numerous studies report different and apparent contradictory properties for the HCV enzymes, a possible result of genetic variability. Even though almost all biochemical research to date has focused on the most common HCV genotypes found in the United States (genotypes 1a and 1b) the various isolates studied often possess key genetic differences.

The first prototype genotype 1a strain was the H strain [7], which was used to study polyprotein processing. The prototype 1b strain was the BK strain. As discussed above, the first HCV isolates, including the H and BK strains, were not infectious in the chimpanzee model. Thus, currently neither the H strain nor the BK strain is studied extensively. The prototype strains are now the infectious clones of the HCV virus. There are only a few infectious clones, and they undoubtedly do not represent even a small fraction of HCV genetic diversity. The first two infectious clones reported were modifications of the H strain, the H77 clone reported by Kolykhalov and colleagues [6] and the H77c clone described by Yanagi *et al.* [5]. Infectious genotype 1b strains include HC-J4 [36], Con1 [37] and HCV-N [38]. The only reported non-genotype 1 infectious clone was made from the HC-J6(CH) genotype 2a strain [39]. Several of the infectious clones can be studied both in animals and as replicons. Strain H77c from genotype 1a has been used to construct the full-length binary replicon system [23, 24]. Using genotype 1b strains, sub-genomic and genomic replicons have been constructed using the Con1 strain [17] and HCV-N strain [20, 21].

CURRENT HCV THERAPY

All currently approved HCV therapies are based on the use of two drugs, interferon and ribavirin (see [40, 41] for reviews). Numerous different forms of interferon are now used routinely to treat HCV [3]. Interferons are cytokines that act to modulate the immune system, and their antiviral actions are likely the result of the induction of cellular proteins that lead to destruction of the virus or infected host cells. There is little evidence that interferon-induced factors directly affect the action of the HCV replicase. However, two HCV proteins have been suggested to help the virus escape the effects of interferon, the E2 [42] and NS5A [43] proteins. Both have been shown to inhibit the actions of the interferon-induced factor protein kinase R (PKR). Mutations in either E2 or NS5A, which accumulate due to ribavirin induced mutagenesis (see below), could disrupt HCV/PKR interactions and enable the immune system to clear the virus.

Ribavirin likewise modulates the immune system by influencing the fate of T-cells. Exposure to ribavirin shifts the fate of T-cells from primarily Type 2 T cells, which tend to permit chronic infections, to Type 1 T cells, which activate cytotoxic T lymphocytes. Although the molecular

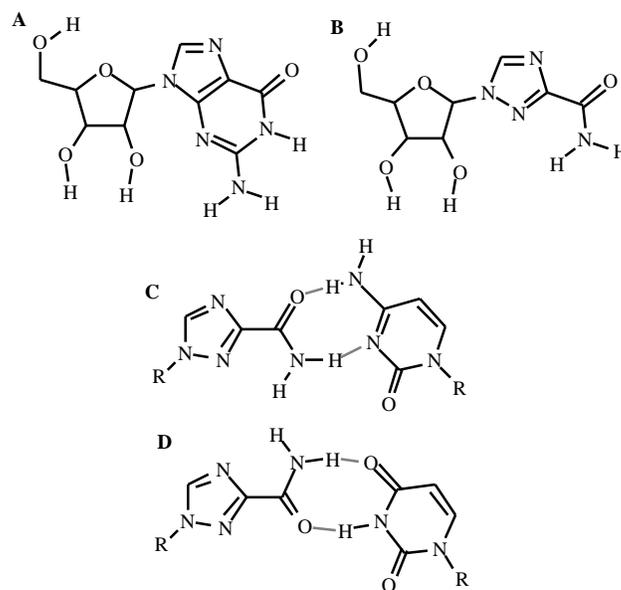


Fig. (2). Potential ambiguous base pairing of ribavirin in RNA. Ribavirin (1-(β-D-ribofuranosyl)1,2,4-triazole-3-carboxamide) (**B**) is a guanosine analogue (**A**) that can base pair in duplex RNA with either cytosine (**C**) or uracil (**D**).

mechanism is still unclear, a preponderance of clinical evidence supports this theory of ribavirin action (see [44] for a review). Ribavirin could also modulate the immune system in a manner similar to immunosuppressant drugs because it inhibits inosine 5'-monophosphate dehydrogenase (IMPDH). IMPDH is the enzyme catalyzing the rate-limiting step in guanine nucleotide synthesis. Its inhibition subsequently reduces cellular nucleotide pools leading to a suppression of cellular proliferation necessary for a robust immune response [45]. Reduction of GTP pools in such a manner could also have an inhibitory effect on the replicase because NTPs are the precursors for viral RNA synthesis.

Because it is a nucleoside analogue, ribavirin may also directly affect viral proteins. Unfortunately, many researchers prematurely ruled out this idea because ribavirin binds polymerase and helicase relatively weakly even though this is not the active form of the drug in the cell. In the cell, ribavirin is modified by cellular kinases, the most important metabolite being ribavirin 5'-triphosphate (RTP). Recently, two different groups showed that RTP interacts with both the HCV polymerase [46] and HCV helicase [47]. In fact, HCV polymerase can use RTP as a substrate for RNA synthesis. When incorporated into RNA, the pseudo-base of ribavirin (1,2,4-triazole-3-carboxamide) can direct the incorporation of either cytosine or uracil (Fig. (2)), leading to transcription errors. NS5B has been shown to incorporate ribavirin into RNA [46]. A mutagenic effect of ribavirin has been documented in experiments using poliovirus [48, 49] and GB virus B, a close relative of HCV [50]. Mutations have also been found to accumulate in full-length HCV replicons when cells expressing these replicons are grown in the presence of ribavirin [24]. Ribavirin's mutagenic properties could cause the virus to undergo "error catastrophe" or "lethal mutagenesis" where the majority of virus offspring are not viable [51]. Although there is still no consensus as to whether ribavirin functions by influencing the fate of T-cells

or as a viral mutagen, numerous reviews recently have been published that argue strongly in favor of the lethal mutagen hypothesis [51-55].

HCV NS5B RNA-dependent RNA Polymerase

Although the gene for the HCV RNA polymerase was identified shortly after the first HCV genome was isolated, the NS5B protein was not characterized until many years later because NS5B is difficult to study biochemically. Comprehensive HCV NS5B reviews have been published elsewhere [56-59]. The discussion below is intended to supplement those excellent resources by placing recent studies in their appropriate context, and identifying possible genotypic differences in NS5B that could help explain some discrepancies in the current literature.

Recombinant HCV NS5B

Although initial reports [60] claimed that soluble full length NS5B could be expressed in *E. coli*, almost all subsequent studies showed that such constructs invariably generate the recombinant protein primarily as insoluble inclusion bodies. The production of large quantities of NS5B in an active form in *E. coli* normally requires the deletion of part of the C-terminus of the protein, which contains several hydrophobic residues [61-63]. N-terminal deletions are not tolerated [64]. Although 21 C-terminal amino acids are normally deleted in order to produce the protein in *E. coli*, the mutagenesis of only 4 conserved leucines is sufficient for solubilization [63]. The extreme C-terminus of NS5B has been demonstrated to be a transmembrane region, and membrane-protein interactions localize the replicase in the endoplasmic reticulum [65, 66], as reviewed elsewhere [67].

NS5B is frequently purified as a fusion protein with a modified C-terminus or N-terminus. Such studies need to be cautiously interpreted because the fusion peptides have been shown to influence the enzyme's activity. For example, an N-terminally tagged GST-NS5B fusion protein is more active as a fusion protein than it is after the GST protein is removed [61]. Likewise, a C-terminal His tag placed on NS5B has been shown to decrease the apparent K_m of the enzyme for its NTP substrates over 10-fold [68].

Because the polymerase from genotype 1a is sometimes unstable or has low activity, only a few studies have focused on genotype 1a polymerases. By far the most work has been done using the NS5B polymerase isolated from genotype 1b strains. The polymerase from the first HCV isolate (HCV-1) was the first genotype 1a polymerase expressed and purified in *E. coli* [60]. The polymerase from the 1a infectious clone H77c was later purified and characterized [63, 69]. Recombinant HCV genotype 1b polymerase (isolate BK) was first purified from insect cells [68, 70]. Later, the BK NS5B was expressed in and purified from *E. coli* as a recombinant protein [63]. Genotype 1b polymerase has also been extensively characterized from strain J4 [71, 72], the HCR6 strain [73, 74], and the Con1 infectious clone [75]. Many other studies have analyzed NS5B polymerase from genomes isolated from patients who were predominately infected with genotype 1b, but do not report the sequences of the proteins analyzed [61, 76-82]. Ferrari *et al.* compared the activity of genotype 1a H77c polymerase to that isolated from the genotype 1b BK isolate [63]. They demonstrated

that the H77c polymerase was about 10-fold less active than the corresponding protein from genotype 1b.

There are undoubtedly differences between the NS5B protein expressed in *E. coli* and those present in eukaryotic cells. When expressed in eukaryotic cells, NS5B is phosphorylated [83] and forms a replication complex with the other non-structural proteins [84]. The roles of potential posttranslational modifications of the HCV polymerase have not yet been elucidated.

Purified recombinant NS5B is capable of synthesizing a full-length viral genome [71], yet only a small portion of the enzyme is catalytically active [85]. For this reason, NS5B has a very low specific activity compared with most other polymerases studied. The rate-limiting step seems to be the ability of the enzyme to bind RNA templates. Once bound, synthesis is quite processive, and the turnover rate in this initial burst of RNA synthesis approaches 200 nucleotides per minute [85, 86]. Purified NS5B catalyzes the template-dependent synthesis of RNA from virtually any RNA template including those derived from the viral genome. Although initial reports indicated that NS5B requires a primer to initiate RNA synthesis [68], several studies have confirmed that the enzyme is capable of RNA synthesis *de novo*, given only a template and NTPs [79, 82, 87]. In addition to RNA-dependent RNA polymerase activity, some preparations of HCV NS5B have been reported to catalyze the addition of nucleotides to the ends of RNA fragments. This terminal nucleotidyl transferase activity was identified in the original NS5B isolates [70], but was later shown by site directed mutagenesis to be a contaminating enzyme [68].

NS5B: Structure and Function

Three groups have reported NS5B structures in the absence of substrates, at 2.8 Å [88], 1.9 Å [89], and 2.5 Å [90] resolution (see [91] for a review). Each of the crystallized enzymes was derived from a genotype 1b strain. Unlike other polymerases that resemble a right hand and bind DNA or RNA in a cleft between the fingers and thumb, HCV NS5B has an enlarged thumb and expanded fingers region that completely encircles the active site and limits substrate accessibility to the catalytic site in the palm domain (Fig. (3A)). The fingers region can be subdivided into two subdomains; the helix rich, -fingers, and the sheet rich, -fingers. The fingers may be flexible because they are disordered in the structure of the related 3D RNA-dependent RNA polymerase from poliovirus [92]. In the HCV polymerase, the fingers and thumb are linked by an extension of the fingers, the "fingertips." Notably, the fingers of HCV are similar to those of HIV reverse transcriptase [93], suggesting that this fold may be unique for enzymes that read an RNA template. This region of DNA-dependent polymerases, as opposed to RNA-dependent polymerases, is rich in alpha helices not sheets [94].

Both Lesburg *et al.* [89] and Bressanelli *et al.* [88] were able to model RNA into their respective NS5B structures without major conformational changes in the fingers and thumb domains, leading them to speculate that the polymerase was in the "closed" conformation. Other DNA and RNA polymerases only adopt this "closed" confirmation in the presence of a primed template and the proper NTP that is to be next inserted. In the absence of proper substrates, all

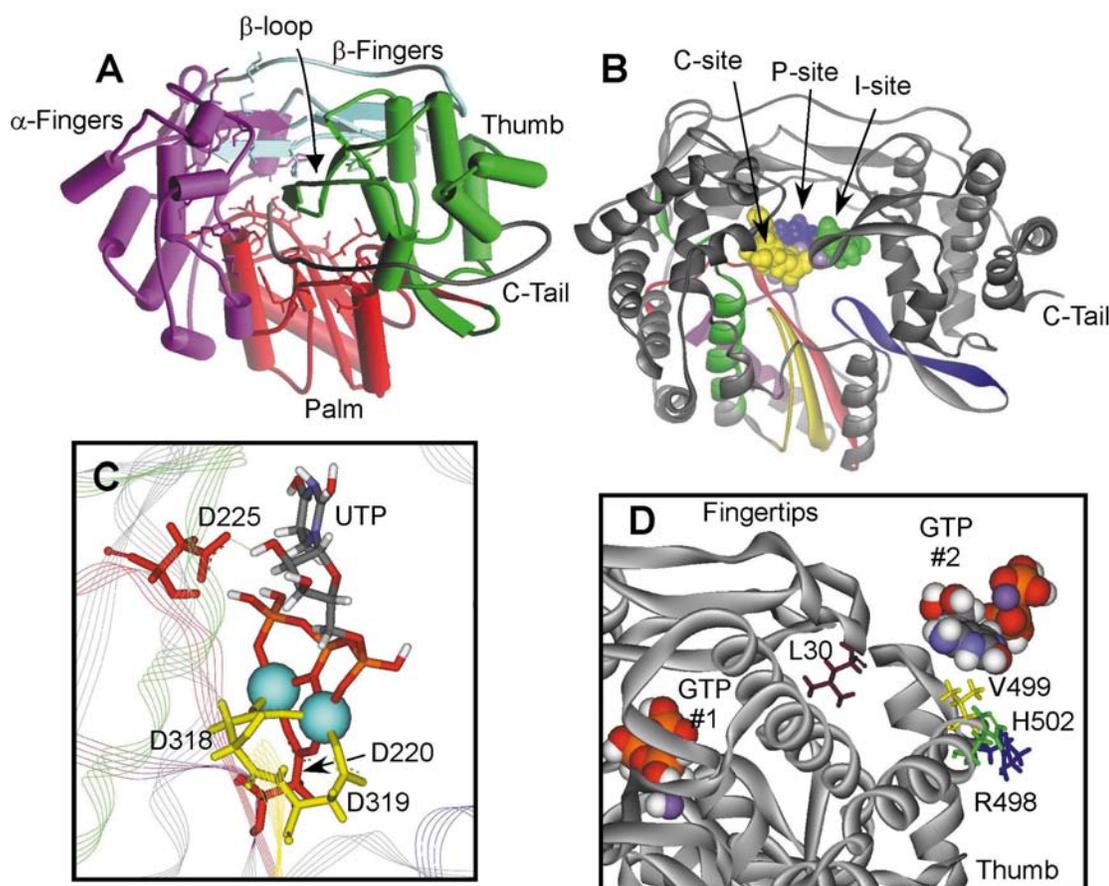


Fig. (3). The HCV NS5B RNA-dependant RNA polymerase. (A) NS5B structure determined by Ago *et al.* [90] showing the domain organization: α -fingers (purple), β -fingers (blue), palm (red), thumb (green), and C-terminal domain (gray). The amino acid side chains that were proposed by Bressanelli *et al.* [88] to interact with RNA or NTPs are shown as sticks. Note the β -loop and C-tail that both extend into the active site. (B) The structure of the NS5B:UTP complex [97] in which 3 nucleotides (space-fill) occupy the C-site (yellow), P-site (blue) and I-site (green). Metal ions are shown as gray spheres. The conserved RNA-dependent RNA polymerase motifs are colored: motif A (red), motif B (green), motif C (yellow), motif D (purple), and motif E (blue). (C) View of UTP bound in the C-site showing the 2 catalytic metal ions (blue) coordinated by conserved aspartates in motif A (red) and motif C (yellow). D225 contacts the 2 OH of the ribose. (D) The NS5B:GTP complex [97] showing one GTP bound in the C-site and a second bound to the surface of the protein. Key residues in the putative allosteric site are noted Leu30 (brown) Val499 (yellow), His502 (green), and Arg498 (blue). Coordinates for (A) are from Protein Data Bank accession code [1QUV](#). Coordinates for (B) and (C) are from Protein Data Bank Accession code [1GX6](#). Coordinates for (D) are from Protein Data Bank accession code [1GX5](#).

other polymerases have been observed in an “open” conformation, with the fingers rotated away from the thumb. It is widely held that the closing of the polymerase only in the presence of the NTP that forms the proper Watson-Crick hydrogen bonded base pair is the primary mechanism whereby polymerases maintain a high fidelity. Thus, the observation that HCV NS5B indiscriminately adopts the closed conformation could account for the low fidelity of the HCV enzyme.

New evidence has been presented that suggests that the original HCV NS5B structures observed to date actually represent an “open” conformation. This would mean that the fingers and thumb do not separate during polymerization and additional conformational changes would be necessary for catalysis. Ng *et al.* determined the structure of the rabbit hemorrhagic disease virus (RHDV, a (+) ssRNA viruses in the *Caliciviridae* family) RNA dependent RNA polymerase in both the “closed” and “open” forms [95]. Serendipitously,

both forms were present in the asymmetric unit of the crystal. Only the closed form of RHDV polymerase binds two metal ions in the configuration necessary for the polymerization reaction. In the open conformation, the active site residues are not arranged to coordinate the two required metal ions in an active configuration. Interestingly, the HCV structures align best with the open conformation of RHDV polymerase [95], suggesting that structures of ternary NS5B/RNA/NTPs complexes will reveal important additional mechanistic insights.

The NS5B substrate complexes will likely resemble the recently determined structures of the RNA-dependent RNA polymerase from bacteriophage ϕ 6 [96]. The structure of the ϕ 6 double stranded bacteriophage RNA dependent polymerase recently has been determined in the presence and absence of NTPs and oligonucleotides [96]. The structure of ϕ 6 in the absence of substrates is strikingly similar to the HCV NS5B even though the two viruses are not

evolutionarily related. In their seminal study, Butcher *et al.* [96] crystallized the ϕ 6 polymerase alone, in the presence of NTP, in the presence of an oligonucleotide template, and as a ternary complex in the presence of a template and NTP. The oligonucleotide binds in a different location in the presence and absence of NTPs. In the absence of NTPs, the 3' OH of the template is not bound in the active site used for phosphodiester bond formation. The site to which the terminal template nucleotide binds is called the "specificity pocket" and likely determines which RNA sequences are selected as templates. When NTPs bind, the template translocates in the 5' direction to the active site where it base pairs with incoming nucleotides. The NTP that forms the 5' end of the new RNA binds one site, termed the priming site (P-site) by Butcher *et al.* The other NTP binds in the catalytic site (C-site). These two nucleotides stack against a hydrophobic residue Y630, in the C-terminal domain of the polymerase. Thus the C-terminal domain acts as a "protein stacking platform" to ensure proper initiation. After initiation, the stacking platform must then move to allow duplex RNA to exit the active site. In the absence of template, a third NTP binding site is apparent that overlaps the C-site. Butcher *et al.* call this the interrogation site (I-site) and suggest that it enables incoming NTPs to aid template selection [96].

The next step towards understanding the mechanism of the HCV polymerase in such detail was taken recently, when new structural models of HCV polymerase in complex with nucleotides were reported [97]. These structures reveal the position of the P-site, C-site, I-site, and divalent metal cations. No major conformational changes were observed in the presence and absence of NTPs, and the metal ions are visualized, suggesting all the observed NS5B structures are catalytically competent. However, the NS5B/NTP structures have not yet been rigorously compared with the RHDV polymerase structures [95], so whether they represent the "open" or "closed" conformation is still unclear. The NTPs bound to NS5B occupy the P-, C-, and I-sites identified in the ϕ 6 structure and thus conserved residues necessary for NTP coordination have been clearly identified (Fig. (3)). However, a stacking platform for the priming NTP is not evident in the NS5B/NTP structures [97].

Oligomeric state of NS5B- Bressanelli *et al.*'s recent report of the NS5B/NTP structures [97] was primarily focused on the elucidation of the various GTP binding sites on NS5B. High concentrations of GTP stimulate RNA synthesis by NS5B [98]. It had been widely assumed that high concentrations of GTP accelerates initiation simply because GTP is the initiating NTP in *de novo* RNA synthesis assays, and initiation is the rate limiting step. When NS5B polymerase is crystallized in the presence of GTP, however, a second GTP-binding site can be seen on the protein far from its catalytic center (Fig.(3D)). The second site bridges the fingertips with the thumb domains of the protein. The discovery of the surface GTP binding site suggests that GTP might be an allosteric regulator of the enzyme.

One of the hallmarks of allosteric proteins is that they function as oligomers. There is increasing evidence that, like poliovirus polymerase (see [99] and references therein), HCV viral RNA is synthesized by a replicase containing more than one polymerase molecule. Using chromatography, GST pull downs, and co-precipitation experiments, Qin *et al.*

[77] showed that NS5B forms a homomeric interaction. Point mutations that disrupt this interaction affect the ability of the polymerase to synthesize RNA. Interestingly, one of the mutation maps to His-502 [77], which forms the regulatory GTP binding site [97]. His-502 was previously identified as critical for activity by scanning mutagenesis [100]. Similarly, Wang *et al.* [81] have used kinetics and cross-linking to demonstrate oligomerization of NS5B. They also report that an interface is readily apparent in an unpublished crystal structure of NS5B. Because these interfaces might involve the putative allosteric GTP binding site, the molecular details of these interactions are anxiously awaited.

A third group has recently confirmed that NS5B forms dimers in the presence of an RNA template. In an analysis of the role of a residue at the tip of the fingers domain (Leu30), Labonte *et al.* showed that whereas the wildtype protein forms only monomers and dimers, a mutant with Leu30 changed to Ser (L30S) forms monomers, dimers and higher order structures. The L30S mutant was 60 times less active than the wildtype enzyme in a polymerase assay [101]. Leu30 is part of a hydrophobic pocket formed where the fingers of NS5B contact the thumb. A polar residue in position 30 could potentially cause the two domains to separate. As is apparent from the NS5B structure (Fig. (3)), separation would disrupt the GTP binding site on the surface of the molecule. If this site were indeed an allosteric regulation site, then such changes would undoubtedly affect the enzyme's activity and quaternary structure. This is exactly what Labonte *et al.* observed [101]. Thus, the proposal that GTP is an allosteric regulator of NS5B seems increasingly likely.

RNA dependent RNA polymerase conserved motifs- Five sequence motifs are conserved in viral RNA-dependent RNA polymerases [102, 103] and they all cluster in the palm subdomain (Fig. (3B)), where catalysis occurs. The motifs are normally designated A-E from their relative positions in the polymerase gene. The A motif is nearest to the N-terminus of NS5B. Motifs A-D are present in all classes of polymerases, whereas motif E is unique to RNA-dependent RNA polymerases. As seen in the structure of NS5B in the presence of UTP (Fig. (3C)), Asp220 in Motif A and the two aspartates in the GDD signature sequence in Motif C (Asp318, Asp319) coordinate the two magnesium ions that are necessary for nucleotidyl transfer. The role of the conserved aspartates and metals is to properly align the nucleotide bound in the catalytic site so that the phosphate can be attacked by the 3' OH of the nucleotide (or RNA) bound in the P site. Replacement of any of these three aspartates abolishes polymerase activity [64, 68] and full-length viral clones with these substitutions are not infectious [104]. The side chain of another conserved aspartate in motif A (Asp225) also helps coordinate the nucleotide in the C-site by making contact with the 2' OH group. Mutation of Asp225 to either Asn or Gly completely abolishes polymerase activity [68].

Motif B does not make direct contact with bound NTPs, but is probably important to orient the active site. The conserved residue Asn291 forms a hydrogen bond with Asp225, which in turn contacts the 2' OH of the incoming NTP. Mutation of Asn291 to lysine abolishes the enzyme's activity [68]. Mutations in other residues in motif

B, including Gly283, Thr286, Thr287 also affect activity, but it is difficult to speculate about the roles of those residues because they are some distance from the known substrate binding sites [68].

The role of Motif D is still unclear. Only one amino acid has been mutated in this motif, and curiously, this mutation leads to a more active polymerase. When Arg345 is changed to a lysine, the purified recombinant protein is more active [68] and replicons with this substitution produce more colonies in cell culture [105]. This information is biologically interesting because almost all viral RNA-dependent RNA polymerases have a lysine in this position, whereas HCV has the less ideal arginine residue [102]. Thus, it appears that HCV has evolved a less active polymerase than related viruses. A slow rate of RNA replication could be desirable to help the virus escape host antiviral responses. A molecular explanation for the effect of Arg/Lys345 on polymerase action is not readily apparent because R345 is on the surface of the protein far from the known NTP binding sites.

Motif E appears to be important for coordinating the NTP that will be incorporated at the 5' end of the new RNA. This NTP is bound in the P-site, and Ser365 of Motif E donates a hydrogen bond to its phosphate [97]. In the NS5B/NTP structure another amino acid, Arg386, also helps coordinate the NTP bound in the P-site. Arg386 donates 2 hydrogen bonds to the phosphate. Site directed mutagenesis had previously revealed that Arg386 is critical for *de novo* initiation [106] but not for primer extension [107].

Many of the above mutations that affect polymerase activity recently have been introduced into the sub-genomic replicon system by Cheney *et al.* [105]. Not surprisingly, those mutations that negatively affected activity led to the production of fewer colonies, whereas the introduction of the R345K mutation, which leads to a more active polymerase, made replicons dramatically more efficient.

Protein stacking platform- In the initiation complex formed by the ϵ 6 RNA-dependent RNA polymerase [96], residues in the C-terminal domain help position the nucleotide that will eventually form the 5' end of the newly synthesized RNA. The C-terminal domain essentially blocks the template binding cleft to ensure terminal initiation at the 3' end of the template. In ϵ 6, residue Y630 stacks against the base of the nucleotide bound in the P-site. In the structures of the NS5B/NTP complexes, the area where Y630 stacks is occupied by water possibly because the protein lacks the 55 NS5B C-terminal amino acids (1-55). Thus, the stacking platform could be part of the missing region of the protein. In structures of less truncated NS5B proteins [89, 90], the C-terminus of the ϵ 21 polymerase protrudes into the active site (Fig. (3A)).

Recent studies support a role for the NS5B C-terminal domain in aiding initiation. Adachi *et al.* have measured the ability of several deletion mutants to synthesize RNA on polyA/oligoU templates and on a template made of the 3' UTR [108]. The deletion mutants were designated according to how much of the C-terminus was deleted (*i.e.* 21, 39, 47 *etc.*). In their assays, a 47 polymerase mutant was dramatically more active than 21 or 39 in assays that measured primer extension. Similar activations were observed with site directed mutants in which only amino acids Leu547, Trp550, and Phe551 were changed to

alanine. These three amino acids form a hydrophobic pocket near the known P-site where they could conceivably provide a stacking platform analogous to that seen in the ϵ 6 structure. In another study, Ranjith-Kumar *et al.* used similar deletion mutants to show that the presence of residues 539-570 is critical for *de novo* RNA synthesis [109]. In their study, efficient Mn^{2+} -dependent *de novo* synthesis was only detected in deletion mutants that retained residues 539-570 of the C-terminus. Mn^{2+} is well known to form more stable nucleotide DNA complexes than in Mg^{2+} [110]. Thus, because *de novo* initiation is metal ion dependent, the metal that coordinates the priming NTP in P-site likely also contacts the C-terminal domain. Metal ion binding has been subsequently reported to quench the intrinsic fluorescence of NS5B [111], suggesting that metals may bind near Trp550 in the C-terminal domain of NS5B.

Besides the C-terminus of the protein, another unique element of NS5B protrudes into the active site. This extended ϵ -hairpin on the thumb subdomain is probably involved in completing the protein-stacking platform necessary for proper initiation (Fig. (3A)). Based on alignments with the HIV-RT primer template complex, Zhong *et al.* proposed that Y448 at the tip of this hairpin could make contact with the 3' terminus of the template used to direct RNA synthesis [106]. Later, the same group found that a mutant protein lacking this hairpin more efficiently utilizes a symmetrical duplex RNA substrate. In that later study, Hong *et al.* proposed that the ϵ -hairpin assures terminal initiation by preventing the 3'-end of template RNA from sliding through the active site of the enzyme [107]. The importance of the ϵ -hairpin in the viral lifecycle was highlighted in a subsequent report by Cheney *et al.* [105], which showed that replicons lacking all or parts of this ϵ -hairpin replicate poorly in cell culture. Thus, residues in this ϵ -hairpin likely act together with residues in the C-terminus to provide a stacking platform for replication initiation. After initiation, the platform must move from the active site to allow processive RNA synthesis. How this subsequent conformational change takes place is still unclear. Perhaps another nonstructural or host protein acts as an elongation factor to assist the transition from an initiation mode to an elongation mode of RNA synthesis.

NTP tunnel- The small space between the fingers and thumb probably provides access for the NTP substrates [88, 89]. This space is called the "NTP tunnel" and is lined with the basic amino acids, Lys151, Lys155, Arg48, and Arg222. These positively charged residues are likely involved in delivering negatively charged NTPs to the C-site, P-site, and I-site. Indeed, in the NS5B/UTP structure, Arg48, Lys51, Lys151, Lys155, and Arg158 are positioned to provide electrostatic interactions with the bound NTP in the putative interrogation site. Using site-directed mutagenesis, Labonte *et al.* have shown that Arg-222 is a critical residue, whereas mutation of Lys-151 has a lesser effect on enzyme activity [101].

Specificity pocket- The structure of the ϵ 6 RNA dependent RNA polymerase [96] explained how that enzyme only initiates at the 3' cytidylate of ϵ 6 negative strand RNA. When the ϵ 6 polymerase binds single stranded RNA, the 3' terminus of the template binds in a specificity pocket that will only accommodate cytosine. Amino acids in the pocket will bind cytosine rather than uracil, and the pocket is too

small to accommodate a purine. Whether or not such a pocket exists on the HCV polymerase is still unclear, but sequence specific interactions between NS5B and RNA seem to be important in the viral life cycle.

There have been numerous reports that the NS5B polymerase preferentially binds to and synthesizes RNA from certain sequences [69, 72, 73, 78, 87, 112-114]. RNA synthesis rates are highly dependent on both the sequence and the secondary structure of the template RNA. These reports are usually interpreted as evidence for certain viral "promoter" sequences or "origins" of replication. It is difficult to draw a basic consensus from all these studies because they each used different NS5B constructs, often derived from different genotypes.

A comparison of some of the studies, however, highlights the role of the C-terminal region of NS5B in template selection and RNA initiation. Studies using the full-length protein [72, 78] have concluded that efficient synthesis requires a stem loop RNA structures. For example, Oh *et al.* [72] showed that NS5B initiates synthesis primarily in the 3 loop of the 3 UTR. Although they were unable to confirm these results using the HCV 3 UTR, Kao *et al.* [78] obtained similar results using a 35 nucleotide stem loop RNA templates derived from brome mosaic virus. When 21 mutants are used [69, 115], the stem loop structures appear less important and *de novo* synthesis from the ends of templates can be detected. In addition, the 3' end of the negative strand genome (the complement to the 5' UTR) is a better substrate than the 3' UTR [69, 115]. As discussed above, part of C-terminus of NS5B anchors the protein to cell membranes and the other part may provide a stacking platform. Immediately after translation of the polyprotein, NS5B is likely not yet associated with the membranes. During that time, the C-terminus might direct the protein to initiate genome synthesis at the final stem loop in the 3' UTR. Later, after the protein is properly inserted into membranes, the extreme C-terminus is removed from the active site and NS5B can initiate from the ends of RNA.

Another region of the protein that determines template specificity appears to be distinct from the RNA binding site used for reading RNA templates during RNA syntheses. Biroccio *et al.* [114] used the Systematic Evolution of Ligand by Exponential Amplification (SELEX) procedure to isolate short RNA sequences that bound tightly to the 55 mutant NS5B polymerase. The 55 polymerase lacks the entire flexible C-terminal tail. The selected RNA molecules (aptamers) shared a common feature in that they were able to form stem loop structures reminiscent of those found at the ends of the HCV genome. The aptamers inhibited RNA synthesis in a non-competitive manner. To demonstrate that the aptamers bound at a site distinct from the active site, a mutant enzyme was selected that did not interact with the aptamer. Interestingly, one mutation (R498E) mapped to the surface thumb domain near the putative GTP allosteric regulatory site (Fig. 3D) [114].

Genotypic Differences

Because of genotypic variation, HCV polymerase studies must be compared cautiously. For example, some conclusions reached by Reigadas *et al.* [69] differ sharply with those of Oh *et al.*'s work [72] regarding the importance of the conserved region in the 3' UTR. These differences may

arise because one protein lacks 21 amino acids from the C-terminus (as suggested above). Alternatively, they may be simply due to genotypic differences; Oh *et al.* [72] used a genotype 1b polymerase, whereas Reigadas *et al.* [69] used a genotype 1a polymerase. As mentioned above, dramatic differences between polymerases isolated from different genotypes have been reported. Nearly all constructs of 1a polymerase have been found to be less active than constructs containing 1b polymerase [63]. Likewise, the inability of the polymerase used by Kao *et al.* [78] to synthesize products using the 3' UTR may be due to the fact that it was a variant from patient quasispecies rather than a polymerase derived from an infectious clone [72]. Bressanelli *et al.* [97] have pointed out that variation in the surface GTP binding site, notably at position 499, could account for some of the discrepancies reported in different NS5B studies. In some genotype 1b isolates residue 499 is a valine. In the NS5B:GTP structure (Fig. 3D), V499 contacts the guanine base of GTP. In most other genotypes, residue 499 is an alanine. In the 1b strains used by Luo *et al.* [82] and Oh *et al.* [71, 72], amino acid 499 is a threonine. Variation at residue 499 could affect initiation, stimulation by GTP, or the ability of the replicase to form oligomers.

Even within individual patients, different HCV quasispecies appear to express polymerases with different activities. Luo *et al.* [82] analyzed several different clones obtained from a patient chronically infected with genotype 1b. The resulting proteins had very different activities. The most active clone, which not surprisingly lacked the C-terminal 18 amino acids, differed from the genotype 1b BK "wildtype" polymerase at 12 positions. Many of these differences map in or around known functional motifs, such as C316N, which is in the NTP binding site near the metal ion coordinating aspartates of Motif C.

Genotypic variation in NS5B might also be clinically important. In one intriguing study, Horiike *et al.* [116] screened the HCV RNA of patients receiving antiviral therapy. All patients either had genotype 1b, which normally does not respond to therapy, or genotype 2a, which is more responsive to therapy. Interestingly, all genotype 1b patients who had a N316C substitution in NS5B responded to therapy. Amino acid 316 in genotype 2a is a Cys, and is located in Motif 3 of the active-site containing palm domain. Conversely, 55% of genotype 2a patients infected with the strains carrying Arg at NS5B position 250, instead of the His normally present in genotype 2a, did not respond to treatment [116].

HCV Polymerase as a Drug Target

Ever since the landmark discovery of acyclovir, the nucleoside analogue that acts as a chain terminator to specifically halt Herpes Simplex Virus replication, viral polymerases have been very popular targets for antiviral drugs. With respect to NS5B, any of the structural features described above could theoretically be exploited for drug design. For example, Bressanelli *et al.* have suggested that compounds similar to UTP that interact with leu159 might lock the enzyme in a non-productive binary complex [97]. Although no inhibitors have been reported from such rational design, traditional screening efforts have led to the discovery of several potent NS5B inhibitors that could be valuable lead compounds for drug development.

Numerous compounds have been reported in various patents that inhibit NS5B (see [59] for structures and appropriate patent references). The patented NS5B inhibitors include both nucleoside analogs, and non-nucleoside compounds [59]. Some of the data regarding NS5B inhibitors is presently being published in the scientific literature. For example, Dhanak *et al.* [117] recently reported a series of benzo-1,2,4-thiadiazine derivatives as inhibitors of NS5B that were identified using high-throughput screening. The compounds inhibited RNA synthesis catalyzed by purified recombinant NS5B in a non-competitive manner with respect to the NTP substrates, and do not bind RNA, suggesting that they bind NS5B at a site distinct from the polymerase active site. The non-nucleoside RNA-dependent RNA polymerase inhibitors also potently inhibit RNA replication in a replicon system with an IC_{50} in the sub-micromolar range [117].

HCV HELICASE

The other nonstructural protein that is a clear component of the HCV replicase is the NS3 protease/helicase. NS3 and NS5B form a stable complex when co-expressed in cells [84] and functionally interact to synthesize the viral genome [115]. NS3 aids rapid RNA synthesis by resolving secondary RNA structures and duplex RNA intermediates. This role was recently established in a study by Piccininni *et al.* [115]. They examined the synthesis of RNA by NS5B on templates derived from the HCV genome. When NS3 was added to the reaction, both templates were used to direct RNA synthesis, and RNA synthesis was dramatically stimulated on all templates. The stimulation was ATP-dependant and NS3 mutants lacking ATPase activity did not stimulate RNA synthesis. Interestingly, the RNA products synthesized by the reconstituted replicase were longer than the original template, suggesting that helicase might allow the polymerase to switch from one strand to another. The HCV helicase has been the prime focus of other reviews [118, 119]. Therefore, the following discussion will attempt to place recent site-directed mutagenesis and mechanistic studies in context with available structural data to facilitate the rational targeting of functional domains necessary for helicase action.

Current models for helicase action- Two main models will be used below to describe the HCV helicase mechanism. In these models, helicase is described as either rolling along RNA [120] or crawling like an inchworm [121]. The inchworm model assumes that the protein functions as a monomer that grips both duplex RNA and ssRNA. The inchworm translocates along ssRNA through a series of conformational changes. On the other hand, the rolling model assumes that the helicase must function as a dimer with one subunit preferentially binding duplex RNA and the other ssRNA.

The rolling model was developed to describe data gathered over many decades with a prototypical helicase from *E. coli*, the Rep helicase (see [122] for review). Rep is a superfamily 1 helicase, but it shares structural homology with superfamily 2 helicases like HCV. The Rep helicase has been crystallized in the presence of DNA and nucleotide analogues. Rep crystallizes with two monomers in the asymmetric unit and the two monomers are in different conformations [120]. The rolling model suggests that the two

conformations have different affinities for either ssDNA or duplex DNA. Upon ATP binding, the conformation of one subunit switches from a preferred ssDNA binding conformation to a conformation that prefers to bind duplex DNA. This new affinity for duplex DNA causes that subunit to translocate toward the duplex DNA. When ATP is hydrolyzed, the subunit bound to duplex DNA again changes conformation to the form that prefers to bind ssDNA. This change causes the protein to break the double helix so it can again bind ssDNA. In the meantime, the other subunit binds ATP and translocates toward the duplex region and the cycle is repeated.

In contrast, the PcrA helicase, a Rep homologue from *Bacillus stearothermophilus*, crystallizes as a monomer [121], and recent data clearly support an inchworm model for PcrA action (see [123] for a review). PcrA adopts a closed and open conformation, and the conformational changes are suggested to help force DNA through a monomeric enzyme. Residues in the ssDNA binding cleft act to bookend DNA, allowing it to slip through one end when the protein is open and the other end when closed. The net result is a unidirectional translocation of the protein along the DNA backbone.

Alignments of the Rep and PcrA structures with those of HCV helicase reveal similarities among all three enzymes [118, 124]. The enzymes are not evolutionarily conserved, however. Both Rep and PcrA are classified as superfamily 1 helicases, whereas HCV is a superfamily 2 helicase. As discussed below, some data suggest that HCV helicase could act as an inchworm whereas other evidence favors a rolling model.

Recombinant HCV Helicase

The full-length NS3 HCV protease/helicase is often difficult to express in bacterial cells and is frequently purified from insect cells. Fortunately, the helicase portion of NS3 can be separated from the N-terminal protease, and readily expressed and purified as a recombinant protein. Consequently, the helicase domain has been analyzed from a variety of genotypes including the genotype 1a strains, HCV-1 [47, 125-131], H [132, 133], and H77c [134], genotype 1b strains [134-139] and even a genotype 2a strain [134]. Lam *et al.* have directly compared the helicases isolated from infectious clones of HCV genotypes 1a, 1b, and 2a and noted some minor differences between the enzymes. These differences were less dramatic than genotypic differences seen with the polymerases isolated from different genotypes, suggesting that the helicase activity might be more strictly conserved than the polymerase. Some differences could nevertheless influence viral life cycle. For example, genotype 1b helicase appears to hydrolyze ATP more slowly than the others, and genotype 2a binds nucleic acids more tightly and unwinds duplex nucleic acids more rapidly [134].

Fusion proteins are frequently attached to the HCV helicase in order to increase protein expression levels and simplify purification. The casual use of fusion peptides makes HCV helicase studies difficult to compare. Some studies use a helicase lacking fusion proteins [135-139], whereas other studies utilize a helicase with a N-terminal His tag [140-142], or a N-terminal GST tag [143]. Some investigators prefer a C-terminal His tag [132-134, 144].

Finally, other studies have used a protein with both ends modified that possesses a T7 gene 10 tag at the N-terminus and a His tag at the C terminus [47, 125-131, 145-148]. To further complicate the situation, not all studies utilize the same portion of the NS3 protein. Whereas most studies have defined the helicase as residues 166-631 of the mature NS3 protein, others have used shorter constructs lacking an additional 13 [149], 15 [140, 142, 143], or 24 [150, 151] amino acids from the N-terminus. Other studies have used a slightly longer protein with an additional 16 N-terminal amino acids [145-148]. It is not clear how all of these modifications might influence the characteristics of the enzyme.

The helicase fragments and the full-length NS3 protein are all able to utilize not only ATP but also the other seven canonical nucleoside triphosphates (NTPs) to fuel strand displacement. In addition, HCV helicase unwinds not only RNA, but also DNA and DNA:RNA hybrids. In the process of strand displacement NS3 translocates in a 3'-5' direction on the strand to which it is bound [152, 153]. Interestingly, the HCV helicase is a more effective DNA helicase than RNA helicase [154]. The ability to function on DNA has led to speculation that NS3 could modify host gene expression, a proposal supported by the facts that HCV infection frequently leads to hepatocellular carcinoma and NS3 possesses the ability to transform NIH 3TC cells [155].

HCV Helicase: Structure and Function

Three groups have independently reported structures of the HCV helicase. Yao *et al.* [149] and Kim *et al.* [132] crystallized the enzyme from genotype 1a, whereas Cho *et al.* [150] used an enzyme from genotype 1b. The HCV helicase enzyme is a Y-shaped molecule composed of three domains separated by two deep clefts (Fig (4A)). One of the structures [132] contains a DNA poly(U) oligonucleotide that is bound in the cleft separating Domains 1 and 2 from Domain 3. This complex also contains a sulfate ion that binds at a site spatially equivalent to the γ -phosphate of the NTP bound to similar helicases [120, 121]. In the HCV helicase, there are six motifs shared with other helicases, which are commonly numbered I through VI [156]. All of the conserved sequence motifs are in the first two domains and cluster around the cleft separating Domains 1 and 2 (Fig.(4A)). Although they share no apparent sequence homology, the structures of Domains 1 and 2 are similar, and they both resemble the fold of the ATPase domain of the recombination protein RecA [157]. Domain III forms a novel structure not seen in other helicases [158]. Domains I and II have been expressed and purified in the absence of the rest of the protein for structural analysis using NMR [159]. The solution structure of domain II has been reported and is similar to the crystal structures [160]. In addition, sequence-specific backbone assignments have been reported for Domain I [161], which should permit precise localization of the NTP binding site.

The two clefts in the Y-shaped HCV helicase are both wide enough to accommodate ssDNA or RNA. In fact, the first studies which described the structure of the HCV helicase in the absence of nucleic acids, showed models of helicase/RNA complexes where the 3' ssDNA tail passes through the cleft between Domains 1 and 2 [149, 150]. These models were based on mutagenesis studies that indicated

residues in Domain 2 are critical for nucleic acid binding. While the structure of HCV helicase bound to ssDNA [132] shows that ssDNA clearly binds to the cleft that separates Domain 3 from Domains 1 and 2, it does not rule out the possibility that the other ssDNA strand binds in the other cleft when the duplex is unwound. Furthermore, the polarity of frayed DNA is unclear because there is yet no direct proof that the bound oligonucleotide represents a 3' tail necessary for initiation or is the complementary anti-parallel strand that is released when the duplex is unwound. The strongest evidence that the bound oligonucleotide represents the strand on which HCV helicase translocates comes from alignments with PcrA complexes in which part of the duplex DNA is visible. Such alignments suggest that the duplex portion of DNA (or RNA) lies near the C-terminus of Domain 3 (lower right side of Fig. (4A)).

Domain 1 (ATPase domain)- In the HCV helicase, motifs I, II, and III are present in Domain 1. As viewed in Fig. (4A), motif II appears in the front of Domain 1, motif I is in the top center, and motif III is in the back. Motif I (GSGKS) forms a Walker-type nucleotide binding site and contains a conserved Lys that forms a phosphate-binding loop (P-loop) surrounding the phosphates of ATP in related proteins [120, 121]. Not surprisingly, mutagenesis of Lys210 to Asn [146], Ala [130, 141, 162], or Gln [141] completely abolishes ATP hydrolysis and helicase activity. In contrast, when Kim *et al.* [129] changed this Lys to a negatively charged Glu, their purified protein retained RNA-stimulated ATPase activity even though it lacked RNA helicase activity. Wardell *et al.* [163] found a similar result when they changed K210 to Ala, suggesting that this Lys may actually be dispensable and a nearby residue may substitute in its role in the P-loop.

The second helicase superfamily 2 motif contains the DECH signature sequence characteristic of the "DEAD-box" family of proteins (Fig.(4B)). As seen in the structure of the PcrA helicase, the conserved Asp in this motif coordinates a metal ion necessary to precisely coordinate ATP in the binding site [121]. Each residue in the DEXH sequence of HCV NS3 has been altered to yield less active helicases [129, 141, 146, 162, 163]. Most notably, a full-length clone of genotype 1a carrying a D290A mutation is not infectious in chimpanzees [104], proving that an active helicase is necessary in the viral life cycle. Changing the HCV helicase's DECH sequence into a "classic" DEAD box motif by introducing a double mutation C292A/H293D also resulted in an inactive enzyme [146]. Interestingly, a H293A mutation in motif II of HCV helicase results in a protein with a significantly higher basal level of ATPase. Even though the H293A protein retains 60% of the wildtype's helicase activity, the rate of ATP hydrolysis by the H293A mutant is actually inhibited by RNA [129].

Motif III (VLATAT) is located on a loop that connects motif 1 to motif 2 and may help couple ATP hydrolysis to a conformational change that leads to translocation along RNA (or DNA). The mutation of the first Thr in this motif [129, 146] or of the second Thr [146] to Ala leads to decreased activity. These two threonines likely play a structural role because their side chains form a hydrogen bond and T322 accepts a hydrogen bond from H293 in the critical DECH sequence of motif II.

Domain 2 (RNA binding Domain)- Domain 2 is structurally similar to Domain 1, and completes the putative

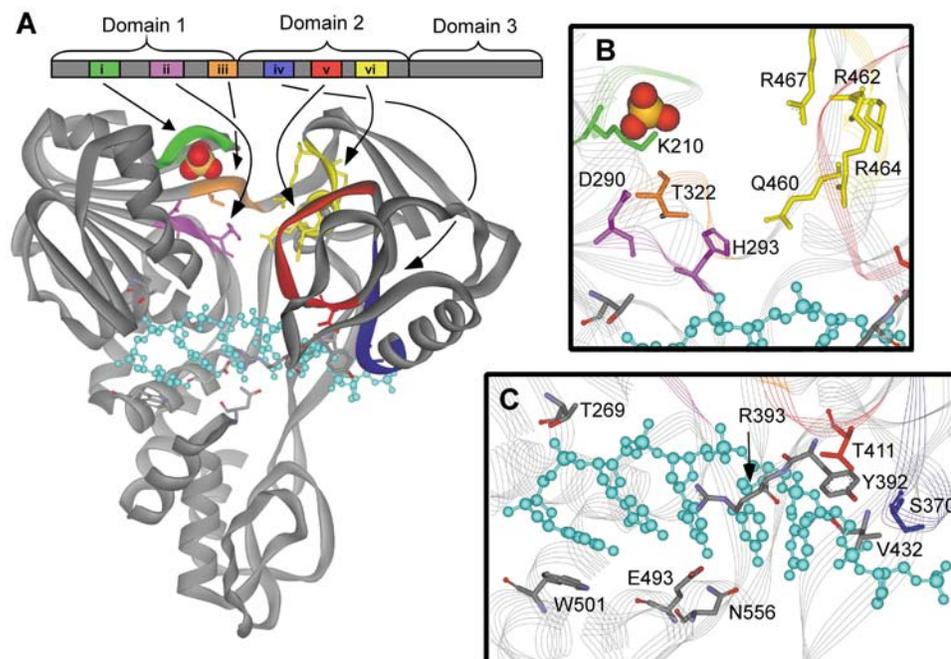


Fig. (4). Structure of the HCV helicase with a bound DNA oligonucleotide. (A) Schematic representation of NS3 helicase residues 190-624 bound to a polyU ssDNA (light blue) with a sulfate ion (space-fill) bound in the putative ATP binding site [132]. Conserved motifs are colored as indicated in the diagram. (B) Key amino acids in the putative ATP binding site. (C) Key amino acids in the nucleic acid binding site are highlighted. The conserved residues R393, E493, and N556 may help position RNA (or DNA) in the binding site so that W501 is properly intercalated between nearby bases. Coordinates are from Protein Data Bank accession code [1A1V](#).

NTP binding site. In addition, Domain 2 contacts the DNA bound in the cleft and could help transmit conformational changes that occur upon NTP hydrolysis at the P-loop to help separate the double helix. Domain 2 is sometimes referred to as the “RNA binding domain,” which is potentially misleading because all three domains make contact with RNA. Conserved motifs IV, V, and VI reside in Domain 2. In Fig. (4A), motif IV (LIFCHSKKK) is on the right side of the protein, motif IV (TDALMTG) lines the front edge of Domain 2, and motif VI (QRRGRTGR) is behind lining the cleft.

Motif IV is present only in superfamily 2 helicases (*i.e.* HCV and eIF4a). The structurally equivalent residues in superfamily 1 helicases (*i.e.* Rep and PcrA) are part of a different consensus sequence. The residue closest to bound DNA in motif IV is the conserved S370, which makes contact with the 5' end of the bound oligonucleotide through a water molecule [132]. The role of Motif IV is still unclear, however, because a S370A helicase mutant possesses characteristics indistinguishable from wild type [133].

Motif V has a more clearly defined role in RNA binding. The first Thr in motif V (T411) forms a hydrogen bond with the phosphate backbone of DNA bound in the HCV helicase structure [132]. When T411 in NS3 helicase is changed to Ala, the enzyme retains its ATPase activity, but binds RNA four-fold less tightly and lacks helicase activity [133]. It is noteworthy that another conserved Thr on Domain 1 (T269)

likewise contacts DNA, and thus a T269A mutant, like the T411A mutant, has an inability to bind and unwind RNA [133]. Fig. (4C) shows a view of these residues in the ssDNA binding site.

Motif VI is frequently referred to as a RNA-binding motif. Motif VI was first proposed to interact with RNA because negatively charged RNA would bind tightly to the positively charged Arg-rich motif VI (Fig. (4B)). Several mutagenesis studies support this notion. The substitution of any of the conserved arginines in the homologous superfamily 2 helicase, eIF-4A, results in the inability to cross-link that helicase to RNA [164]. In HCV, an NS3 Q460H mutant lacks helicase but retains some ATPase activity [163]. When Arg467 is changed to Lys [129, 163] or Ala [118], the proteins do not unwind RNA, and ATPase is decreased over 10-fold. The R464A mutant has a similar phenotype [118, 129, 162]. Interestingly, Kim *et al* [129] report that an R464A mutant retains most of its basal ATPase but does not catalyze any detectable ATP hydrolysis in the presence of RNA. Thus, in the R464A protein, RNA appears to inhibit hydrolysis. Chang *et al* [165] have used mutants containing either R462A or R464A to demonstrate that both R462 and R464 may be involved in RNA binding. In addition to its role in RNA binding, motif VI appears also to be a post-translational modification site. Arg467 is methylated by cellular protein arginine-glycine methyltransferases [166], but it is unclear how methylation affects helicase activity.

Domain 3 (Novel helical domain) - To date, the atomic structures of five helicases similar to HCV helicase have been determined (see [158] for a review). All the structures share similarities in only the regions corresponding to Domains 1 and 2 of the HCV structure. Thus, there is very little information as to the role of Domain 3 in helicase action other than to complete the ssDNA-binding site. This novel domain is likely responsible for the characteristics that distinguish HCV helicase from related proteins. Domain 3 is clearly necessary for helicase function. Deletion of 97 amino acids from the C-terminus of NS3 results in an inactive helicase [128, 142]. However, proteins lacking the C-terminal 41 amino acids retain helicase activity [141]. Thus, the minimal helicase domain has been estimated to reside between NS3 amino acids 183 and 581 of NS3 [128].

Evidence for an inchworm model- When they reported the structure of HCV helicase bound to an oligonucleotide, Kim *et al.* [132] proposed a model for helicase action very similar to the inchworm model that was later used to describe the mechanism of the PcrA helicase [121]. They proposed that in the absence of ATP, HCV helicase was present in the open conformation tightly bound to RNA (or DNA) with a residue in Domain 3 (W501) intercalated between the nucleotides like a bookend. Another residue, V432, was proposed to bookend the 5' end of the DNA (Fig. (4C)). When ATP binds, the cleft closes, as in other P-loop containing proteins. An analogous domain closure was later visualized in the PcrA structure [121]. When the cleft closes, W501 releases its grip and slides 1 or 2 base pairs in the 5' direction. After hydrolysis, W501 again grips the 3' end, and the 5' bookend is released as the inchworm widens. The net result is a translocation of the protein in a 3' to 5' direction. The opening and closing of the ATP binding cleft was suggested to be regulated by "gatekeepers" that act to rotate the motifs when ATP binds or is hydrolyzed. Two candidates are H293 in motif II and Q460 in motif IV (Fig. (4B)) [132, 146].

The strongest evidence for the inchworm model of HCV helicase action has come from a series of elaborate kinetic studies performed with the HCV helicase fragment isolated from a genotype 1b strain [135-139]. Porter *et al.* demonstrated in a careful and meticulous study that HCV helicase functions with a step size of 1-2 base pairs, as would be predicted by an inchworm model. Step size refers to the number of base pairs separated in a single catalytic cycle. A rolling protein would take bigger steps, assuming that necessary conformational changes only require the hydrolysis of a single ATP. Porter *et al.* also provide biophysical evidence that the protein functions as a monomer [136].

To further test the ratcheting inchworm model, W501 has been mutated to Ala [131, 133, 140, 146, 167], Phe [131, 133, 140], Leu [133], Glu [131] and Arg [131]. Without a bulky aromatic amino acid at position 501, HCV helicase is unable to unwind RNA [131, 133, 146]. However, when W501 is substituted with Phe, the enzyme retains some helicase activity [131, 133]. Interestingly, a W501A mutant retains some ability to unwind DNA, [131, 167] even though it is devoid of RNA helicase activity. However, W501A unwinds DNA more slowly than the wild type [140]. Because NS3 is more active on DNA templates than RNA

templates [154, 168, 169], this observation could be accounted for simply by the greater sensitivity of the DNA based assays. Alternatively, different residues might be essential for DNA unwinding, or HCV might have evolved a RNA helicase activity from an ancestral DNA helicase by adding a Tyr at position 501. Kim *et al.* [131] explained this difference in DNA vs. RNA unwinding by W501 mutants using molecular modeling to show that RNA but not DNA would be unstably bound to the W501A mutant.

The data is less clear regarding the residue that might bookend the 5' end of the RNA. Kim *et al.* propose that this residue is V432 in Domain 2 [132]. Reduction of helicase activity was seen in V432A and V432G mutants [140, 167]. However, Tai *et al.* [146] report similar DNA binding and only a slight reduction of ATPase and helicase activities in a V432A mutant. Nevertheless, Tai *et al.* found the addition of a V432 mutation to a W501A mutant in a V432A/W501A double mutation resulted in the lowering of RNA-stimulated ATPase from 61% to 8% of the wild type level. Recently, Kim *et al.* reported that a change of V432 influences nucleic acid binding and unwinding, but its replacement does not completely abolish DNA or RNA helicase activity [131]. A V432R mutant binds DNA significantly more tightly than wild type, demonstrating that this new positively charged residue is positioned to interact with the negatively charged phosphate backbone [131]. Another residue in domain 2 has also been implicated in unwinding. Based on molecular modeling, Paolini *et al.* have suggested that Y392 could play a similar role as suggested for V432 (Fig. (4C)). To support this argument, a Y392A helicase mutant is reportedly impaired in its ability to unwind DNA [167].

Evidence for a rolling model- Accumulating evidence indicates that NS3 can form oligomers. Such an oligomerization could be indicative of a rolling model like the one proposed by Cho *et al.* [150]. Based on an analysis of crystal packing interactions, Cho *et al.* proposed that HCV helicase could form a dimer by forming contacts between Domains 1 and 2 of two separate monomers. In other words, the tops of two Y-shaped monomers could join making a single box-like structure. This dimer was predicted to traverse RNA like a "descending molecular see-saw" [150]. Experimental evidence for such a HCV helicase dimer was first presented by Levin & Patel [130], based on kinetics and cross-linking data. For example, when Levin & Patel measured the inhibition of wild type genotype 1a helicase by an inactive K210A mutant, their data could only fit to models in which the two proteins interacted. Later, the same authors found that multiple helicase monomers bound together on DNA, but in a non-cooperative manner [170].

Other evidence for helicase dimer formation has come from yeast two-hybrid assays that suggest that NS3 protein forms a dimer *via* Domain 1 of the helicase [143, 171]. Dimerization of NS3 has also been visualized using analytical gel filtration in the presence of an oligonucleotide [143]. The minimum peptide required for an NS3:NS3 interaction contains Domain 1 residues 162-335, which surround conserved motifs 1, 2, and 3 [143]. Using a reverse two hybrid screen, Khu *et al.* have isolated three mutations in Domain 1 that are unable to form dimers, T266A, Y267S and M288T [143]. These mutations not only affect dimer formation that can be assayed using gel filtration, but also the ability of the proteins to unwind DNA. Incidentally,

Met288 is not a conserved residue and is normally an Ile in all but a few HCV isolates. The ability of the dimerization mutants to unwind RNA was not reported.

RNA/DNA Binding Specificity

When the HCV helicase was first isolated by Suzich *et al.* [172], they noted that the NS3 ATPase was preferentially stimulated by certain nucleic acid sequences. This unique polynucleotide stimulation profile distinguishes the HCV enzyme from related cellular proteins [144] and even helicases isolated from the same family of viruses [172]. Since then, several other studies have illustrated that HCV helicase binds some sequences, particularly those containing uracil, more tightly [126, 173-175], and that NS3 catalyzed ATP hydrolysis is preferentially stimulated by certain nucleic acids [139, 176]. Lam *et al.* recently showed that this template preference is an intrinsic and conserved property of the enzyme [134].

This conserved template specificity could help bring the helicase, and possibly the whole replicase, to a certain region of the HCV genome like the 3' UTR. Using NS3 protein derived from the "HCV 1969 cDNA clone," Banerjee & Dasgupta [175] have shown that NS3 and the helicase domain alone specifically binds both to the 3' UTR and 3'-end of the (-) strand viral transcript (the reverse complement of the 5' UTR). Both events could be significant, allowing the NS5B polymerase to synthesize RNA in regions containing stable secondary structures.

An analysis of the structure of the helicase/DNA complex yields some insights into how the enzyme may read nucleic acid base pairs (Fig. (4C)). In addition to several threonines that contact the DNA backbone [132, 133], Arg393 contacts the phosphate backbone. R393 points like a finger from Domain 2 and could act to clamp nucleic acids in the binding cleft. No side chains interact directly with the bases, but W501 stacks near the first uracil seen in the complex. Two other amino acids in Domain 3 make contact with two adjacent uridines through a tightly bound water molecule (Fig. (4)). The water molecule is 2.29 Å from the 3NH of the 3rd uridine from the 3' DNA end and 2.3 Å from the 3NH of the 4th uridine. The side chain COO⁻ group of E493, which is part of the same alpha helix as W501 (helix 5 of [132]), is only 2.4 Å from this water, and 2.2 Å from the bound water is the side chain NH2 of Asn556. If the ssDNA contains purines at these positions, then E493 and N556 would likely need to move to provide sufficient space, and subsequent movements of helix 5 could cause W501 to no longer ideally intercalate into the helix. Like W501, E493 and N556 are highly conserved residues. There is some data to support the notion that helix 5 could allow HCV helicase to read DNA templates. When W501 is substituted with Phe, the enzyme retains helicase activity but loses its preference for polyU. Lin & Kim state that other polynucleotides stimulate the ATPase of W501F more effectively than PolyU [133]. Further study of the W501F mutant, and mutants with substitutions at E493 and N556 could clearly be very informative.

Helicase Drug Design

Although viral polymerases are the enzymes most commonly exploited by antivirals, helicases have begun to

attract increasingly more attention. The most noteworthy work has emerged from efforts to find new treatments for Herpes Simplex Virus. Groups from both Bayer AG [177] and Boehringer Ingelheim Pharmaceuticals [178] have each reported that new drug candidates designed to inhibit the HSV helicase complex reduce viral replication and disease severity in animal models.

Several groups have reported specific HCV helicase inhibitors. Locatelli *et al.* [179] reported that L-(+)-dCTP, and L-(+)-dTTP each specifically inhibit nucleic acid unwinding by the HCV helicase. Likewise, Borowski, *et al.* have reported that ribavirin triphosphate [47] and imidazo[4,5-d]pyridazine nucleosides [47] inhibit HCV helicase. Human monoclonal antibodies that target the HCV helicase are also being developed as potential HCV therapeutics [180]. This recent progress in the identification of HCV helicase inhibitors has been reviewed in more detail elsewhere [119].

Few HCV helicase inhibitors have been explored as drug candidates primarily because of the similarity of the HCV helicase to cellular helicases. HCV helicase is a member of the superfamily 2 helicase class and shares signature sequences characteristic of DEAD-box helicases [181], typified by eIF4a [182]. Thus, potent HCV helicase inhibitors could inactivate numerous cellular proteins. Recent comparative studies have shown that HCV helicase differs from other DEAD-box proteins in a number of important characteristics [144, 154]. Besides acting both as a DNA and RNA helicase, NS3 is able to utilize not only ATP but also the other seven canonical NTPs to fuel strand displacement. Other DEAD-box proteins have a more narrow substrate specificity. For example, eIF4a hydrolyzes only ATP and dATP [144]. Its distinctive nucleotide stimulation profile also distinguishes NS3 from other superfamily 2 helicases [172]. The NS3 ATPase is more stimulated by poly(U) or poly(dU) than by other homopolymers [134].

With these observations in mind, the structure in Fig. (4C) could serve as a template for a novel class of HCV antiviral drugs, based on nucleic acid analogs designed to bind tightly near E493 and N556. Perhaps compounds with functional groups bridging the 3NH groups of adjacent uridines might bind HCV helicase dramatically more tightly than normal DNA (or RNA). When bound to the enzyme, such molecules could essentially lock the enzyme in the conformation that hydrolyzes NTPs most rapidly. Because the enzyme shows little preference among the various NTPs, such an enhanced NTPase activity would deplete nucleotide pool levels at the HCV replication fork. The resulting local depletion of NTP pools should effectively stall replication by the HCV NS5B polymerase. In addition, the higher affinity of HCV helicase for DNA over RNA would enable the usage of DNA-based oligos, which may not target other cellular DEAD-box proteins.

OTHER COMPONENTS OF THE HCV REPLICASE

Besides the NS3 helicase and NS5B polymerase, other nonstructural HCV proteins are clearly important components of the viral replicase. Obviously, the NS3 protease is present since it is covalently linked to the helicase, and the NS4A protein is a well-established co-factor necessary for protease activation. Both these factors

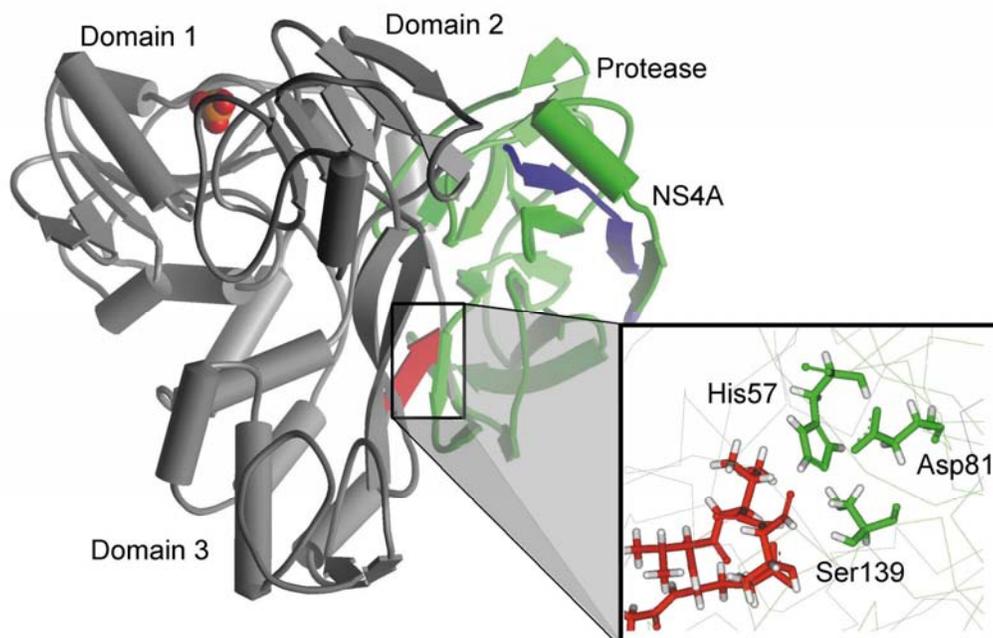


Fig. (5). Structure of the NS3/NS4A complex. The NS3 helicase domains 1-3 (gray) are rotated 45° relative to the orientation shown in Fig. (4) to reveal the location of the protease (green) and the bound NS4A cofactor (blue). The insert shows a view of the catalytic triad at the protease (blue) active site in which the C-terminus of the protein (red) is bound. A sulfate ion (space-fill) occupies the putative ATP binding site. Coordinates are from Protein Data Bank accession code [1CU1](#)[185].

could affect the rate at which the helicase unwinds RNA and therefore, could help control replication rates. Similar roles have been suggested for the NS5A and NS4B proteins.

Role of HCV NS3 protease in helicase action- To assess the role of the protease domain of NS3 in helicase action, the full length NS3 protein must be purified as a recombinant protein. The presence of the protease makes this a much more difficult task because the protein is more apt to form inclusion bodies when expressed in prokaryotic cells. For this reason, many studies have used full-length NS3 proteins expressed in insect or mammalian cell lines. As was the case with NS5B, the NS3 protein from genotype 1b is more easily expressed in *E. coli* than the protein from 1a. Only a few studies have used full length NS3 derived from genotype 1a strains [154, 179, 183]. Most full-length NS3 studies have focused on the 1b strains BK [167, 174], or the J4 infectious clone [115, 153, 168].

Even though numerous laboratories have purified both full-length NS3 and the isolated helicase domains, direct comparisons have not yet noted clear differences. In early studies Heilek *et al.* [141], concluded that the protease domain had little or no effect on helicase activity. In fact, in their assays, the full-length protein seemed to unwind RNA more slowly. Likewise, Gallinari *et al.* concluded that there were no significant differences between the full-length protein and the isolated helicase domain [184]. In addition, as noted above, the protease domain does not appear necessary for the helicase to stimulate polymerase activity [115]. Recent studies have nevertheless noted properties of the full-length helicase that were not previously reported in

studies that used the helicase domains alone. For example, the ability of the helicase to form oligomers is more evident in studies using full length NS3 [143, 183]. Perhaps in light of this new data, a more rigorous comparison of the full length and truncated helicase is warranted.

The reason for a lack of an obvious influence of the protease domain on helicase or ATPase is apparent when one examines the structure of the full length NS3 protein. Yao *et al.* [185] have determined the crystal structure of a protein complex containing full length NS3 and part of NS4 derived from the genotype 1b polyprotein (BK strain). The protease binds to the “back” of the helicase. Such a position leaves the helicase clefts free to bind RNA and ATP, but buries the protease active site between two domains (Fig. (5)). In the complex, the C-terminus of NS3 is bound in the active site of the protease, which is formed from a catalytic triad containing His57, Asp81, and Ser139 (Fig. (5-insert)). In order to cleave the rest of the polyprotein, Yao *et al.* propose that the protease domain swings away from the helicase *via* the flexible linker that connects the two regions [185]. The protease coordinates a zinc ion between residues Cys97, Cys99, Cys145, and His149. The zinc ion does not bind near the active site and likely does not play a direct role in catalysis. This zinc ion may instead play a structural role or help catalyze cleavage of the NS2/N3 junction by the NS2/3 autocatalytic protease, which contains the N-terminus of NS3 and absolutely requires metal for its activity. The HCV proteases are clear drug targets and substantial progress has been made in developing HCV protease inhibitors into new therapeutics (see [3] for a review).

Although there are yet no established effects of the protease on the helicase, helicase actions seem to influence protease activity. PolyU binding to helicase stimulates the protease activity of the full-length complex 5-fold but has no effect on the NS3 protease domain alone. The presence of ATP in such assays does not influence proteolysis rates suggesting that protease stimulation does not seem to be influenced by helicase translocation [176]. The mechanism of this apparent inter-domain communication warrants further investigation.

NS4A: Protease stimulator and possible processivity factor- Tightly bound to the protease domain is the NS4A protein, which is colored blue in Fig. (5). NS4A markedly stimulates protease activity. In crystals of the protease domain in the absence of the NS4 peptide [186], the active site is partially unfolded with a misaligned serine protease catalytic triad. When NS3 binds to NS4A, the NS3 N-terminus becomes ordered and the protease becomes active catalytically [187]. Only amino acids 21-35 of the 54-amino acid long NS4A protein are required for protease activation [188]. NS4A binds by making numerous hydrophobic interactions with NS3 and forms hydrogen bonds with NS3 residues Arg11 and Glu32.

The N-terminus of NS4A that is not involved in protease activation contains almost entirely hydrophobic amino acids. The N-terminus of NS4 likely clamps the NS3 domains together *via* hydrophobic interactions to properly position the NS3-NS4A junction in the protease active site for cleavage. After cleavage, this hydrophobic N-terminus is probably inserted in to cell membranes like the NS5B C-tail. Co-expression of NS4 has been found to direct NS3 to the endoplasmic reticulum [189], the cellular location where viral replication likely takes place. Such an anchoring might act to separate the protease active site out and away from the back of the helicase domain so that it can cleave the rest of the HCV polyprotein.

The NS3/NS4A complex shown in Fig. (5) was a cleverly engineered protein in which the NS4 peptide was tethered to the N-terminus of NS3. In the virus, NS4A is tethered to the C-terminus of NS3. Such an arrangement facilitated the expression and purification of the complex and resulted in a protein with an enhanced helicase activity [190]. This report that NS4A stimulates the helicase contrasts sharply with a report by Gallinari *et al.* that showed an inhibitory effect of NS4A on NS3 catalyzed RNA unwinding [191]. There is other evidence that NS4A aids the helicase, however. In their key study that showed that the HCV helicase was more processive on DNA templates than RNA templates [154], Pang *et al.* compared the activities of a NS3/NS4A complex expressed in insect cells [192] with an N-terminally His tagged, full-length NS3 expressed and purified from *E. coli*. The NS3/NS4A complex required less time to form a functional complex on RNA, but NS4A had no effect on the ability of NS3 to unwind DNA, leading the authors to conclude that NS4A is a processivity factor. Such a role for NS4 is supported by the fact that Darke *et al.* mention that NS4A possesses an RNA binding activity [193]. Based on the structure of the NS3/NS4A complex (Fig. (5)) it is difficult to envision how NS4A interacts with RNA unless there is a major conformational change in the protein.

NS4B and NS5A as replication regulators - Both NS4B [115] and NS5A [76, 100] have been shown to bind NS5B and inhibit its activity in polymerase assays. Like NS5B, the NS4B protein is an integral endoplasmic reticulum membrane protein [194], but its role in the viral lifecycle is still largely a mystery. The NS5A protein has been more intensely studied mainly because the sequence of a portion of NS5A was initially found to predict the response of Japanese patients to interferon [195]. Although the sequence of this interferon sensitivity determining sequence (ISDR) was later shown not to predict treatment response in all patients [196], the resulting studies implicated the NS5A protein in a myriad of cellular processes. Although a similar role has been proposed for the E2 protein [42], NS5A may help HCV evade the interferon response by inhibiting the cellular RNA induced protein PKR [197]. Deletion of the ISDR, prevents NS5A from interacting with PKR [43]. Like NS5B, NS5A is phosphorylated, but responsible kinases are still unknown. The presence of NS4A affects phosphorylation, suggesting there is an interaction between NS4A and NS5A [198].

Host Accessory Proteins- In addition to viral proteins, several host proteins may aid HCV replication in cells. Several groups have identified proteins that bind specifically to the 3'-UTR, where replication begins and to which both NS5B [72, 78, 112] and NS3 [175] bind specifically. These cellular proteins include polypyrimidine tract-binding protein [199], heterogeneous nuclear ribonucleoprotein C [200], heterogeneous nuclear ribonucleoprotein I [201], and human hepatic glyceraldehyde-3-phosphate dehydrogenase [202].

CONCLUDING REMARKS

One of the first viral replicases was isolated almost 40 years ago by a team of investigators led by Sol Spiegelman. Their classic studies were focused on a fragment of bacteriophage Q that was able to rapidly propagate itself in cell free extracts [203, 204]. Q replicase studies and those of similar RNA viruses have revealed countless insights into transcription, translation, and gene expression. The first Q studies caused quite a stir in the popular media, which interpreted the results as the creation of life in a test tube and dubbed the replicase "Spiegelman's Monster." Ironically, the RNA replicase field has shifted much of its attention to study true monsters, like HCV. Appropriately, many HCV patient support groups have adopted a dragon as a symbol of their struggle with this particularly tenacious disease. Such patients, who include one out of every 50 persons alive today, anxiously wait for studies of the HCV replicase to provide the weapons necessary to slay their dragon. Hopefully, their wait will not be a long one. Significant progress has already been made in understanding how HCV replicates its genome, and the development of inhibitors targeting the HCV replicase into antiviral therapeutics should eventually enable us to find a badly needed cure.

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FOOTNOTES

¹The nonstandard abbreviations used are:

(HCV)	=	Hepatitis C virus
(NS)	=	nonstructural
(NTP)	=	nucleoside triphosphate
(5 UTR)	=	5 untranslated region
(3 UTR)	=	3 untranslated region
(ss)	=	single-stranded
(IRES)	=	internal ribosome entry site
(RTP)	=	ribavirin 5 triphosphate
(RHDV)	=	rabbit hemorrhagic disease virus
(GST)	=	Glutathione S-transferase
(P-site)	=	priming site
(C-site)	=	catalytic site
(I-site)	=	interrogation site
(PKR)	=	protein kinase R
(IMPDH)	=	inosine 5 -monophosphate dehydrogenase
(eIF-4a)	=	eukaryotic translation initiation factor 4a
(ISDR)	=	Interferon Sensitivity Determining Sequence

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