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Hepatitis C Virus Subgenomic Replicon Requires an Active NS3 RNA Helicase

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Mutations were introduced into the NS3 helicase region of a hepatitis C virus (HCV) Con1 subgenomic replicon to ascertain the role of the helicase in viral replication. One new replicon lacked two-thirds of the NS3 helicase (Δhel), and six others contained one of the following six amino acid substitutions in NS3: R393A, F438A, T450I, E493K, W501A, and W501F. It has been previously reported that purified R393A, F438A, and W501A HCV helicase proteins do not unwind RNA but unwind DNA, bind RNA, and hydrolyze ATP. On the other hand, previous data suggest that a W501F protein retains most of its unwinding abilities and that purified T450I and E493K HCV helicase proteins have enhanced unwinding abilities. In a hepatoma cell line that has been cured of HCV replicons using interferon, the T450I and W501F replicons synthesized both negative-sense and positive-sense viral RNA and formed colonies after selection with similar efficiencies as the parent replicon. However, the Δhel, R393A, F438A, and W501A replicons encoded and processed an HCV polyprotein but did not synthesize additional viral RNA or form colonies. Surprisingly the same phenotype was seen for the E493K replicon. The inability of the E493K replicon to replicate might point to a role of pH in viral replication because a previous analysis has shown that, unlike the wild-type NS3 protein, the helicase activity of an E493K protein is not sensitive to pH changes. These results demonstrate that the RNA-unwinding activity of the HCV NS3 helicase is needed for RNA replication.

Hepatitis C virus (HCV) is a single-stranded positive-sense RNA virus with a 9.6-kilobase genome that encodes one long polypeptide, which is processed into at least 10 structural and nonstructural (NS) proteins. The structural proteins form the viral capsid and its glycoproteins, while the NS proteins are responsible for the replication of the viral genome. Among the HCV replicative proteins, the NS3 protease/helicase is one of the best characterized. However, the biological role that the helicase portion of NS3 plays during the replication cycle of the virus still remains largely unclear. HCV helicase is composed of the C-terminal two-thirds of NS3. Although the N-terminal protease region, which is responsible for processing HCV proteins NS3 through NS5B, is not absolutely required for unwinding, it facilitates the interaction of NS3 and RNA and accelerates helicase action (6, 8, 15, 36). Structurally (3, 12, 34, 35), HCV helicase is a three-domain protein that shares several conserved motifs with other related cellular and viral helicases and helicase-like motor proteins, all of which are located in two N-terminal helicase domains (domains 1 and 2). The C-terminal domain (domain 3) contains no motifs conserved with other helicases, and structurally similar domains have not been seen in related cellular proteins. One strand of nucleic acid binds in the cleft between domain 3 and the first two domains, and ATP likely binds in the cleft separating domains 1 and 2. It is not clear where the complementary strand or the duplex region binds to the protein.

The HCV helicase possesses the ability to (i) bind and hydrolyze nucleoside triphosphates, (ii) interact with both RNA and DNA, (iii) translocate in a 3′-to-5′ direction, (iv) separate nucleic acid base pairs, and (v) displace nucleic acid binding proteins. To initiate unwinding, HCV helicase requires a single-stranded region with a 3′-end overhang on which to load, and the energy from ATP hydrolysis is believed to fuel both translocation and unwinding. Unlike related helicases, HCV helicase cleaves ATP relatively rapidly in the absence of RNA (or DNA). This basal ATPase activity is stimulated up to 100-fold by nucleic acids depending on the nucleic acid sequence and whether or not the protease region is present (6, 17, 31). Also of interest is the fact that HCV NS3 helicase unwinds DNA more efficiently than RNA duplexes (9, 26) even though HCV replication does not involve any known DNA intermediate during its replication cycle.

The most logical biological role for HCV helicase is to assist the NS5B RNA-dependent RNA polymerase with viral replication by resolving RNA secondary structures and/or double-stranded replication intermediates. There is even evidence for the coordinated action of NS3 and NS5B (10, 28, 36). However, it is equally possible that cellular helicases perform this function, and recently HCV NS5B has been shown to recruit and interact with the cellular RNA helicase p68, which in turn assists in synthesis of minus-strand HCV RNA (7). Besides unwinding viral RNA, the motor action of HCV helicase could also perform other cellular functions such as assisting translation, coordinating polyprotein processing, disrupting RNA-protein interaction, packaging RNA in viral capsids, and even separating cellular DNA to alter host gene expression. This study was initiated to explore these possibilities.

The need for the ATPase function of HCV NS3 in viral replication has been demonstrated by changing NS3 residues D290 and E291 to alanines (DE→AA) in an HCV infectious clone. When DE→AA HCV RNA is transfected into cells, a

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polyprotein is translated and processed, but when it is injected into a chimpanzee, no infection occurs (14). Based on other work (19, 24, 32, 33), the DE→AA mutation would undoubtedly abolish the ability of NS3 to cleave ATP and hence all motor functions of HCV helicase. To examine the role of the helicase in more detail, this study examined the effect of other NS3 mutations in a subgenomic replicon system (1). The subgenomic replicon is a simpler system than the chimpanzee as there is no production of virions, and it facilitates a more rigorous examination of polyprotein translation, processing, minus-strand transcription, and plus-strand RNA synthesis. The chosen amino acid substitutions (R393A [17], F438A [17], T450I [16], E493K [5], and W501A/F [13, 20, 27, 30, 32]) have been previously shown to influence RNA unwinding, but, unlike the DE→AA mutation, they do not abolish ATP hydrolysis, DNA unwinding, or RNA binding. Three of the residues (R393, F438, and T450) are located within domain 2, while the other two (E493 and W501) are located in helicase domain 3. The enzymatic impact of the mutations is clear from previous work. Three of the mutations, R393A and F438A (17) and W501A (13, 20, 27, 30, 32), lead to proteins that have no ability to unwind RNA but that retain some ability to unwind DNA. The W501F protein (13, 20, 30) retains an ability to unwind RNA that is somewhat diminished compared to the wild type. The T450I (16) and E493K (5) mutations lead to helicases that bind nucleic acid more tightly and unwind duplexes better in standard unwinding assays.

In this study, hepatoma cells were transfected with replicons containing the above mutations within the NS3 protein in order to examine the role of NS3 helicase in HCV replication in vivo. Not surprisingly, cells containing R393A, F438A, and W501A replicons did not synthesize viral RNA or form colonies after selection, demonstrating that the helicase must unwind RNA for HCV replication. A cellular helicase cannot replace NS3 helicase, and the ability of HCV helicase to unwind DNA is not sufficient to support replication. Mutants that retain the ability to unwind RNA (T450I and W501F) support RNA replication and colony formation. Curiously, the E493K replicon did not form colonies even though that helicase can unwind nucleic acid in vitro (5). The enzymatic impact of the E493K mutation is that it allows the protein to bind RNA tightly over a broad pH range. While HCV helicase normally unwinds poorly in vitro above pH 7.5, E493K retains an ability to function at high pH (5). Our findings provide the first conclusive evidence that RNA unwinding catalyzed by HCV helicase is needed for viral RNA synthesis and support the idea that this activity might be regulated by pH changes.

MATERIALS AND METHODS

Materials. The highly permissive cell line for HCV RNA replication Huh-7.5 (2) and plasmids expressing blasticidin-selectable Con1 strain subgenomic replicons (4, 25) containing the NS5A S2204I adaptive mutation without (pBDL429P+S+) or with (pGDD-/Bsd) a defective NS5B polymerase were generous gifts from Charles Rice and Brett Lindenbach (Rockefeller University, New York, NY). The replicon lacking the polymerase mutations contains two unique restriction sites (introduced by silent mutagenesis) flanking the NS3 region and is referred to here as S2204I-Bsd. The isogenic replicon with the polymerase mutations is referred to as GDD$^-$. Dulbecco's modified Eagle medium (DMEM), heat-inactivated fetal bovine serum (FBS), nonessential amino acids (NEAA), trypsin-EDTA, and blasticidin were purchased from Invitrogen (Carlsbad, CA). Phosphate-buffered saline (PBS) was purchased from Cambrex (Verviers, Belgium). All RNase-free solutions were purchased from Ambion (Austin, TX). DNA oligonucleotides were synthesized by Integrated DNA Technologies (Corralville, IA).

Cell culture. Huh-7.5 cells were propagated in complete medium (DMEM supplemented with 10% heat-inactivated FBS and 0.1 mM NEAA). Blasticidin was added at a final concentration of 3 μg/ml to cells containing subgenomic replicons. Both naive and replicon-containing Huh-7.5 cells were passaged twice a week after treatment with 0.05% trypsin-EDTA and were seeded at a dilution of 1:3 to 1:4.

Mutagenesis. A SacII-SpeI fragment containing the NS3 region of pBD L429P+S+ was subcloned into a pGEM-5Zf(+) vector from Promega (Madison, WI), which was digested with the same restriction enzymes. The ligated product, pGEM-Con1NS3, was used to construct each helicase mutant (R393A, F438A, T450I, E493K, W501A, and W501F) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Note that amino acid numbering of the NS3 helicase starts from the first amino acid in the full-length NS3 protein. To perform site-directed mutagenesis, the following oligonucleotides were used: conR393A(+), 5′-GCT GTA GCA TAT TAC GCG GGC CTT GAT GTA TCC GTC-3′; conR393A(−), 5′-GAC GGA TAC ATC AAG GCC CGC GTA ATA TCG TAC AGC-3′; conF438A(+), 5′-CAG ACA GTC GAC GCC AGC CTG GAC CCG-3′; conF438A(−), 5′-CGG GTC CAG GCT GGC GTC GAC TGT CTG-3′; conT450I(+), 5′-CCA TTG AGA CGA CGA TCG TGC CAC AAG ACG-3′; conT450I(−), 5′-CGT CTT GTG GCA CGA TCG TCG TCT CAA TGG-3′; conE493K(+), 5′-CCT CGG TTC TGT GCA AGT GCT ATG ACG CG-3′; conE493K(−), 5′-CGC GTC ATA GCA CTT GCA CAG AAC CGA GG-3′; conW501A(+), 5′-CGG GCT GTG CTG CGT ACG AGC TCA CGC-3′; conW501A(−), 5′-GCG TGA GCT CGT ACG CAG CAC AGC CCG-3′; conW501F(+), 5′-GCG GGC TGT GCT TTC TAC GAG CTC ACG CC-3′; conW501F(−), 5′-GGC GTG AGC TCG TAG AAA GCA CAG CCC GC-3′. The NS3 deletion mutant (Δhel) which encodes an NS3 protein lacking amino acids 330 to 627 was constructed using the oligonucleotides Δhel (+), 5′-CCT CCG GGA TCG GTC GAG GTC GTC ACG AGC-3′, and Δhel (−), 5′-GCT CGT GAC GAC CTC GAC CGA TCC CGG AGG-3′. The Δhel deletion was confirmed by sequencing, and the Δhel region was digested with SacII and SpeI and reinserted into a similarly digested pBDL429P+S+. After confirming each of the other mutations by sequencing, they were then inserted into the Δhel replicon vector, replacing the Δhel region, using the SacII and SpeI restriction enzyme sites. The final mutagenized plasmids were confirmed by restriction analysis and were sequenced again to verify that each replicon plasmid carried the appropriate mutation.

Replicon RNA transcription. Expression plasmid DNA encoding either wild-type NS3 or its NS3 mutants was linearized with ScaI. Linearized DNA was digested with proteinase K at 60°C for 2 h; the DNA was then purified by extracting first with phenol and then phenol-chloroform and was subsequently recovered using ethanol precipitation. Linearized DNA was then transcribed into RNA with T7 RNA polymerase (20 units) from Epicenter (Madison, WI) at 37°C for 1.5 h in transcription buffer (40 mM Tris, pH 7.5, 10 mM NaCl, 12.5 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 3.75 mM of each nucleoside triphosphate, and 12 units of SUPERasin [Ambion]). HCV RNA was purified after treatment with the RNase-free DNase using the QIAGEN (Valencia, CA) RNeasy minikit. RNA concentrations were determined by UV spectrophotometry, and the integrity of the RNA was checked using 1% agarose gel electrophoresis.

Transfection. Huh-7.5 cells were transfected with replicon RNA using a procedure based on one previously described (25). Trypsinized Huh-7.5 cells were washed twice with ice-cold RNase-free PBS. Cells were counted and suspended at 1.5×10^7 cells/ml in ice-cold RNase-free PBS. A total of 6×10^6 Huh-7.5 cells were mixed with 1 μg HCV replicon RNA, transferred to a 2-mm-gap electroporation cuvette, and pulsed immediately (820 V, 99 μs, five pulses at 1.1-s intervals) using a BTX ECM 830 square wave electroporator from Genetronics (San Diego, CA). Cells were allowed to recover at room temperature for 10 min before being diluted in complete medium to a final volume of 10 ml.

Blasticidin selection. Cells were seeded into 100-mm-diameter dishes at 6×10^5, 6×10^4, 6×10^3, and 6×10^2 cells per dish together with cells transfected with GDD$^-$ RNA transcripts such that the total number of cells in each dish was maintained at 6×10^5. Seventy-two hours after electroporation, selection was started with 3 μg/ml of blasticidin (4, 25). Blasticidin selection was carried out twice a week. After 3 weeks of antibiotic selection, cells were fixed with 7% formaldehyde for 10 min and stained with 1.25% crystal violet in 20% ethanol for 2 h. After being washed with water, stained colonies were counted to determine efficiency of HCV replication (colony-forming efficiency [CFU/μg HCV RNA]). Selection was repeated three times with three independent batches of cells for each replicon.

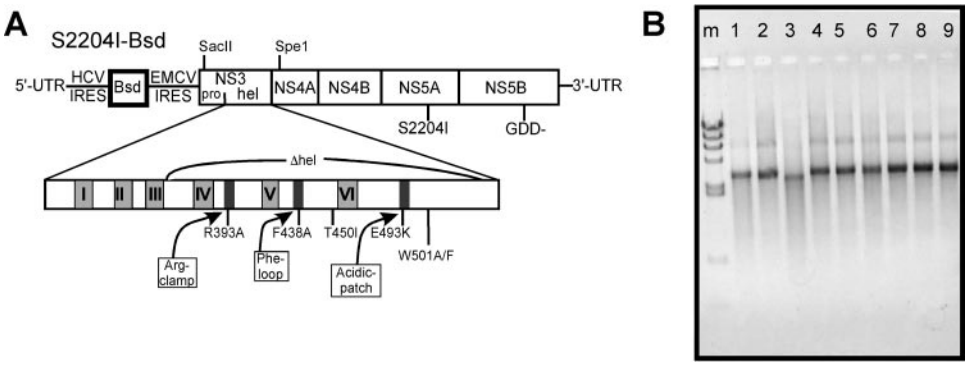

FIG. 1. (A) Schematic representation of the Con1 strain blasticidin-selectable subgenomic replicon S2204I-Bsd. The HCV 5′ UTR is fused to the HCV IRES sequence, followed by the blasticidin S deaminase gene, the encephalomyocarditis virus (EMCV) IRES, the HCV NS3 to NS5B coding region, and the HCV 3′ UTR. The GDD$^-$ variant with a lethal mutation in the NS5B active site (G2737A, D2738A, or D2739G) was used as a negative control. All replicons contain the adaptive mutation (S2204I) within the NS5A region. Site-directed mutagenesis was used to engineer mutations (R393A, F438A, T450I, E493K, W501A, and W501F) into the NS3 helicase region, and their locations relative to the conserved superfamily 2 helicase motifs are indicated. (B) In vitro-transcribed HCV RNA replicons analyzed on a 1% agarose gel with λ DNA digested with HindIII as markers (lane m). Lane 1, S2204I-Bsd; lane 2, GDD$^-$; lane 3, Δhel; lane 4, R393A; lane 5, F438A; lane 6, T450I; lane 7, E493K; lane 8, W501A; lane 9, W501F.

Real-time RT-PCR. Total cellular RNA was extracted from Huh-7.5 cells using the TRIZOL reagent (Gibco-BRL). DNA was synthesized from equal amounts of cellular RNA using SuperScript II RNase H- reverse transcriptase (RT) (Invitrogen) in the presence of 100 nM of one of two primers complementary to the HCV 5′ untranslated region (UTR). Positive-strand HCV RNA was synthesized using a 5′-UTR antisense primer (5′-CCA AAT CTC CAG GCA TTG AGC-3′), and negative-strand HCV RNA was synthesized using a 5′-UTR sense primer (5′-CCT CTA GAG CCA TAG TGG TCT-3′). Primer sequences were the same as those used in reference 2. After 50 min at 50°C, reverse transcriptase was inactivated. Ten percent of the reaction mixture was then used for real-time PCR using the LightCycler FastStart DNA Master SYBR Green I kit (Roche). Reaction mixtures containing both 5′-UTR sense and antisense primers (500 nM) and hot-start *Taq* polymerase were heated to 95°C for 10 min, followed by 40 cycles of PCRs of 15 s at 95°C and 1 min at 60°C. In vitro-transcribed HCV RNA of known concentrations was used to generate a standard curve. Real-time PCR signals were analyzed using the LightCycler software (Roche; version 5.32), and the sizes and uniqueness of PCR products were verified by performing both melting curves and agarose gel electrophoresis.

Western blot analysis. Electroporated Huh-7.5 cells were transfected with 20 μg of HCV RNA as described above, and cells were seeded into 100-mm plates. Four hours after electroporation, cells were washed once with PBS and gently scraped off the plate. Cells were harvested by centrifugation and lysed using 5% sodium dodecyl sulfate (SDS). Protein samples were separated on 10% SDS-polyacrylamide gels and subsequently transferred to nitrocellulose membranes. Transferred proteins were incubated with TBST blocking buffer (Tris-buffered saline with 0.1% Tween 20) containing 5% dry milk for 1 h. Intracellular HCV NS3 and NS5A proteins were detected with either an anti-NS3 monoclonal antibody (C65859M) or an anti-NS5A monoclonal antibody (C65388M) (both were purchased from Biodesign, Saco, ME). After being washed twice with TBST, the membranes were incubated with secondary antibody conjugated to horseradish peroxidase in blocking buffer containing 2% dry milk. After 1 h, the membrane was washed four times with TBST and bound antibodies were detected by chemiluminescence using the SuperSignal West Pico chemiluminescent substrate from Pierce (Rockford, IL). The nitrocellulose membranes then stripped with Western Re-probe buffer from Geno Tech (St. Louis, MO), blocked again, and reprobed using a β-actin antibody from Sigma (St. Louis, MO).

RESULTS

Con1 replicons containing NS3 helicase mutations. Six single-amino-acid substitutions (R393A, F438A, T450I, E493K, W501A, and W501F) were introduced into a subgenomic replicon (Con1 strain) using site-directed mutagenesis (Fig. 1A). To facilitate mutagenesis and sequencing, a truncated replicon (Δhel) was first constructed by deleting the region encoding NS3 amino acids 330 to 627. Since this replicon lacks most of HCV helicase domains 2 and 3, it should lack any helicase activity and was used as a negative control in all experiments. A restriction fragment containing the NS3 coding sequence was excised, altered using site-directed mutagenesis, sequenced, and then reinserted into the Δhel replicon expression plasmid. Flanked on each side by the HCV 5′ and 3′ UTRs, the parent replicon contains the blasticidin S deaminase gene, which confers resistance to blasticidin upon expression, followed by the coding region of the HCV replicative proteins NS3 to NS5B (referred to as S2204I-Bsd) (4, 25). Expression of the blasticidin S deaminase gene is under the control of the HCV internal ribosome entry site (IRES), and the translation of the HCV NS proteins is directed by a second IRES element derived from the encephalomyocarditis virus. All replicons contain one adaptive mutation (S2204I) within the NS5A region, which allows for higher transduction efficiency when compared with the original sequence (1). In addition, an NS5B polymerase-defective replicon in which the critical GDD motif in the polymerase active site is replaced by AAG (referred to as GDD$^-$) was used to balance cell numbers in plates and to normalize HCV RNA levels. After mutagenesis of the replicon plasmid DNA, the new replicons were transcribed into RNA, and the integrity of the transcribed RNA was examined using agarose gel electrophoresis (Fig. 1B).

R393A, F438A, E493A, and W501A replicons do not form blasticidin-resistant colonies. Electroporated Huh-7.5 cells were transfected with replicon RNA, and cells were plated at four different densities by diluting with cells transfected with GDD$^-$ replicons such that the total number of seeded cells per plate remained unchanged. After 3 weeks of antibiotic selection, blasticidin-resistant colonies were stained with crystal violet and counted to determine transduction efficiency (Fig. 2). As expected, cells with the GDD$^-$ replicon failed to form colonies. Likewise, no blasticidin-resistant colonies were formed with cells containing the Δhel replicon.

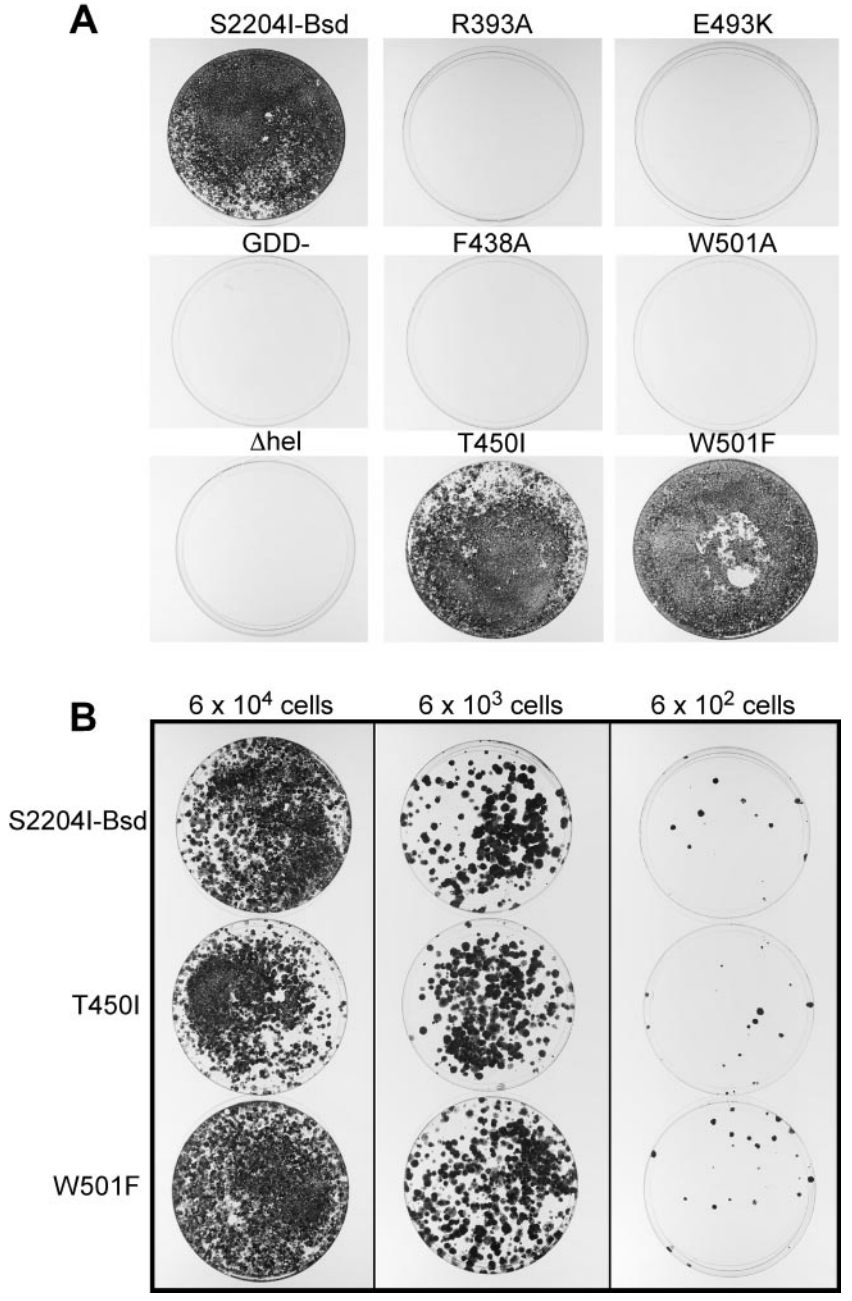

FIG. 2. Colony formation efficiencies of cells transfected with replicons containing either wild-type NS3 helicase or its mutants. (A) Huh-7.5 cells containing S2204I-Bsd, Δhel, GDD$^-$, R393A, F438A, T450I, E493K, W501A, or W501F replicons. Cells were initially plated at a density of 6×10^5 cells/dish. (B) Huh-7.5 cells containing S2204I-Bsd, T450I, and W501F replicons were diluted with cells containing GDD$^-$ replicons 10-fold (6×10^4 cells plus 5.4×10^5 GDD$^-$ cells), 100-fold (6×10^3 cells plus 5.94×10^5 GDD$^-$ cells), and 1,000-fold (6×10^2 cells plus 5.994×10^5 GDD$^-$ cells). Blasticidin-resistant colonies were stained with crystal violet after 3 weeks of antibiotic selection.

Among cells transfected with replicons containing one of the six single-amino-acid substitutions, only cells with T450I or W501F replicons produced colonies (Fig. 2A). Cells transfected with R393A, F438A, E493K, or W501A replicons formed no blasticidin-resistant colonies. In addition, the HCV replication efficiencies of cells containing either T450I or W501F replicons were determined (Fig. 2B). Cells containing T450I replicons had a colony-forming efficiency of 1.4×10^5 CFU/μg HCV RNA, which was comparable to cells transfected with the S2204I-Bsd replicon (1.2×10^5 CFU/μg HCV RNA). Cells containing W501F replicons had slightly higher transduction efficiencies (1.8×10^5 CFU/μg HCV RNA) when compared with those transfected with the S2204I-Bsd replicon.

To ensure that the above helicase mutations were not toxic to cells, cells transfected with the various replicons were allowed to propagate in the absence of blasticidin and their morphology was examined. Cells containing replicons with either wild-type helicase or its mutants appeared similar in phe-

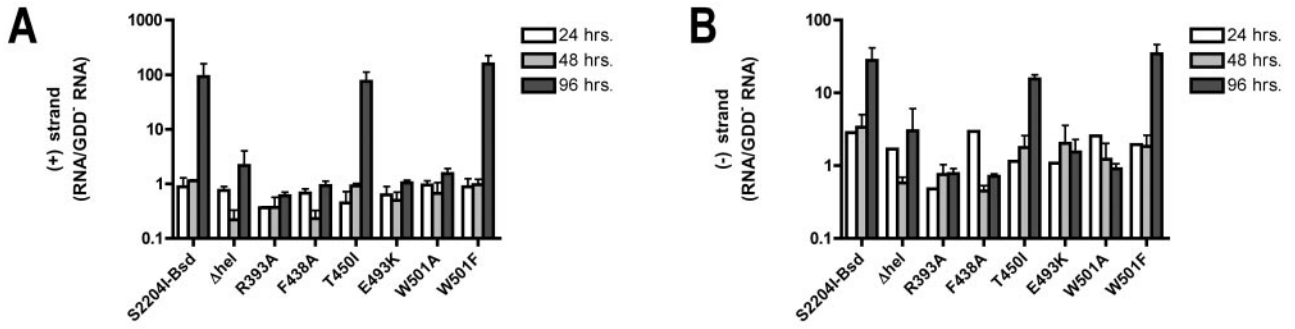

FIG. 3. Intracellular HCV RNA. Total RNA was extracted from cells at the indicated time points and reverse transcribed into cDNA using primers annealing either to the plus or minus strand of the HCV 5′-UTR region. Quantification of HCV RNA was determined by using real-time PCR. (A) HCV plus-strand RNA in cells containing either wild-type helicase or its mutants. (B) HCV minus-strand RNA in cells containing either wild-type helicase or its mutants. RT-PCR was repeated three times.

notype and grew to comparable levels of confluence after 7 days (data not shown), indicating that none of these mutations is cytotoxic to Huh-7.5 cells.

RNA helicase activity is needed for viral-RNA replication. Upon cellular entry, HCV RNA is translated, and the resulting polyprotein is subsequently processed by cellular and viral proteases. The HCV NS5B RNA-dependent RNA polymerase transcribes the genome into minus strands, which are then used as templates to synthesize more positive strands for packaging into viral capsids. In order to examine whether the NS3 helicase mutations affect RNA synthesis by the NS5B polymerase, positive- and negative-strand HCV RNA was measured in Huh-7.5 cells after they were transfected with replicons in the absence of blasticidin.

Total cellular RNA was isolated from replicon-containing Huh-7.5 cells 24, 48, and 96 h after electroporation and reversed transcribed into its cDNA sequence using either the sense or antisense primers specific for a 97-nucleotide fragment within the conserved 5′-UTR region (nucleotides 134 to 230). The quantity of HCV RNA produced was measured in three independent batches of cells using real-time PCR to determine the amount of HCV RNA molecules per microgram of cellular RNA. After 24 h, it was found that $\sim 4 \times 10^7$ HCV RNA molecules/μg total cellular RNA were detected in cells transfected with the S2204I-Bsd replicon. Slightly less plus-strand RNA was found in GDD$^-$, Δhel, F438A, and W501A cells, which had $\sim 3 \times 10^7$ HCV plus-strand RNA molecules/μg cellular RNA. Cells transfected with replicons containing the other helicase mutations (R393A, T450I, E493K, and W501F) retained about 1×10^7 HCV RNA molecules/μg cellular RNA. The amount of HCV RNA plus strands decreased for all replicons after an additional 24 h, indicating HCV RNA degradation by cellular nucleases. However, in those replicons that were active, there was more HCV RNA than there was in cells with the GDD$^-$ replicon.

Figure 3 shows the level of HCV RNA in cells containing replicons with either the wild-type helicase or its mutants relative to the HCV RNA level in cells transfected with the GDD$^-$ replicon. Since GDD$^-$ replicons do not generate additional HCV RNA (22), the GDD$^-$ replicon was used to normalize data obtained with other replicons (Fig. 3). The amount of HCV RNA detected in each sample was divided by the amount detected in cells bearing the GDD$^-$ replicon under the same conditions. If the resulting value was greater than 1, then additional HCV RNA was synthesized. At 96 h, only cells with replicons that could form blasticidin-resistant colonies retained plus-strand RNA above background levels, and these cells had about 100 times more HCV RNA than cells with the GDD$^-$ replicon (Fig. 3A).

The amount of minus-strand HCV RNA detected in Huh-7.5 cells transfected with S2204I-Bsd or its helicase mutants was 10- to 40-fold lower than plus-strand HCV RNA, consistent with the notion that less minus-strand RNA is formed in cells and that it is used as a template to generate multiple plus-sense strands. Cells transfected with replicons containing wild-type helicase, T450I, or W501F had a significant level of HCV RNA after 96 h (more than 5×10^5 HCV RNA molecules/μg cellular RNA). In cells containing all the other replicons, which did not form blasticidin-resistant colonies, less then 3×10^4 HCV RNA molecules/μg cellular RNA were detected. This was roughly the same number detected in cells with the GDD$^-$ replicon and likely represents DNA synthesized nonspecifically from the PCR primer. The levels of negative-strand HCV RNA produced by each replicon normalized against the GDD$^-$ replicon RNA levels are shown in Fig. 3B.

All replicons express and process HCV proteins. Besides a role in RNA synthesis, another possible cellular function of the NS3 helicase is to assist polyprotein translation by resolving RNA secondary structure. Failure of the Huh-7.5 cells to form colonies when transfected with replicons containing the NS3 helicase mutants could be due to interference of the mutated NS3 helicase with subsequent polyprotein processing by the NS3 protease. In order to determine the effect of NS3 helicase mutations on NS3 protease activity, cleavage of the NS3 polyprotein was assessed by detecting the expression of two mature HCV proteins: NS3 and NS5A. A *cis* cleavage event is necessary to produce mature NS3, and two *trans* cleavage events are required to produce mature NS5A. Huh-7.5 cells were electroporated with 20 μg of replicon RNA and were then lysed 4 h later for Western blot analysis.

None of the helicase point mutants were found to significantly affect the amount or size of intracellular NS3 or NS5A, suggesting that NS3 RNA helicase activity is not needed for polyprotein translation or processing (Fig. 4). Only the Δhel

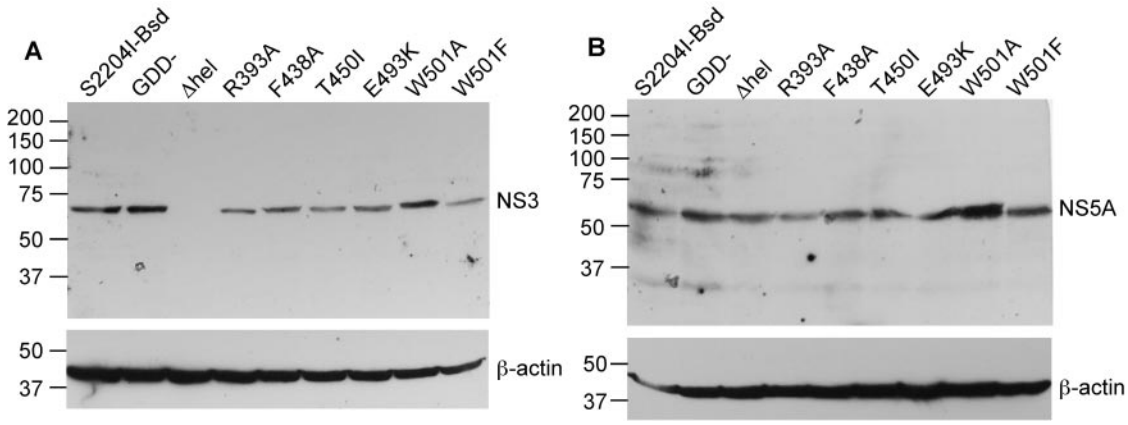

FIG. 4. HCV NS3 and NS5A proteins in Huh-7.5 cells containing replicons. Twenty micrograms of HCV RNA was electroporated into Huh-7.5 cells. At 4 h, cells were washed with PBS, scraped off the plates, lysed with 5% SDS, and analyzed with a 10% SDS gel. Antibodies against either (A) NS3 or (B) NS5A proteins were used for immunoblotting. Blots were stripped and reprobed with a β-actin antibody.

mutant lacked a detectable NS3 protein of the proper size, and this is most likely due to the fact that the epitope detected by the monoclonal NS3 antibody is no longer present (Fig. 4A). The NS3 protease was active in *trans* in all replicons, as revealed when NS5A was examined. All HCV NS3 helicase mutants generate a correctly cleaved NS5A protein, indicating that the replicon-expressed polyprotein was appropriately processed upon cellular entry (Fig. 4B).

Long-term effect of T450I and W501F mutations. Hepatoma Huh-7 cells containing HCV subgenomic replicons maintain them for a long time in the presence of antibiotic selection (29). In order to examine the long-term effect of Huh-7.5 cells containing the helicase mutant T450I and W501F replicons, cells were sustained by continual blasticidin selection during repeated passages twice a week. Figure 5 shows the level of HCV RNA production as well as presence of both NS3 and NS5A proteins as examined by Western blotting. Results show that cells containing the NS3 helicase mutations behave similarly to the wild type. Similar amounts of HCV RNA and HCV NS3 and NS5A proteins were detected.

DISCUSSION

The primary goal of this study was to examine the biological impact of changing five residues (amino acids 393, 438, 450, 493, and 501) in the HCV NS3 helicase that have clearly defined roles in the RNA-unwinding reaction catalyzed by NS3. The most thoroughly studied is W501, which intercalates between the nucleic acid bases to prevent the protein from sliding along RNA (or DNA) in the absence of ATP (13, 20, 27, 30). Changing W501 to Ala has been shown to abolish RNA unwinding but not all of HCV helicase's ability to unwind DNA (13). However, when Phe replaces W501, the mutant retains RNA-unwinding ability (13, 20, 30). Blasticidin-resistant colonies were formed only with the W501F mutant, indicating that the aromatic side chain at residue 501 is crucial for viral replication.

Both R393 and F438 are found within domain 2 of the NS3 helicase, while E493 is located in domain 3 along the same helix as W501. R393 and E493 contact DNA in the nucleic acid binding cleft (12), whereas F438 is part of a hydrophobic patch

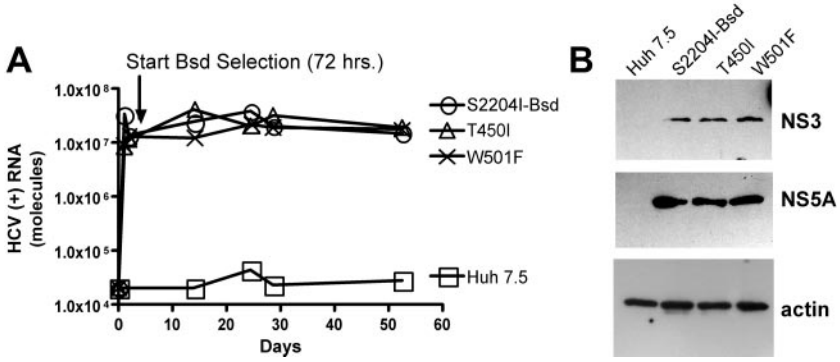

FIG. 5. Long-term effects of the T450I and W501F mutations. (A) Amount of plus-strand HCV RNA detected per microgram of RNA isolated at different times from cells transfected with S2204I-Bsd, T450I, or W501F replicons. (B) Western blot analysis of NS3 and NS5A protein expression in Huh-7.5 cells 25 days after electroporation. In both panels Huh-7.5 cells without replicons that were grown over the same period of time (without blasticidin) are shown for comparison.

that provides a pivot point for the rotation of domain 2 relative to domains 1 and 3 (34). It is widely held that this rotation is modulated by ATP hydrolysis and allows the protein to move along RNA like an inchworm. An F438A mutation results in a protein that does not release DNA upon ATP binding possibly because this critical conformational change is perturbed (17). On the other hand, an R393A mutation results in a protein that rapidly falls from RNA upon translocation so that it is unable to unwind RNA (17). E493 is part of a negatively charged patch that repels RNA when ATP binds and RNA is free to clear the W501 bookend. The main impact of the E493K mutation is to abolish the low-pH requirement of the helicase (5, 18). An E493K mutation leads to a protein that binds RNA extraordinarily tightly and unwinds duplexes in a reaction that is not sensitive to pH changes like the wild type (5, 18).

It was not particularly surprising that R393A, F438A, and W501A did not form colonies because purified NS3 helicases containing an Ala substitution for either R393 or F438 do not unwind RNA, even though they retain some ability to unwind DNA and retain the same level of nucleic acid stimulated-ATPase activity as the wild type (17). The failure of cells containing replicons with R393A, F438A, and W501A mutations to form colonies indicates that a helicase which can hydrolyze ATP, bind nucleic acids, and unwind DNA is not sufficient to support HCV replication. It was, however, somewhat surprising that cells transfected with the E493K replicon do not form colonies. This negative result supports the notion that changes in cellular pH could influence HCV replication (18). In the presence of ATP, HCV helicase normally binds RNA tightly at low pH but dramatically more weakly at high pH. This phenomenon causes the protein to unwind RNA better at low pH because it does not fall from the nucleic acid as rapidly. An E493K-ATP complex, in contrast, binds RNA tightly throughout the entire physiological pH range (5). There are a couple of possible interpretations of the inability of the E493K replicon to synthesize RNA. One is that it is critical that HCV helicase is active only when pH falls below the neutral range. Another is that in order for the protein to function it must efficiently release itself from RNA. More work will be necessary to explore the role of E493 and the acidic patch surrounding this residue.

T450 also contacts the backbone of DNA that binds in the cleft separating domain 2 from domains 1 and 3 (12). Unlike R393, F438, E493, and W501, which are conserved in almost all HCV isolates sequenced to date, T450 is in a region that varies among the HCV genotypes. NS3 amino acid 450 is a Thr in genotype 1 strains but is an in Ile in some (but not all) helicases isolated from genotype 2a strains (16). When residue 450 in HCV genotype 1a strain H77c was changed to an Ile, the T450I helicase mutant unwound DNA faster (16). Since recent genotype 2a isolates replicate more efficiently than genotype 1 (11, 21, 37), it is possible that T450I could represent an adaptive mutation. The results shown in Fig. 2 suggest that this is likely not the case because T450I replicons do not form colonies more efficiently.

Overall, the observations made in this study demonstrate that RNA unwinding by HCV helicase is needed for viral replication. Helicases that retain some ability to bind RNA, translocate, or unwind DNA are not able to support HCV replication, and cellular proteins cannot substitute. The data presented here also confirm the biological importance of many years of detailed mechanistic work that has been conducted using the HCV helicase. It has long been speculated that the virus requires a bulky hydrophobic residue at NS3 position 501 in order to unwind RNA (13, 20, 27, 30, 32), and the above results confirm that hypothesis. Likewise, the results confirm the necessity of the Arg-clamp, Phe-loop (17), and the acidic patch on NS3 (6) in the replication of HCV. It is of practical importance that each of these motifs is conserved in all known HCV isolates, but they are not shared with related cellular proteins. Such molecular architecture could aid rational drug design, and, interestingly, a potent inhibitor of HCV helicase binding near W501, E493, and R393 was recently reported (23). It will be interesting to examine such compounds in the replicon system.

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