A library of infectious hepatitis C viruses with engineered mutations in the E2 gene reveals growth-adaptive mutations that modulate interactions with scavenger receptor class B type I

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An E2 gene infectious HCV library reveals growth adaptive mutations that modulate interactions with the scavenger receptor class B type I

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Running title: Adaptive HCV E2 mutations modulating SR-BI interaction
ABSTRACT

While natural hepatitis C virus (HCV) infection results in highly diverse quasispecies of related viruses over time, mutations accumulate more slowly in tissue culture, in part because of the inefficiency of replication in cells. To create a highly diverse population of HCV particles in cell culture and identify novel growth-enhancing mutations, we engineered a library of infectious HCV with all codons represented at most positions in the ectodomain of the E2 gene. We identified many putative growth adaptive mutations and selected nine highly represented E2 mutants for further study: Q412R, T416R, S449P, T563V, A579R, L619T, V626S, K632T, and L644I. We evaluated these mutants for changes in particle to infectious unit ratio, sensitivity to neutralizing antibody or CD81 large extracellular loop (CD81-LEL) inhibition, entry factor usage, and buoyant density profiles. Q412R, T416R, S449P, T563V and L619T were neutralized more efficiently by anti-E2 antibodies and T416R, T563V and L619T by CD81-LEL. Remarkably, all nine variants showed reduced dependence on scavenger receptor class B type I (SR-BI) for infection. This shift from SR-BI usage did not correlate with a change in the buoyant density profiles of the variants, suggesting an altered E2-SR-BI interaction rather than changes in the virus-associated lipoprotein-E2 interaction. Our results demonstrate that residues influencing SR-BI usage are distributed across E2 and support the development of large scale mutagenesis studies to identify viral variants with unique functional properties.
IMPORTANCE

Characterizing variant viruses can reveal new information about the lifecycle of Hepatitis C virus (HCV) and the roles played by different viral genes. However, it is difficult to recapitulate high levels of diversity in the laboratory because of limitations in the HCV culture system. To overcome this limitation, we engineered a library of mutations into the E2 gene in the context of an infectious clone of the virus. We used this library of viruses to identify nine mutations that enhance the growth rate of HCV. These growth enhancing mutations reduced the dependence on a key entry receptor, scavenger receptor class B type I (SR-BI). By generating a highly diverse library of infectious HCV, we mapped regions of the E2 protein that influence a key virus-host interaction and provide proof-of-principle for the generation of large-scale mutant libraries for the study of pathogens with great sequence variability.
INTRODUCTION

Hepatitis C virus (HCV) infection causes a major global health burden, as approximately 3% of the world's population is affected (1). Up to 30% of individuals with HCV will resolve infection spontaneously, whereas the majority develop chronic infections that can cause fibrosis, cirrhosis, and hepatocellular carcinoma (2). HCV is a member of the hepacivirus genus of the Flaviviridae family of RNA viruses, and is a positive-sense, enveloped virus with a 9.6 kilobase genome. The genome comprises a single open reading frame encoding an approximately 3,000 amino acid polyprotein flanked by 5′- and 3′-noncoding regions. HCV primarily infects and replicates in hepatocytes, and its lifecycle is tied intimately to the lipoprotein biosynthesis pathway. HCV particles incorporate host lipoproteins forming a "lipoviroparticle" with unique properties when compared to other Flaviviridae members (3, 4).

HCV is divided into 7 genotypes and 67 subtypes and exists as a quasispecies within a host (5). Natural HCV isolates exhibit considerable diversity in their genomic sequences, however it is difficult to recapitulate the extent of this variability in vitro. Recent advances have made it possible to infect cells in culture with human-derived HCV isolates and observe genome replication, but new infectious particles still cannot be generated readily from clinical samples (6). The study of cell culture infectious HCV relies on a few, relatively slow-growing infectious clones (7–13). These clones do not acquire growth adaptive mutations rapidly during passaging despite the high error rate of the HCV polymerase, possibly because of the limited production of new infectious virions.

HCV has two envelope glycoproteins, E1 and E2. The E2 gene is a protein of particular variability and is under selection pressure from adaptive immune responses during an extended chronic infection (14, 15). In conjunction with virus-associated lipoprotein, the envelope proteins mediate attachment and entry of the virus into host cells (16). E1 and E2 form non-covalently linked trimers of disulfide-linked heterodimers on the virion surface (17, 18). Two partial crystal structures of the "core" of the E2 protein have provided some insight into E2
structural biology (19, 20). E2 core has a compact, globular structure consisting mainly of random coils and β-strands. While several structural features of E2 have well-described functions in the HCV lifecycle, the contributions of other regions of the protein are less well understood (21–24).

To study an HCV population containing an array of single amino acid variants, we created a genotype 2a JFH-1 isolate library with maximal diversity in the E2 gene. We generated a site-directed saturation mutagenesis (SDSM) library from a JFH-1-derived infectious clone, which allowed us to explore almost the entire sequence space for single-codon substitutions in E2. We used the library to identify novel E2 growth adaptive mutations. After only three passages, nine mutations in different regions of the protein were identified and confirmed to have growth kinetic advantages: Q412R, T416R, S449P, T563V, A579R, L619T, V626S, K632T, and L644I. These mutants had varying profiles with respect to neutralizing monoclonal antibody (NMAb) and CD81 large extracellular loop (CD81-LEL) sensitivity. However, all of these mutations yielded a reduced dependence on scavenger receptor class B type I (SR-BI) for entry even though they were distributed across several regions of E2. Our study provides insight into the molecular determinants of the interaction between SR-BI and E2 and acts as proof-of-principle for the use of large scale variant libraries to address questions in HCV biology.
METHODS AND MATERIALS

Cell culture. Huh7.5 cells were obtained as a gift (C. Rice, Rockefeller University) and maintained in Dulbecco’s Modified Eagle Medium (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Equitech-Bio), MEM nonessential amino acids, and penicillin/streptomycin (Corning).

Plasmid library and virus production. The plasmid pLJ83, which encodes the JFH-1 HCV infectious clone bearing adaptive mutations outside of the E2 gene, was a generous gift (G. Luo, University of Alabama, Birmingham) (25) and used for our SDSM library. Unless noted in the text, all amino acid residues numbers refer to the JFH-1 genome (26). Three hundred and twenty complementary primer sets containing random nucleotides at all three positions of a single codon within the E2 gene were designed and ordered from Integrated DNA Technologies. All residues between amino acid positions 384 and 721 in the polyprotein were targeted, excluding cysteines (positions 429, 452, 459, 488, 496, 505, 510, 554, 566, 571, 585, 589, 601, 611, 624, 648, 656, and 681). Each primer set was used to amplify variants by polymerase chain reaction (PCR) from pLJ83 using a Phusion® high fidelity polymerase kit (New England Biolabs). The PCR products were digested with DpnI (New England Biolabs) and transformed in library-grade MegaX® DH10B T1R electrocompetent cells (Thermo Fisher Scientific).

Plasmids were prepared from bacterial cultures using plasmid Maxi-prep kits (Qiagen) from sets of ten sequential codon libraries generated by SDSM. The 32 pooled plasmid libraries corresponded to the following amino acids: 384-393, 394-403, 404-413, 414-423, 424-434, 435-444, 445-455, 456-466, 467-476, 477-486, 487-498, 499-509, 511-520, 521-530, 531-540, 541-550, 551-561, 562-573, 574-583, 584-595, 596-606, 607-617, 618-628, 629-638, 639-649, 650-660, 661-670, 671-680, 682-691, 692-701, 702-711 and 712-721. The plasmids were linearized by digestion with NruI (New England Biolabs), purified, and genomic RNA was synthesized using a MEGAscript T7 transcription kit (Ambion). Infectious HCV was generated after transfection of Huh7.5 cells as follows: 2 µg of in vitro-derived viral RNA was electroporated into
4 x 10^6 Huh7.5 cells, and virus was harvested 48, 72, 96, and 120 h later. HEPES pH 7.3 (10 mM) was added to the virus-containing supernatants for storage at 4°C prior to concentration with Amicon Ultra 100,000 kDa MWCO centrifugal filters (EMD Millipore). Concentrated virus stocks were stored at -80°C and titered using the 50% tissue culture infectious dose (TCID_{50}) method (27).

**Amplicon sequencing of plasmid libraries.** The 32 sets of pooled plasmids described above were analyzed using next generation sequencing to determine the diversity of the library. An approximately 2 kilobase fragment corresponding to the E1 and E2 genes was amplified by PCR from each of the 32 plasmid pools and submitted for Ampicon-seq library preparation and Illumina HiSeq sequencing (single end 50 basepair reads) at the Genome Technology Access Center at Washington University. Reads were aligned to the pLJ83 sequence using Bowtie2. Reads containing insertion or deletions were not considered, and the remaining reads were counted to determine codon frequency using a custom script.

**Focus-forming assay.** Infectious HCV was detected using a focus-forming assay (FFA) on Huh7.5 cells. Briefly, 6.4 x 10^3 Huh7.5 cells were plated in individual wells of a 96-well plate treated with of poly-L-lysine one day prior to infection. Three days later, cells were rinsed with PBS, fixed and permeabilized with cold methanol, rinsed again and incubated in blocking solution (PBS containing 1% BSA and 0.2% skim milk). Subsequently, the cells were incubated with 100 ng/mL anti-NS5A mAb (9E10, a gift of C. Rice, Rockefeller University (9)), rinsed again, and incubated with 1:2000 dilution of horseradish peroxidase conjugated goat anti-mouse IgG (Sigma). Virally-infected cell foci were visualized using TrueBlue™ peroxidase reagent (KPL) and quantified using an S5 Biospot™ Macroanalyzer (Cellular Technologies Ltd).

**Selection of mutations in E2 that confer increased HCV growth.** Parallel cultures of 3.8 x 10^4 Huh7.5 cells were infected with 1 x 10^4 TCID_{50} of each of 32 HCV mutant pools containing engineered diversity at ten sequential positions in E2. Input viruses were removed 6 h post-infection and newly generated viruses were harvested 7 days later and used to infect...
new Huh7.5 cells. This process was performed twice for a total of three passages. After the third passage, viral RNA was harvested using QIAamp® viral RNA mini kits (Qiagen) from all 32 final passage viruses, cDNAs were synthesized using the Superscript III® first strand synthesis system (Invitrogen), and DNA fragments corresponding to the E1-E2 fragment were amplified by PCR. Each PCR product was sequenced across the region of the E2 gene containing the sites of engineered mutations in the original pool. If changes to the consensus sequence were observed, the exact combinations of nucleotide substitutions at any given codon were determined using a Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) to generate individual bacterial colonies bearing unique E1-E2 sequences. Nine mutations identified from the TOPO cloning results were introduced into pLJ83 using conventional site-directed mutagenesis. Following verification of the complete genome sequence of the mutant plasmids, all nine clonal mutant viruses were generated as described above. Three of the nine selected mutations (S449P, L619T and L644I) also were introduced into a JFH-1-derived chimeric infectious clone encoding the structural genes of the genotype 1 H77 isolate (H77/JFH-1) at homologous positions in the E2 gene (28). The corresponding mutations in the H77 strain were S449P, L615T and L640I. Virus was produced exactly as described above, except an XbaI restriction enzyme digestion was performed prior to in vitro RNA production.

Alignment of genotype 2 E2 amino acid sequences. To determine if the substitutions selected were present in circulating HCV strains, an alignment of genotype 2 E2 sequences was generated using the NIAID Virus Pathogen Database and Analysis Resource (ViPR) (http://www.viprbrc.org/) (29).

HCV growth analysis. Huh7.5 cells were infected at a multiplicity of infection (MOI) of 0.1 with parent virus or clonal growth-adapted mutants. Input viruses were removed 6 h post-infection and samples collected every 24 h for seven days. Viral yield in the supernatant was quantified by FFA. The FFA protocol increased the apparent number of infectious units of growth adapted virus in a sample slightly because of the more rapid growth and spread to new
cells by the mutants. We therefore performed a second growth kinetic analysis using the TCID$_{50}$ method of virus titration to quantify virus. Huh7.5 cells were infected at an MOI of 0.05 with each of the clonal growth-adapted mutants as described above, and viral yield in supernatant was collected 5 days post-infection and calculated.

**HCV genome quantification.** qRT-PCR was used to quantify genome copy number in virus-containing supernatants. Viral RNA was harvested using QIAamp® Viral RNA Mini Kit (Qiagen). All reactions were prepared using a TaqMan® RNA-to-C$_T$™ (Applied Biosystems), with forward primer 5′-GATAAACCACTCTATGCCCG-3′, reverse primer 5′-CTATCAGGCAGTACCACAAGG-3′, and probe 5′-56-FAM/CTTTCGCAACCAACCTACTCG/-36-TAMSp/-3′. Run conditions were 48°C for 15 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. *In vitro* transcribed genomic transcripts were used as standards.

**Inhibition of HCV infection by anti-E2 NMAbs and CD81 large extracellular loop.** Two-hundred TCID$_{50}$ of parent or mutant viruses were pre-incubated with a dilution series of anti-E2 antibody or CD81-LEL for 1 h at 37°C and then added to Huh7.5 cells. Infection was quantified 72 h later by FFA, and EC$_{50}$ values determined after performing non-linear regression analysis. Anti-E2 antibodies HC84.26 (a gift of S. Foung, Stanford University) and H77.39 have been described (30, 31). To generate soluble CD81-LEL, residues 114-203 of human CD81 were cloned into a Pet28(+) derived vector with a thrombin cleavable C-terminal BirA biotinylation site and 6xHis tag. CD81-LEL was produced by isopropyl β-D-1-thiogalactopyranoside induction in BL21-DE3 E. coli cells and purified by oxidative refolding from inclusion bodies (32). Briefly, bacterial cell pellets were resuspended in a 1:1 mixture of solution buffer (50 mM Tris [pH 8.0], 25% sucrose, 10 mM dithiothreitol [DTT]), and lysis buffer (50 mM Tris [pH 8.0], 1% Triton X-100, 100 mM NaCl, 10 mM DTT). The sample was lysed by sonication and centrifuged, and the pellets were washed three times with wash buffer (50 mM Tris [pH 8.0], 0.5% Triton X-100, 100 mM NaCl, 1 mM DTT) and once in wash buffer without
Triton X-100 to obtain purified inclusion bodies. The purified inclusion body pellets were resuspended in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA), and aliquots of the slurry were solubilized in 6 M guanidine-HCl, 10 mM Tris (pH 8.0), and 20 mM β-mercaptoethanol. Solubilized aliquots were diluted rapidly into a stirring reservoir of oxidative refolding buffer (400 mM L-Arginine, 100 mM Tris [pH 8.0], 0.5 mM oxidized glutathione, 5 mM reduced glutathione) followed by overnight incubation. The refolded CD81-LEL then was purified by size-exclusion chromatography.

**Inhibition of infection by antibodies against HCV entry factors.** Huh7.5 cells were treated with serially diluted anti-CD81, anti-claudin-1 (CLDN1), or anti-SR-BI antibodies for 1 h at 37°C and then infected with 200 TCID50 of parent or mutant viruses. Infection was quantified 72 h later by FFA, and EC50 values were determined after non-linear regression analysis. Anti-CD81 (clone JS-81) was purchased from BD Biosciences, and anti-CLDN1 (clone 5.16v5, a gift of I. Hotzel, Genentech), and anti-SR-BI (clone mAb16-71, a gift of A. Nicosia, CEINGE Biotecnologie Avanzate, Naples, Italy) were obtained from colleagues (33, 34).

**CRISPR/Cas9 mediated gene editing of SR-BI.** To perform CRISPR/Cas9 mediated gene editing, we generated expression plasmids encoding the U6 promoter in front of SR-BI specific guide RNAs (35). Two rounds of overlapping PCR were performed by amplifying a guide RNA encoding plasmid (provided by George Church, Harvard University, Boston, MA, Addgene plasmid # 41819): in the first round, PCR products were generated encompassing the U6 promoter through the 5′ end of the guide RNA (consisting of the specific target sequence) with the ME-O-1122 oligonucleotide (5′-CGGGCCCCCCTCGAGTGTACAAAAGCAGGCT-3′) and an SR-BI target sequence specific reverse oligonucleotide (see list below). A second PCR product was generated encompassing a region from the SR-BI target sequence through the end of the guide RNA coding sequence with a forward direction CLDN1 target sequence specific oligonucleotide (ME-O-1138; 5′-GCTTCATTCTCGCCTTCCGTTTTAGAGCTAGAAATA-3′). These products were re-amplified with only the outside oligonucleotides, ME-O-1122 and -
1123, to produce single PCR products flanked by XhoI and EcoRI sites at the 5′ and 3′ ends, respectively, that were cloned into pBlueScript. Four separate SR-BI specific gRNA plasmids were created with the following forward and reverse oligonucleotide combination: ME-O-1241/1242; 5′-CTGCTCCGCAAAGCAGCGC GTTTTAGAGCTAGAAATA-3’ / 5’-GGCCCTACGTGTACAGGT GTTTTAGAGCTAGAAATA-3’ / 5’-CACCTGTACACGTAGGGCC CGGTGTTTCGTCCTTTCC-3’, ME-O-1243/1244; 5′-GGCCCTACGTGTACAGGT GTTTTAGAGCTAGAAATA-3’ / 5’-CACCTGTACACGTAGGGCC CGGTGTTTCGTCCTTTCC-3’, ME-O-1245/1246; 5′-CCCTCCAAGTCCACCGCT GTTTTAGAGCTAGAAATA-3’ / 5’-AGCCGTGGGACTTGGAGGG CGGTGTTTCGTCCTTTCC-3’, and ME-O-1247/1248; 5′-AGCCGTGGGACTTGGAGGG CGGTGTTTCGTCCTTTCC-3’ / 5’-GATCCACCTCGTGGACAAG GTTTTAGAGCTAGAAATA-3’ / 5’-CTTGTCCACGAGGTGGATC CGGTGTTTCGTCCTTTCC-3’, which target nucleotides 5259-5277, 51409-51427, 53942-53960, and 57068-57086 of the SR-BI human genomic locus (relative to Genbank accession number NG_028199), respectively.

Huh7.5 cells were transiently transfected with expression plasmids encoding a human codon optimized Cas9 protein from *Streptococcus pyogenes* (provided by George Church, Harvard University, Boston, MA, Addgene plasmid # 41815) (35) and a pooled mixture of the above SR-BI guide RNA expression plasmids. Transfected cells were passaged for one to two weeks to allow the turnover of previously translated target protein. Cells were then subjected to fluorescence activated cell sorting (FACS) for loss of SR-BI following staining with the anti-SR-BI1 antibody (mAb16-71) and a goat anti-human Alexa-647 antibody (Invitrogen, Carlsbad, CA). Knockout of SR-BI efficiency prior to sorting was 11-15%. Single cell clones were obtained by limiting dilution cloning in 96-well plates. Individual clones were expanded and assayed for SR-BI expression by flow cytometry with the mAb16-71 antibody, and the capacity to support HCV infection, as previously described (9). We chose Huh7.5 SR-BI KO clone 16 as a representative single cell clone for further experiments.
Lentiviral transduction of SR-BI\(^{\text{KO}}\) Huh7.5. Transgene expression through lentiviral transduction was used to complement SR-BI\(^{\text{KO}}\) cells. To express GFP alone, the self-inactivating lentiviral provirus TRIP-GFP-linker was used (31). For SR-BI expression, the TRIP-GFP-hu-SR-BI-linker provirus was used. VSV-G pseudotyped lentiviral stocks were produced in 293T cells, as previously described (31, 36). Supernatants were collected 2 days post transfection and used to infect gene-edited Huh7.5 cells, also as previously described (31, 36). Cells were expanded for at least two weeks prior to analysis.

Infection of SR-BI gene-edited Huh7.5 cells. SR-BI\(^{\text{KO}}\)+GFP or SR-BI\(^{\text{KO}}\)+GFP+SR-BI cells (1 x 10\(^5\)) were plated in wells of a poly-L-lysine treated 96-well plate one day prior to infection. Seventy-five TCID\(_{50}\) of virus per well were added, and three days later the plates were developed by FFA. Relative infection was calculated by determining the percent of foci present in SR-BI\(^{\text{KO}}\)+GFP wells compared to SR-BI\(^{\text{KO}}\)+GFP+SR-BI wells for each virus.

Virus attachment to gene-edited Huh7.5 cells. SR-BI\(^{\text{KO}}\)+GFP or SR-BI\(^{\text{KO}}\)+GFP+SR-BI cells (5 x 10\(^5\)) in suspension were incubated with 5 x 10\(^4\) TCID\(_{50}\) of virus for 3 h on ice. Cells were washed 6 times with chilled media and cellular RNA was harvested using a Qiagen RNeasy® Mini Kit. Viral genome copies were measured by qRT-PCR as described above. Relative attachment was calculated by determining the percentage of viral RNA bound to SR-BI\(^{\text{KO}}\)+GFP cells compared to SR-BI\(^{\text{KO}}\)+GFP+SR-BI cells for each WT and mutant virus.

Sucrose density gradient ultracentrifugation. HCV was ultracentrifuged using 5-50\%(w/v) sucrose gradient ultracentrifugation. Sucrose solutions were prepared in TEN buffer (0.01 M Tris, pH 8.0; 1 mM EDTA, and 100 mM NaCl) using a Gradient Master (Biocomp). The viruses were ultracentrifuged for 17 h at 4°C and 105,000 x g (Beckman SW41 Ti) and 1 ml fractions were collected and titered by FFA. The number of genome copies in each fraction was determined by RT-PCR as described above. Fraction densities were measured using an ABBE-3L refractometer (Thermo Fisher Scientific).
Statistical analysis. Statistical analyses were performed using GraphPad Prism software. Differences in mean EC\textsubscript{50} values, relative infections, titers or RNA:TCID\textsubscript{50} values between the mutants and parent virus were analyzed by one way-ANOVA followed by a Dunnett's test.
RESULTS

Isolation of adaptive JFH-1 variants by passage of a library containing mutagenized E2 genes. Several growth adaptive mutations have been previously identified in E2 and studying the properties of these mutants is an established method of linking structural features of E2 with functions in the virus lifecycle (37–41). Past attempts to identify growth adaptive mutations in the HCV genome have relied on serial passage of a clonal virus stock for weeks or months until adaptive mutations arise (25, 38–50). To identify adaptive mutations more expediently, we created a library of infectious HCV with a fully mutagenized E2 ectodomain (Fig 1A). Three hundred and twenty primer sets containing three random nucleotides (NNN) corresponding to one codon in the ectodomain of E2 were designed. Using these primers and a JFH-1 infectious clone plasmid, we performed separate SDSM reactions with each primer pair yielding 320 plasmid libraries. To simplify production of infectious virus, we grouped the plasmid libraries into 32 sets of 10 adjacent codons for all downstream steps. To confirm that each plasmid library contained 64 engineered variants (all nucleotide combinations at each codon) we performed amplicon sequencing of the 32 sets of 10 pooled single codon plasmid libraries (Fig 1B). Under ideal conditions we expected a 10% mutation rate for a single codon within the ten plasmid pool containing variability at that position; our library had an average mutation rate of 6.0%. To produce infectious HCV, we transcribed RNA genomes in vitro from the pooled plasmids and electroporated each of 32 RNA stocks into Huh7.5 cells. By the end of this process, we had created 32 viral "pools" with each containing maximum variability at ten positions in E2.

Following virus production, cultures of Huh7.5 cells were inoculated with $10^4$ TCID$_{50}$ of each of the 32 mutant pools at a MOI of 0.3. Input virus was removed 6 h after infection and newly produced virus was collected 7 days later. This output virus was used to infect Huh7.5 cells serially twice more after which Sanger sequencing of HCV E2 RT-PCR products was performed. Eight of the 32 third passage (P3) viruses had changes in the consensus sequence...
of E2 within the region containing engineered variability. To determine the specific substitutions that were enriched by passaging, we generated cDNA from P3 viruses, and TOPO-cloned the E1-E2 genome fragment amplicons produced by PCR. We sequenced the E2 gene for 24 to 48 resultant bacterial colonies from each of the 8 P3 viruses with consensus sequence changes (Table 1).

Based on these results, nine variants that corresponded to the dominant amino acid substitution in each of the P3 viruses were selected for further study: Q412R, T416R, S449P, T563V, A579R, L619T, V626S, K632T, and L644I. Four of these residues (Q412, T416, S449 and T563) are at or very near to positions in E2 that have been reported as adaptive mutations (38–41, 45), with the remainder not having been described to affect growth. Sequencing of individual clones suggested that multiple substitutions at a given position might confer growth advantage over the parent virus with the exception of S449, which had only proline substitutions. S449P also was unique, as it eliminated a predicted N-linked glycosylation site at N448.

**Clonal mutant viruses show enhanced growth kinetics.** We used conventional site directed mutagenesis to introduce the nine dominant mutations into the parental infectious clone. Following verification by complete genome sequencing of the plasmids, mutant viruses were generated after electroporation of in vitro derived RNA into Huh7.5 cells and titered using the TCID\(_{50}\) method. To validate the significance of the mutations, multi-step viral growth analyses were performed by FFA after infection of Huh7.5 cells (MOI of 0.1) with each mutant and the parental virus (Fig 2A). Whereas each of the mutations conferred a growth advantage compared to the parental virus, we noticed that the input virus titer (t = 0) for all of the mutant viruses appeared higher than the parent JFH-1 in this assay. Because we inoculated with equivalent input amounts based on the TCID\(_{50}\) assay, we hypothesized that the difference at t = 0 of the input virus reflected the more rapid growth of the adaptive mutant strains over the course of the FFA compared to WT virus. Given this potential confounding issue, we performed
a second confirmatory viral growth assay. Cultures of Huh7.5 cells were infected at an MOI of 0.05 (as determined by TCID$_{50}$ assay) with the mutant and parent viruses, and supernatants were collected five days later and titered by TCID$_{50}$ assay (Fig 2B). Using this second method, the mutants exhibited a 5- and 10-fold growth advantage compared to the parental viruses. In this assay, the V626S mutant did not show a statistically significant growth advantage compared to the parental strains. Nonetheless, it may still be a growth adaptive mutation based on accumulated data (Table 1, Fig 2).

**Particle to infectious unit ratios for E2 mutants.** We next explored whether there were functional differences in the properties of growth-adapted viruses compared to the parent JFH-1 strain. We initially assessed the relative specific infectivity by measuring the RNA:TCID$_{50}$ ratio of each mutant virus (Table 2). A more efficient assembly process or increased stability of infectious virions could contribute to increased peak titers. As HCV growth kinetic assays are long, small differences may compound over time and account for at least part of the adaptive growth phenotype. Using the tittered samples described in Fig 2B, viral RNA was harvested and genome copy determined via RT-PCR using in vitro derived-RNA from infectious clone plasmids as standards. The greatest difference between parent and a mutant virus was observed for T563V, and even this fold-change was less than 4-fold. All mutant viruses, apart from K632T, had a lower RNA:TCID$_{50}$ ratio than the parental virus, although these differences did not achieve statistical significance.

**Buoyant density profiles of growth adapted mutants are similar to parent.** HCV has a unique buoyant density profile when compared to many other Flaviviridae family members (e.g., flaviviruses). Because of lipoprotein incorporation, infectious HCV particles have a lower density and broader infectivity peak (3, 4). Some E2 growth variants have altered buoyant density profiles relative to their parental strains (37, 40), and such differences may reflect alterations in lipoprotein incorporation or changes to the subset of HCV particles (high or low density) that display peak infectivity for a variant. To determine whether our panel of mutants...
had shifts in buoyant density profiles, we performed sucrose gradient ultracentrifugation and quantified the density, viral titer, and HCV genome copy number in each fraction collected (Fig 3). The mutants all behaved similarly to parent virus, suggesting they have similar lipoprotein utilization or incorporation.

**Some adaptive mutations enhance CD81-LEL and NMAb sensitivity.** E2 is the major viral target of neutralizing antibodies during infection. Many of the most effective broadly NMAbs block interaction between E2 and CD81, one of the key HCV entry receptors (30, 31, 51, 52).

Some growth-adaptive mutations in E2 were shown to increase inhibition by soluble CD81-LEL and NMAbs (37, 39, 40). To assess whether the identified mutations affected the binding of E2 to CD81, we performed inhibition of infection assays with a soluble CD81-LEL (Fig 4A). A subset of adaptive mutant viruses (T416R, T563V, and L619T) were inhibited more efficiently by CD81-LEL than the parent JFH-1 virus (>5-fold decrease in EC\(_{50}\), \(P < 0.05\)), and thus an enhanced CD81-E2 interaction might contribute to the growth advantages of these viruses.

However, most of the mutants were not more sensitive to CD81-LEL inhibition, which contrasts with some published growth-adaptive variants.

We next performed dose-response inhibition of infection assays with two broadly neutralizing anti-E2 NMAbs, H77.39 (Fig 4B) and HC84.26 (Fig 4C), both of which block interactions between E2 and CD81 (30, 31). H77.39 maps to an epitope centered on amino acids 415 and 417 and part of a larger epitope between positions 412-421. Residues within this region, especially W420, can influence CD81 interactions (24, 39). HC84.26 maps to conserved residues within the CD81 binding site between residues 441 and 446 and to residue 620 (H77 residue numbers). Q412R, T416R, S449P, T563V, and L619T viruses were neutralized more efficiently by H77.39 than the parent JFH1 (>5-fold decrease in EC\(_{50}\), \(P < 0.0001\)) (Fig 4B) and T416R, S449P and T563V viruses were inhibited to a greater extent (>5-fold decrease in EC\(_{50}\), \(P < 0.05\)) by HC84.26 (Fig 4C). However, A579R, V626S, K632T and L644I viruses were unchanged or exhibited modestly shifted profiles with anti-E2 NMAbs and CD81-LEL.
HCV containing E2 adaptive mutations require CD81 and CLDN1 for infection. HCV entry is a complex process that requires several host factors including heparan sulfate proteoglycans (53, 54), low-density lipoprotein receptor (54–56), SR-BI (21, 57), CD81 (51, 58), CLDN1 (59), and occludin (OCLN) (60). Some E1 and E2 mutations cause altered entry factor usage or reduce dependence on a particular receptor (37, 39, 40, 61). To test whether our adaptive mutations changed CD81 or CLDN1 receptor dependency, we performed neutralization assays with antibodies against these host entry factors. However, all of the adaptive mutant viruses were inhibited by anti-CD81 and anti-CLDN1 similarly compared to the parental strain, suggesting little change in dependency on these receptors (Fig 5).

Growth-adaptive mutants are less-dependent on SR-BI for infection. Markedly different neutralization profiles were observed for the mutants when an anti-SR-BI antibody was used (Fig 6), as each variant was more resistant to antibody inhibition. Whereas the parental JFH-1 had a lower asymptote of 14% relative infection (i.e. resistant fraction), some mutant viruses appeared to be entirely resistant to anti-SR-BI treatment of Huh7.5 cells. Even the most sensitive mutant virus had a lower asymptote at 34% relative infection, 2.5-fold greater than parent. To confirm changes to SR-BI dependency, we generated SR-BI KO Huh7.5 cells using CRISPR/Cas9 gene editing (Fig 7). A clonal SR-BI KO Huh7.5 line, clone 16, was selected for further study. Clone 16 was confirmed to lack SR-BI expression (Fig 7A) and exhibited a moderately impaired capacity to support HCV cell entry (Fig 7B). Importantly, this phenotype was restored completely by trans-complementation from a lentiviral vector (Fig 7B). Paired infections of control (GFP) and SR-BI (SR-BI + GFP) trans-complemented SR-BI KO cells with WT and mutant HCV corroborated the anti-SR-BI antibody data: the mutants showed greater infection in cells lacking SR-BI compared to the parental virus (Fig 7C). Thus, growth adaptation in Huh7.5 cells resulted in the emergence of viruses with less dependency on SR-BI for infection. To assess whether the mutations had a similar effect on other HCV genotypes, we generated homologous mutations to JFH-1 S449P, L619T and L644I in a chimeric infectious
clone encoding the structural genes of the genotype 1 H77 isolate (H77/JFH-1). We performed paired infections of control and SR-BI trans-complemented SR-BI\(^{K0}\) cells with the H77/JFH-1 mutants and again observed less dependence on SR-BI for infection with the mutant viruses (Fig 7D). Finally, we explored whether deletion of SR-BI from Huh7.5 cells differentially affected the attachment of parent and mutant viruses. Notably, the attachment of all viruses to the SR-BI gene-edited cells was similar, suggesting the mutations likely affect a post-attachment interaction step that requires SR-BI (Fig 7E).

Our results suggested that all of the growth adaptive mutations identified in our screen modulated the SR-BI dependence of HCV for entry into target cells. When we mapped the positions of the growth adaptive mutations on the E2 core structure (19), we found that four of the residues (L619T, V626S and K632T, and L644I) were proximal to one another, with K632T, and L644I directly adjacent in an anti-parallel beta hairpin (Fig 7F). This clustering suggests a possible role for this region of E2 in an interaction with SR-BI.
DISCUSSION

The study of single amino acid viral variants has facilitated advances in our understanding of host-pathogen interactions. However, classical methods of producing viral variants rely on stochastic errors of the viral polymerase to generate mutations. Such approaches can be an inefficient means of producing new mutants even when selective pressure is applied. This approach may fail to explore all possible amino acid substitutions, as multiple nucleotide changes at a single codon are less likely. The limitation of relying on randomly occurring mutations is particularly problematic for HCV, as it is relatively slow growing in culture. By molecularly engineering variability into E2 and producing our SDSM library we circumvented the need to wait months for mutations to arise in culture. Where previous attempts to identify new culture adaptive mutations yielded few mutations, we selected for many variants within three tissue culture passages.

Previous studies of growth-enhancing E2 mutations were limited to a few sites with E2, between positions 412-421 and at positions 451 and 563 (38–41). These mutations increased the sensitivity of the virus to inhibition by CD81-LEL and anti-HCV NMAbs targeting the E2-CD81 interaction. These mutations may elicit a more open E2 conformation, which promotes interactions with CD81 or other entry factors at the cost of greater exposure of the NMAbs that block access to the CD81 binding site on E2. Additionally, some of these prior mutations altered the dependence of HCV on SR-BI and caused a shift in the buoyant density profile of the infectious viruses. In comparison, our panel of mutations were distributed more evenly across the E2 protein. Four substitutions (Q412R, T416R, S449P and T563V) were proximal to the previously described positions, however the remaining five mutations (A579R, L619T, V626S, K632T, and L644I) were at unique positions within E2.

The increased sensitivity to NMAb and CD81-LEL inhibition by growth-enhancing E2 mutations may represent a trade-off made by the virus in the face of humoral immune pressure, with a compromise of growth kinetics to facilitate immune evasion. Consistent with this
hypothesis, an alignment of all genotype 2 E2 amino acid sequences in the ViPR database (http://www.viprbrc.org/) revealed that our adaptive mutations were present at low frequencies or absent from most HCV isolates (Table 3). A subset of our mutations, Q412R, T416R, S449P, T563V, and L619T, were more sensitive to NMAb inhibition than the parental virus. Of these, T416R, T563V and L619T also were more sensitive to inhibition by CD81-LEL. T416R falls within the epitope of H77.39 and correspondingly is neutralized more efficiently by this antibody. The S449P mutation eliminates a predicted N-linked glycosylation site at N448 near the CD81 binding site, which might enhance NMAb potency by reducing steric hindrance of the glycan. At present, the effects of other residues on the potency of neutralization by H77.39 and HC84.26 are more challenging to model. Structural data for the binding of these antibodies to E2 are not available, and some of the residues are not visible in the crystal structures of the E2 core. Residues that are not proximal to the footprints of H77.39 and HC84.26 could affect neutralization potency indirectly, perhaps via altered lipoprotein association or changes in the global conformation of E2.

Q412R and S449P were more sensitive to H77.39 and HC84.26 antibody neutralization without showing increased inhibition by CD81-LEL. This suggests that increased exposure of the CD81 binding site is not responsible exclusively for enhanced anti-E2 neutralization. We also identified growth adaptive mutations in E2 (e.g., A579R and K632T) that did not render the virus more susceptible to neutralizing antibodies, although further studies are warranted. These mutants could be more sensitive to NMAbs targeting different epitopes. Alternatively, these mutations may adapt the virus to Huh7.5 cells but not to cell targets in vivo.

Mutations in E1 and E2 can alter the dependence of HCV on particular receptors or even cause a shift in receptor utilization (37, 39, 40, 61). We tested whether our growth adapted viruses had different entry factor utilization. Antibodies to CD81 and CLDN1 showed little difference in potency between the mutant and parental viruses; thus, the mutant viruses remained dependent on CD81 and CLDN1 for entry. A different pattern was observed when
cells were pretreated with anti-SR-BI antibodies. Parental virus was neutralized efficiently with a 90% reduction at high antibody concentrations. However, all of the growth adapted viruses were resistant to the effects of anti-SR-BI, a finding which was corroborated using CRISPR/Cas9 generated SR-BIKO and trans-complemented cells. Additionally, H77/JFH-1 clones bearing homologous mutations to JFH-1 S449P, L619T and L644I, similarly were resistant to SR-BI deletion, suggesting the mutations have a conserved role in modulating the SR-BI interaction. As changes to lipoprotein incorporation could contribute to the observed SR-BI phenotype of some growth adaptive mutants, we analyzed buoyant density profiles for relative infectivity. However, all of our growth adaptive mutants had virtually identical profiles relative to parental virus. This observation suggests that the altered SR-BI usage may not be a function of changes to lipoprotein incorporation. Consistent with this idea, HCV uses SR-BI at multiple points in the entry pathway, as an attachment factor via interactions with virus associated lipoprotein and also during post-attachment steps by directly interacting with E2 (62). As deletion of SR-BI did not differentially affect attachment of parent or variant HCV to Huh7.5 cells, these mutations may alter a post-attachment interaction with SR-BI.

While it is known that deletion of HVR1 abrogates binding of E2 to SR-BI, the details of this interaction remain unclear. Our findings, along with data from previous studies, suggest that reduced SR-BI dependence for infection is a common feature of growth-enhancing E2 mutations (at least in cell culture) and that residues distributed across the E2 protein can modulate the interaction with SR-BI. As four of the sites of growth adaptive mutations we identified with altered SR-BI dependency were proximal to each other, we speculate this could represent part of a possible SR-BI binding site in E2. The molecular basis for why our mutants show less dependency on SR-BI awaits more detailed structural or biochemical resolution of the E2 and SR-BI interaction.

In summary, we developed a novel E2 JFH-1 mutant library with significant diversity and used it to identify new growth adaptive mutations for study. The properties of these mutants are
summarized in Table 4, and varied with respect to NMAb and CD81-LEL inhibition sensitivity and SR-BI-dependence. More broadly, these results demonstrate the utility of libraries of variant viruses to address questions in HCV biology, especially as it relates to receptor interactions and possibly, immune escape. The SDSM method provides a depth of variability that can be employed rapidly for discovery of novel HCV variants with unique functional profiles.
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adaptation of hepatitis C virus and in vivo viability of an adapted variant. J. Virol. 81:13168–79.


FIGURE LEGENDS

Figure 1. Generation of a SDSM library of infectious HCV and selection of growth adaptive mutations. A. Summary of the strategy used to generate the SDSM E2 library. Three-hundred and twenty degenerate (NNN) primer sets were used to randomize each codon in the ectodomain of E2, excluding cysteine residues. These plasmid libraries were grouped into sets of 10 sequential codons. E1-E2 fragments amplified by PCR from these plasmid pools were used to generate 32 pools of mutant viruses. B. Amplicon sequencing results for the plasmid libraries. The frequency of mutant codons at each position within the library is shown. The ideal mutant frequency is 10% using our approach (red dashed line).

Figure 2. Growth kinetics of viruses bearing the dominant mutations identified by serial passage of E2 mutant pools. A. Huh7.5 cells were infected with each of the nine mutants selected for study and parent virus at a MOI of 0.1 based on titers calculated using the TCID$_{50}$ method. Input virus was removed 6 h after infection and samples collected every 24 h for 7 days. Supernatant viral titers were analyzed by FFA. B. Cultures of Huh7.5 cells were infected as in (A) at a MOI of 0.05. Inoculum (black bars) and titers of virus from supernatant at day 5 (grey bars) were determined using the TCID$_{50}$ method. Error bars represent standard error of the mean (SEM) and dashed lines indicate the limit of detection of the assays. Asterisks indicate differences that are statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant). The results are the average of three independent experiments performed in duplicate.

Figure 3. Growth enhancing mutations do not change buoyant density profiles. Virus-containing media was layered over 5-50% sucrose gradients and ultracentrifuged at 105,000 x g for 17 hours at 4°C. Twelve 1 mL fractions were collected. The density of the liquid in each fraction was determined using a refractometer, and viral titers and genome copies in each fraction were quantified by FFA and RT-PCR, respectively. In each panel, the percent of total infectious virus collected in each fraction is displayed in the top graph and the percent of
total genome copies in the bottom, with a control plot for the parental virus shown in red. The mutants are displayed in the panels as follows: Q412R (A); T416R (B); S449P (C); T563V (D); A579R (E); L619T (F); V626S (G); K632T (H); and L644I (I). Panel (J) includes a comparison between parent JFH-1, the T416R mutant and J6/JFH-1 viruses run as a control to confirm differences in buoyant density profiles could be measured using our experimental approach. Each point represents the mean of three independent experiments with error bars representing SEM.

Figure 4. A subset of growth enhanced mutants are more sensitive to CD81-LEL and NMAb inhibition. Parental or mutant viruses were pre-incubated with a dilution series of CD81-LEL (A) or anti-E2 antibodies H77.39 (B) or HC84.26 (C) for 1 h at 37°C and then added to Huh7.5 cells. Infection was quantified 72 h later by FFA. EC50 values were determined by non-linear regression analysis. The left panels are representative dose response curves for parental virus and the mutants. The fold decrease in EC50 value for the antibody treatment relative to parental virus is plotted in the right panel. Asterisks indicate differences that are statistically significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant). Error bars represent SEM. The results are the average of three independent experiments performed in triplicate or quadruplicate.

Figure 5. The dependence on CD81 and CLDN1 for growth adapted mutants is similar to the parental virus. Huh7.5 cells were pre-incubated with a dilution series of anti-CD81 (A) or anti-CLDN1 (B) for 1 h at 37°C and then infected with parental and growth adapted mutant viruses. Infection was quantified 72 h later by FFA and EC50 values determined by non-linear regression. For each mutant virus, the fold change in EC50 value for the antibody treatment relative to parental virus is plotted. Asterisks indicate differences that are statistically significant (*, P < 0.05; **, P < 0.01; ns, not significant). Error bars represent SEM. The results are the average of three independent experiments performed in triplicate or quadruplicate.
Figure 6. Growth enhanced mutants are less dependent on SR-BI for infection.

Huh7.5 cells were pre-incubated with a dilution series of anti-SR-BI for 1 h at 37°C and then infected with parental and mutant viruses. Infection was quantified 72 h later by FFA. In each panel, the dose-response curve for a single mutant virus is displayed with the same control dose-response curve for parental virus. The mutants are displayed in the panels as follows: Q412R (A); T416R (B); S449P (C); T563V (D); A579R (E); L619T (F); V626S (G); K632T (H); and L644I (I). Error bars represent SEM. The results are the average of three independent experiments performed in quadruplicate.

Figure 7. Generation of SR-BI deficient Huh7.5 cells. A. Parental Huh7.5 cells or Huh7.5 SR-BI KO clone cell populations were stained with isotype control or SR-BI specific antibodies and analyzed by flow cytometry. B. The relative titers of a single stock of HCV were determined by limiting dilution assay on parental Huh7.5 cells or Huh7.5 SR-BI KO clonal cells either not expressing a transgene (‘mock’) or transduced with lentiviral particles to express GFP or SR-BI. Values represent the mean TCID₅₀/mL value and the error bars indicate SEM of three independent assays. C. SR-BI KO Huh7.5 cells stably expressing GFP or GFP and SR-BI were infected with parental or variant virus. The relative infection of GFP-only cells compared to GFP and SR-BI co-expressing cells is shown. Error bars represent SEM. Asterisks indicate differences that are statistically significant (*, \(P < 0.05\); ***, \(P < 0.001\); ****, \(P < 0.0001\)). The results are the average of three independent experiments performed in quadruplicate. D. SR-BI KO Huh7.5 cells stably expressing GFP or GFP and SR-BI were infected as described in (C) using parental H77/JFH-1 and H77/JFH-1 variant viruses bearing corresponding mutations to JFH-1 S449P, L619T, and L644: S449P, L615T and L640L, respectively. E. SR-BI KO Huh7.5 cells stably expressing GFP or GFP and SRBI were incubated with parental or variant virus or 3 h on ice. Cells were washed extensively, cellular RNA was harvested, and HCV genome copies were quantified by qRT-PCR. The relative attachment to GFP-only cells compared to GFP and SR-BI co-expressing cells is shown. Error bars represent SEM. No statistically significant
differences were observed. The results are the average of three independent experiments performed in duplicate. **F.** Ribbon diagram of the E2 core structure (PDB ID: 4MWF) (19) illustrating the locations of the adaptive mutations identified in this study (red spheres) and residues essential for CD81 binding (blue spheres, amino acid positions 529-535). Q412 and T416 were excluded as they fall within a disordered region.
Table 1: Mutations selected by serial passage of HCV E2 mutant pools

<table>
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<tr>
<th>Positions of mutations in E2 of P0 virus</th>
<th>Positions of P3 putative adaptive mutations identified by consensus sequencing</th>
<th>Amino acid substitutions identified by colony sequencing (% of colonies bearing the substitution)</th>
<th>Other sites of mutations identified by colony sequencing</th>
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<td>L644</td>
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* Consensus sequencing suggested only a proline substitution.

Each pool of E2 mutants was passaged three times in tissue culture. Subsequently, viral RNA was harvested, cDNA generated, and E1-E2 amplified by PCR and Sanger sequenced across the sites of engineered mutations within E2. Eight of the thirty-two pools had changes to the E2 consensus sequence: 404-413, 414-423, 445-455, 563-573, 574-583, 618-628, 629-638, and 639-649. The E1-E2 fragments from these samples were cloned, and plasmids from individual colonies were sequenced to determine the specific amino acid substitutions selected from each pool.
Table 2: Genome copy number to TCID<sub>50</sub> ratios for growth enhanced mutants

<table>
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<td>2.4 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
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Huh7.5 cell cultures were infected at a MOI of 0.05 with each mutant and the parental virus. The inoculum was washed off and 5 days post infection nascently produced virus was collected and titered by the TCID<sub>50</sub> method. Genome copies were measured by RT-PCR. Values are the mean of three experiments performed in duplicate.
Table 3: Number of genotype 2 sequences in the ViPR database bearing each amino acid at the site of adaptive mutation.

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</tbody>
</table>

All HCV genotype 2 E2 amino acid sequences in the ViPR database were aligned and the number of sequences containing each residue at the sites of our adaptive mutations was determined.
Table 4: Summary of properties of the growth adaptive E2 variants

<table>
<thead>
<tr>
<th>Virus</th>
<th>Residue</th>
<th>RNA: TCID&lt;sub&gt;50&lt;/sub&gt;*</th>
<th>H77.39*</th>
<th>HC84.26*</th>
<th>CD81LEL*</th>
<th>anti-CD81**</th>
<th>anti-CLDN1**</th>
<th>anti-SRBI***</th>
<th>SRBI&lt;sup&gt;KO&lt;/sup&gt;***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q412R</td>
<td>Q412</td>
<td>1.7</td>
<td>19</td>
<td>8.4</td>
<td>2.1</td>
<td>2.2</td>
<td>2.0</td>
<td>82%</td>
<td>58%</td>
</tr>
<tr>
<td>T416R</td>
<td>T416</td>
<td>2.3</td>
<td>63</td>
<td>6.5</td>
<td>5.5</td>
<td>2.8</td>
<td>1.4</td>
<td>72%</td>
<td>57%</td>
</tr>
<tr>
<td>S449P</td>
<td>S449</td>
<td>2.0</td>
<td>46</td>
<td>11</td>
<td>1.5</td>
<td>0.91</td>
<td>1.9</td>
<td>100%</td>
<td>59%</td>
</tr>
<tr>
<td>T563V</td>
<td>T561</td>
<td>3.3</td>
<td>26</td>
<td>9.7</td>
<td>6.2</td>
<td>1.4</td>
<td>1.3</td>
<td>55%</td>
<td>55%</td>
</tr>
<tr>
<td>A579R</td>
<td>(N577)</td>
<td>3.8</td>
<td>2.8</td>
<td>1.0</td>
<td>1.1</td>
<td>2.6</td>
<td>1.5</td>
<td>34%</td>
<td>33%</td>
</tr>
<tr>
<td>L619T</td>
<td>L615</td>
<td>2.6</td>
<td>28</td>
<td>6.0</td>
<td>10</td>
<td>3.3</td>
<td>1.7</td>
<td>100%</td>
<td>61%</td>
</tr>
<tr>
<td>V626S</td>
<td>L622</td>
<td>1.5</td>
<td>4.9</td>
<td>2.6</td>
<td>1.6</td>
<td>2.6</td>
<td>2.7</td>
<td>57%</td>
<td>44%</td>
</tr>
<tr>
<td>K632T</td>
<td>K628</td>
<td>1.2</td>
<td>1.2</td>
<td>1.7</td>
<td>0.44</td>
<td>2.1</td>
<td>4.4</td>
<td>36%</td>
<td>44%</td>
</tr>
<tr>
<td>L644I</td>
<td>L640</td>
<td>1.3</td>
<td>3.8</td>
<td>4.5</td>
<td>1.7</td>
<td>1.2</td>
<td>2.7</td>
<td>53%</td>
<td>58%</td>
</tr>
</tbody>
</table>

*Fold reduction  
**Fold increase  
***Resistant fraction  

A summary of the properties of the mutants identified in this study. Fold decrease relative to parent is listed for RNA:TCID<sub>50</sub>. The fold change in EC<sub>50</sub> (increase or decrease) is listed for all dose-response inhibition assays. For the SR-BI antibody dose response or infection of SR-BI<sup>KO</sup> cells, the fraction of virus resistant to SR-BI blockade or deletion is listed.
A

CTCATT AACACCAAC
T7

pLJ83

PCR amplify with degenerate primers

Encodes culture adapted JFH-1 infectious clone

CTCATT NNNACCAAC
T7

pLJ83

Group into sets of 10 sequential codons

320 plasmid libraries of 64 variants

Next generation sequencing to measure engineered diversity

412  Q  L  I  N  T  G  S  W  H  421
... NNN CTC ATT AAC ACC AAC GGC AGT TGG CAC ...
... CAG NNN ATT ACC ACC AAC AAC GGC AGT TGG CAC ...
... CAG CTC NNN AAC ACC AAC GGC AGT TGG CAC ...
... CAG CTC ATT AAC NNN AAC AAC GGC AGT TGG CAC ...
... CAG CTC ATT ACC ACC NNN AAC AAC GGC AGT TGG CAC ...
... CAG CTC ATT AAC ACC AAC NNN AAC AAC GGC AGT TGG CAC ...
... CAG CTC ATT AAC ACC AAC AAC NNN AAC AAC GGC AGT TGG CAC ...
... CAG CTC ATT AAC ACC AAC AAC AAC NNN AAC AAC GGC AGT TGG CAC ...

32 combined plasmid libraries of 640 variants

T7 transcription and electroporation into Huh7.5 cells

32 pools of infectious HCV E2 mutants

B

Frequency of Mutant Codon (%)

0  2  4  6  8  10  12  14  16  18

400 420 440 460 480 500 520 540 560 580 600 620 640 660 680 700 720

Amino Acid Position in JFH-1 Polyprotein