

Figure 2. Effects of antiviral inhibitors on the replication of S310 subgenomic replicons. Three cell clones (clone 6, 9 and 10) carrying genotype 3a S310 replicons and one cell clone each harboring genotype 1b Con1 and genotype 2a JFH-1 replicons were treated with the indicated concentrations of (A) interferon alpha, (B) HCV protease inhibitor BILN-2061, (C) the non-nucleoside polymerase inhibitor JTK-109, and (D) the nucleoside polymerase inhibitor PSI 6130 for 72 hours and replication levels were measured by quantifying intracellular HCV RNA. Results are means \pm standard deviations of 3 replicates.

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Reprint requests

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DDBJ/EMBL/GenBank accession numbers: S310/A: AB691595, S310/B: AB691596, SGR-S310/A: AB691597, SGR-S310/B: AB691598, SGR-S310/Luc: AB691599.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Methods

Cell Culture

The human hepatoma cell line HuH-7¹ and its derivative cell line Huh-7.5.1² were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, minimal essential medium nonessential amino acids, 100 U/mL penicillin, 100 μ g/mL streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate at 37°C in a 5% CO₂ incubator.

Primary human hepatocytes (PHH) were isolated from an encapsulated liver sample.³ Isolated PHH were seeded in 12-well plates and cultured at 37°C in Lanford medium before infection.

PHH Infection With HCV-Positive Sera

Three days post seeding, PHH were inoculated with HCV-positive sera. After 16 h of inoculation, monolayers were washed with William's E medium and fresh Lanford medium was added. Cells were harvested at 72 h post infection. Total RNA was isolated using a guanidinium isothiocyanate solution (RNABle; Eurobio, Courtaboeuf, France) and intracellular levels of HCV RNA were quantified using the SuperScript III Platinum One-Step quantitative reverse transcription polymerase chain reaction (RT-PCR) system (Invitrogen, Carlsbad, CA) and a LightCycler480 real-time PCR system (Roche Diagnostics, Meylan, France).

HCV Genotype 3a Clone

Clone S310 was isolated from a 71-year-old female patient suffering from post liver transplantation HCV recurrence. She was diagnosed with HCV genotype 3a infection at the age of 59 years and underwent liver transplantation 4 years later due to liver cirrhosis. HCV-RNA titer was 2.8×10^6 copies/mL. Total RNA extracted from 100 μ L serum using the acid-guanidinium isothiocyanate-phenol-chloroform method (Isogen-LS; Nippon Gene, Tokyo, Japan) was precipitated with isopropanol, washed with ethanol, and dissolved in 10 μ L nuclease-free water. An aliquot of 4 μ L was subjected to reverse transcription using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript III; Invitrogen) at 42°C for 50 min and then at 50°C for 10 min.

Isolation of HCV

The sequences of 4 isolates of genotype 3a (accession numbers AF046866, D28917,⁴ X76918, and D17763⁵) that were obtained from the HCV database (<http://hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html>) were aligned and PCR primers were designed based on the conserved sequences. These primers were used to amplify the complementary DNA (cDNA) of S310 into 9 overlapping fragments by nested PCR (nt 1–370, nt 127–1284, nt 1117–1997, nt 1704–3352, nt 3152–5080, nt

4869–6842, nt 6601–8129, nt 7988–9145, and nt 9082–9576; nucleotide numbers refer to the positions on S310, with nt 1 being the first nucleotide of the 5' UTR). The sequence of these primers is shown in Supplementary Table 1. Two microliters of cDNA was subjected to PCR using Pyrobest DNA polymerase (Takara Bio, Kyoto, Japan) and the outer set of primers, and this first-round PCR product (2 μ L) was further amplified by a second round of PCR using the inner set of primers. PCR conditions for the first and second rounds of PCR consisted of 35 cycles each of denaturation at 98°C for 20 s, annealing at 55°C for 1 min, and extension at 72°C for 3 min. A fragment encompassing the 5' end of the viral genome (nt 1–370) was amplified by 5'RACE. Briefly, cDNA was synthesized with a 5' UTR primer (antisense), tailed with a dCTP homopolymer by using terminal deoxynucleotidyl transferase, and amplified by PCR (5' RACE System for Rapid Amplification of cDNA Ends; Invitrogen) using TaKaRa LA Taq polymerase (Takara Bio). The PCR products of all fragments were separated by agarose gel electrophoresis, cloned into the pGEM-T EASY vector (Promega, Madison, WI) and sequenced using the Big Dye Terminator Mix and an automated DNA sequencer. The consensus sequence of 5 to 9 isolated cDNA clones was adopted for each fragment. Two major populations of the virus were identified in the patient's serum that differed in 4 amino acids in the NS3 protein (aa 1039, 1183, 1463, and 1504), and these populations were designated as S310/A and S310/B (DDBJ/EMBL/GenBank accession number: AB691595 and AB691596, respectively). To assess the complexity of the HCV population in the patient's serum, the hypervariable region sequences of 10 clones were determined.

Computer Analysis

A phylogenetic tree was constructed using the neighbor-joining method to examine the relationship between the polyprotein region of S310 and that of other HCV genotype 3a isolates available in the database. In order to analyze the diversity in each subgenomic region, the genetic distance was calculated between all possible pairs of genotype 3a isolates and between S310/A and other isolates using MacVector software (MacVector, Inc., Cary, NC). The ratios of these 2 values (mean genetic distance between S310/A and other isolates/mean genetic distance among all genotype 3a isolates) were compared.

Construction of Replicons

Based on the consensus sequence of S310, we assembled pS310/A and pS310/B, which contained the full-length S310/A and S310/B cDNA, respectively, downstream of the T7 RNA polymerase promoter. Briefly the 9 amplicons described here were combined by overlapping PCR and ligated with pGEM-T EASY vectors to generate 6 plasmids (A through F) in such a way that each plasmid contained a unique restriction enzyme

cleavage site toward the 3' end of the viral fragment, which overlapped with the 5' end of the next fragment. For this purpose, we took advantage of the EcoRI restriction site that is present in the polycloning site of the plasmid toward the 5' end of the viral fragment. Plasmid A contained the T7 promoter sequence followed by one G-nucleotide and nt 1-3352 of S310, while plasmids B, C, D, and E contained nt 1704-4307, nt 4044-6013, nt 5424-7755, and nt 7276-9425, respectively. Plasmid F contained the fragment constructed by combining the C-terminal end of NS5B (nt 9182-9402) and the variable and poly U/UC regions of the S310/A 3'UTR (nt 9403-9610) with the last 44 nucleotides of JFH-1. Restriction sites for EcoRI and XbaI were introduced upstream of the T7 promoter sequence and downstream of the conserved region, termed the *X-region*, of the 3'UTR, respectively, and the restriction sites of these enzymes that were present within the cDNA were removed by PCR-based mutagenesis. In the neomycin-based subgenomic replicons (SGR-S310/A and SGR-S310/B, accession number: AB691597 and AB691598, respectively), the cassette containing the neomycin phosphotransferase gene and the EMCV IRES replaced the region of S310 that encompasses amino acids 20-1032. Firefly luciferase-based subgenomic replicons (SGR-S310/Luc, accession number: AB691599) were generated from SGR-S310/A by replacing amino acids 20-1032 of S310/A with the cassette containing firefly luciferase and the EMCV IRES from pSGR-JFH1/Luc.⁶

RNA Synthesis

RNA was synthesized by *in vitro* transcription as described previously.⁷ Briefly, the plasmids carrying the cDNA described here were linearized with the XbaI restriction enzyme and 5' overhangs were removed by treating with mung bean nuclease. Reaction mixtures were further incubated at 50°C for 1 h with 2 μ L 20 mg/mL proteinase K and 10 μ L 10% sodium dodecyl sulfate to degrade nucleases, and templates were purified with 2 rounds of phenol-chloroform extraction and ethanol precipitation. Three micrograms of templates were subjected to *in vitro* transcription using a MEGascript T7 kit (Ambion, Austin, TX) according to the manufacturer's recommendations. Synthesized RNA was treated with DNase I (Ambion) and then purified using ISOGEN-LS (Nippon Gene). The quality of the synthesized RNA was examined by agarose gel electrophoresis.

RNA Transfection

In vitro transcribed RNA or total cellular RNA isolated from replicon cells was introduced into cells by electroporation. Trypsinized cells were washed twice with serum-free Opti-MEM I (Invitrogen) and 3.0×10^6 cells were resuspended in 400 μ L cytomix buffer.⁸ RNA was delivered into cells by a single pulse of 260 V and 950 μ F using the Bio-Rad Gene Pulser II apparatus (Bio-Rad,

Hercules, CA). Transfected cells were immediately suspended in culture medium and transferred to the appropriate plates. For G418 selection of colonies, the transfected cells were seeded in 10-cm dishes, each containing 8 mL culture medium. G418 (500 μ g/mL; Nacalai Tesque, Kyoto, Japan) was added to the culture medium at 24 h after transfection. Culture medium supplemented with G418 was replaced every 3 days. Three weeks after transfection, cells were fixed with buffered formalin and stained with crystal violet or replicon colonies were picked and expanded.

Analysis of G418-Resistant Cells

G418-resistant colonies were collected and used for further analysis. Colonies were independently isolated using cloning cylinders (Asahi Techno Glass Co., Tokyo, Japan) and were expanded until they were 80%-90% confluent in 10-cm dishes. Expanded cells were harvested for nucleic acid and protein analysis. Total RNA was isolated from the cells using the ISOGEN reagent (Nippon Gene). Another aliquot of the cell pellet was dissolved in RIPA buffer containing 0.1% sodium dodecyl sulfate for Western blot analysis. For immunofluorescence analysis of viral proteins, cells were seeded on 12-well slides.

Quantification of HCV RNA by Real-Time RT-PCR

Copy numbers of HCV RNA were determined by real-time detection RT-PCR, as described previously,⁹ using the ABI Prism 7700 Sequence Detector System (Applied Biosystems Japan, Tokyo, Japan). The concentration of total RNA in the cells was determined using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Rockford, IL).

Northern Blot Analysis

Isolated RNAs (3 μ g) from replicon cells were separated on a 1% agarose gel containing formaldehyde, transferred to a positively charged nylon membrane (Hybond-N+; GE Healthcare UK Ltd., Buckinghamshire, UK) and immobilized using a FUNA-UV-LINKER (Funakoshi, Tokyo, Japan). Hybridization was carried out with a [α -³²P]dCTP-labeled DNA probe using Rapid-Hyb buffer (GE Healthcare UK Ltd.). The DNA probe was synthesized from a BsrGI-MfeI fragment of the S310 clone that contained NS3-5B genes using the Megaprime DNA labeling system (GE Healthcare UK Ltd.).

Indirect Immunofluorescence

Untransfected HuH-7 cells or S310 replicon-replicating cells were grown on a glass slide for 24 h and fixed in acetone-methanol (1:1 [vol/vol]) for 10 min at -20°C. Cells were then incubated in immunofluorescence buffer (phosphate-buffered saline, 1% bovine serum albumin, 2.5 mM EDTA). S310 patient serum was added at

a dilution of 1:200 in immunofluorescence buffer. After incubation for 1 h at room temperature, cells were washed and then incubated with an Alexa Fluor488-conjugated goat anti-human IgG antibody (Invitrogen) in immunofluorescence buffer. The glass slide was washed and a cover glass was mounted using PermaFluor mounting solution (Thermo Scientific, Cheshire, UK). Cells were examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Western Blot Analysis of HCV Proteins

The protein samples were separated on 12.5% polyacrylamide gels and subsequently transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA). Transferred proteins were incubated with 2% skim milk. Anti-NS3 mouse monoclonal antibody (clone 8G2, Abcam, Cambridge, UK) and peroxidase-labeled sheep anti-mouse IgG (Cell Signaling Technology, Danvers, MA) were used to detect HCV proteins. The signals were detected with a chemiluminescence system (ECL Prime; GE Healthcare UK Ltd.).

Identification of Mutations

cDNA was synthesized from total RNA that was extracted from replicon-expressing cells at 2 different times. These cDNAs were amplified into 5 overlapping fragments that spanned the 5'UTR and the NS3-NS5B region using LA Taq DNA polymerase (Takara Bio) and the primers described in Supplementary Table 1. The sequence of each amplified DNA was determined. The mutations identified were subsequently introduced into SGR-S310/A and SGR-S310/Luc by PCR-mediated mutagenesis.

Luciferase Assay

Five micrograms of RNA, prepared by in vitro transcription of S310/SG-FLuc constructs with or with-

out adaptive mutations, were introduced into 3.0×10^6 Huh-7.5.1 cells by electroporation. Cells were harvested with Cell Culture Lysis Reagent (Promega) at 4, 24, 72, and 96 h post electroporation, and luciferase activity was determined by use of a Luciferase Assay System (Promega) and the Lumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany).

Inhibition of S310 Replicon Replication by Specific Inhibitors

S310 replicon cell clones 6, 9, and 10 and the genotype1b Con1 and 2a JFH-1 replicon cells,¹⁰ were seeded into 24-well plates at a density of 5.0×10^4 cells/well. On the next day, the culture medium was replaced with medium containing 0.1% dimethyl sulfoxide with or without various concentrations of interferon alfa (Dainippon-Sumitomo, Osaka, Japan), the specific NS3 protease inhibitor, BILN-2061 (Boehringer Ingelheim Ltd., Québec, Canada), or the NS5B inhibitors, JTK-109 (Japan Tobacco, Inc., Osaka, Japan) and PSI-6130 (Pharmasset, Inc., Princeton, NJ). After 72-h incubation, cells were harvested and HCV RNA was quantified as described.

Supplementary References

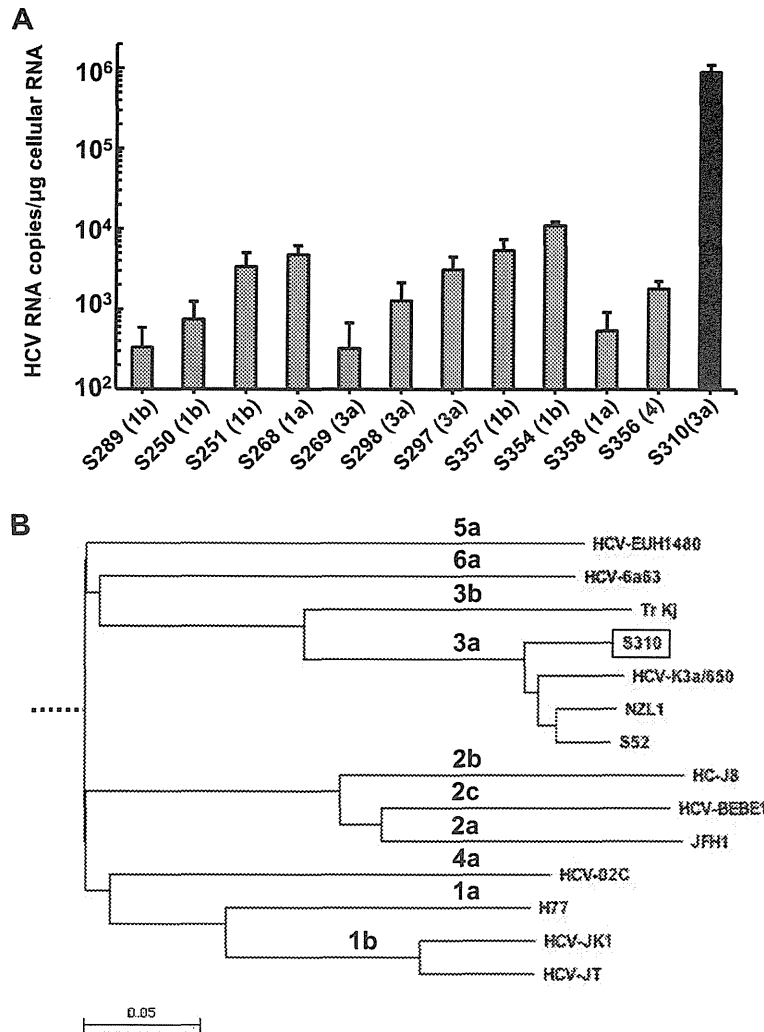
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Supplementary Table 1. Primers for Amplification of the S310 HCV Strain

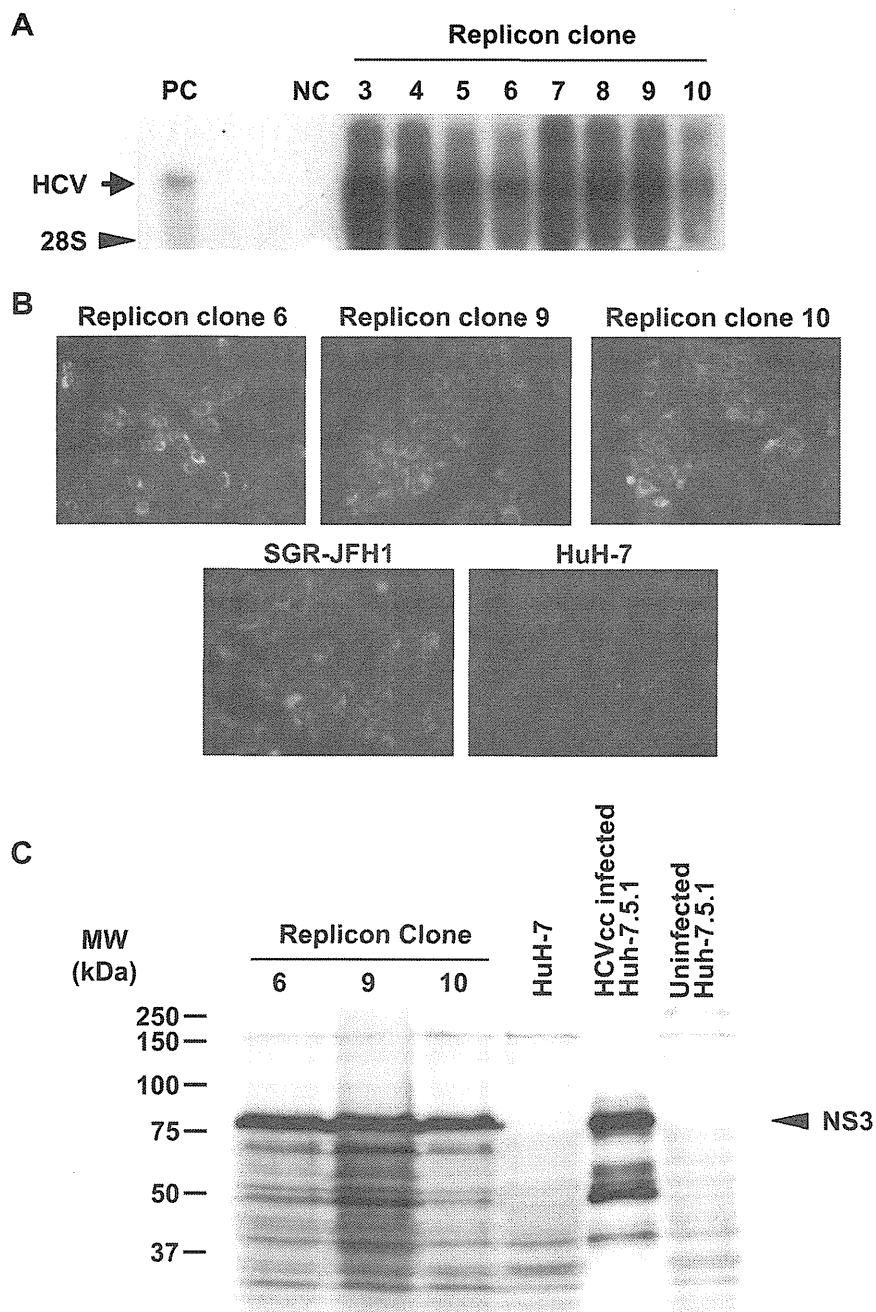
	Fragment		Primer sequence (5'→3')
1 (5' RACE) ^a	Outer	Antisense	CTTGACGTCCTGTGGGCGA
	Inner	Antisense	TTTTCTTTGGGGTTTAGG
2	Outer	Sense	GTCTTCACGCGGAAAGCGC
	Inner	Antisense	CACCCAAACCACCGACCAC
		Sense	CCGGGAGAGCCATAGTGGTC
3	Outer	Antisense	TCCTGAAAGATGGCCTGGGTA
		Sense	CTTGGCCCTCTATGGTAA
4	Inner	Antisense	GATGTTTCCTGAAGCAGTCG
		Sense	AGTCATGTGGACCTATTAGT
5	Outer	Antisense	CACCCAAACCACCGACCAC
		Sense	ATGGCTCGTGGCACATCAA
6	Inner	Antisense	TAGTCATCAGCAGGTCCCAA
		Sense	GCTCAGCAGTGC AAGCCCA
7	Outer	Antisense	CGCAAAGAATATCTCCGCAAG
		Sense	ATTTTTGACATCACTAAGCTAC
8	Inner	Antisense	AGTGTGGCTTAAGCCGCA
		Sense	AATACTTCCAGATGATCATACT
9	Outer	Antisense	GTGACAGAAAGTGGGCAT
		Sense	GTTCCCGCAGCCAACGT
10	Inner	Antisense	GTCTCTCAACATCGAGGT
		Sense	CGGTGAAAGACCGTCTGGA
11	Outer	Antisense	CAGGGGAGTTGAGATCCT
		Sense	GGCCGCGTACATGTGCTAAC
12	Inner	Antisense	CCGCAGACAAGAAAGTCCGGGT
		Sense	CTATGGCGCGTGGCTGCCA
13	Outer	Antisense	ACCCCCAGGTCAGGGTACAC
		Sense	CATAACCTAGTCTATTCAACG
14	Inner	Antisense	TGGTCTTGGTGCGTACCG
		Sense	GCTCCGTCTGGGAGGACTTGC
15	Outer	Antisense	CTCGTGCCCGATGTCTCAA
		Sense	TGCTCCTCCAACGTCTCCGT
16	Inner	Antisense	GCGGCTCACGGACCTTTCAC
		Sense	GTCGCGGGGACACTCAGGAA
		Antisense	ACTAGGGCTAAGATGGAGCC

RACE, rapid amplification of complementary DNA ends.

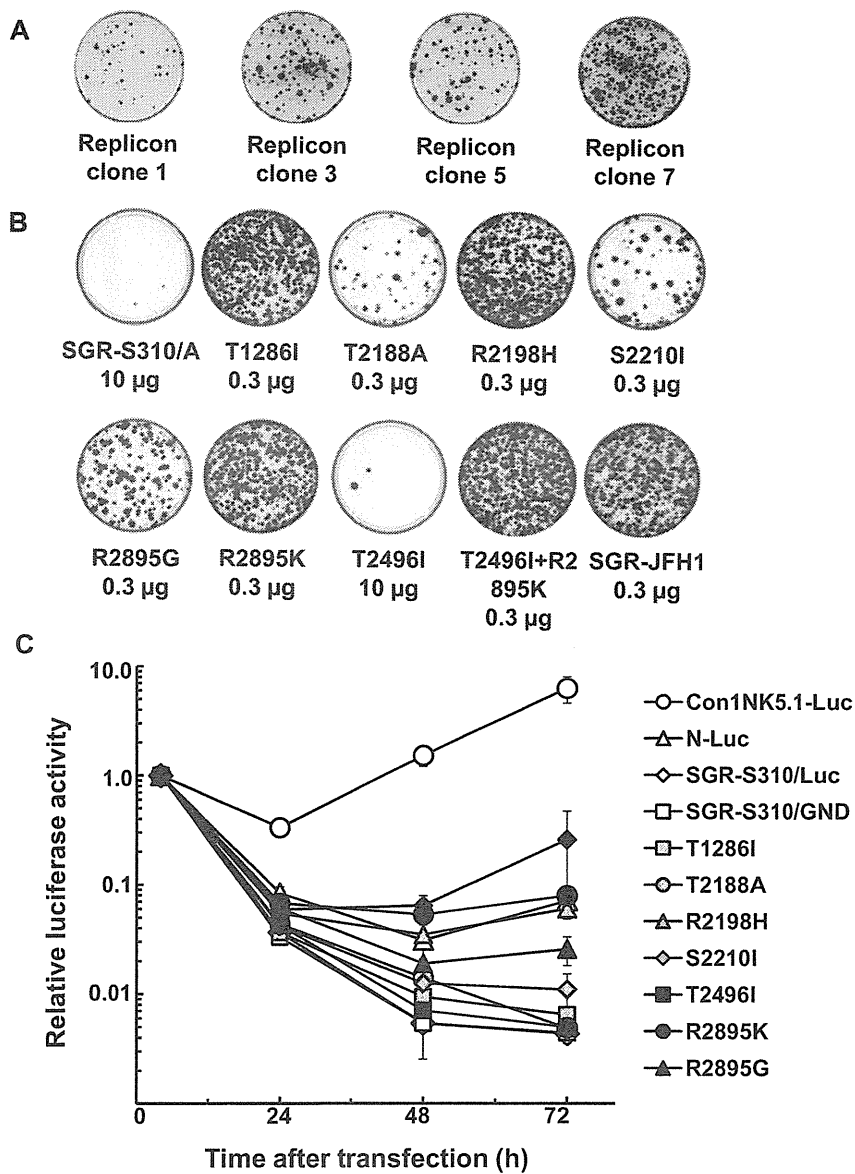
^aForward primers used were those in the 5'RACE kit (Abridged Universal Amplification Primer (AUAP) for the first round of PCR and Universal Amplification Primer (UAP) for the second round of PCR).



Supplementary Figure 1. Infection of PHH with HCV patient sera and phylogenetic tree analysis of the S310 strain. (A) PHH were exposed to sera of patients infected with genotype 1b (S289, S250, S251, S357, S354), 1a (S268, S358), 3a (S269, S298, S297, S310), and 4 (S356) for 16 h (25 µL/well, except for S310, 10 µL). Intracellular HCV RNA was quantified 72 h post inoculation. Experiments were done in triplicate and data are presented as means ± standard deviation. (B) The phylogenetic tree was constructed using the polyprotein region of S310 and HCV strains of different genotypes. The HCV strains analyzed and their corresponding GenBank accession numbers are: K3a/650; D28917, NZL1; NC_009824, S52; GU814263, EUH1480, HCV-6a63; DQ480514, Tr KJ; D49374, HC-J8; D10988, BEBE1; D50409, JFH-1; AB047639, HCV-02C; DQ418784, H77; AF009606, HCV-JK1; X61596 and HCV-JT; D11168. The root of the tree was tentatively taken as the midpoint of the longest path. The length of the horizontal bar indicates the number of nucleotide substitutions per site.



Supplementary Figure 2. Detection and quantification of HCV RNA and proteins in replicon cells. (A) Total RNA (3 μ g) from replicon cells was analyzed by Northern blot; 5.0×10^7 copies of in vitro-transcribed RNA were loaded in parallel as a positive control (PC), while total RNA from untransfected HuH-7 cells served as the negative control (NC). Replicon RNA was detected using a [α - 32 P]dCTP-labeled DNA probe. Arrow and arrowhead indicate the positions of the replicon RNA and 28S ribosomal RNA, respectively. (B) Subcellular localization of viral proteins determined by immunofluorescence. S310 replicon cell clones, JFH-1 replicon cells, and untransfected HuH-7 cells were grown on glass slides for 24 h. After fixation, cells were incubated with patient serum. (C) Western blot analysis. Cell lysates were prepared from replicon clones 6, 9, and 10, untransfected HuH-7, and HCVcc (J6/JFH1)-infected Huh-7.5.1 cells and uninfected Huh-7.5.1 cells. Protein (10 μ g) was resolved by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and viral nonstructural protein NS3-specific bands were detected using an anti-NS3 mouse monoclonal antibody (clone 8G2). Arrow indicates the position of NS3.



Supplementary Figure 3. Analysis of the effect of mutations on the colony-forming efficiency and transient replication of the subgenomic replicon S310. (A) Total RNA was isolated from the indicated replicon cell clones and 10 µg RNA was introduced into 3 million naïve HuH-7 cells by electroporation. After 3 weeks of G418 selection (500 µg/mL), colonies were stained. (B) Three million HuH-7 cells were electroporated with the indicated amounts of transcribed RNA and colonies were selected by a 3-week G418 selection. The JFH-1 subgenomic RNA was included as a positive control. (C) Huh-7.5.1 cells were transfected with the transcribed RNA from pSGR-S310/Luc and pSGR-S310/Luc constructs with mutations (GND mutation in NS5B, T1286I, T2188A, R2198H, S2210I, T2496I, R2895K, R2895G, and T2496I+R2895K) and Con1-NK5.1/Luc and N/Luc replicon. Transfected cells were harvested at the indicated time points and at 4 h post transfection. Relative luciferase activity (arbitrary units) was measured in the cell lysate and was normalized to the activity at 4 h post transfection. Assays were performed in triplicate, and data are presented as means ± standard deviation.

