Impaired Replication of Hepatitis C Virus Containing Mutations in a Conserved NS5B Retinoblastoma Protein-Binding Motif

Running head: NS5B Rb-binding Mutants

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HCV down-regulates the retinoblastoma tumor suppressor protein (Rb), a central cell cycle regulator which is also targeted by oncoproteins expressed by DNA tumor viruses. HCV genome replication is also enhanced in proliferating cells. Thus, it is possible that HCV interactions with host cell cycle regulators, such as Rb, have evolved to modify the intracellular environment to promote viral replication. To test this hypothesis and to determine the impact of viral regulation of Rb on HCV replication, we constructed infectious viral genomes containing mutations in the Rb-binding motif of NS5B which ablate the ability of HCV to regulate Rb. These genomes underwent replication in transfected cells, but produced variably reduced virus yields. One mutant, L314A, was severely compromised for replication, and rapidly mutated to L314V, thereby restoring both Rb regulation and replication competence. Another mutant, C316A, also failed to down-regulate Rb abundance and produced virus yields that were about one third that of virus with wt NS5B sequence. Despite this loss of replication competence, purified NS5B-C316A protein was 2- to 3-fold more active than wt NS5B in cell-free polymerase and replicase assays. Although siRNA knockdown of Rb did not rescue the replication fitness of these mutants, we conclude that the defect in replication fitness is not due to defective polymerase or replicase function, and is more likely to result from the inability of the mutated NS5B to optimally regulate Rb abundance and thereby modulate host gene expression.
INTRODUCTION

Chronic infection with hepatitis C virus (HCV) is associated with an increased risk for development of hepatocellular carcinoma (11). However, unlike most viruses associated with cancer, HCV has an RNA genome and an exclusively cytoplasmic life-cycle. The precise mechanisms by which HCV infection leads to carcinogenesis are unclear. Although chronic inflammation is suspected to play a role, transgenic mice that express a low abundance of the entire HCV polyprotein do not exhibit hepatic inflammation and yet have an increased incidence of hepatocellular carcinoma (10). These data suggest that HCV proteins may have a direct oncogenic effect. Consistent with this, several structural and nonstructural proteins encoded by HCV (core, NS3, NS5A and NS5B) have been shown to regulate host cell-cycle regulators and tumor suppressor proteins, including p53 and the retinoblastoma protein (Rb) (7, 8, 14, 17) (reviewed recently in 16).

Rb has many important functions in cell-cycle control, including the repression of transcription factors needed for the transition from G1/G0 to S phase. Rb is targeted by a number of DNA oncoviruses including adenovirus (27), simian virus 40 (SV40) (3) and human papillomavirus (HPV) (5). These small DNA viruses exploit host cell machinery to facilitate genome replication, and encode oncoproteins that can sequester Rb, or down-regulate its abundance. This results in the release of the Rb-imposed repression of various transcription factors that are needed to express genes required for DNA replication, such as proliferating cell nuclear antigen and thymidine kinase (4). In this way, the DNA oncoviruses can induce expression of cellular genes that promote their replication. The regulation of Rb by these DNA viruses is likely to contribute substantially to their oncogenic potential.
Although HCV does not require the host DNA replication machinery to replicate its RNA genome, several *in vitro* observations suggest that HCV RNA replication and genome accumulation are enhanced in proliferating cells (19, 20, 26). This is an intriguing feature of HCV, since its primary target cell, the hepatocyte, is generally quiescent and, while very active metabolically, demonstrates very slow turnover in the non-infected liver. Our previous studies have shown that HCV has evolved a mechanism to down-regulate the Rb protein. The HCV RNA-dependent RNA polymerase (RdRp), NS5B, interacts with Rb, targeting it for E6-associated protein (E6AP)-dependent ubiquitination and proteasomal degradation, thereby promoting cell cycle progression (17, 18). The interaction between NS5B and Rb thus represents an interesting parallel with the oncoproteins of DNA tumor viruses. Like many cellular and viral proteins, HCV NS5B interacts with Rb through a conserved Rb-binding motif (LxC/NxD) homologous to Rb-binding domains found in DNA virus oncoproteins (17, 18). Surprisingly, this Rb-binding motif overlaps with the Gly-Asp-Asp sequence in NS5B that co-ordinates the binding of divalent metals near the active site of the polymerase.

In order to better understand the role of Rb regulation in the HCV life-cycle, we investigated the impact of mutations within the LxC/NxD motif of NS5B on HCV RNA replication and virus production. We show that viruses with mutations in the NS5B Rb-binding site are incapable of regulating Rb abundance and also variably impaired in replication fitness. Additional data suggest that the defect in replication fitness is not due to a direct loss of polymerase or replicase complex activity, but may reflect the inability of these viruses to regulate Rb abundance and thereby modulate host gene expression.
MATERIALS AND METHODS

Cells. Human hepatoma Huh-7 cells, and the Huh-7 derivatives, Huh-7.5 and FT3-7 were cultured as described previously (28, 29).

Plasmid construction, HCV genome transfection and virus production. Plasmid pHJ3-5, previously referred to as pH-NS2/NS3-J(Y361H/Q1251L) (29), encodes a chimeric infectious HCV genome consisting of sequence encoding the structural proteins of the genotype 1a H77S virus within the background of the genotype 2a JFH-1 virus. This plasmid contains mutations in E1 (Y361H) and NS3 (Q1251L) that enhance virus production (13). The HCV genome encoded by this plasmid was selected as the “wild-type” (wt) HCV for the purpose of this study, and will be referred to as HJ3-5 in this study. For mutagenesis of the NS5B sequence, the JFH-1 sequence from nucleotide 8014 to the end of the genome was released from pHJ3-5 by digestion with HpaI and XbaI and the fragment was inserted into pUC20 at Smal and XbaI sites in the polylinker. The resulting subclone served as a template for mutagenesis of NS5B using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutated NS5B sequence was digested with SnaBI and XbaI and inserted back into similarly digested, full length pHJ3-5. The sequences of mutated regions in the resulting plasmids were confirmed by direct DNA sequencing. Mutations were named with reference to the amino acid number of the JFH-1 NS5B protein.

HCV infectivity assay. HCV RNAs were transcribed in vitro using the Megascript T7 kit (Ambion, Austin, TX), electroporated into cells, and cell culture supernatants containing infectious virus were harvested as described previously (29). The titer of infectious virus released from cells following RNA transfection or virus infection was determined using a focus-forming assay, as described previously (30). Briefly, 100 µL aliquots of cell culture supernatants were
used to inoculate naïve Huh-7.5 cells seeded 24 hours earlier in 8-well chamber slides at \(4 \times 10^4\) cells/well. Cells were incubated at 37°C, 5% CO\(_2\) and fed with 200 µL medium after 24 h. At 48 h after virus inoculation, cells were washed twice in 1x PBS, fixed in methanol:acetone (1:1) for 9 min. and washed again with 1x PBS. Cells were then incubated for 3 h at 37°C with monoclonal antibody to the core protein (C7-50; Affinity Bioreagents, Golden, CO) diluted 1:500 in 3% BSA, 1x PBS. Cells were washed three times in 1x PBS before incubating for 1 h at 37°C with 1:200 dilution of fluorescein-isothiocyanate conjugated goat anti-mouse immunoglobulin G antibody (Southern Biotech, Birmingham, AL). Cells were washed three times in 1x PBS and mounted in Vectashield (Vectorlabs, Burlingame, CA).

**Analysis of viral RNA replication and sequences of progeny viruses.** Total RNA was extracted from transfected cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Viral RNA abundance was measured by quantitative real-time reverse transcription PCR (qRT-PCR) as previously described (13). Primers DRM59 (GGGCGTTAACCACATCAAGTCCGTG; corresponding to JFH-1 nucleotides 8007 to 8032) and DRM61 (TGGCGCCCCAAGTTTTCTGAG; nts 9151-9131) targeting the NS5B region were used to amplify a fragment of approximately 1.1 kb containing the Rb-binding motif. RT-PCR products were sequenced directly.

**Expression and purification of proteins.** Wild-type and mutant JFH-1 NS5B proteins were expressed in *E. coli* and purified as described previously (24). Briefly, *E. coli* BL21(DE3) cells transformed with NS5B constructs in pET303 plasmids were grown in LB medium to an OD\(_{600}\) of ~1.0 and induced for expression by adding 1 mM IPTG for 24 h at 16°C. The cells were subsequently pelleted by centrifugation and lysed by sonication in Buffer L (50 mM Tris (pH 7.6), 300 mM NaCl, 1 mM β-mercaptoethanol, 10% glycerol, 1 mM PMSF and 20 mM...
imidazole). The clarified lysate was applied to a 1 ml His-Trap affinity purification column (GE Healthcare). The column was washed with the sample application buffer and the bound proteins were eluted in the same buffer containing 500 mM imidazole. The fractions containing the protein were pooled and the salt concentration was adjusted to 100 mM before applying the sample on to polyU-agarose (Sigma, St. Louis, MO). The bound proteins were eluted in Buffer A (20 mM Tris [pH 7.6], 10% glycerol, 3 mM DTT and 1 mM PMSF) containing 400 mM NaCl. The proteins were run on SDS-PAGE along with known concentrations of BSA to determine the concentrations.

**RdRp activity assays.** RdRp assays contained 1 pmole of linear template RNA or 30 pmoles of circular (C) template RNA and 0.04 µM of NS5B protein (2, 23). The assays were carried out in 20 µL reactions containing 20 mM sodium glutamate (pH 8.2), 0.5 mM dithiothreitol, 4 mM MgCl₂, 12, 0.5 % (v/v) Triton X-100, 200 µM ATP and UTP, and 250 nM α-[P³²]-CTP (MP Biomedicals). GTP was used at a final concentration of 0.2 mM unless specified. MnCl₂ was included in all the assays at a final concentration of 1 mM. Reaction mixes also contained 20 mM NaCl that came from the protein storage buffer. RNA synthesis reactions were incubated at 25°C for 60 min. The reactions were stopped by phenol/chloroform extraction followed by ethanol precipitation of the RNA in the presence of glycogen and 0.5 M ammonium acetate. Products were separated by electrophoresis on denaturing (7.5 M urea) 20% polyacrylamide gels. Gels were wrapped in plastic and quantification of radiolabeled bands was performed using a PhosphorImager (GE Healthcare).

**Circularization of RNA.** The protocol to circularize linear RNA was as described previously (23). Briefly, 2 nmoles of a 16-mer linear RNA named “L” was incubated with T4 RNA ligase (Ambion, Austin, TX) according to manufacturer’s specifications for 3 h at 37°C in
a 20 µL reaction. An aliquot of the reaction was run on a 20% polyacrylamide-7.5 M urea
denaturing gel along with the linear RNA as control. The gel was stained briefly in Toluidine
Blue and the RNA was visualized, showing a clear electrophoretic mobility difference between
the linear (L) and the circularized (C) molecules (data not shown). The circularized RNA was
excised from the gel and eluted using 0.3 M ammonium acetate overnight, then purified by
phenol-chloroform extraction and ethanol precipitation. The concentration of the RNA was
measured by its absorbance at 260 nm.

**Immunoblot analysis.** Preparation of protein extracts, SDS-PAGE and subsequent
immunoblotting were done as described previously (18) using mouse monoclonal antibodies
against β-actin (AC-15; Sigma, St Louis, MO), Rb (G3-245; BD Biosciences, San Jose, CA),
and core (C7-50; Affinity BioReagents, Golden, CO) and rabbit polyclonal antibodies against
NS5B (A266-1; Virogen, Watertown, MA or in replicase assays, ab35586; Abcam, Cambridge,
MA) and β-tubulin (ab6046; Abcam, Cambridge, MA) and goat polyclonal antibodies against
NS3 (ab21124; Abcam). The endoplasmic reticulum marker Rab1b was detected using rabbit
polyclonal antibody G-20 (sc-599; Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblots
were visualized either by chemiluminescence or by direct detection of infrared fluorescence on
an Odyssey Infrared Imaging System (Li-Cor, Lincoln, NB).

**Indirect immunofluorescence microscopy.** Infected or mock-infected cells were seeded
onto glass chamber slides at 6 x 10^4 cells per well and allowed to grow for 24 h. Cells were
washed twice with 1 x PBS and fixed in methanol:acetone (1:1) at -20°C for 10 minutes. Fixative
was removed and slides air-dried in a fume hood for 1 h. For the images shown in Fig. 3C, cells
were fixed in 4% paraformaldehyde for 30 minutes and permeabilized in 0.2% Triton X-100, 1 x
PBS for 12 minutes. Slides were washed twice in 1 x PBS before blocking for 1 h in 10% goat
serum (Sigma, St Louis, MO) in 1 x PBS. The slides were then washed twice in 1 x PBS and incubated overnight at 4°C with primary antibodies diluted in 1% BSA, 1 x PBS. A mouse monoclonal antibody was used to detect Rb (G3-245; BD Biosciences, San Jose, CA), and either a human polyclonal anti-HCV serum or rabbit polyclonal antibody to NS5A (a gift from Dr. Craig Cameron) were used for detection of HCV antigens. Slides were washed 3 times for 10 min. each in 1 x PBS and incubated in darkness for 1 h at 37°C with secondary antibodies diluted in 1% BSA, 1 x PBS. Secondary antibodies were Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) or Texas Red-conjugated donkey anti-human IgG (Jackson Immunoresearch Laboratories, West Grove, PA). The slides were washed 3 times for 10 min. each in 1 x PBS, counterstained with DAPI and mounted in Vectashield (Vectorlabs, Burlingame, CA). Fluorescence images were obtained using a Zeiss Axiohot II fluorescence microscope. Confocal fluorescence images were obtained using a Zeiss LSM 510 UV META laser scanning confocal microscope in the UTMB Infectious Disease and Toxicology Optical Imaging Core. Recorded digital images and related controls were uniformly enhanced for brightness and contrast using Photoshop CS2 (Adobe Systems, San Jose, CA).

**Isolation of heavy membrane fractions containing HCV replication complexes.**

Heavy membrane fractions were isolated from cells infected with virus following electroporation of viral RNAs, and from stable Huh-7 cell lines containing dicistronic, subgenomic genotype 1a or 2a HCV replicon RNAs. Cells were grown to 70-90% confluence, trypsinized, washed twice with PBS, and resuspended in hypotonic lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 5 mM DTT, and EDTA-free protease inhibitors (Roche, Mannheim, Germany). Cells were kept at 4°C for 20 min., and disrupted by repetitive passage (40X) through a a 25 gauge needle.
Nuclei were removed by centrifugation (1,000 x g, 10 min, 4°C). Post-nuclear homogenates were centrifuged at 16,000 x g for 30 min at 4°C. Pellets of heavy membranes containing HCV replication complexes (P16) were resuspended in hypotonic buffer containing 10% glycerol, and stored at -80°C.

**Cell-free HCV RNA synthesis by membrane-bound replicase complexes.** Aliquots of P16 fractions were incubated at 37°C for 3 h in standard transcription mixtures containing 50 mM HEPES, pH 7.9, 5 mM MgCl₂, 50 mM KCl, 10 mM DTT, 15 µg/mL actinomycin D, 1 mM spermidine, 800 U RNasin (Promega, Madison, WI) per mL, 1% DMSO, 1 mM concentration of GTP, ATP, and UTP, 10 µM CTP, and 1 mCi of [α-³²P]CTP per mL. Total RNA was extracted using the RNeasy Mini Kit, precipitated with isopropanol, and resolved by 1% agarose gel containing glyoxal (NorthernMax-Gly, Ambion, Austin, TX).

**Gene knock-down by RNA interference.** siRNAs were purchased from Dharmacon (Lafayette, CO). An siRNA oligonucleotide SMARTpool, containing four siRNA oligonucleotides specific for the human retinoblastoma gene (L-003296-00) and four individual siRNAs (#5, #6, #7 and #9; D-003296-05, -06, -07, and -09) were used to target Rb. A pool of four siRNAs (D-001206-13) that are not specific for the Rb message and individual control siRNAs #1 and #2 (D-001230-01 and D-001220-01) were used as negative controls. For knockdown experiments, FT3-7 or Huh-7.5 cells were seeded at 30% confluence in 6-well plates and transiently transfected with 50 nM siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Duplicate transfections were used for either HCV infection, or supertransfection with DNA plasmids and preparation of protein extracts for immunoblot analysis at 72-120 h after transfection.
Plasmid DNA transfection and reporter gene assays. FT3-7 cells were seeded at 6 x 10^4 cells per well in 48 well plates 48 h following siRNA transfection. Cells were transfected with plasmid DNA using Lipofectamine 2000 according to the manufacturer’s instructions. The reporter plasmids, pMAD2-Luc, p107-Luc (18) or pIFN-β-Luc (12) (gift of John Hiscott), were used to transfect cells at 0.2 µg/well, together with pCMV-β-galactosidase at 0.1 µg/well. Cells were harvested at 48 h post transfection and lysed in reporter lysis buffer (Promega, Madison, WI). Quantification of luciferase and β-galactosidase activities was accomplished with commercial enzyme assay kits according to the manufacturer’s instructions (Promega, Madison, WI).

RESULTS

Replication competence of HCV mutants with substitutions in the Rb-binding domain of NS5B. The down-regulation of Rb by HCV is dependent upon the viral RdRp, NS5B, which interacts with Rb through a conserved LxC/NxD motif near the active site of the polymerase (18). Since HCV RNA replication is enhanced in proliferating cells (20, 26), we hypothesized that the NS5B interaction with Rb may have evolved to create an intracellular environment permissive for viral replication. To test this hypothesis, we constructed a series of HCV genomes containing mutations in the conserved Rb-binding domain of NS5B, within the background of a chimeric viral genome, HJ3-5 (hereinafter considered wt), encoding the nonstructural proteins of JFH-1 virus that replicates efficiently in cell culture (Fig. 1A). The mutations were selected based on prior studies that demonstrated their ability to ablate NS5B binding to Rb (18). We examined the effect of the mutations on replication competence and infectious virus yield in viral RNA transfection and virus infection experiments. Hub-7 human hepatoma cells were electroporated with synthetic RNAs representing each of the mutated viral
genomes. Cells were harvested and the supernatant fluids were collected at 96 h post electroporation to assess virus yield. Viral RNA abundance in cell lysates was determined by real-time quantitative RT-PCR (qRT-PCR; Fig. 1B), while the titer of infectious virus released from the cells was determined by a fluorescent focus assay (Fig. 1C).

Four of the mutants contained single amino acid substitutions in NS5B, while one contained two substitutions (Fig. 1A). Since the Rb-binding domain overlaps with the divalent metal-coordinating Gly-Asp-Asp residues in the HCV RdRp (18), the mutations within the Rb binding domain could also negatively influence polymerase activity. Indeed, viral RNA accumulation was reduced in cells infected with each of the five mutants, as determined by the qRT-PCR assay (Fig. 1B). The L314A mutant produced only one tenth the RNA abundance achieved by the wt RNA by 120 h after transfection, while replication of the double mutant, L314A/C316A, was severely compromised and resulted in no detectable increase in viral RNA in this assay (Fig. 1B). Consistent with these results, virus production was also reduced with each of the mutants (Fig. 1C), with the most dramatic reduction being observed with the L314A mutant and the double mutant, L314A/C316A. As expected, D318N, which is mutated in the NS5B active site, was unable to produce infectious virus. The C316A mutant, with a substitution in the center of the Rb-binding domain of NS5B, had the least effect on virus production, with virus yields averaging 33% of that from the parental wt RNA. This mutant was further examined in a multi-cycle infection analysis (Fig. 1D). Virus production by C316A-infected cells was reduced throughout the 144 h assay period when compared to cells infected in parallel at the same m.o.i. as the parental wt virus.

Viruses with mutations in the LxCxD domain do not down-regulate Rb abundance.

Previously, we have shown that ectopic expression of wt NS5B in human hepatoma cells results
in an interaction of NS5B with Rb, targeting Rb for proteasome-dependent degradation in an
E6AP-dependent fashion (17, 18). In contrast, ectopic expression of mutant NS5B with
substitutions in the LxCxE-like Rb-binding motif does not induce changes in Rb abundance (18).
We have also shown that Rb is down-regulated in HCV-infected Huh-7 cells (17). It was
therefore of interest to determine whether HCVs with mutations in the Rb-binding motif of
NS5B are able to down-regulate Rb. We initially selected the C316A mutant for study, since it
was the least impaired for replication and therefore most likely to be comparable to the parental
wt virus in terms of the abundance of viral proteins expressed within infected cells. FT3-7 cells
were infected at an m.o.i. of 1 with either wt or C316A virus. After 3 days, the cells were
trypsinized and seeded into 8-well chamber slides, and one day later fixed and immunostained
for NS5A and Rb as described in the Methods. NS5A was detected in almost all cells in both the
wt and C316A-infected cultures. Fluorescence microscopy demonstrated that Rb was sharply
reduced in abundance in cells infected with wt virus, but not in cells infected with the C316A
mutant (Fig. 2A).
We also examined cells infected with wt, C316A or L314A virus by confocal microscopy
(Fig. 2B, left panel). Cells were infected at low m.o.i. (<0.01) and passaged for 8 days prior to
fixation and antibody labeling of Rb and HCV antigens. While Rb was found to be
predominantly nuclear in its localization in cells infected with each of these viruses, a substantial
amount of Rb could be detected within the cytoplasm of many cells infected with the wt virus
(Fig. 2B, left panel arrows, and right panel). This finding is consistent with previous observations
demonstrating the cytoplasmic accumulation of Rb in cells expressing NS5B, a phenomenon that
is enhanced by inhibition of the proteasome and is likely to be due to the trapping of Rb in
cytoplasmic complexes with NS5B (17). Importantly, this abnormal, partially cytoplasmic
distribution of Rb was never observed in mock-infected cells or in cells infected with the L314A or C316A viruses in which Rb was detected exclusively within the nucleus.

Immunoblots of cell lysates confirmed the down-regulation of Rb in cells infected with wt virus, and the lack of an effect on Rb abundance in cells infected with the C316A mutant (Fig. 2C). Importantly, the absence of down-regulation of Rb in the C316A-infected cells could not be attributed to reduced expression of NS5B, as the abundances of NS5B in the wt and C316A-infected cells were comparable by 120 h after infection (Fig. 2C). Thus, consistent with previous results from experiments involving ectopic expression of NS5B (18), mutations that ablate the Rb-binding motif in NS5B result in infectious viruses that can no longer down-regulate Rb abundance.

**Stability of the LxCxD domain mutants.** To assess the stability of the C316A mutation, total RNA was isolated from cells 96 hrs after transfection with the mutant genome, and used as template to amplify a 1.1 kb fragment of the NS5B coding region (nts 8007-9151 of the JFH-1 virus; Genbank AB047639). The sequence of this fragment retained the engineered mutation and contained no additional changes (data not shown). The stabilities of the other mutations engineered in the Rb-binding domain were also examined over several passages of cell-free virus mutants. With the exception of L314A, each of the mutations was stable for at least three passages after electroporation. In cells infected with the L314A mutant, an Ala to Val change was detectable by passage 1 and became present in the majority of the viral sequences examined by passage 2 (data not shown). This new mutant contained a single nucleotide transition (C to T) at nucleotide 8607. In order to determine the effect of the L314V mutation on virus production, it was reconstructed within the wt genome. In FT3-7 cells, the yield of L314V virus was approximately 50% that of the wt virus, representing a dramatic increase in yield compared to
L314A virus (~1%) (Fig. 3A). The L314V virus yield was consistently greater than that obtained with the C316A virus in 3 independent experiments, although the differences in the yields of these two mutants were modest. Consistent with these results, L314V RNA accumulation was greater than C316A but lower than wt when measured by qRT-PCR (Fig. 1B). These data suggest that the selection of the Ala to Val mutation in cells infected with the L314A mutant was due to the enhanced replication efficiency of the L314V virus.

To determine whether the L314V revertant had regained the ability to regulate Rb, we assessed Rb abundance in Huh-7.5 cells 3 or 4 days after infection with the virus. Compared with mock-infected cells, Rb abundance was down-regulated in immunoblots of lysates from L314V-infected cells (Fig. 3B). This was confirmed by fluorescence microscopy of cells infected at low m.o.i. with the L314A mutant, L314V revertant, or wt virus. These studies revealed substantial decreases in nuclear Rb abundance in most cells infected with the wt or L314V virus, while no difference was apparent in the nuclear Rb signal in cells infected with L314A, or in non-infected cells adjacent to those infected with wt or L314V virus (Fig. 3C). Some L314V-infected cells showing weak HCV antigen expression appeared to retain a normal nuclear Rb abundance, but in aggregate these data indicate that the L314V revertant had at least partially regained the ability to down-regulate Rb.

Mutations that ablate Rb-binding do not necessarily impair RdRp activity. It was important to determine whether the mutations in NS5B affected polymerase activity directly, or acted in an indirect fashion to impair replication of the virus through the failure of the LxCxD motif mutants to modulate Rb abundance. To address this question, we purified bacterially expressed wt and mutant NS5B, and compared their activities in an in vitro polymerase assay.
The wt JFH-1 NS5B, identical to that in the wt HJ/3-5 virus, was expressed without the C-terminal 21 residues (named J-Δ21). Four derivatives with mutations as above, L314A, L314V, C316A, and the double mutant L314A/C316A, were purified to ~ 90% homogeneity (Fig. 4A) after separation on a Ni-NTA column and a poly-U column. The L320A mutant could not be purified from the E. coli proteins despite repeated attempts and was not analyzed further. The RNA synthesis activities of the four proteins were first assessed using a 19-nt linear RNA template named LE19. LE19 was designed to report on four distinct activities of the HCV RdRp (Fig. 4B, top): RNA synthesis by *de novo* initiation (generating a 19-nt RNA product), terminal nucleotide addition (generating 20- and 21-nt RNAs), extension from a primed template (generating a 32-nt RNA that is formed from a partial duplex of two LE19 molecules), and a template switch product (of 38-nt and sometimes longer) (9, 22, 24). J-Δ21 has all four of the activities that have been previously documented in the genotype 1b RdRp (25) (Fig. 4C, lane 1). In the absence of GTP, the putative initiation nucleotide, the *de novo* initiation product decreased more severely than the primer extension product, which does not require GTP with the LE19 template (Fig. 4C, lane 3).

Reproducible results were obtained with the L314A, L314V, C316A, and L314A/C316A mutants in 5 independent assays (Fig. 4D). The results revealed that the C316A mutant produced higher levels of both the *de novo* initiated and the primer-extension products than the wt J-Δ21 protein. The L314A mutant had slightly lower RNA synthetic activity than the wt protein, while the double mutant, L314A/C316A, which demonstrated a severely debilitated replication phenotype (Fig. 1B and C), was severely defective in RNA synthesis *in vitro* (Fig. 4C, lanes 21-23). Thus, while it is apparent that some mutations in the Rb-binding domain cause reduced polymerase activity, this is not true for all mutants. The results obtained with the C316A mutant
demonstrate in particular that the Rb binding function of NS5B is not important for polymerase activity.

We challenged the RdRps with two additional templates to assess whether the mutations may have caused more subtle defects. First, each RdRp was tested with the template LE19p, which has a puromycin covalently linked to the 3’ terminal hydroxyl of LE19, thus rendering it competent for de novo initiation but not primer extension. With the LE19P template, J-Δ21 and each of the mutant RdRps with single amino acid substitutions retained the ability to initiate RNA synthesis by the de novo mechanism (Fig. 4C, lanes 2, 7, 12 and 17). The L314A/C316A mutant was inactive. Next, we assessed the ability of the HCV RdRp to direct RNA synthesis from linear (L) or circular (C) 16-nt templates. Since a circularized template cannot thread a 3’ terminus into the RdRp active site, this template can only be recognized by the RdRp in an open conformation, which then closes for initiation (2). Again, both the wt J-Δ21 and the mutant proteins with single amino acid substitutions retained the ability to generate products, while L314A/C316A was far less active (Fig. 4C, lanes 5, 10, 15, 20, and 25). The products generated from a circularized RNA were different than those from the linear version of the same RNA, demonstrating that the J-Δ21 RdRp can undergo a transition between the open and closed conformations (2). Thus, with respect to each of the NS5B activities we assessed in these experiments, mutations that disrupt the interaction of NS5B with Rb do not necessarily have a negative effect on RNA synthesis in vitro.

Effects of Rb-binding site mutations on HCV replicase activity. The finding that the C316A mutant was approximately twice as active in RNA synthesis in vitro was unexpected, given that the virus carrying this mutation is impaired for replication (Fig. 1B) and produces less virus in either RNA-transfected or virus-infected cells (Figs. 1C and 1D). Therefore, we
compared RNA synthesis in cell-free reactions containing replicase complexes isolated from wt or C316A virus-infected cells. For these experiments, FT3-7 cells were electroporated with wt or C316A genomic RNA, and passaged until they were 70-90% infected as judged by immunofluorescence detection of core antigen. Cells transfected with the replication-incompetent D318N genomic RNA were passaged in parallel as a control. We sequenced viral RNA extracted from the cells, and confirmed that neither the wt nor C316A virus had acquired mutations in the NS5B-coding region during passage. The cells were then harvested, and heavy membrane fractions containing HCV replicase complexes were isolated in order to measure replicase activity (Fig. 5A), as described in the Methods. In this assay, the viral RNA polymerase uses endogenous viral RNA as template. For comparison between the different heavy membrane preparations, [\(^{32}\)P]-CTP incorporation into RNA was assessed quantitatively by Phosphoimager analysis (Fig. 5B), and then normalized to NS5B abundance as determined in immunoblots (Fig. 5C). As an additional measure of nonstructural protein abundance in these replicase preparations, we also analyzed immunoblots for NS3 protein (Fig. 5C). In three experiments, the replicase complexes from cells transfected with the C316A mutant synthesized an average of 2.21 (±0.48) fold more labeled product than replicase complexes from the wt RdRp (Figs. 5A and B), consistent with the enhanced \textit{in vitro} synthetic activity of the recombinant C316A protein described above (Fig. 4C). The increased activity of the C316A mutant relative to wt was evident across the range of protein concentrations tested in the cell-free reaction (Fig. 5B). As expected, there was no RNA synthesis by heavy membranes fractions prepared from D318N-transfected cells.

\textbf{Effects of Rb knockdown on HCV infection.} The results obtained thus far indicated that NS5B mutations that ablate the interaction with Rb do not necessarily reduce the ability of
the enzyme to direct HCV RNA synthesis. Given that the same mutations impair HCV RNA replication and virus production, we hypothesized that the lack of regulation of Rb might in some way limit the efficiency of virus replication. To test this, we used RNA silencing to knockdown the intracellular abundance of Rb, and then examined the impact of this on replication of the virus. Transfection of FT3-7 cells with a pool of siRNAs targeting Rb led to undetectable levels of Rb when assessed by immunoblots 3 to 5 days later (Fig. 6A). Surprisingly, however, analysis of the culture media from cells infected with wt or C316A virus (m.o.i. of 2) three days after siRNA knockdown of Rb revealed a modest decrease, and not an increase, in the yield of both wt and C316A virus compared to cells treated with control siRNAs (Fig. 6B). A similar experiment was performed using four individual siRNAs targeting Rb, and two non-targeting control siRNAs. Again, cells transfected with siRNAs targeting Rb demonstrated a negligible abundance of Rb compared to the control siRNA-transfected cells at 4 days post-transfection (Fig. 6C). With three of the four siRNAs, the virus yield was reduced ~50% compared to control siRNA transfected cells, although one of the Rb-specific siRNAs caused a ~90% reduction in virus yield (Fig. 6D, #9). Similar results were obtained in 3 independent experiments.

As an alternative experimental strategy, cells were first infected at m.o.i 0.1 with wt, C316A or L320A virus, and the cells grown for 7 d prior to transfection with either the pool of siRNAs specific for Rb or the pool of non-targeting control siRNAs. Cell culture supernatants were harvested 3 d after transfection with siRNA (10 d post-infection) and the titer of virus released from the cells was determined. Immunoblots of cell lysates confirmed efficient knockdown of Rb as before (data not shown). Again, for each of the 3 viruses, the amount of virus released from cells transfected with Rb-specific siRNAs was lower than that from cells transfected with the non-targeting control siRNAs (Fig. 6E).
To further examine the effect of Rb gene knockdown on HCV replication, we determined the effect of Rb knockdown on the spread of HCV infection in Huh-7.5 cells. Cells were infected with serial dilutions of the wt virus 3 days after transfection with Rb-specific or control siRNAs, and then analyzed 3 days later for the number and size of foci of infected cells identified by core protein-specific immunofluorescence. Immunoblotting confirmed efficient knockdown of Rb (Fig. 7A). Consistent with the results shown in Fig. 6, Rb knockdown resulted in a reduction in the number of infectious foci to less than 50% that developing in control cells (Fig. 7B). Furthermore, foci of infected cells were approximately 50% smaller in the cells in which Rb expression had been silenced, compared with cells transfected with control siRNA (Fig. 7C).

To confirm that siRNA-mediated Rb knockdown had, as expected, resulted in activation of E2F-responsive promoters in these experiments, we determined the activities of two cellular promoters that are subject to Rb regulation using luciferase reporter assays. The promoters for MAD2 and p107 are both suppressed by Rb through an inhibitory effect of the tumor suppressor on E2F transcription factors (18). Consistent with this, MAD2 and p107 promoter activity was increased by 3.4- and 2-fold, respectively, in cells transfected with Rb-targeting siRNA, compared to cells transfected with control siRNA (data not shown). Furthermore, we did not observe an effect on the IFN-β promoter with either siRNAs, consistent with the absence of a major effect of Rb on this promoter. These results confirm that the siRNAs used in these experiments were effective in down-modulating both Rb abundance and Rb function.

Overall, these studies indicate that siRNA-mediated Rb knock-down reduces HCV replication in Huh-7 cells, despite the fact that NS5B interacts with Rb and targets it for degradation in infected cells (17, 18). There are several possible explanations that may account for this apparent paradox, as discussed below.
DISCUSSION

The HCV RdRp, NS5B, interacts with and down-regulates the cellular abundance of Rb, an important tumor suppressor and cell cycle regulator (18). Here, we have shown that viruses containing mutations in NS5B that disrupt the Rb-binding motif are impaired for replication in cultured Huh-7 hepatoma cells (Fig. 1B and C). Importantly, we have also shown that the loss of fitness was not necessarily accompanied by reduced RdRp function in cell-free RNA synthesis assays. The discordant changes that we observed in viral fitness and RdRp activities of these NS5B LxCxD domain mutants suggest that the Rb-binding activity of NS5B is not required for polymerase function per se, but rather that the regulation of Rb by NS5B may have evolved as a mechanism to orchestrate cellular gene expression in such a manner as to create an intracellular environment that is conducive to HCV replication. We attempted to demonstrate this by assessing the impact of siRNA-mediated knockdown of Rb on the replication of two Rb-binding mutants (C316A and L320A), but found that this reduced rather than enhanced replication fitness (Fig. 6). Thus, although we believe that a role in modulating cellular gene expression remains the most likely explanation for the regulation of Rb by HCV, there is no simple, straightforward relationship between Rb abundance and viral replication fitness.

Role of the Rb-binding site in NS5B. Since the LxCxE-like motif overlaps with the GDD active site motif of NS5B, it was important to examine whether mutations in the LxCxD motif directly affect NS5B activity. To do this, we first measured RNA synthesis in vitro by bacterially-expressed wt and mutant NS5B proteins. Although C316A virus was moderately impaired in replication competence (Fig. 1B and C), the NS5B-C316A RdRp was twice as active and retained all of the in vitro activities of the wt NS5B protein (Fig 4). We also examined RNA synthesis by fully-formed membrane-bound replicase complexes extracted from virus-infected
cells, and confirmed that replicase containing NS5B with the C316A substitution was at least as active (if not more active) for RNA synthesis as the JFH-1 replicase from the parental wt virus used in these studies (Fig. 5). These results confirm that Rb-binding is not directly important for NS5B polymerase activity and thus has evolved for other reasons. A very likely scenario is that NS5B may function in two or more populations: one that participates in RNA synthesis as part of the replicase and a second population that regulates cellular processes, including the abundance of Rb. This is consistent with estimates from biochemical studies that only a small proportion of NS5B is actively engaged in RNA synthesis in HCV replicon cells (21).

The genomes of each of the LxCxD domain mutants that we constructed contained two or more nucleotide changes from the wt RNA sequence. As a result, they were generally stable and did not revert to wt sequence on passage. However, the L314A mutant, which was severely handicapped in replication (Fig. 1B and 1C), underwent additional mutation to L314V on passage, thereby restoring replication fitness to close to that of wt (Fig. 3A). Interestingly, the increased replication fitness of L314V was associated with restoration of the ability of the virus to down-regulate Rb, as assessed by immunoblotting and fluorescence microscopy (Fig. 3B and C). However, the data do not allow us to say whether these two attributes of L314V, enhanced replication fitness and restored ability to regulate Rb, are in any way related to each other.

Although the L314V mutation fully corrected the modest defect in function observed with the NS5B L314A mutant in the cell-free polymerase assays (both in de novo initiation and primer extension, Fig. 4A), it did not restore viral fitness completely to wt levels (Fig. 1B and 3A).

The Rb-binding motif is LxCxD in most HCV strains, and LxNxD in a subset of genotype 1b viruses (18). Despite the fact that the C316A mutant appears to possess increased polymerase activity compared to the wt protein (Fig. 4C and 4D), 316A is not found commonly...
in the RdRp of naturally occurring HCV strains. This suggests that any benefit it might confer
upon the enzymatic activity of the NS5B polymerase may be outweighed by the abolition of Rb-
binding in the context of viral replication. Mutations at L314 and C316 have been found in HCV
replicons selected for resistance to a benzofuran inhibitor of NS5B, HCV796, but in agreement
with the present study, these mutants were found to have reduced fitness compared to the wt
replicon (6). The impact of these resistance mutations on the ability of NS5B to bind Rb is not
known.

**Rb abundance and optimal HCV replication.** If the ability of NS5B to down-regulate
Rb has evolved because it facilitates virus replication, it might be expected that HCV replication
would be increased in cells in which Rb abundance is reduced by other means. However, down-
regulation of Rb by siRNA knockdown resulted in no increase, and in fact a modest decrease, in
replication of both wt and mutated HCV lacking a functional Rb-binding domain in NS5B (Figs.
6 and 7). The role of Rb in HCV infection is thus likely to be quite complex, and any effects on
the efficiency of HCV replication in Huh-7 cells may be interwoven with other processes
regulated by Rb. An important caveat in these experiments is that the only available cell culture
systems that are sufficiently robust to study HCV infection \textit{in vitro} use Huh-7-derived cell lines.
Huh-7 cells are known to express high levels of a mutant p53 gene (1) and are thus likely to be
defective in other aspects of normal cell cycle regulation. They also contain a constitutively-
activated NF-κB signaling pathway. Although the expression of wt NS5B, but not NS5B
containing Rb-binding motif mutations, was able to down-regulate Rb and activate E2F-
responsive promoters in Huh-7 cells (18), cell culture systems that more closely mimic primary
hepatocytes will be required to fully understand the effects on Rb abundance and cell cycle
regulation exerted by HCV. Importantly, our previous studies failed to find any difference in cell
cycle regulation in Huh-7-derived cell lines containing replicating, genome-length RNA
replicons and their interferon-cured progeny (26). In contrast, we found previously that ectopic
expression of NS5B (to an abundance similar to that in replicon cells) stimulates cell
proliferation and S phase entry in U2OS osteosarcoma cells that possess functional Rb and p53
pathways (18).

In vivo, down-regulation of Rb might benefit HCV in a number of ways. In addition to its
role as a central cell-cycle regulator, Rb interacts with several transcription factors. Microarray
studies have shown that down-regulation of Rb leads to both up-regulation of genes required for
cell-cycle progression and down-regulation of genes involved in the modulation of immune
functions (15). Over 500 different genes were differentially regulated by the acute loss of Rb in
these studies. It is possible that the interaction between NS5B and Rb acts to fine-tune the level
of Rb protein in the cell. That is, too much Rb (or too much hypophosphorylated Rb) may be
detrimental to HCV infection due to its block in cell cycle progression. However, too little Rb
could lead to altered gene expression that also disfavors HCV RNA replication. Given the
possibility of such a scenario, it is not surprising to find that RNA silencing of the Rb gene
would fail to regulate Rb abundance at the level that is optimal for viral replication. This is
particularly so in Huh-7 cells, in which pathways regulating the cell cycle are intrinsically
abnormal. Further studies will be needed to sort through these various possibilities, and to
ascertain the mechanism(s) responsible for the presumed benefit to viral fitness that underlies the
evolution of Rb regulation by NS5B.
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LEGENDS TO THE FIGURES

Figure 1. Mutations in the Rb-binding motif of NS5B reduce RNA replication and replication of HCV. (A) (top) Alignments of amino acids 307-325 of JFH-1 NS5B (present in the inter-genotypic chimeric HJ3-5 virus used as wt in these studies) and the Rb-binding domains of the E7 protein of HPV type 18 and pp71 of HCMV strain AD169. The Rb-binding motif is shown in bold font, while the overlying GDD motif in the NS5B polymerase is shaded. (bottom) Amino acid sequences of the NS5B Rb-binding motif mutants used in this study. The mutated residues are underlined. (B) qRT-PCR analysis of HCV RNA accumulated in FT3-7 cells. The cells were transfected with wt HJ3-5 transcript or mutant derivatives as shown and harvested for analysis after 4, 48, 72, 96 and 120 h. Results are shown as the fold-increase in viral RNA abundance relative to the replication-incompetent D318N RNA which was transfected in parallel. (C) Infectious virus released by RNA-transfected FT3-7 cells. Serial dilutions of cell culture supernatant fluids (harvested from FT3-7 cells 96 h after the initial transfection with wt or mutant transcripts) were used to inoculate naïve Huh-7.5 cells. Cells expressing HCV core protein were detected by immunofluorescence analysis and infectious foci were counted to determine the number of focus-forming units per mL (FFU/mL). The limit of detection in this assay is 10 FFU/mL. D) Growth kinetics of mutant C316A were compared to wt in a multi-cycle infection assay. FT3-7 cells were infected with wt or C316A virus at a moi of 0.05. Cell culture supernatants were collected every 12 h from 24 to 144 h post infection. Infectious virus release was measured as described immediately above. Results shown represent the means of three independent infections.

Figure 2. Analysis of Rb abundance in cells infected with either wt or LxCxD domain mutant viruses. (A) Immunofluorescence analysis of cells infected with wt or C316A virus. FT3-7
cells were infected at moi 1 and incubated for 4 days. Infected cells were fixed and immunostained for NS5A and Rb. (B) Laser-scanning confocal microscopy of cells infected with wt, C316A and L314A viruses. (left panel) Cells were labeled with a murine anti-Rb mAb (green) or human polyclonal anti-HCV sera (red). Nuclei were labeled with DAPI. The arrows indicate the presence of cytoplasmic Rb in cells infected with wt virus, a phenomenon not observed in uninfected cells or cells infected with mutant virus. (right panel) Expanded view of the wt virus-infected cells showing merged DAPI and Rb images with cytoplasmic Rb. (C) Infrared immunoblot analysis of Rb in lysates from cells infected with wt or C316A virus. (left panel) Cells were infected at m.o.i. 1-2 and lysates collected on the day indicated post-infection were analysed by immunoblotting using antibodies specific for Rb, NS5B, and β-tubulin (loading control). (right panel) Quantitation of the infrared immunoblot showing the abundance of Rb relative to β-tubulin at various times following infection with the wt or C316A virus.

Figure 3. Analysis of a partial revertant of the L314A mutant. (A) Virus released into cell culture media following transfection with HJ3-5, L314A, L314V, C316A or D318N RNA. (B) Infrared immunoblot analysis of Rb in lysates from cells infected with L314V. (left panel) Cells were infected with L314V or mock-infected and lysates collected on the day indicated post-infection were analyzed by immunoblotting using antibodies specific for Rb, NS5B and β-tubulin (loading control). (right panel) Quantitation of the infrared immunoblot showing the abundance of Rb relative to β-tubulin at the indicated time points. (C) Immunofluorescence analysis of cells infected with HJ3-5, L314A or L314V. Huh-7.5 cells were infected at m.o.i 0.1 and incubated for 4 days before fixation and staining for Rb or NS5A.
Figure 4. Effect of mutations in NS5B on RNA synthesis in vitro. (A) SDS-PAGE of the purified proteins. The proteins were expressed in *E. coli* and purified through two affinity columns (see Materials and Methods). Lane M contains Benchmark protein molecular weight marker (Invitrogen). (B) Sequence of template LE19 that can direct *de novo* initiated RNA synthesis by NS5B. LE19 can also form a partially duplexed dimer that can direct primer extension to result in a 32-nt product. The single-stranded RNA named “L” is the molecule that can be ligated to form the circularized RNA named “C” (see text for additional details on templates). (C) Autoradiograph of the products of RdRp assay run on a 20% polyacrylamide-7.5 M urea denaturing gel. The templates used are specified above each lane and the GTP where present is at 0.2 mM final concentration. The sizes of the RNA products are denoted at the left and lane numbers are shown at the bottom of the image. (D) Quantification of the *de novo* initiation (19-nt) and PE (32-nt) products of the RdRp assay using LE19 as template. Four replicate reactions were used to quantify the products; results are shown as mean ± standard deviation. Both the *de novo* initiation and PE products generated by all the mutant polymerases were normalized to that of wild type whose activity was set at 100%.

Figure 5. *In vitro* RNA synthesis assay from heavy membrane fractions containing functional HCV replication complexes. (A) Heavy membrane fractions isolated from cells infected with wt or C316A virus, or mock infected cells were incubated in a transcription reaction containing α-32P-labeled CTP. Heavy membrane fractions from genotype 1a and 2a subgenomic replicon-bearing cell lines (Sg-Rep) were included as positive controls. Labeled products were resolved on a denaturing agarose gel and visualized by autoradiography. (B) Quantification of products from panel A by Phosphorimagery analysis. (C) Immunoblot
analysis of HCV proteins NS3, NS5B and core in the heavy membrane fractions used in panel A. The blots were also probed for Rab1b, an endoplasmic reticulum marker, as a loading control for the heavy membrane fractions.

**Figure 6.** Effects of Rb knockdown by siRNA on virus replication. (A) Immunoblot analysis of lysates from FT3-7 cells transfected with a pool of siRNAs targeting Rb or pooled control siRNAs. Rb was efficiently knocked down to undetectable levels at 3, 4 and 5 d post transfection (top). The same blot was also probed for actin as a loading control (bottom). (B) FT3-7 cells transfected with pools of siRNA targeting Rb or a similar pool of control siRNAs were infected with either wt or C316A virus at 3 d post transfection at moi 2. Cell culture supernatants were harvested at 2 days post infection. Virus released into supernatant fluids of infected cells was determined. Immunoblot analysis of protein from a parallel transfection performed at the same time is shown in (A). (C) Immunoblot analysis of lysates from FT3-7 cells transfected with individual siRNAs targeting Rb or non-specific control siRNAs. Lanes are numbered 1: Rb siRNA pool, 2: Rb siRNA #5, 3: Rb siRNA #6, 4: Rb siRNA #7, 5: Rb siRNA #9, 6: control siRNA pool, 7: control siRNA #1, 8: control siRNA #2. (D) FT3-7 cells transfected with various siRNAs for Rb or control siRNAs were infected with wt HJ3-5 virus at 3 d post transfection at moi 2. Infectious virus in cell culture supernatants was quantitated by focus-forming assay. Immunoblot analysis of protein from a parallel transfection performed at the same time is shown in (C). (E) FT3-7 cells were infected with either wt, C316A or L320A virus at m.o.i. 0.1. At 6 dpi, cells were seeded into 6 well plates and transfected with a pool of siRNAs specific for Rb or pooled control siRNAs. At 10 dpi, cell culture supernatants were harvested from the transfected cells and virus titers were determined.
Figure 7. (A) Immunoblot analysis of lysates from Huh-7.5 cells transfected with a pool of siRNAs targeting Rb or pooled control siRNAs. Cells were harvested at 3 days post transfection. Parallel transfections were trypsinized and seeded into 8-well chamber slides after 2 d (4 x 10^4 cells per well) and infected with serial dilutions of wt HJ3-5 virus at 3 days post transfection. Cells were fixed and stained for core antigen at 2 dpi. The number of focus forming units counted on cells transfected with control siRNAs was greater than 2-fold higher than on cells transfected with Rb-specific siRNAs (B). Examples of foci are shown in (C) to demonstrate the size of foci.
A

Consensus: LxCxD/E
HPV type 18 E7: QNEIPVDLLCHEQLSDSEELHCMV AD169 pp71: LVDALETACSDPNTIHKJFH1 NSSB: GIVAPTMVCGD0LVVISEND318N: GIVAPTMVCGDOLVVISENSSB Mutants

B

Fold Increase in HCV RNA

C

FFU/ml

10^9

10^8

10^6

10^4

10^2

10

wt D318N L314A L316A L314A/C316A L320A

D

FFU x 10^3/ml

3

2

1

0

0 24 48 72 96 120 144

Hrs

wt L314A

L320A L314A/C316A L314V

Hrs
### Table D

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<tr>
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<td>70 ± 15</td>
<td>57 ± 21</td>
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<tr>
<td>L314V</td>
<td>106 ± 10</td>
<td>119 ± 21</td>
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<tr>
<td>C316A</td>
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### Diagrams

**Diagram A**
- M, JΔ21, L314A, L314V, C316A, C316A/L314A

**Diagram B**
- LE19 5' UGUU AUAC 3'
- AU
- UG
- AU
- AU
- U
- U
- L
- 5'p-AUUUCA G
- 3' CAUGUUU U

**Diagram C**
- LE19P: +, +, +, +, +
- GTP: +, +, +, +, +
- L/C: L, C, L, C, L, C

**Diagram D**
- Lanes: 1-25
- Bases: 16, 19, 21, 22, 23, 30, 31, 32, 34