The Hepatitis C Virus NS3 Protein: A Model RNA Helicase and Potential Drug Target

David N. Frick

Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595, USA

Abstract

The C-terminal portion of hepatitis C virus (HCV) nonstructural protein 3 (NS3) forms a three domain polypeptide that possesses the ability to travel along RNA or single-stranded DNA (ssDNA) in a 3' to 5' direction. Fueled by ATP hydrolysis, this movement allows the protein to displace complementary strands of DNA or RNA and proteins bound to the nucleic acid. HCV helicase shares two domains common to other motor proteins, one of which appears to rotate upon ATP binding. Several models have been proposed to explain how this conformational change leads to protein movement and RNA unwinding, but no model presently explains all existing experimental data. Compounds recently reported to inhibit HCV helicase, which include numerous small molecules, RNA aptamers and antibodies, will be useful for elucidating the role of a helicase in positive-sense single-stranded RNA virus replication and might serve as templates for the design of novel antiviral drugs.

Introduction

Hepatitis C (HepC) is a disease that affects about 170 million people worldwide. HepC is frequently called a "silent" killer because it causes few symptoms while the pathogen slowly destroys the liver. After a couple decades of unknown infection, when they might transmit the blood-borne virus to others, many HepC patients develop fibrosis, cirrhosis, or liver cancer. At this late stage, a liver transplant is the only option for survival, and as a result, HCV infection is presently the most common cause for liver transplantation in many countries.

HCV vaccines and treatments have been delayed because the virus is extraordinarily difficult to work with in the laboratory. Although HCV accounts for the vast majority of viral hepatitis not caused by hepatitis A or B viruses, HCV was identified almost two decades after either HAV or HBV, and it was only last year that HCV could be cultivated in cell culture with reliability (Lindenbach *et al*., 2005; Zhong *et al*., 2005). HCV is primarily comprised of a single long open reading frame encoding an approximately 3,000 amino acid long protein that is cleaved into 10 mature structural (core, E1, E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The non-structural proteins are responsible for replication and packaging of the viral genome into capsids formed of structural proteins. Since the vast majority of today's antiviral drugs exert their actions through enzymes involved in viral replication, much attention has focused on studying the non-structural HCV proteins. HCV encodes a polymerase that specifically synthesizes new viral RNA (NS5B), and two proteases that cleave the polyprotein, the NS2/NS3 autocatalytic protease and the NS3-NS4A serine protease. Several compounds that influence the activity of the NS3 protease and the NS5B RNA-dependent RNA polymerase are currently in clinical trials, and will likely become the next generation of anti-HCV drugs.

Even though the NS3-NS4A protease and NS5B have been so far the most successful HCV encoded drug targets, neither was the first HCV enzyme that was purified, characterized and examined at an atomic resolution. The first HCV protein crystallized was the portion of NS3 that acts as a helicase, an enzyme that tracks along a nucleic acid strands displacing annealed strands or RNA-binding proteins. The mature NS3 protein comprises 5 domains: the N-terminal 2 domains form the serine protease along with the NS4A cofactor, and the C-terminal 3 domains form the helicase. The helicase portion of NS3 can be separated form the protease portion by cleaving a linker. Since the protease portion is more hydrophobic, removing it allows the NS3 helicase fragment to be expressed as a more soluble protein at higher levels in *E. coli*. The fragment of NS3 possessing helicase activity here referred to as "HCV helicase" was shown over a decade ago to hydrolyze ATP (Suzich *et al*., 1993) to fuel a reaction that unwinds duplex RNA (Kim *et al*., 1995) or DNA (Tai *et al*., 1996).

There are numerous reasons why the HCV helicase has not attracted as much attention as the NS5B polymerase and the NS3-NS4A protease. First, until recently the mechanism of action of any helicase was not clear. Second, the HCV helicase resembles helicases and similar motor proteins encoded in all human cells. Third, the role that the HCV helicase plays in viral replication is still not clear. This review focuses on recent progress made in these areas and in identifying inhibitors that target the HCV helicase.

Structure of HCV helicase

Unlike other systems where mechanistic experiments were carried out long before protein-substrate interactions were viewed at an atomic resolution, the first crystal structures of HCV helicase were solved only a few years after the protein was first purified (Yao *et al*., 1997; Cho *et al*., 1998; Kim *et al*., 1998; Yao *et al*., 1999; Mackintosh *et al*., 2006). These structures are shown in Fig. 1. The helicase portion of NS3 forms three domains. When viewed as a Y-shaped molecule, the N-terminal domain (domain 1) and the middle domain (domain 2) are above the C-terminal domain (domain 3). In two structures (Kim *et al*., 1998; Mackintosh *et al*., 2006), short DNA oligonucleotides are bound to the helicase in the cleft that separates domain
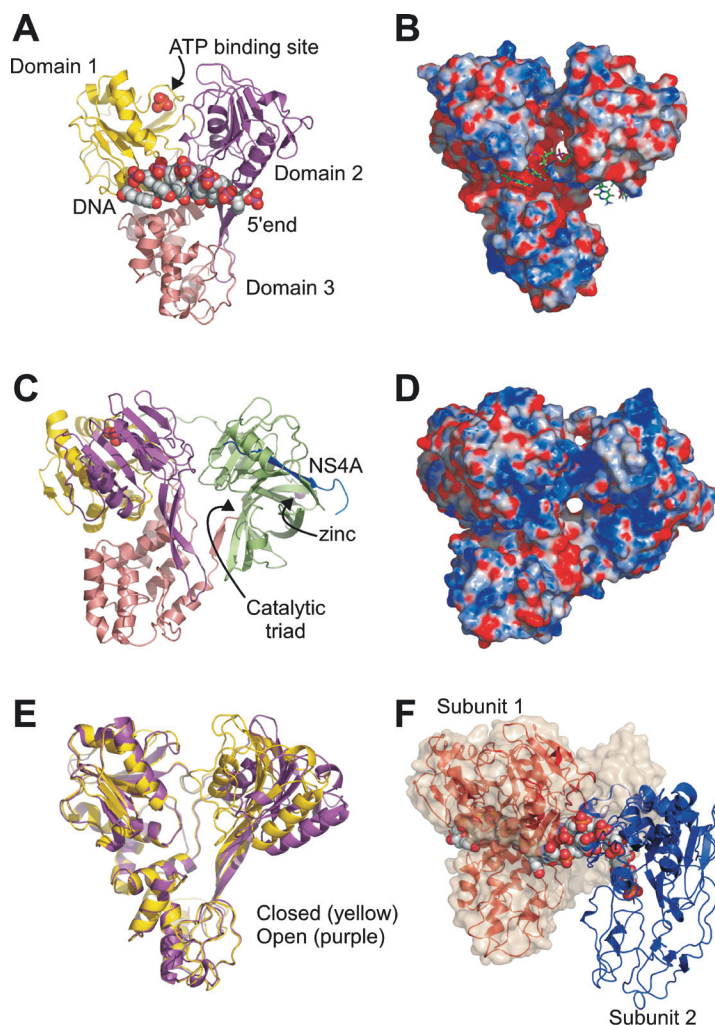
For correspondence: David_Frick@NYMC.edu

Fig. 1 HCV Helicase Structures. (A). PDB file 1A1V, showing the HCV helicase with a bound DNA oligonucleotide and sulfate ion (Kim *et al*., 1998). The N-terminal RecA-like domain (domain 1) is colored yellow, the C-terminal RecA-like domain (domain 2) is purple, and domain 3 is pink. DNA and a sulfate ion (which occupies the ATP binding site) are depicted as spheres. (B) An electrostatic surface of the protein in 1A1V calculated without the DNA using the program APBS (Baker *et al*., 2001). Note the DNA is held in a negatively-charged pocket. (C) A full-length NS3 complex with the central portion of NS4A covalently tethered to the NS3 N-terminus (Howe *et al*., 1999), as seen in PDB file 1CU1 (Yao *et al*., 1999). Helicase domains are colored as in *panel A* with the protease colored green and NS4A blue. The protein is rotated about 90° relative to *panel A*. (D) An electrostatic surface of the protein as viewed in *panel C*. Note that the positively-charged cleft surrounding the protease, which could provide additional RNA binding sites. (E) Comparison of HCV helicase in the closed conformation (PDB file 1HEI (Yao *et al*., 1997)) and the open conformation (PDB file 8OHM (Cho *et al*., 1998)). Proteins are superimposed along domains 1 and 3. (F) Two NS3 helicase fragments (red, blue) bound to the same oligonucleotide (Mackintosh *et al*., 2006). Subunit 1 (red) of the helicase: DNA complex (PDB file 2F55) was aligned with the backbone the helicase portion of 1CU1. The surface of 1CU1, which includes the helicase and protease domains, is shown as transparent amber surface. All structures were rendered using the program Pymol (DeLano Scientific LLC, San Francisco, CA).

3 from domains 1 and 2 (Fig. 1A). In several structures, a sulfate molecule is seen bound between domains 1 and 2, in a position where ATP has been seen in high-resolution structures of similar helicases (Soultanas *et al*., 1999; Velankar *et al*., 1999; Bernstein *et al*., 2003) (Fig. 1A, C). When the entire NS3-NS4A complex is viewed with the ATP and DNA binding sites in the front, the protease is in the back, with its active site buried on the back of the helicase domains (Yao *et al*., 1999) (Fig. 1C). Behind the protease is its NS4A cofactor, which positions the catalytic triad of the protease so it will cleave the NS3–4A junction (Kim *et al*., 1996). The zinc ion needed for the NS2–3 auto-catalytic protease lies on the same side of the protease as NS4A (Fig. 1C). These crystal structures, along with high resolution NMR structures of HCV helicase domain 2 (Liu *et al*., 2001; Liu *et al*., 2003), have greatly influenced proposals explaining how helicases function and have guided experiments designed to test these ideas.

Structural variations
Each of the available HCV helicase crystal structures is shown in one of the panels of Fig. 1. Kim *et al*.'s structure (PDB accession code 1A1V (Kim *et al*., 1998)) is shown in Fig. 1 panels A and B, and Yao *et al*.'s structure (PDB 1CU1 (Yao *et al*., 1999)) is in panels C and D. In panel E, Cho *et al*.'s structure (PDB 8OHM (Cho *et al*., 1998)) and Yao *et al*.'s structure (PDB 1HEI (Yao *et al*., 1997)) are aligned for comparison. Finally, Mackintosh *et al*.'s (Mackintosh *et al*., 2006) structure of a helicase dimer (PDB 2F55) bound to a single oligonucleotide is shown in Fig. 1F. The main difference between the available HCV structures concerns the position of domain 2 relative to domains 1 and 3. Domains 1 and 3 share more of an interface than domain 2 shares with either of the other domains. Domain 2 is connected to domains 1 and 3 *via* flexible linkers, which allow domain 2 to freely rotate relative to domains 1 and 3. In some structures, domain 2 is rotated away

from domain 1 in an "open" conformation, while in other structures domain 2 is closer to domain 1 in a "closed" conformation (Fig. 1E). The pivot point for these rotations is provided by additional contacts between domain 3 and an extended β-hairpin originating from domain 2. An animation showing the rotation of domain 2 is available in the Database of Macromolecular Movements (http://www.molmovdb.org/cgi-bin/morph.cgi?ID=109065–518) (Echols et al., 2003).

How well these structures represent the diverse array of NS3 proteins encoded by all varieties of HCV is not clear because natural variation in the amino acid sequence of NS3 undoubtedly impacts its structure. The known HCV genotypes have remarkably different nucleotide sequences, and the corresponding amino acid substitutions likely would affect protein folding. HCV helicase from only three, very similar, genotypes has been examined at the atomic level. Both Yao et al. (Yao et al., 1997) and Kim et al. (Kim et al., 1998) examined an enzyme isolated from the same genotype 1a H strain, Yao et al. (Yao et al., 1999) examined the helicase from the genotype 1b BK strain, and Cho et al. (Cho et al., 1998) used an enzyme from another genotype 1b strain. Although there are many differences between the structures, no obvious differences appear to be genotype specific; the genotype 1a structures are as different from each other as they are from the genotype 1b structures.

Nevertheless, variation in HCV helicase residues clearly influences its activity, as evidenced by the fact that adaptive mutations in HCV replicons frequently arise in the helicase region (Blight et al., 2000; Krieger et al., 2001; Grobler et al., 2003). To concisely depict helicase sequence variability among various HCV genotypes, a consensus sequence of the NS3 peptide is superimposed on a cartoon of the helicase structure in Fig. 2. Figure 2 was generated by rendering an alignment of all the NS3 sequences deposited in the hepatitis C virus (HCV) database project (http://hcv.lanl.gov/) using a program called Weblogo (http://weblogo.berkeley.edu/) (Crooks et al., 2004). The original alignment can be downloaded from the HCV database by choosing "NS3" under the subheading "alignments." Weblogo depicts an alignment as a sequence logo (Schneider and Stephens, 1990), in which each NS3 residue is represented as a stack of one letter amino acid codes. The height of each stack corresponds to the amino acid conservation at that position. When the residue is invariant, only one letter is shown, and the most common substitutions are noted when the residue is variable.

Lam et al. (Lam et al., 2003b) have explored the impact of genotypic variation on the various activities of HCV helicase by examining recombinant proteins that were isolated from infectious clones of HCV genotype 1a (Yanagi et al., 1997), 1b (Yanagi et al., 1998), and 2a (Yanagi et al., 1999). Although there are some differences between the genotypes, the proteins are surprisingly similar. The main difference between genotype 1 and 2 strains can be attributed to variation at residue 450, which is normally a Thr (Fig. 2), but in the genotype 2a infectious clone it is an Ile (Yanagi et al., 1999). When Thr^{450} alone
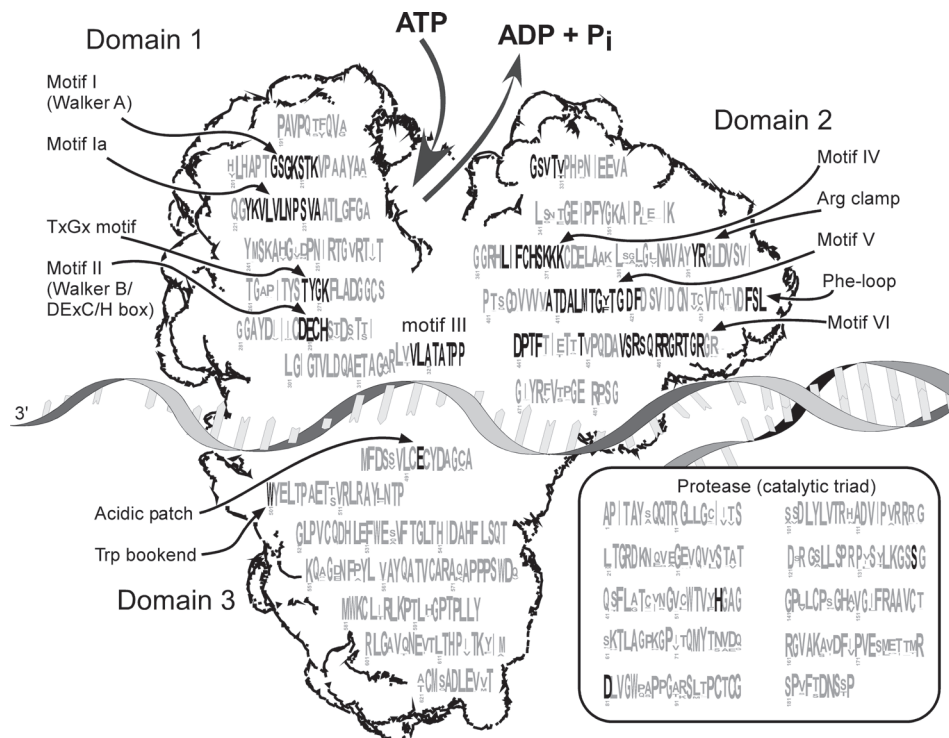

Fig. 2 HCV NS3 sequence conservation. A sequence logo (Schneider and Stephens, 1990) of an NS3 sequence alignment is overlaid on a cartoon of the HCV helicase. Each residue of NS3 is depicted as a stack of letters, the height of which correlates with how well it is conserved in 138 NS3 sequences deposited in the HCV database (http://hcv.lanl.gov/). The height of the letters in each stack correlates with how frequently that amino acid occurs at that position. Conserved superfamily 2 helicase motifs and other key residues are noted and highlighted with bold type.

is changed to Ile, the protein binds ssDNA differently and unwinds DNA faster, suggesting that the interaction of Thr[450] with DNA observed in the crystal structure somehow modulates the rate of helicase movement. Upon close examination of sequences that were later deposited in the HCV database, it appears now that only the particular genotype 2a strain used in our study (Lam *et al.*, 2003b) contains the Ile substitution. Since this was an infectious clone isolated from a chimpanzee (Yanagi *et al.*, 1999), we now believe that T450I is an adaptive mutation that permits the virus to efficiently replicate in chimpanzees, but it is not normally seen in HCV infecting humans.

Conserved motifs

The sequence logo in Fig. 2 also reveals that there are numerous stretches of amino acids that do not vary in known isolates. The numbered sequence motifs are shared with related helicases (Gorbalenya and Koonin, 1993; Hall and Matson, 1999). Some of these motifs, such as the DExC/H-box portion of motif II and motif IV are characteristic only of helicases closely related to HCV helicase, while others, such as the Walker A motif (Motif I), are conserved among all helicases and in a wide variety of other proteins that hydrolyze ATP. Other motifs are found only in HCV and closely related viruses, including the Arg-clamp, the Phe loop (Lam *et al.*, 2003a), and all motifs in domain 3.

It was not possible to precisely depict all the conserved residues in Fig. 2 relative to their position within each domain of HCV helicase, but most are close. Diagrams noting exact motif positions on actual crystal structures have been published elsewhere (Hall and Matson, 1999; Kwong *et al.*, 2000; Lam *et al.*, 2003a; Frick, 2004). Motifs I, Ia, II, III, IV, V, and VI, which are conserved in similar helicases encoded by both viruses and cellular organisms, line the ATP binding cleft, and some of these motifs project residues into the nucleic acid binding site. These seven helicase motifs essentially form the motor which converts the chemical energy derived from ATP hydrolysis into a mechanical force that drives helicase movements leading to the disruption of DNA or RNA base pairs.

The roles of most of the key conserved residues in motifs I through VI have been investigated using site-directed mutagenesis. The various studies are tabulated in Table 1 along with a phenotype of each mutant. The phenotype listed in Table 1 simply depicts whether helicase activity (i.e. DNA or RNA unwinding), ATPase, or nucleic acid binding properties of each mutant is unchanged (normal), diminished, or enhanced. Mutations in motifs I-VI normally impact the ability of the protein to both unwind DNA and hydrolyze ATP, showing that the two activities are coupled. However, sometimes mutations result in decreases of ATP hydrolysis rate, but not a similar corresponding decrease in DNA unwinding. The results have been interpreted by some authors as evidence that ATP hydrolysis is not absolutely required for unwinding, but they could instead be due to the different sensitivities of ATPase and helicase assays under the particular conditions utilized.

Outside motifs I to VI, domains 1 and 2 have regions that are both variable and conserved. With a hope of discovering regions that might provide binding sites for novel anti-HCV therapeutics, our lab examined the role of two motifs in domain 2 that are conserved in all HCV isolates but not related proteins. The rationale was that compounds that bind such sites would be relatively non-toxic because similar sites are not present on related cellular helicases. The first motif identified centered on Arg[393], a residue that contacts the nucleic acid backbone. When Arg[393] is changed to Ala, the protein still catalyzes RNA-stimulated ATP hydrolysis but does not unwind DNA or RNA. The R393A protein also binds DNA weaker both in the presence and absence of a non-hydrolyzable ATP analog, suggesting that this Arg-clamp motif functions to tether the protein to the nucleic acid strand on which it is translocating (Lam *et al.*, 2003a).

The second motif characteristic of only helicases from HCV strains forms a loop connecting two β-sheets that extend from domain 2. The β-loop structure is composed of residues Thr[430] to Ala[452], and a pair of residues, Phe[438] and Phe[444], and is located in a highly conserved region at the loop's tip. The turn of the loop is composed of NS3 amino acids 438 to 444. At the time, the function of this "Phe-loop" was a curiosity. Kim *et al.* (Kim *et al.*, 1998) had proposed that this loop functions like a DNA binding loop found in ssDNA binding proteins. Alternately, Yao *et al.* (Yao *et al.*, 1997) proposed that Phe[438] and Phe[444] could pack into a hydrophobic pocket together with Phe[531], Phe[536], and Trp[532], allowing the loop to take on a more structural role. Both Phe[438] and Phe[444] were altered to Ala to assess these two very different possibilities. Mutagenesis of the Phe's that flank the Phe-loop demonstrates that the loop is not involved in nucleic acid binding. Rather, Phe[438] and Phe[444] are important both for proper protein folding and for modulating conformational changes leading to the release of DNA upon ATP binding (Lam *et al.*, 2003a).

All helicases crystallized to date contain domains that resemble domains 1 and 2, but none share a domain that resembles domain 3. In some helicases, such as PcrA (Subramanya *et al.*, 1996) and Rep (Korolev *et al.*, 1997), two domains replace domain 3, one which extends from domain 1 (called domain 1B) and one that extends from domain 2 (called domain 2B). In several helicase structures that share domains similar to domains 1 and 2 of HCV helicase, such as the RecQ protein (Bernstein *et al.*, 2003), DnaG (Singleton *et al.*, 2001), and eukaryotic translation initiation factor 4A (Caruthers *et al.*, 2000) domain 3 is missing entirely, suggesting that domain 3 might not be required for HCV helicase movements. This is not the case, however, and although its role in unwinding is only beginning to be understood, domain 3 is clearly essential. Deletion of 97 amino acids from the C-terminus of NS3 results in an inactive helicase (Jin and Peterson, 1995; Kim *et al.*, 1997a). Two key residues in domain 3 are Trp[501], which stacks against a nucleic acid base to act like a bookend (Lin and Kim, 1999; Preugschat *et al.*, 2000; Kim *et al.*, 2003), and Glu[493] which helps repel nucleic acids from the binding cleft upon ATP binding (Frick *et al.*, 2004a).

Mechanism of action

There is presently no consensus on exactly how the HCV helicase unwinds RNA. Debate about the HCV helicase mechanism continues largely because in some

Table 1. HCV helicase mutants.

Mutant	Motif	Phenotype	Reference(s)
A204V	I	A^n, B^+, H^-	(Tai et al., 2001)
K210A	I	A^-, B^n, H^-	(Heilek and Peterson, 1997; Levin and Patel, 1999; Min et al., 1999; Wardell et al., 1999)
K210N	I	A^-, B^+, H^-	(Tai et al., 2001)
K210Q	I	A^-, H^-	(Heilek and Peterson, 1997)
K210E	I	A^-, B^n, H^-	(Kim et al., 1997b; Chang et al., 2000)
S231A	Ia	A^+, B^+, H^n	(Lin and Kim, 1999)
T266A		H^-, No dimer	(Khu et al., 2001)
Y267S		H^-, No dimer	(Khu et al., 2001)
T269A	TxGx	A^-, B^-, H^-	(Lin and Kim, 1999)
D290A	II	A^-, B^n, H^-	(Levin and Patel, 1999; Min et al., 1999; Wardell et al., 1999)
D290N	II	A^-, B^n, H^-	(Tai et al., 2001)
E291A	II	A^-, B^n, H^-	(Wardell et al., 1999; Tai et al., 2001)
E291Q	II	A^-, B^n, H^-	(Tai et al., 2001)
C292G	II	A^-, B^n, H^n	(Kim et al., 1997b)
C292S	II	A^-, B^n, H^-	(Kim et al., 1997b; Wardell et al., 1999)
C292A	II	A^-, B^n, H^-	(Tai et al., 2001)
M288T		H^-, No dimer	(Khu et al., 2001)
H293A	II	A^+, B^+, H^-	(Heilek and Peterson, 1997; Kim et al., 1997b; Tai et al., 2001)
H293K	II	A^-, B^+, H^-	(Tai et al., 2001)
H293Q	II	A^-, B^+, H^-	(Tai et al., 2001)
T322A	III	A^-, B^+, H^-	(Kim et al., 1997b; Tai et al., 2001)
T324A	III	A^-, B^n, H^-	(Tai et al., 2001)
H369A	IV	A^n, B^n, H^n	(Frick et al., 2004a)
H369K	IV	A^+, B^+, H^-	(Frick et al., 2004a)
S370A	IV	A^n, B^n, H^n	(Lin and Kim, 1999)
Y392A		A^n, B^-, H^-	(Paolini et al., 2000)
R393A	Arg-clamp	A^n, B^-, H^-	(Lam et al., 2003a)
T411A	V	A^+, B^-, H^-	(Lin and Kim, 1999)
V432A		A^-, B^n, H^-	(Paolini et al., 2000; Preugschat et al., 2000; Tai et al., 2001)
V432D		A^-, B^-, H^n	(Kim et al., 2003)
V432R		A^+, B^+, H^n	(Kim et al., 2003)
F438A	Phe-loop	A^n, B^+, H^-	(Lam et al., 2003a)
F444A	Phe-loop	A^-, B^-, H^-	(Lam et al., 2003a)
T450I		A^n, B^+, H^+	(Lam et al., 2003b)
Q460A	VI	A^-, B^n, H^-	(Kwong et al., 2000)
Q460H	VI	A^-, B^+, H^-	(Kim et al., 1997b; Wardell et al., 1999)
R461A	VI	A^-, B^n, H^-	(Kim et al., 1997b; Kwong et al., 2000)
R461Q	VI	A^-, B^-, H^-	(Tai et al., 2001)
R462A	VI	A^+, B^n, H^n	(Kwong et al., 2000)
R462L	VI	A^-, B^-, H^-	(Kim et al., 1997b; Chang et al., 2000)
G463A	VI	A^-, B^n, H^n	(Kim et al., 1997b)
R464A	VI	A^-, B^n, H^-	(Kim et al., 1997b; Min et al., 1999; Chang et al., 2000; Kwong et al., 2000)
T465N	VI	A^-, B^n, H^n	(Kim et al., 1997b)
G466A	VI	A^-, B^n, H^-	(Kim et al., 1997b)
R467A	VI	A^-, B^n, H^-	(Kwong et al., 2000)
R467K	VI	A^-, B^n, H^-	(Kim et al., 1997b; Wardell et al., 1999)
E493K		A^+, B^+, H^+	(Frick et al., 2004a)
E493Q		A^+, B^+, H^+	(Frick et al., 2004a)
W501A		A^n, B^-, H^-	(Lin and Kim, 1999; Paolini et al., 2000; Preugschat et al., 2000; Tai et al., 2001; Kim et al., 2003)
W501L		A^n, B^-, H^-	(Lin and Kim, 1999)
W501F		A^n, B^n, H^n	(Lin and Kim, 1999; Preugschat et al., 2000; Kim et al., 2003)
W501E		A^n, B^-, H^-	(Kim et al., 2003)
W501R		A^n, B^-, H^-	(Kim et al., 2003)

A^n, Normal ATPase; A^+, Enhanced ATPase; A^-, Poor ATPase; H^n, Normal duplex unwinding; H^+, Enhanced duplex unwinding; H^-, Poor duplex unwinding; B^n, Normal nucleic acid binding; B^+, Enhanced nucleic acid binding; B^-, Poor nucleic acid binding

experiments, HCV helicase appears to function as a monomer, but in others it appears to be a dimer or a higher order oligomer. Below, attempts will be made to reconcile this and some other controversies, but first, to understand the intricacies of these molecular models, it will be necessary to review a few fundamental characteristics of all helicases.

Basic properties of all helicases
All helicases can be divided into two basic groups. Some form rings that encircle DNA (or RNA) while others, like HCV helicase, do not form rings. Both ring and non-ring helicases, are primarily associated with one strand of a double helix and can be classified based on the polarity of that strand. The protein either shifts from the 3'-end to the 5'-end or from the 5'-end to the 3'-end on the strand to which it is mainly bound. The most common method to diagnose the direction of movement is to determine whether the helicase requires a 5'-ssDNA tail or a 3'-ssDNA tail to initiate unwinding. 5'-3' helicases need a 5'-ssDNA tail, and 3'-5' helicases require a 3'-ssDNA tail. HCV helicase is a 3'-5' helicase (Tai *et al*., 1996; Morris *et al*., 2002). As a consequence, if the oligonucleotide bound to HCV helicase in PDB file 1A1V (Fig. 1A) (Kim *et al*., 1998) represents the strand on which HCV helicase translocates, then the duplex portion of the helix would likely be positioned to the right of the protein in Fig. 1A (see cartoon in Fig. 2). Helicases are thirdly classified based on their evolutionary relationships. Gorbalenya and Koonin have used protein sequence comparisons to classify most helicase families into one of three large superfamilies (Gorbalenya and Koonin, 1993). Non-ring helicases are generally members of helicase superfamily 1 (SF1) or superfamily 2 (SF2), while ring helicases are in superfamily 3 (SF3) or in other families not in the three main superfamilies. HCV helicase is a member of SF2, and like all helicases in SF2 shares conserved motifs I through VI described above.

The ring formed by ring helicases usually is composed of six identical subunits assembled in a head-to-tail manner. The rings surround the strand on which the helicase is translocating and the complementary strand passes outside the ring (Egelman *et al*., 1995). ATP binds between the subunits, to the head of one subunit and the tail of an adjacent protomer. There are consequently six ATP binding sites per hexameric ring (Singleton *et al*., 2000). Each subunit of a ring helicase contains a single domain that resembles a domain first seen in the structure of a protein called RecA, which plays a key role in *E. coli* DNA recombination (Story and Steitz, 1992). In ring helicases, ATP hydrolysis leads to rotation of the RecA-like domains which in turn leads to movements of positively-charged loops that protrude into the center of the ring. The positively charged loops bind DNA (Notarnicola *et al*., 1995; Washington *et al*., 1996), and the sequential interaction of the DNA-binding loops with DNA is thought to lead to ring helicase movement (Singleton *et al*., 2000).

In non-ring helicases, like HCV, there are two RecA-like domains in a single protein subunit, and ATP binds between these subunits. In HCV helicase, domains 1 and 2, fold into similar structures although they share no apparent sequence homology. The core of both domains is composed of a series of beta sheets sandwiched between sets of alpha helices. Both domains 1 and 2 are similar to RecA, form the ATP binding site, and contact DNA. The main structural difference between domains 1 and 2 is that domain 2 contains two long beta sheets that project towards domain 3 (the Phe-loop discussed above), which are not present in domain 1.

Mechanism of ATP hydrolysis
Although the position of ATP bound to HCV helicase has not yet been visualized, the mechanism of its hydrolysis most likely resembles that seen in other helicases. The approximate configuration of ATP in the binding site can be seen by comparing a HCV helicase structure with one of a similar helicase that has been crystallized in the presence of a non-hydrolyzable ATP analog. Figure 3A shows the results of a structural alignment of HCV helicase (PDB file 1A1V) with the SF2 helicase RecQ bound to ATPγS (PDB file 1OYY) (Bernstein *et al*., 2003). Shown only are the ATPγS (from 1OYY), the HCV helicase, and its bound oligonucleotide (both from 1A1V). Residues that likely play key roles in ATP hydrolysis are highlighted as sticks.

The configuration of residues at the ATP-binding site depicted in Fig. 3A is reminiscent of that seen in all other helicases that have been studied bound to NTPs (Sawaya *et al*., 1999; Soultanas *et al*., 1999; Velankar *et al*., 1999; Singleton *et al*., 2000; Bernstein *et al*., 2003; Gai *et al*., 2004; James *et al*., 2004). ATP and a required metal ion cofactor (depicted as Mg^{2+} in Fig. 3A) normally bind to a helicase in the cleft that separates two adjacent RecA-like domains. The most critical residues for ATP binding arise from the Walker A and B motifs (Walker *et al*., 1982). The Walker A motif of HCV helicase forms a phosphate binding loop (P-loop) with the conserved Lys[210] likely contacting the γ phosphate of ATP. The Walker B motif contains acidic residues that coordinate the positively charged divalent metal cation, which in turn contacts the phosphates of ATP. In the alignment in Fig. 3A, Asp[290] seems to be ideally suited to coordinate the catalytic metal. In or near the Walker B motif of helicases and related proteins, there is normally a residue which acts as a catalytic base by accepting a proton from the water molecule that attacks the γ phosphate of ATP. Normally, the catalytic base in this class of enzymes is a glutamate (Goetzinger and Rao, 2003; Orelle *et al*., 2003), and Glu[291] seems to be properly positioned to perform this function.

A more detailed analysis of HCV structures suggests that the roles of particular residues might be somewhat more complicated than assumed above. For example, to function as a catalytic base, the pK_a of Glu[291] would need to be much higher than that of a typical Glu in a protein. However, electrostatic analysis of all HCV helicase structures reveals that neither Glu[291], nor any nearby Glu, has an abnormally high pK_a. In contrast, Asp[290] has a pK_a as high as 10 in some structures and as low as 3 in others. Interestingly, in structures in the open conformation (such as 8OHM), the pK_a of Asp[290] is low, and in the closed conformation (ex. 1A1V), the pK_a of Asp[290] is higher than 7, suggesting that Asp[290] picks up a proton (like a catalytic base) when the protein changes from the open to the closed conformation. Thus, Asp[290] could serve as
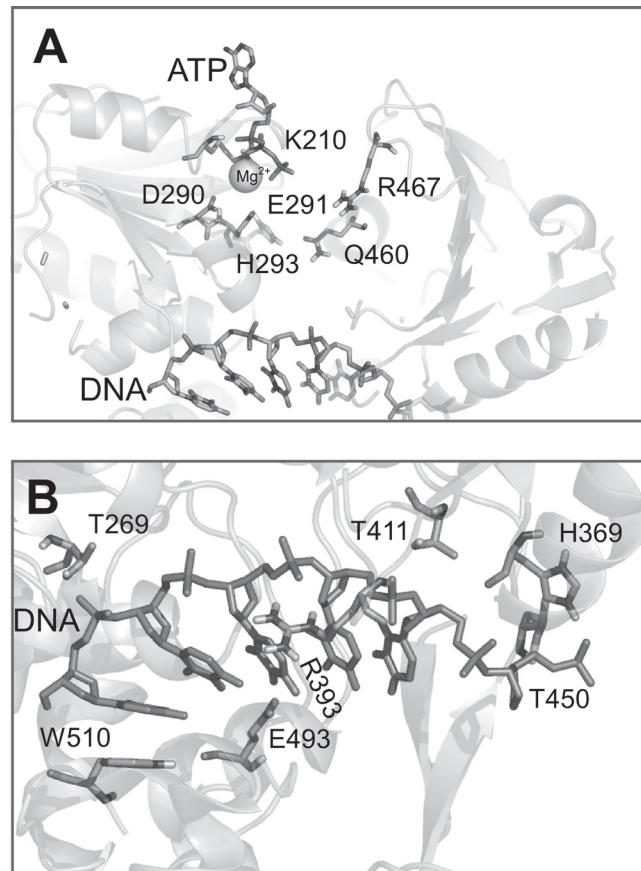
Fig. 3 Key residues in HCV helicase. (A) HCV helicase residues likely involved in modulating ATP binding and hydrolysis. The approximate position of ATP bound to HCV helicase is revealed by a structural alignment of HCV helicase (PDB file 1A1V) (Kim *et al*., 1998) with the SF2 helicase RecQ bound to ATPγS (PDB file 1OYY) (Bernstein *et al*., 2003). (B) Key residues contacting the oligonucleotide bound to HCV helicase in PDB file 1A1V (Kim *et al*., 1998).

a catalytic base instead of, or in addition to, coordinating the magnesium-ATP complex (Frick *et al*., 2004a).

Once the water molecule is activated, it acts as a nucleophile, most likely attacking the terminal phosphate of ATP. The pentavalent transition state, where the γ phosphate is bound to 5 oxygen atoms, then breaks down into ADP and inorganic phosphate. In similar ATP hydrolyzing enzymes, the transition state is stabilized by one or more positively charged residues, normally arginines, that function in concert with the lysine of the Walker A motif. In many enzymes, including helicases, these "arginine fingers" are frequently part of adjacent protein subunits. For example, in small GTPases like Ras, the Arg-finger that activates GTP hydrolysis is part of the GTPase-Activating Protein (GAP) (Ahmadian *et al*., 1997). In F_1ATPase, an Arg-finger on the alpha subunit stabilizes the transition state (Nadanaciva *et al*., 1999). Recently, it was shown that in ring helicases, the Arg-finger and P-loop are part of different polypeptide chains in the hexamer (Crampton *et al*., 2004), demonstrating why ring helicases need to oligomerize to cleave ATP. In HCV helicase, several arginines are present in domain 2, and they line the ATP binding cleft. These residues are part of conserved motif IV and include Arg[461], Arg[462], Arg[464], and Arg[467]. In the model shown in Fig. 3, Arg[467] and Arg[464] are nearest the phosphates of ATP. Either could rotate even closer to ATP if the cleft between domains 1 and 2 closes. Of the two, only Arg[467] is shown because it is of the most interest. Arg[467] is methylated by cellular protein arginine-methyltransferase I (Rho *et al*., 2001). Although it is still unclear how methylation influences helicase activity, such a modification should eliminate all activity if Arg[467] acts as an Arg-finger. Site-directed mutagenesis supports this contention. When Arg[467] is changed to Lys (Kim *et al*., 1997b; Wardell *et al*., 1999) or Ala (Kwong *et al*., 2000), the proteins do not unwind RNA, and ATPase activity is decreased over 10-fold. An R464A mutant has a similar effect (Kim *et al*., 1997b; Min *et al*., 1999; Kwong *et al*., 2000).

Many of the other conserved residues help to properly position the above groups by forming networks of hydrogen bonds, ionic bonds, and hydrophobic interactions. In addition, some conserved residues help coordinate the rotation of domain 2. Two such amino acids are noted on Fig. 3A. His[293] in motif II (domain 1) and Gln[460] in motif VI (domain 2) are near each other in many structures and could interact. Kim *et al*. called these residues "gatekeepers" and propose that they might provide a switch modulating the opening and closure of the cleft between domains 1 and 2 upon ATP binding (Kim *et al*., 1998). Mutation of either residue has a profound and interesting effect on ATPase. Mutation of Gln[460] abolishes detectable ATPase, but an H293A mutation results in a protein with a significantly *higher* level of ATPase in the absence of RNA, and the protein still unwinds RNA. In the presence of RNA, the H293A mutant hydrolyzes ATP

slower than wildtype, to such an extent that RNA appears to *inhibit* ATP hydrolysis (Kim *et al*., 1997b). These data further support the idea that rotation of domain 2 is related to ATP hydrolysis, and that domain closure leads to a completion of the active site and hydrolysis of ATP. The question of how ATP hydrolysis is translated into helicase movement on nucleic acid still remains unanswered, however. The first two models that were applied to HCV helicase to explain its movements suggest that either the protein operates as a monomer like an inchworm (Kim *et al*., 1998) or as a dimer, which rolls along nucleic acid (Cho *et al*., 1998).

The inchworm and rolling models

If ATP binding and hydrolysis leads to movement of domain 2 relative to domain 1, then this conformational change would in turn impact interactions between residues in these two domains with RNA. A close-up of the DNA binding site of structure 1A1V is shown in Fig. 3B, with key amino acids highlighted. Unlike SF1 helicases, which have many interactions with nucleic acid bases (Velankar *et al*., 1999), most of the contacts occur with protein side chains and the sugar-phosphate backbone of DNA (Kim *et al*., 1998). One key hydrogen bond is donated from domain 1 residue Thr^{269}, which is part of the conserved TxGx motif, and an analogous interaction arises from motif V residue Thr^{411} in domain 2. Mutagenesis of either residue affects both RNA binding affinity and unwinding rates (Lin and Kim, 1999). Also noted on Fig. 3B are residues Thr^{450}, Arg^{393}, Trp^{501}, and Glu^{493}, whose roles were alluded to above.

Unlike ring helicases that need to oligomerize to cleave ATP because the Arg-finger is located on the opposite side of a protein monomer relative to the Walker A motif, all the residues necessary for ATP hydrolysis are present in a single polypeptide chain in HCV helicase. Monomeric models for HCV helicase action state that upon rotation, ATP binding leads to a closure of the cleft between domains 1 and 2 by a rotation of domain 2 relative to the rest of the protein, a movement first observed in the structures of Yao *et al*. (Yao *et al*., 1997). Such conformational changes conceivably could cause the protein to act like an inchworm to move along RNA. Most monomeric models are variations on the "ratcheting inchworm" model first proposed for HCV helicase by Kim *et al*. (Kim *et al*., 1998). Based on the observation that the oligonucleotide appears to be locked into the binding cleft because a residue in domain 3, Trp^{501}, is stacked against the 3'-terminal base, Kim *et al*. proposed that ATP binding, and the subsequent closure of the cleft between domains 1 and 2, will lead to a ratcheting of Trp^{501} past 1 or 2 nucleotides. Consequently, the protein would move towards the 5'-end of the bound nucleic acid. After ATP is hydrolyzed and Trp^{501} is again locked into place acting as a bookend, the cleft opens and RNA slides through the other side of the protein. Kim *et al*. proposed that the residue that acts as a 5'-bookend, analogous to the 3'-bookend Trp^{501}, might be Val^{432} in domain 2 (Kim *et al*., 1998).

The dimeric models for HCV helicase action are essentially variations on Wong and Lohman's rolling dimer hypothesis that was used to explain the actions of a dimer formed by the *E. coli* Rep helicase upon DNA binding (Wong and Lohman, 1992). In the rolling dimer, each subunit alternates between a form that prefers to bind ssDNA and a form that preferentially binds a double helix. Switching between the states is modulated by ATP binding and hydrolysis. In theory, both forms are bound to a DNA fork, with one subunit bound to the ssDNA tail, and the other bound to the duplex region. When the trailing subunit changes conformation so that it prefers to bind duplex DNA, it will roll toward the double helix causing the subunit bound to the duplex to wrench one strand away from its complement so that it can then bind the resulting ssDNA (for review see (Lohman and Bjornson, 1996)). A modified rolling model was applied to the HCV helicase by Cho *et al*. (Cho *et al*., 1998), who observed that two HCV helicase monomers could pack together. Cho *et al*. called their model a "descending molecular see-saw" and proposed that RNA could thread through a long cleft formed between domains 1 and 2 of adjacent subunits (Cho *et al*., 1998). However, the later structure by Kim *et al*. (Kim *et al*., 1998) showing DNA bound in another cleft (Fig. 1A), coupled with the fact that ATP likely binds in the cleft between domains 1 and 2 (Fig. 3), makes such an orientation seem unlikely.

More recently Mackintosh *et al*. have crystallized two HCV helicase monomers bound to the same oligonucleotide revealing an interface between the two subunits. When this interface is perturbed using site-directed mutagenesis, HCV sub-genomic replicons fail to replicate in cells but there are only small effects observed in unwinding assays, suggesting that the interface is more important for inter-protein interactions than for unwinding (Mackintosh *et al*., 2006). When this structure is aligned with the structure of the full-length NS3 protein (Fig. 1F), it is apparent that much of second subunit occupies the same space as the protease domains of NS3 (the NS3 fragment used in the Mackintosh *et al*. study lacks the protease). Thus, in order for the helicase to oligomerize in such a manner, the protease domain would need to rotate away from the helicase, as has been proposed by Yao *et al*. (Yao *et al*., 1999).

Evidence for a functional monomer (the inchworm model)

Ever since the HCV helicase portion of NS3 was first purified, it was apparent that it behaved as a monomer and did not need to oligomerize to cleave ATP. Initial studies found HCV helicase to act as a monomer in solution based on gel filtration (Preugschat *et al*., 1996) and analytical ultracentrifugation (Porter *et al*., 1998). As discussed above, the monomeric enzyme has all the residues necessary to catalyze ATP hydrolysis, and as a result, no decrease in turnover number (k_{cat}) is observed when HCV helicase is diluted (Levin and Patel, 2002). In contrast, diluting a ring helicase leads to a loss of the ability to hydrolyze ATP at low protein concentrations (Guo *et al*., 1999).

Soon after the ratcheting inchworm model was introduced for HCV helicase (Kim *et al*., 1998), it was tested by several groups using site-directed mutagenesis (Table 1). Most initial interest focused on the residues that act as bookends, or the teeth of the ratchet. Several

groups have confirmed the importance of Trp[501] in both nucleic acid binding and unwinding (Lin and Kim, 1999; Paolini *et al*., 2000; Preugschat *et al*., 2000; Kim *et al*., 2003). Without a bulky aromatic amino acid at position 501, HCV helicase is unable to unwind RNA (Lin and Kim, 1999; Tai *et al*., 2001; Kim *et al*., 2003) but retains some ability to unwind DNA (Kim *et al*., 2003), albeit more slowly than wild type (Preugschat *et al*., 2000). 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 Val[432] in Domain 2 (Kim *et al*., 1998), but Paolini *et al*. have suggested that Tyr[392] could play a similar role (Paolini *et al*., 2000). Some of these reports have suggested that mutation of these residues leads to decreases in helicase activity (Paolini *et al*., 2000; Preugschat *et al*., 2000; Tai *et al*., 2001; Kim *et al*., 2003).

More recently, other predictions made by the inchworm model have been tested. One basic prediction is that binding of ssDNA to the cleft separating domain 3 from domains 1 and 2 activates ATP hydrolysis. Supporting this theory, several mutations have been made in the DNA binding cleft that decrease the affinity of the protein for both DNA and RNA and affect rates of ATP hydrolysis (see Table 1) (Kim *et al*., 1997b; Lin and Kim, 1999; Tai *et al*., 2001). However, there exists an alternate explanation for such results. The mutant proteins might not fold properly or may be less stable than the wildtype, explaining the decreased binding and unwinding activity. In contrast to these negative results, our lab has recently found that substitution of one of two residues in the DNA binding cleft, His[369] or Glu[493], enhances binding and lowers the amount of nucleic acid needed to stimulate ATP hydrolysis. For example, an E493K mutant enhances binding to RNA in the presence of ATP by several orders of magnitude. This positive result provides the clearest evidence that DNA/RNA binding to this region activates ATP hydrolysis (Frick *et al*., 2004a).

Another prediction made by the inchworm model is that the ssDNA bound between domain 3 and domains 1 and 2 is the strand on which the helicase is translocating in a 3' to 5' direction. We have tested this idea by analyzing a helicase in which Arg[393] is changed to Ala. Without this Arg-clamp in the DNA binding cleft (Fig. 3B), the protein cannot unwind DNA or displace proteins bound to ssDNA. The R393A protein retains full RNA-stimulated ATPase activity, and still binds ssDNA with the same stoichiometry as wildtype, albeit more weakly. These data provide strong evidence that the protein moves along the strand seen in the crystal structure in a 3' to 5' direction and the duplex region would lie as diagrammed in Fig. 2 (Lam *et al*., 2003a).

The inchworm model lastly predicts that ATP binding should modulate the affinity of the protein for RNA (or ssDNA). Early kinetic studies of nucleic acid stimulation of ATP hydrolysis suggest that, indeed, ATP binding weakens the affinity of the protein for RNA (Preugschat *et al*., 1996). However, it was difficult to confirm this observation by directly measuring dissociation constants because HCV helicase only weakly interacts with most common non-hydrolyzable ATP analogs. A breakthrough came when Levin *et al*. found that the presence of BeF$_3$ tightly locks the reaction product, ADP, on the enzyme (Levin *et al*., 2003). In other systems, BeF$_3$ coordinates like the γ phosphate of ATP, so that ADP(BeF$_3$) is essentially a non-hydrolyzable ATP analog (Xu *et al*., 1997). Using ADP(BeF$_3$), Levin *et al*. showed that when ATP binds HCV helicase, affinity for DNA falls by almost two orders of magnitude. More recently, Lam *et al*. have shown that nucleic acid binding to HCV helicase is pH dependent in the presence of ADP(BeF$_3$) but not in the absence of the analog, demonstrating that a conformational change occurs upon ATP binding (Lam *et al*., 2004), as is also predicted by the inchworm model.

Evidence for a functional oligomer (the rolling model)
While the evidence that HCV helicase acts as a monomer is convincing, there is also evidence that multiple subunits interact with each other to efficiently unwind RNA. Yeast two-hybrid assays provide the most persuasive evidence that NS3 interacts with itself (Flajolet *et al*., 2000; Khu *et al*., 2001). In such experiments, the minimum peptide required for an NS3-NS3 interaction contains only domain 1 residues 162–335. This peptide surrounds conserved motifs I, II, and III (Khu *et al*., 2001), suggesting domain 1 would interact with domain 1 of another monomer. Three residues, which were identified using a reverse two hybrid screen, are critical for dimer formation, Thr[266], Tyr[267] and Met[288] (Khu *et al*., 2001). Met[288] is not conserved and is normally an Ile in all but a few HCV isolates (Fig. 2). Mutations of these residues not only influence dimer formation that can be assayed using gel filtration, but also the ability of the protein to unwind DNA (Khu *et al*., 2001).

Oligomerization of NS3 seems to be dependent on nucleic acids. Before the yeast two-hybrid data were reported, Levin and Patel demonstrated that DNA aids the ability to chemically crosslink HCV helicase into high molecular weight species (Levin and Patel, 1999). Dimerization of NS3 has also been visualized using analytical gel filtration, but only in the presence of an oligonucleotide (Khu *et al*., 2001). Nucleic acid binding data can sometimes be fit to models that do not take into account subunit interactions (Porter, 1998b; Porter, 1998a; Porter *et al*., 1998; Levin and Patel, 2002), but under certain conditions, cooperative models fit the data better (Locatelli *et al*., 2002; Frick *et al*., 2004b). Taken together, these data suggest that two or more HCV helicase protomers cooperatively assemble onto ssDNA (or RNA) in a controlled manner.

Other evidence for oligomerization has emerged from measurements of rates of HCV helicase catalyzed DNA and RNA unwinding. Notably, unwinding rates are not linearly dependent on the amount of protein present in the reaction, but rather, accelerate greatly once a critical protein concentration is reached (Lam *et al*., 2003a; Frick *et al*., 2004b). By measuring unwinding under single-turnover conditions, several groups have presented kinetic models explaining this cooperativity (Levin *et al*., 2004; Serebrov and Pyle, 2004; Tackett *et al*., 2005). As reviewed elsewhere (Bianco, 2004), these models all take into account the interaction of multiple protomers aligned on a ssDNA (or RNA) strand and attempt to calculate the number of base pairs unwound in a single turnover event (called "step size"). The theory holds that

an oligomer would unwind many base pairs (10 or more) in a single event while a monomer would only unwind a few base pairs at a time. Levin *et al.* (Levin *et al.*, 2004) have calculated a step size of 9 base pairs using DNA, and using a long RNA substrate, Serebrov and Pyle have determined that 18 base pairs are unwound by HCV helicase in a single step (Serebrov and Pyle, 2004). These values support a rolling dimer model that undergoes a periodic cycle of pausing and unwinding and stand in stark contrast with an older calculation by Porter *et al.* that only a few base pairs of fluorescently-labeled DNA are unwound by HCV helicase in a single turnover event (Porter *et al.*, 1998). This periodic cycle of pausing and unwinding was recently confirmed in a collaborative study between Pyle's group and Carlos Bustamante's group using optical tweezers to examine the action of single molecules of NS3 on RNA (Dumont *et al.*, 2006). Notably, however the single molecule study did *not* confirm that NS3 need to oligomerize to unwind RNA.

Other mechanisms explaining helicase movement
Because neither the inchworm nor the rolling model fully explains all elements of helicase action, additional models

have been recently proposed. One such model states that HCV helicase acts like a Brownian motor (Astumian, 1997, Levin *et al.*, 2005). A Brownian motor exploits random movements that constantly occur on the molecular level (Brownian motion) and an asymmetrical path to shift an object in a single direction. As diagrammed in Fig. 4A, collisions that occur between HCV helicase, water, and other small molecules constantly transfer small amounts of momentum to the protein so that it wobbles slightly relative to the RNA to which it is bound. In the absence of ATP, the helicase is constrained in a certain location due to molecular barriers. However, when HCV helicase binds ATP, it releases its grip on RNA by changing conformation so that it is free to move along RNA. Random collisions will then be more likely to transfer enough momentum that the protein clears the barrier constraining it to its original position. The key to the Brownian motor model is the asymmetry of the path on which the motor is traveling. Because the path is asymmetrical in the Brownian motor model, the protein will be more likely to move in one direction than the other, and the net result of many movements will be movement in a single direction. If the path were symmetrical, then the protein would be equally
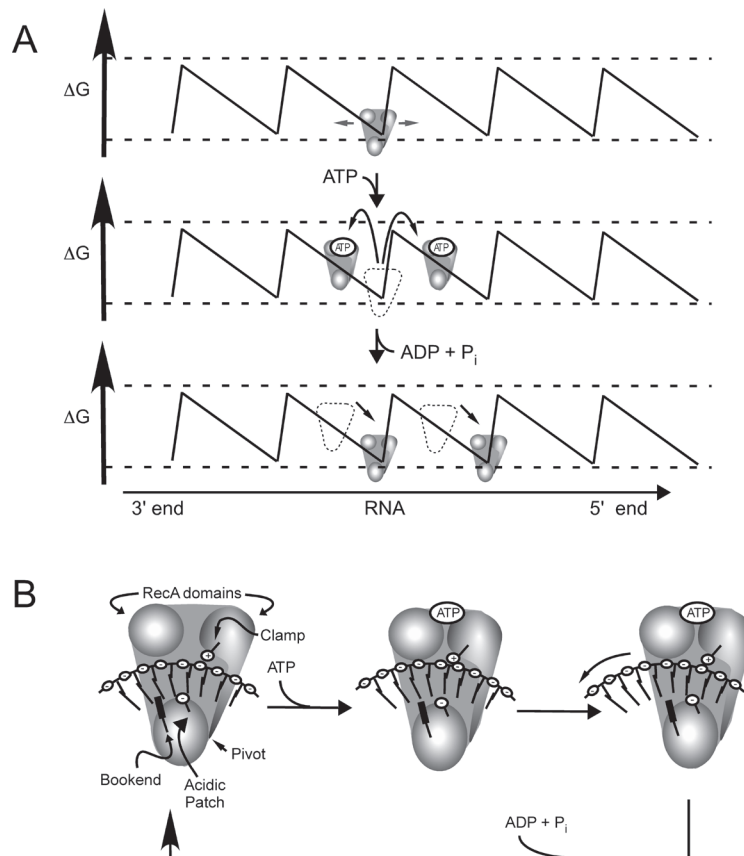

Fig. 4 Two possible mechanisms for HCV helicase translocation on RNA. (A) The Brownian motor model (Levin *et al.*, 2005). In the absence of ATP, HCV helicase is confined in a single location on an asymmetrical path of RNA. When ATP binds, binding releases the protein from RNA, allowing random movement (Brownian motion) to transport the helicase either in a 5' or 3' direction. Because the path is asymmetrical, molecules moving in the 3' direction will return to their original position, whereas molecules moving in the 5' direction will change positions. Net movement will be in a 5' direction. (B) The propulsion-by-repulsion model (Frick *et al.*, 2004a; Lam *et al.*, 2004). ATP binding rotates domain 2 so that a positively charged Arg-clamp (Lam *et al.*, 2003a) moves the RNA so that it clears Trp[501], which is holding the RNA in a negatively charged cleft. When ATP is bound, the protein repels RNA past Trp[501] so that the protein moves in a 5' direction until ATP is hydrolyzed and the protein returns to its original conformation.

likely to return to the original position as it would be to move to either adjacent position, and the net result would be no movement.

In Fig. 4, this irregularity is depicted as an asymmetrical free energy diagram. If the helicase moves in a 5' direction, then it will likely clear a free energy peak and descend to the base of another valley towards the 5' end of the RNA. On the other hand, if the protein moves toward the 3' end of the RNA by the same amount, it will not clear the peak and will return to the position at which it began the cycle. Thus, even though movements occur randomly in either direction and many molecules will remain in the same location, the net result will be that most molecules will move in the same 3' to 5' direction. This model has been applied to HCV helicase by Levin *et al.* (Levin *et al.*, 2003), who recently observed that HCV helicase has a higher affinity for a partially duplex DNA substrate with 3'-ssDNA tails, than it does for either ssDNA alone, or DNA with a 5'-ssDNA tail. They propose that interaction with the fork of the DNA leads to asymmetry of the free energy diagram (Levin *et al.*, 2005). While movements towards the fork likely play some role in helicase movement, the fact remains that two groups have independently observed translocation of HCV helicase in a 3' to 5' direction in systems containing no duplex portion on the ssDNA substrate (Morris *et al.*, 2002; Lam *et al.*, 2003a). Thus, if the Brownian model holds true for HCV helicase, then all asymmetry in the free energy diagram should be intrinsic to the helicase and the nucleic acid strand on which it is traveling.

Our lab has proposed another model to explain HCV helicase movement that suggests that HCV helicase utilizes electrostatic forces to move along DNA and RNA (Frick *et al.*, 2004a; Lam *et al.*, 2004). This "propulsion-by-repulsion" model (Fig. 4B) is based on two observations. First, DNA is tightly bound in a pocket of the enzyme that is highly negatively charged (see Fig. 1B). Second, release of DNA from the enzyme is pH dependent; the enzyme binds weaker to DNA in the presence of ATP at a higher pH. The first observation hints that there is a potential energy buildup when the protein is locked onto DNA in the absence of ATP. The second observation suggests that ionizable residues come in contact with DNA upon ATP binding. We have shown using mutagenesis that one of these key residues is Glu[493] in the ssDNA binding cleft (Frick *et al.*, 2004a). In our model, ATP binding leads to a conformational change such that the nucleic acid bases can clear the Trp[501] bookend (Lam *et al.*, 2004). In the absence of ATP, RNA cannot exit the enzyme because it is blocked by Trp[501] and clamped in the cleft by the Arg-clamp on domain 2 (Lam *et al.*, 2003a). When ATP binds, domain 2 rotates bringing with it the positively-charged Arg-clamp. The Arg-clamp attracts the negatively-charged phosphodiester backbone so that RNA moves free from the bookend. The negatively-charged RNA is then repelled by the negatively charged binding cleft, so it moves through the protein until ATP is hydrolyzed, and the protein clamps it tightly again. In such a model, the step size of the helicase would depend on the nature of the nucleic acid on which the protein is translocating explaining, in part, why different step sizes have been calculated using different substrates (Porter *et al.*, 1998;

Levin *et al.*, 2004; Serebrov and Pyle, 2004; Dumont *et al.*, 2006).

Role of the protease domain and NS4A
Reviewing the HCV helicase literature is perplexing because frequently the data reported in one study differs from that reported in other studies. Rates of ATP hydrolysis, DNA/RNA unwinding, and dissociation frequently differ by more than 10-fold. The most likely explanation for such differences (when the same protocol is used) is that most labs utilize different recombinant versions of HCV helicase. Many of these proteins are quite different because they either (1) include different portions of NS3, or (2) have been isolated from different HCV strains. Because full-length NS3 is difficult to express in *E. coli*, a truncated protein containing only NS3 residues 166–631 is frequently used. However, some studies have used helicase constructs with more or fewer N-terminal NS3 residues. Furthermore, some studies use a helicase lacking fusion proteins, whereas other studies utilize a helicase with a N-terminal or C-terminal His-tags, a T7-tag, a GST-tag, or combinations of multiple tags. Frequently, the tags are not removed before analysis.

We have compared numerous NS3 constructs in our laboratory to try to understand why different studies have reported such different results. Initially, we thought that such variation might be due to intrinsic differences between the HCV genotypes. Our comparison of three helicases isolated using the same procedure from three different genotypes noted some differences, but these tended to be small (less than 2-fold) and did not explain the widely divergent data in the literature (Lam *et al.*, 2003b). We then set out to compare the effect of fusion proteins, which are attached to the helicase to aid expression and purification, and found that these modifications led to major changes in activity (Frick *et al.*, 2004b). While modifications to the C-terminus did not effect most assays, modification to the N-terminus did, suggesting that the protease domain and the conformation of the region linking it to the helicase could have a major role in aiding the cooperative assembly of the protein on RNA and in unwinding (Frick *et al.*, 2004b).

In our hands (Frick *et al.*, 2004b), full-length NS3 (with NS4A) unwinds RNA better than versions lacking the protease, but hydrolyzes ATP slower, suggesting that it is a more efficient molecular motor. Some of the effects of the protease could be substituted for by GST or His-tag fusion proteins, but several could not, suggesting that RNA makes specific contacts with the protease region. As discussed above, it is not clear where the complementary strand or the duplex region of RNA interacts with HCV helicase. Nevertheless, an electrostatic analysis of the full-length protein (Fig. 1D) reveals that a positively-charged cleft is formed between the protease and domain 2 of the helicase. Residues in this cleft could tether the protein to the negatively-charged phosphate backbone of RNA. It is possible that a similar cleft could be formed when the protease is replaced with a fusion protein, explaining why such proteins have a higher apparent processivity than the helicase domain alone (Frick *et al.*, 2004b).

Not all other studies have noted as clear differences when the protease domain is removed from NS3. For

example, even though Kuang *et al.* found that a NS3-NS4A complex unwinds RNA better than an isolated helicase domain, they also noted NS3 lacking NS4A is a poor helicase relative to an isolated helicase domain, suggesting that the protease without its NS4A cofactor might actually inhibit helicase movements (Kuang *et al.*, 2004). Similarly perplexing data showing relatively poor helicase activity for full-length NS3 have been reported by others (Heilek and Peterson, 1997; Gallinari *et al.*, 1998). The poor helicase activity of some full-length NS3 constructs could be explained by the conformational flexibility of the protein. In order to cleave the rest of the polyprotein, Yao *et al.* proposed that the protease domain swings away from the helicase *via* the flexible linker that connects the two regions (Yao *et al.*, 1999). If this occurs, then the putative RNA binding cleft proposed above would be disrupted and the helicase would more rapidly dissociate from RNA substrates.

A model in which RNA binds a cleft between domain 2 of the helicase and the protease would also provide a plausible role for the NS4A peptide in facilitating helicase action. NS4A could hold the protein in a conformation so that the RNA binding cleft between the protease and helicase remains intact, explaining why some investigators find that a NS3-NS4A complex unwinds RNA better than NS3 alone. For example, Pang *et al.* (Pang *et al.*, 2002) compared the activities of a NS3-NS4A complex expressed in insect cells (Sali *et al.*, 1998) with a His-tagged, full-length NS3 protein expressed and purified from *E. coli,* and found that the NS3-NS4A complex requires less time to form a functional complex on RNA. Based on the structure of the NS3-NS4A complex (Fig. 1C), it is difficult to envision a direct interaction between NS4A and RNA, as has been proposed by others (Silverman *et al.*, 2003). Thus, we prefer a model where NS4A stabilizes the formation of an RNA binding cleft on NS3 (Frick *et al.*, 2004b).

In addition to being a better RNA helicase, we also find that the full-length protein oligomerizes more readily than the truncated protein lacking a protease domain, indicating that the protease domain properly configures the protein for oligomerization. As evidence, we find twice as many protomers of full-length NS3 bound to a single oligonucleotide as recombinant proteins containing the helicase domain only (Frick *et al.*, 2004b). This key observation explains why early studies using isolated helicase domains lacking the protease failed to detect cooperative assembly (Preugschat *et al.*, 1996; Levin and Patel, 2002; Lam *et al.*, 2003a), while later studies using the full-length NS3 often detect oligomers (Khu *et al.*, 2001; Locatelli *et al.*, 2002; Frick *et al.*, 2004b).

HCV helicase inhibitors

Because compounds that inhibit a helicase encoded by herpes simplex virus (HSV) have been recently shown to moderate disease symptoms (Crute *et al.*, 2002; Kleymann *et al.*, 2002), there has been great interest in finding inhibitors of HCV helicase. Many compounds that inhibit HCV helicase have been reported, and they can be broadly classified as small molecules, nucleic acids, or antibodies. There are, however, many obstacles that must be overcome before developing helicase inhibitors into viable antiviral agents. The main problem will likely be

toxicity because the motor domains of HCV helicase are conserved in a vast array of cellular proteins. Consequently, there is more focus on finding inhibitors that bind sites that are not conserved with cellular enzymes, such as the RNA binding site(s) described above and possible allosteric regulatory sites. Even if these inhibitors are never developed into drugs, they should still be useful for elucidating the role of HCV helicase in the viral lifecycle.

Small molecules
Many of the small molecules that were initially examined as HCV helicase inhibitors were nucleoside analogs. Although nucleoside analogs might also inhibit cellular proteins by interacting with conserved Walker sequences, there is some potential for these compounds because there is a possibility that nucleotides could bind to a second site on the helicase in addition to the conserved Walker site. Such a site could be formed, for example, if the protein oligomerizes and ATP binds to an interface between the RecA-like domains of adjacent subunits. Porter *et al.* first detected a possible second nucleotide binding site when they studied product inhibition in the presence of NaF. In their studies, about *two* moles of ADP bound per protein monomer (Porter, 1998a). In contrast, as discussed above, when beryllium fluoride is added to the reaction, only one mole of ADP is bound per protomer (Lam *et al.*, 2003a; Levin *et al.*, 2003). One model explaining these data assumes that the helicase functions as a dimer with ATP bound tightly to the interface between domains 1 and 2 and ADP bound more weakly to a second interface. ADP fluoride complexes likely do not resemble the substrate, ATP, as closely as ADP(BeF$_3$), so they might bind both active and allosteric sites. Also in support of a second NTP-binding site, Locatelli *et al.* have demonstrated that nucleotides bind HCV helicase cooperatively (Locatelli *et al.*, 2002).

There is also some evidence that the second potential nucleotide binding site on HCV helicase is more specific than the nucleotide binding site between domains 1 and 2. The alignment in Fig. 3 reveals few contacts are made between HCV helicase and the sugar or base of an NTP, explaining the observed non-specificity of this site. HCV helicase hydrolyzes all eight canonical nucleoside triphosphates (Preugschat *et al.*, 1996; Wardell *et al.*, 1999; Lam *et al.*, 2003b). The seven other (d)NTPs are competitive inhibitors of ATP hydrolysis (Lam *et al.*, 2003b) and most studies find that they all support unwinding. However, one study found that only some NTPs fuel unwinding with efficiency comparable to that seen with ATP (Locatelli *et al.*, 2001). Other (d)NTPs, particularly dATP, were found to be poor substrates and potent inhibitors of unwinding (Locatelli *et al.*, 2001). Such results can be explained if NTP binding to a regulatory site is more specific than NTP binding to the catalytic site. For example, the regulatory site might only bind dATP but not the NTPs that fail to inhibit unwinding.

Regardless of whether nucleoside analogs will ever be developed into anti-HCV therapeutics, the effects of such compounds on HCV helicase have been extensively studied. Examples include ribavirin triphosphate (Borowski *et al.*, 2001), 5'-O-(4-fluorosulphonylbenzoyl)-esters of ribavirin (FSBR), adenosine (FSBA), guanosine

(FSBG) and inosine (FSBI) (Bretner *et al*., 2004), and ring-expanded ("fat") nucleosides and nucleotides (Zhang *et al*., 2003). Much of the data has been previously reviewed (Borowski *et al*., 2002a; Borowski *et al*., 2002b). Generally, such compounds inhibit only at very low ATP concentrations, and are competitive with ATP, so that under physiological conditions little or no inhibition is observed.

Compounds resembling nucleoside bases have also been reported to be HCV helicase inhibitors. For example, tetrachlorobenzotriazole (TCBT) and tetrabromobenzotriazole (TBBT) were recently analyzed as helicase inhibitors. Both compounds inhibit unwinding catalyzed by helicases from related viruses (such as West Nile virus) with IC_{50}'s in the low micromolar range, but only TBBT inhibits RNA unwinding by HCV helicase (IC_{50} ~60 μM). Neither compound inhibits helicase-catalyzed ATP hydrolysis (Borowski *et al*., 2003), but it is still not clear whether these compounds bind a true allosteric site or if they inhibit unwinding by non-specific interactions with the nucleic acid substrate. Also a compound that resembles nucleotides (called QU663) was recently reported to be a potent HCV helicase inhibitor that competes with the nucleic acid substrate but not ATP with a Ki of 0.75 μM (Maga *et al*., 2005)

Many groups have reported non-nucleoside based inhibitors of HCV helicase, primarily in the patent literature. These compounds include a piperidine derivative, heterocyclic carboxamide, antracycline antibiotics (Borowski *et al*., 2002b), paclitaxel, trifluoperazine (Borowski *et al*., 2002a), and aminophenylbenzimidazole derivatives (Phoon *et al*., 2001). Many of these compounds intercalate in nucleic acids and likely act *via* that non-specific mechanism. Whether or not any of these compounds or other small molecules decrease HCV replication measured using replicons or animal models is still yet to be reported.

Nucleic acid based inhibitors
One of the unique properties of HCV helicase is that, unlike other helicases, the protein binds RNA and DNA in a sequence specific manner. Even the first studies of the protein noted that HCV helicase has a distinctive nucleic acid stimulation profile (Suzich *et al*., 1993). This means that ATP hydrolysis is stimulated by some nucleic acid polymers much better than it is stimulated by others. The range is quite dramatic. Poly(G) RNA does not stimulate at any measurable level, and poly(U) RNA (or DNA) stimulates best (up to 50 fold). Interestingly, differential stimulation is not entirely due to differences in binding affinity. Direct binding assays confirm that poly(U) binds HCV helicase tighter than polymers composed of the other bases (Gwack *et al*., 1996), but at saturating nucleic acid concentrations, not all sequences support the same maximum rate of ATP hydrolysis, suggesting that the protein assumes different conformations when bound to different sequences (Lam *et al*., 2003b). RNA specificity has also been proposed to play a role in directing the helicase, and possibly the entire HCV replication complex, to certain regions of the viral genome. For example, HCV helicase specifically binds both to the 3'-UTR and the 3'-end of the negative strand viral transcript (the complement of the 5'-UTR). This might be necessary during the viral lifecycle to allow the NS5B polymerase to synthesize RNA in these regions that contain stable secondary structures (Banerjee and Dasgupta, 2001).

Nucleic acid interactions with HCV helicase depend not only on the base composition but also on the composition of the nucleic acid backbone. It is widely recognized that HCV helicase unwinds a DNA duplex more efficiently than an RNA duplex. The biological reason for this, if there is one, is still a mystery because there is no DNA stage in the viral lifecycle, and replication likely occurs on the endoplasmic reticulum (Wolk *et al*., 2000). However, some reports have detected NS3 in the nucleus (Muramatsu *et al*., 1997; Errington *et al*., 1999), where the helicase could modify host gene expression (Sakamuro *et al*., 1995). Whereas loss of the 2'-OH group from RNA permits the helicase to unwind substrates faster (DNA is unwound faster than RNA), adding a methyl group to this position (2'-O-methyl RNA) weakens helicase interaction with RNA and prevents unwinding (Hesson *et al*., 2000). The effects are strand specific in that the helicase only appears to sense the chemical composition of the strand with the 3'-overhang (the longer strand in the helicase substrate). Composition of the shorter strand does not affect unwinding rates as drastically, suggesting that interactions are made primarily with the nucleic acid sugars in the single-stranded region not in the double-stranded region of the helicase substrate. When the longer strand (with the 3' overhang) is DNA, the shorter strand can be composed of RNA, 2'-O-methyl RNA, morpholino-DNA, or phosphorothioate-DNA without affecting unwinding (Hesson *et al*., 2000; Tackett *et al*., 2001; Pang *et al*., 2002). However, if the shorter strand is composed of peptide nucleic acid (where a N-(2-aminoethyl)glycine backbone replaces the deoxyribose phosphates), then unwinding is slower than with natural substrates (Tackett *et al*., 2001). Whether peptide nucleic acids are poor substrates because of the lack of specific interactions with HCV helicase, or simply because they form more stable duplexes, is still unclear (Tackett *et al*., 2001).

Two groups have tried to exploit the nucleic acid specificity of HCV helicase with a goal of developing RNA-based inhibitors. Both groups have used SELEX (systematic evolution of ligands by exponential amplification) to find RNA aptamers that tightly bind HCV helicase. In the SELEX procedure, an RNA library is screened for sequences that bind a macromolecule. Only those sequences that bind tightly are amplified to create a new library, and the selection process is repeated with the new library. Although directly using RNA as an antiviral drug will be challenging because of its cellular instability, the information derived from aptamer studies could be used to make more stable derivatives or by delivering RNA directly to infected cells using gene therapy.

One set of aptamers specific to HCV helicase was generated by modifying aptamers that bind tightly and inhibit the NS3-NS4A serine protease. Such aptamers were found to bind truncated NS3 lacking the helicase domains using SELEX. They all share the conserved sequence GA(A/U)UGGGAC (Fukuda *et al*., 2000), bind NS3 protease over one thousand times tighter than

random RNA sequences and are effective at inhibiting the HCV NS3 protease (Fukuda *et al.*, 2000; Nishikawa *et al.*, 2003). When positions Arg[130], Arg[161], and Lys[165] are substituted with Ala, the aptamers no longer bind NS3, suggesting that they interact near these NS3 residues, which are located in the region that links the protease to the helicase (Hwang *et al.*, 2000). To create an aptamer that inhibits protease and helicase activity of NS3, a 14-mer uridine tail was added to one of the most effective HCV protease-binding RNA aptamers. The new, longer aptamer interacts with both the protease and helicase domains of the full-length NS3 protein (Fukuda *et al.*, 2004), binds to the helicase portion of NS3 with high affinity (K_d ~4 nM) (Fukuda *et al.*, 2004), and inhibits the NS3 helicase activity with an EC_{50} of ~500 nM. The same group has recently reported a new "advanced dual functional" aptamer in which another aptamer, selected for helicase binding (Nishikawa *et al.*, 2004), is tethered to a protease-binding aptamer using a poly(U) linker. This new aptamer is about five times more effective than either aptamer when they are not covalently linked (Umehara *et al.*, 2005).

A second group has also selected for aptamers using the helicase portion of NS3 as the bait in the SELEX procedure (Hwang *et al.*, 2004). This aptamer (called SE RNA) folds to form four stem loops with GC pairs that are similar to the stem loop located at the 3'-terminal of the negative strand HCV RNA. This observation suggests that the SE aptamer might bind the helicase in a similar manner as the stem loop located at the 3'-terminal of the negative strand HCV RNA (Banerjee and Dasgupta, 2001). SE RNA binds the HCV helicase tightly (K_d ~990 pM), efficiently competes with poly(U), stimulates ATP hydrolysis, and potently inhibits RNA unwinding (IC_{50} ~12.5 nM). When delivered to human liver cells (Huh 7) infected with HCV replicons, the SE aptamer slows HCV RNA synthesis, and interestingly, labeled SE aptamers can also be used as a diagnostic tool to detect the NS3 protein in cells from HCV patients (Nishikawa *et al.*, 2004).

Antibodies
The third, and possibly most ambitious, method that is currently being explored to inhibit HCV helicase is to generate antibody-like molecules that, when expressed intracellularly, will bind and inhibit HCV helicase activities. Almost all HCV patients produce antibodies directed against the NS3 protein, and the vast majority of these bind to the helicase portion of the protein (Chen *et al.*, 1998). Several groups are working toward the goal of introducing recombinant antibodies into cells for "cellular immunization," a procedure which has been used experimentally with HIV (Goncalves *et al.*, 2002). In this approach, HCV infected cells are transfected with a gene expressing a portion of an antibody selected for reactivity with NS3. One method is to use single chain fragment (ScFv) antibodies. A ScFv is composed of the immunoglobulin heavy chain variable domain connected to the variable region of the light chain by a polypeptide linker. Such a molecule can be constructed using PCR. The other principal method uses an antibody fragment (Fab), which contains the complete light chain and the variable and first constant domains of the heavy chain. A Fab is larger and usually more stable than a ScFv.

To construct a ScFv, immunoglobulin specific PCR is first used to construct a library of human antibody fragments using plasma cells from HCV patients as the PCR template. To identify which antibodies react with HCV helicase, the fragments are fused to a bacteriophage coat protein for phage display, and phages with a high affinity for HCV helicase are purified. Tessman *et al.* have used this technique to isolate a series of high affinity ScFv's that specifically interact with HCV helicase (Tessmann *et al.*, 2002). ScFv's that bind HCV helicase have also been constructed by splicing together the variable domains of monoclonal antibodies (Zhang *et al.*, 2000; Sullivan *et al.*, 2002), and after expression and purification, several of these recombinant proteins inhibit HCV helicase-catalyzed DNA unwinding (Sullivan *et al.*, 2002; Artsaenko *et al.*, 2003). One particular ScFv consists of the variable regions of the human monoclonal antibody CM3.B6, which recognizes an epitope that spans conserved SF2 helicase motifs IV and V (Mondelli *et al.*, 1994). The CM3.B6 ScFv has been expressed in HCV infected hepatocytes (HepG2 cells), immunoblots of which reveal an intra-cellular interaction between the antibody and NS3. HCV RNA synthesis within primary hepatocytes infected with HCV is also reduced by 10-fold when the cells contain a vector carrying the CM3.B6 ScFv gene (Sullivan *et al.*, 2002).

Phage display has also been used to isolate an anti-HCV helicase Fab from a patient infected with HCV genotype 1b. Prabhu *et al.* have isolated this human Fab, called HFab-aNS3, and demonstrated that it has HCV antiviral activity (Prabhu *et al.*, 2004). HFab-aNS3 recognizes an epitope that spans motifs I to V of the protein, and when purified and pre-incubated with HCV helicase, HFab-aNS3 abolishes detectable DNA unwinding. Intracellular expression of HFab-aNS3 within replicon-transfected Huh 7 cells suppresses NS3 protein expression and significantly inhibits viral RNA synthesis of both subgenomic and full-length HCV replicons (Prabhu *et al.*, 2004).

Conclusions and future directions
HCV helicase has attracted the attention not only of researchers interested in developing novel antiviral drugs, but also those studying how proteins interact with nucleic acids. As one of only three helicases that have been crystallized bound to an oligonucleotide, HCV helicase has become one of the best model proteins to study how helicases unwind duplexes and move on DNA and RNA. Different theories on how chemical energy stored in ATP is transformed into the mechanical force necessary to move a protein from one position to another are currently hotly debated, and many have been tested using the HCV NS3 protein. Obviously, work will continue until HCV helicase's precise mechanism of action is defined.

While it is still uncertain if HCV helicase functions using an inchworm, rolling, Brownian, or electrostatic mechanism, extensive work has uncovered its basic properties and the roles of several key residues. ATP binds HCV helicase between two RecA-like domains,

causing a conformational change that leads to a decrease in the affinity of the protein for nucleic acids. Key residues contacting ATP include Lys^{210}, which likely coordinates the phosphates, Asp^{290}, which could coordinate a divalent metal ion, Glu^{291}, which might act as a catalytic base, and one or more arginines on the adjacent domain. One strand of RNA binds in a second cleft formed perpendicular to the ATP-binding cleft and its binding leads to stimulation of ATP hydrolysis. RNA and/or ATP binding likely causes rotation of domain 2 of the enzyme relative to domains 1 and 3, and somehow this conformational change allows the protein to move like a motor. Key residues involved in RNA binding include Trp^{501}, which locks the protein in position in the absence of ATP, and Glu^{493}, which repels RNA when ATP binds.

Clearly, the biological role of HCV helicase needs to be investigated in more detail. It has long been a mystery why RNA viruses that replicate outside the nucleus encode a helicase. A protein that resolves duplex RNA and DNA structures and displaces proteins bound to nucleic acids, like HCV helicase, could be valuable to [1] provide single stranded templates to the viral polymerase, [2] resolve secondary structure blocking translation or transcriptions, [3] strip proteins from viral RNA, or [4] regulate cellular gene expression. Based on three lines of information, the HCV helicase most likely assists RNA dependent RNA replication by tracking along RNA and resolving double stranded intermediates that form either as secondary structures in a single strand or between (+) sense and (-) sense RNA molecules. First, one group has shown *in vitro* that NS3 stimulates the ability of NS5B to synthesize long RNAs (Piccininni *et al*., 2002). Second, the motor action of NS3 on RNA is clearly established. Third, recent studies with subgenomic replicons show that HCV genomes with mutations in the helicase domains of NS3 replicate HCV RNA poorly in cells (Lam and Frick, 2006; Mackintosh *et al*., 2006). More specifically, our study with subgenomic replicons determined that replicons lacking a functional helicase translated and processed the polyprotein normally and showed reduction in both (–) strand and (+) strand RNA synthesis (Lam and Frick, 2006). Similarly, when an antibody against HCV helicase is co-expressed in cells expressing HCV replicons, there is not only diminished positive strand synthesis but also less synthesis of HCV negative strand RNA and HCV proteins, suggesting that the helicase plays numerous complex and important roles in the viral lifecycle (Prabhu *et al*., 2004).

While presently it appears that HCV protease and HCV polymerase inhibitors will be developed as the next generation of anti-HCV drugs, compounds inhibiting HCV helicase might also someday prove therapeutically useful. Both NS5B and NS3 protease have clearer roles in HCV replication, and unlike helicases, the mechanisms of serine proteases and RNA polymerases have been understood for decades. Consequently, it is not surprising that HCV helicase inhibitor development lags behind that for the other HCV enzymes. As long as its mechanism and role in replication are not clearly understood, development of antiviral drugs targeting HCV helicase will remain difficult. Nevertheless, rapid progress is being made in the helicase field, and it will not be surprising if HCV helicase inhibitors someday enter clinical trials.

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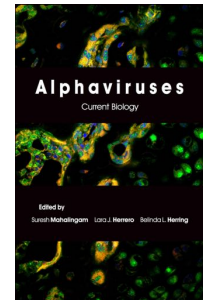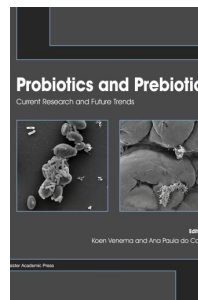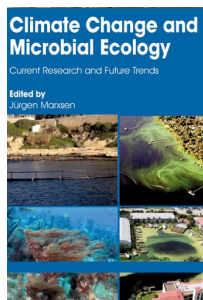
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