BRIEF REPORTS

Replication of Hepatitis C Virus Genotype 3a in Cultured Cells

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Hepatitis C virus (HCV) genotype 3a is widespread worldwide, but no replication system exists for its study. We describe a subgenomic replicon system for HCV genotype 3a. We determined the consensus sequence of an HCV genome isolated from a patient, and constructed a subgenomic replicon using this clone. The replicon was transfected into HuH-7 cells and RNA replication was confirmed. We identified cell culture-adaptive mutations that increased colony formation multiple-fold. We have therefore established a genotype 3a replicon system that can be used to study this HCV genotype.

Keywords: Virology; Experimental Model; HCVGT3; In Vitro Culture System.

Hepatitis C virus (HCV) infection leads to chronic infection and advanced liver diseases in most infected adults.[1] Of the 6 major HCV genotypes, genotypes 1 and 2 are the most prevalent in North America, Europe, and Japan,[2,3] and are the most highly studied. However, other genotypes display specific characteristics. For example, genotype 3a infection can result in hepatic steatosis[4] and telaprevir and boceprevir are less effective against genotype 3a.[5] Therefore, the pathogenesis and inhibitor sensitivity of all HCV genotypes should be studied. Although HCV subgenomic replicons are useful for understanding viral/host factors involved in HCV replication and inhibitor sensitivity, only HCV replicons for genotypes 1a, 1b, and 2a have been established.[6–9] Here, we report on the robust genotype 3a replication system.

An almost complete HCV genome was recovered from the serum of a patient with post-transplantation recurrent HCV infection. This serum exhibited higher infectivity than other tested sera toward primary human hepatocytes (Supplementary Figure 1*A*). The isolate, named S310, contained the following structural elements: a 5′UTR (nt 1-339), an open reading frame encoding 3021 aa (nt 340-9402), and a 3′UTR (nt 9403-9654). Only the last 44 nt of the X-region (nt 9611-9654) could not be recovered. Two major virus populations were found; S310/A contained Ala, Thr, Thr, and Ile, and S310/B contained Thr, Ala, Ala, and Thr, at the 7th, 151st, 431st, and 472nd aa of the NS3 protein, respectively. S310 was clustered into genotype 3a by phylogenetic analysis (Supplementary Figure 1*B*). The complexity of the virus quasispecies in the serum was analyzed by sequencing the hypervariable region. Identical amino acid sequences in all 10 hypervariable region clones indicated a very low degree of diversity. The hypervariable region sequence of the JFH-1 strain also exhibited monoclonality,[10] which can be important for efficient replication in cultured cells.

Subgenomic replicons SGR-S310/A and SGR-S310/B were constructed and their replication efficiency was evaluated by G418-resistant colony-formation assay. After 3 weeks, a small number of colonies were visible for both replicons (Figure 1*A*). Because more colonies were observed in SGR-S310/A than in SGR-S310/B, we focused on SGR-S310/A (henceforth called SGR-S310). Ten cell colonies of SGR-S310 were isolated and analyzed for HCV replication. The mean RNA titer was $9.1 \times 10^7 \pm 4.6 \times 10^7$ copies/μg total RNA (Figure 1*B*). HCV RNA (approximately 8 kb) was detected by Northern blotting (Supplementary Figure 2*A*). Viral proteins in the replicon cells were detected by immunofluorescence and Western blotting (Supplementary Figure 2*B* and 2*C*). To determine whether the G418 resistance of the cells was transmissible by cellular RNA transfection, we electroporated total cellular RNA isolated from 4 replicon clones into naïve HuH-7 cells. Multiple G418-resistant colonies appeared after transfection of the RNA isolated from the replicon clones (Supplementary Figure 3*A*), but not from the naïve HuH-7 cells. These results indicate that the replicon RNA in the parental colonies could replicate in naïve cells. Thus, the G418-resistant colonies that were isolated from cells electroporated with SGR-S310 synthetic RNA contained replicating viral RNA.

Replicating genomes have been shown to accumulate cell culture adaptive mutations, which increase their replication potential. To examine whether SGR-S310 acquired mutations, the complete HCV sequences from 10 replicon clones were sequenced. At least one nonsynonymous mutation was detected in the NS3-NS5B region of each replicon clone (Figure 1B). The following mutations were identified: T1286I in the NS3 helicase (6 of 10

Abbreviation used in this paper: HCV, hepatitis C virus.

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A

SGR-S310/A 10 μg | SGR-S310/B 10 μg | SGR-JFH1 0.1 μg

B

Replicon cell clone	Nucleotide mutation	Amino acid substitution	Affected protein	Replicon RNA amounts (copies/μg RNA)
1	5272 (6932) G→A	2198 R→H	NS5A	2.1×10^8
2	7362 (9022) A→G	2895 R→G	NS5B	9.9×10^7
3	2536 (4196) C→T	1286 T→I	NS3	9.5×10^7
4	2536 (4196) C→T	1286 T→I	NS3	9.9×10^7
5	2536 (4196) C→T	1286 T→I	NS3	5.6×10^7
6	2536 (4196) C→T	1286 T→I	NS3	4.0×10^7
7	2536 (4196) C→T	1286 T→I	NS3	8.3×10^7
8	2536 (4196) C→T	1286 T→I	NS3	8.7×10^7
9	6166 (7826) C→T	2496 T→I	NS5B	9.3×10^7
	7363 (9023) G→A	2895 R→K	NS5B	
10	5241 (6901) A→G	2188 T→A	NS5A	5.1×10^7

C

Replicon constructs	Protein	Colony formation efficiency (cfu/μg RNA)	Transient replication efficiency (72h/4h†)
SGR-S310/A	None	0.4×10^0	4.4×10^{-3}
T1286I	NS3	2.9×10^3	6.5×10^{-3}
T2188A	NS5A	2.3×10^2	4.9×10^{-3}
R2198H	NS5A	3.7×10^3	6.0×10^{-2}
S2210I	NS5A	6.2×10^2	1.1×10^{-2}
T2496I	NS5B	0.2×10^0	5.0×10^{-3}
R2895K	NS5B	2.3×10^3	7.9×10^{-2}
R2895G	NS5B	9.8×10^2	2.6×10^{-2}
T2496I+R2895K	NS5B	4.1×10^3	2.6×10^{-1}
Con1-NK5.1	NS3+NS5A	9.1×10^2	6.3×10^0
N	None	NA††	7.1×10^{-2}
SGR-JFH1	None	5.3×10^3	1.5×10^2

Figure 1. S310 subgenomic replicon analysis. (*A*) Three million HuH-7 cells were electroporated with 10 μg RNA from SGR-S310/A or SGR-S310/B or 0.1 μg RNA from SGR-JFH1. G418-selected colonies were fixed and stained after 3 weeks. (*B*) Non-synonymous mutations identified in the replicon genomes and HCV RNA titers in the replicon clones. Nucleotide positions within the S310 subgenomic replicon and within the full-length S310 genome (in parentheses) are given. (*C*) Replication potential of the adaptive mutants as determined by the colony-formation assay using Neo-replicons and by the transient replication assay using Fluc-replicons. †72 h/4 h, transient replication efficiency was determined as a ratio of luciferase activity in the transfected cells between 72 h and 4 h post transfection. ††NA, not available.

clones); T2188A or R2198H in NS5A (2 clones); an R2895G substitution in NS5B (1 clone); and T2496I in NS5A plus R2895K in NS5B (1 clone). These mutations and the S2210I mutation (corresponding to S2204I in genotype 1 replicon)[7,8] were introduced, individually or in combination, into the parental SGR-S310 and the colony-formation efficiencies of the mutant replicons were tested. All mutations, except T2496I, increased the colony formation, indicating an adaptive phenotype (Figure 1*C*, Supplementary Figure 3*B*). Transient replication efficiency was also tested using firefly luciferase reporter replicons. SGR-S310/Luc did not replicate in Huh-7.5.1 cells, whereas the adaptive mutants displayed varying degrees of replication (Figure 1*C*, Supplementary Figure 3*C*). Adaptive mutations T2496I and R2895K, when combined together, most efficiently enhanced the colony formation as well as transient replication (Figure 1*C*). Interestingly, T1286I and R2895G found in our study correspond to the Con1 adaptive mutations T1280I and R2884G, respectively.[11,12] T2188A or R2198H in NS5A were identified in 2 replicon clones and are located close to S2210I. Indeed, S2210I also enhanced SGR-S310 replication, suggesting that this region might be important for HCV replication. S310 replicons with adaptive mutations were compared with genotype 1b (Con1 and N) and 2a (JFH-1) replicons. Colony-formation efficiencies of most S310 adaptive replicons were at levels comparable with Con1 and JFH-1 (Figure 1*C*, Supplementary Figure 3*B*). In contrast, S310 adaptive replicons replicated less efficiently than Con1-NK5.1 and JFH-1 replicons in transient replication assays. However, genotype 1b N replicon replicated at a level similar to some S310 adaptive replicons (Figure 1*C*, Supplementary Figure 3*C*). Future studies will dissect the detailed mechanisms that underlie the effects of these mutations.

Successful generation of a genotype 3a replicon provided a unique opportunity to compare the susceptibility of genotype 3a (SGR-S310), 1b (Con1[13]), and 2a (JFH-1/4-1[13]) replicons to HCV inhibitors. Interferon-alfa dose-dependently decreased the replication of all tested genotypes (Figure 2*A*), whereas a protease inhibitor, BILN-2061, was more effective against replicons from genotypes 1b and 2a than 3a (Figure 2*B*). The non-nucleoside polymerase inhibitor JTK-109 was more potent against genotype 1b and 3a (Figure 2*C*). However, the nucleoside polymerase inhibitor, PSI-6130, equally inhibited all genotypes (Figure 2*D*).

In conclusion, we established a subgenomic replicon for genotype 3a, which should be useful for understanding the specific characteristics of this genotype and for the screening of antiviral chemicals that are effective against this genotype. Construction of a full-length infectious S310 clone is in progress.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2012.09.017.

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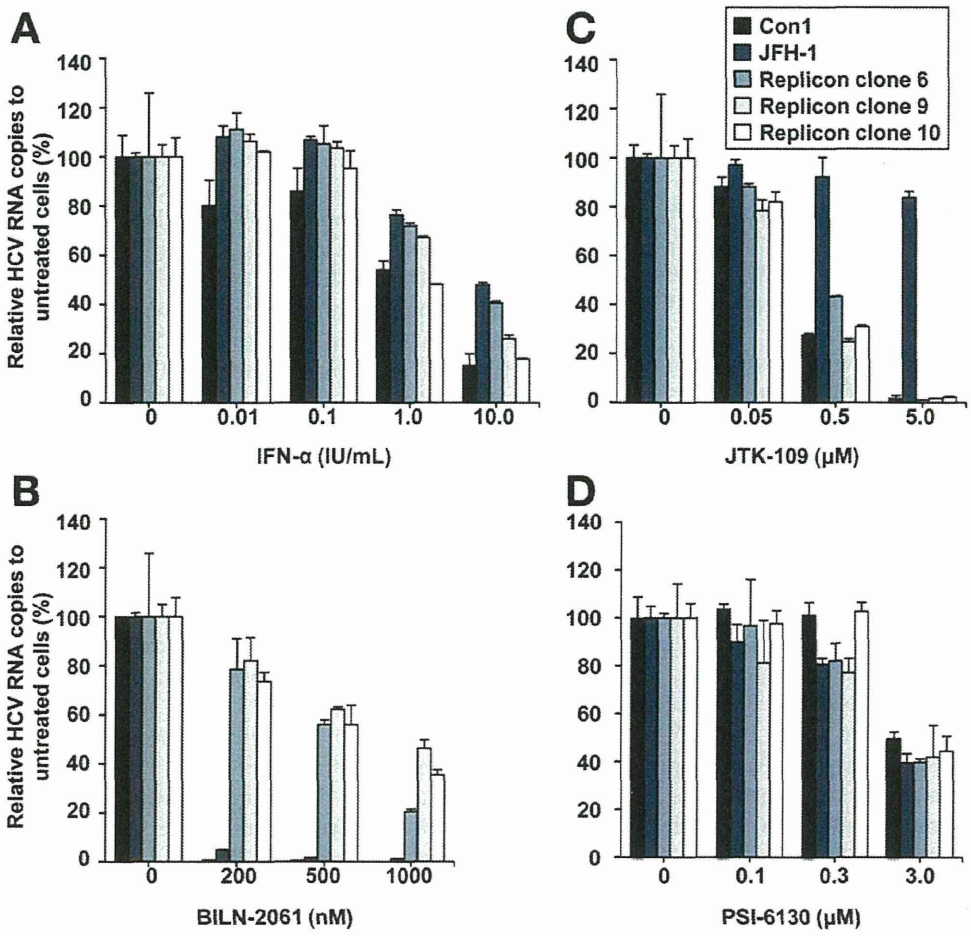

Figure 2. Effects of antiviral inhibitors on the replication of S310 subgenomic replicons. Three cell clones (clone 6, 9 and 10) carrying genotype 3a S310 replicons and one cell clone each harboring genotype 1b Con1 and genotype 2a JFH-1 replicons were treated with the indicated concentrations of (A) 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 ± 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-7[1] and its derivative cell line Huh-7.5.1[2] 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 μg/mL streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate at 37°C in a 5% CO_2 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 Platinium 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 μL 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 μL nuclease-free water. An aliquot of 4 μL 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 D17763[5]) 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 μL) 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 μL 20 mg/mL proteinase K and 10 μL 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 μL cytomix buffer.[8] RNA was delivered into cells by a single pulse of 260 V and 950 μF 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 μg/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 μg) 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-^{32}P]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 without 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.

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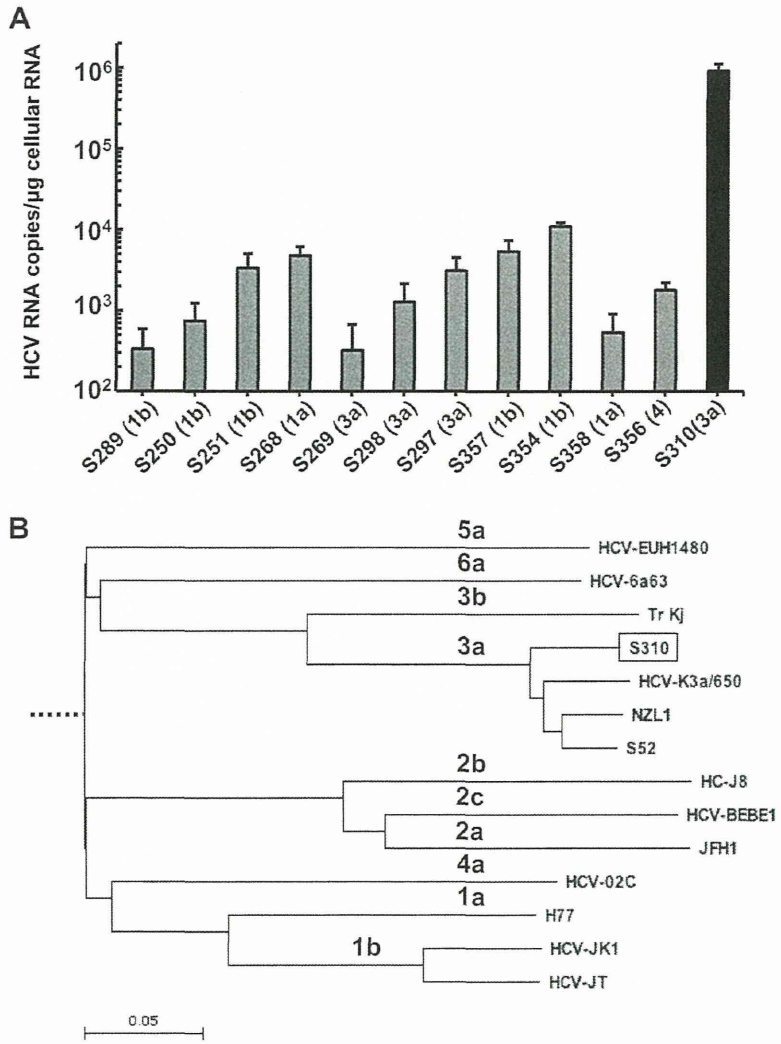
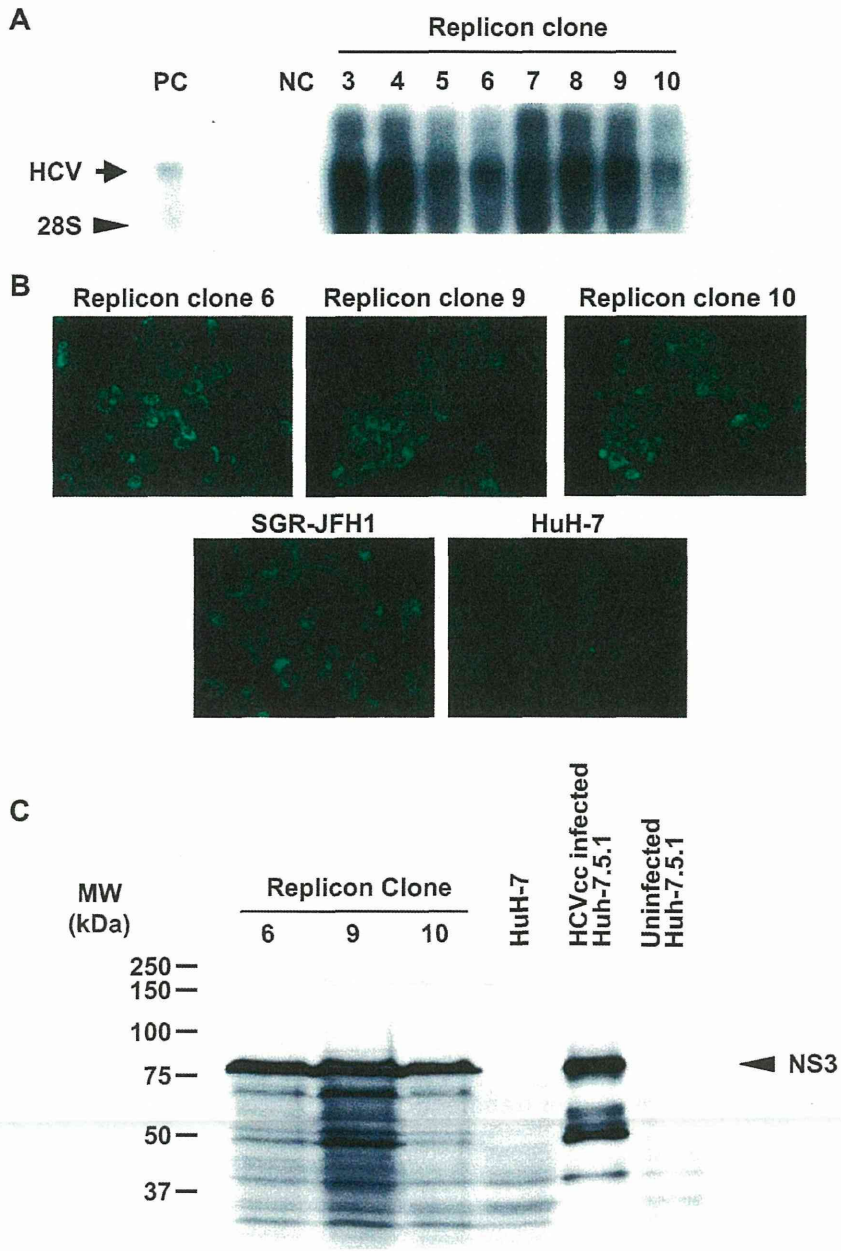
Supplementary Table 1. Primers for Amplification of the S310 HCV Strain

Fragment			Primer sequence (5′→3′)
1 (5′ RACE)[a]	Outer	Antisense	CTTGACGTCCTGTGGGCGA
	Inner	Antisense	TTTTTCTTTGGGGTTTAGG
2	Outer	Sense	GTCTTCACGCGGAAAGCGC
		Antisense	CACCCAAACCACCGACCAC
	Inner	Sense	CCGGGAGAGCCATAGTGGTC
		Antisense	TCCTGAAAGATGGCCTGGGTA
3	Outer	Sense	CTTGGCCCCTCTATGGTAA
		Antisense	GATGTTTCCTGAAGCAGTCG
	Inner	Sense	AGTCATGTGGACCTATTAGT
		Antisense	CACCCAAACCACCGACCAC
4	Outer	Sense	ATGGCTCGTGGCACATCAA
		Antisense	TAGTCATCAGCAGGTCCCAA
	Inner	Sense	GCTCAGCAGCTGCAAGCCCAT
		Antisense	CGCAAAGAATATCTCCGCAAG
5	Outer	Sense	ATTTTTGACATCACTAAGCTAC
		Antisense	AGTGTTGGCTTAAGCCGCA
	Inner	Sense	AATACTTCCAGATGATCATACT
		Antisense	GTGACAGAAAGTGGGCAT
6	Outer	Sense	GTTTCCCGCAGCCAACGT
		Antisense	GTCTCTCAACATCGAGGT
	Inner	Sense	CGGTGAAAGACCGTCTGGA
		Antisense	CAGGGGAGTTGAGATCCT
7	Outer	Sense	GGCCGCGTACATGTGCTAAC
		Antisense	CCGCAGACAAGAAAGTCCGGGT
	Inner	Sense	CTATGGCGCGTGGCTGCCA
		Antisense	ACCCCCAGGTCAGGGTACAC
8	Outer	Sense	CATAACCTAGTCTATTCAACG
		Antisense	TGGTCTTGGTGCGTACCG
	Inner	Sense	GCTCCGTCTGGGAGGACTTGC
		Antisense	CTCGTGCCCGATGTCTCCAA
9	Outer	Sense	TGCTCCTCCAACGTCTCCGT
		Antisense	GCGGCTCACGGACCTTTCAC
	Inner	Sense	GTCGCGGGGACACTCAGGAA
		Antisense	ACTAGGGCTAAGATGGAGCC

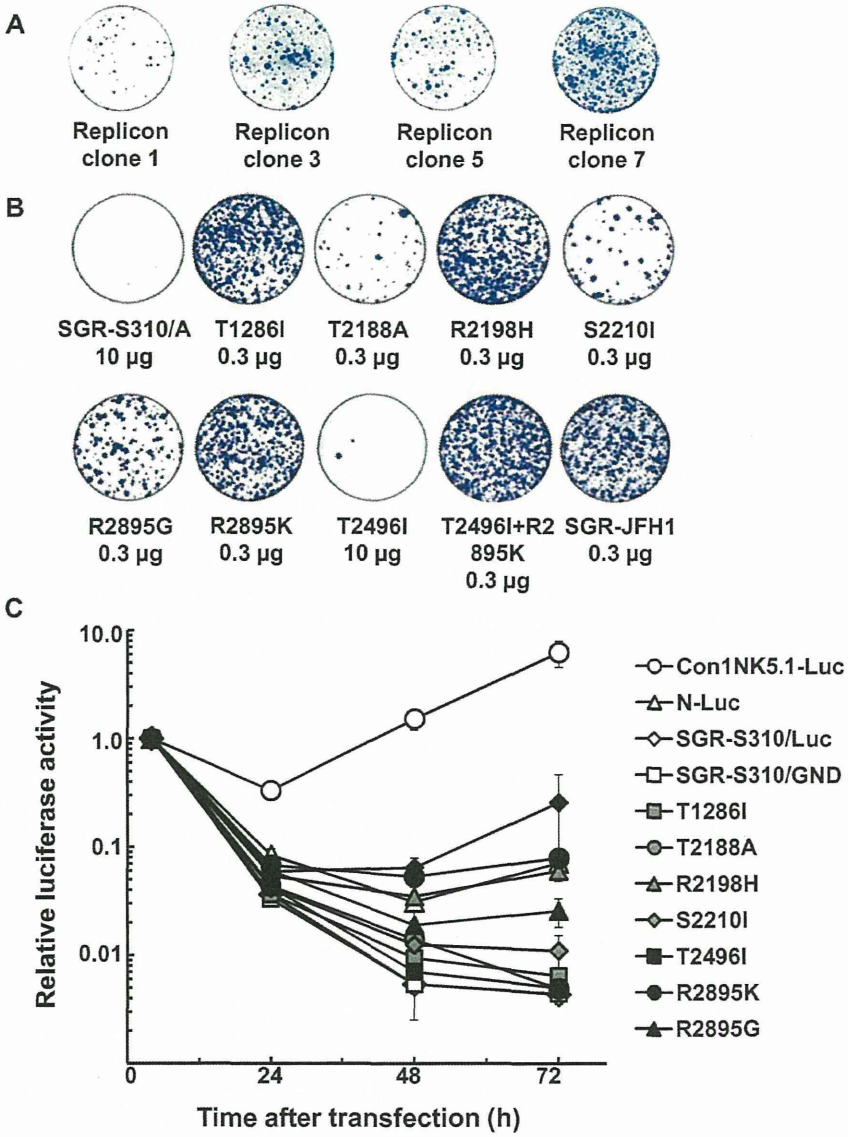
RACE, rapid amplification of complementary DNA ends.
[a]Forward primers used were those in the 5′RACE kit (Abridged Universal Amplification Primer (AUAP) for the first round of PCR and Universal Amplification Primer (UAP) for the second round of PCR).



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 μg) 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 μg) 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 naive 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.