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Figure S1

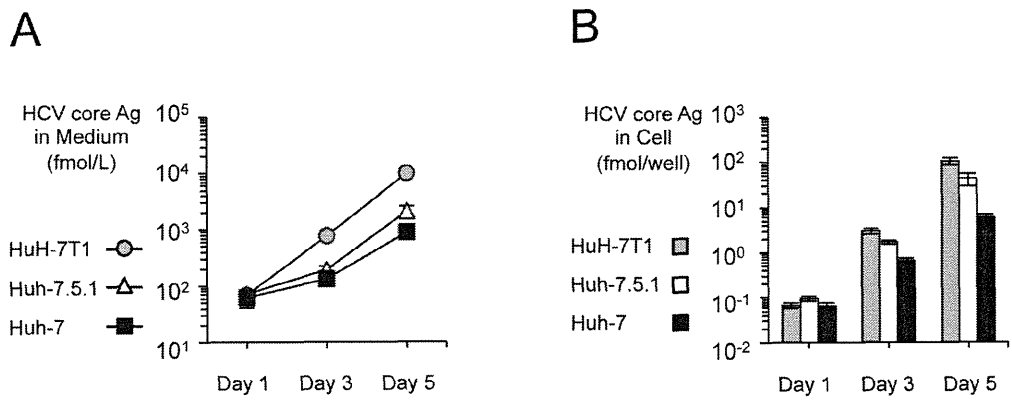

A
HCV core Ag in Medium (fmol/L)
10^5
10^4
10^3
10^2
10^1
HuH-7T1
Huh-7.5.1
Huh-7
Day 1
Day 3
Day 5
B
HCV core Ag in Cell (fmol/well)
10^3
10^2
10^1
10^0
10^{-1}
10^{-2}
HuH-7T1
Huh-7.5.1
Huh-7
Day 1
Day 3
Day 5

Figure S2

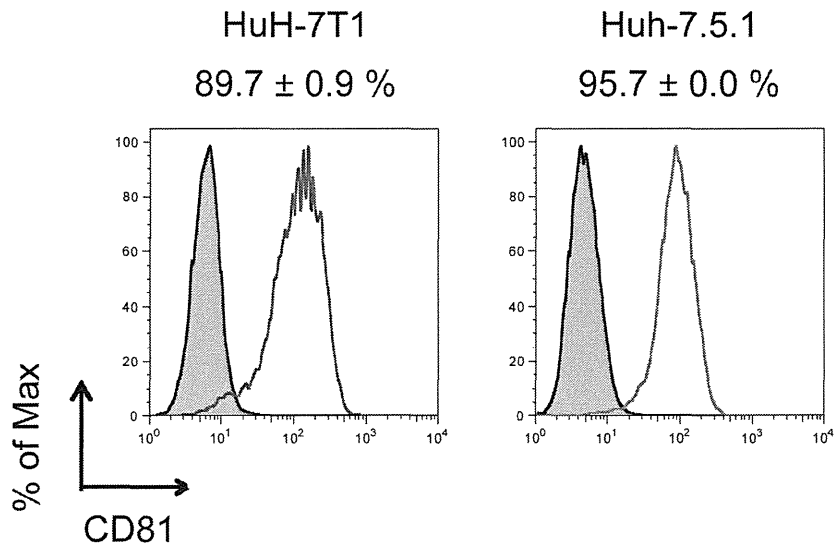


Figure S3

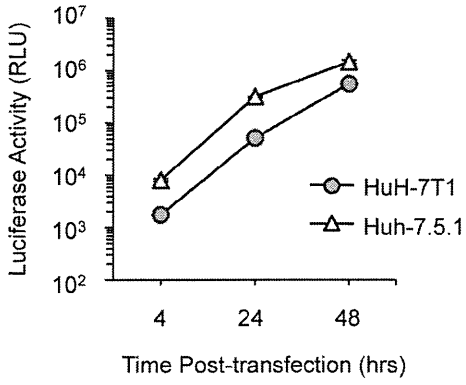

Luciferase Activity (RLU)
10^7
10^6
10^5
10^4
10^3
10^2
4
24
48
Time Post-transfection (hrs)
HuH-7T1
Huh-7.5.1

Figure S4

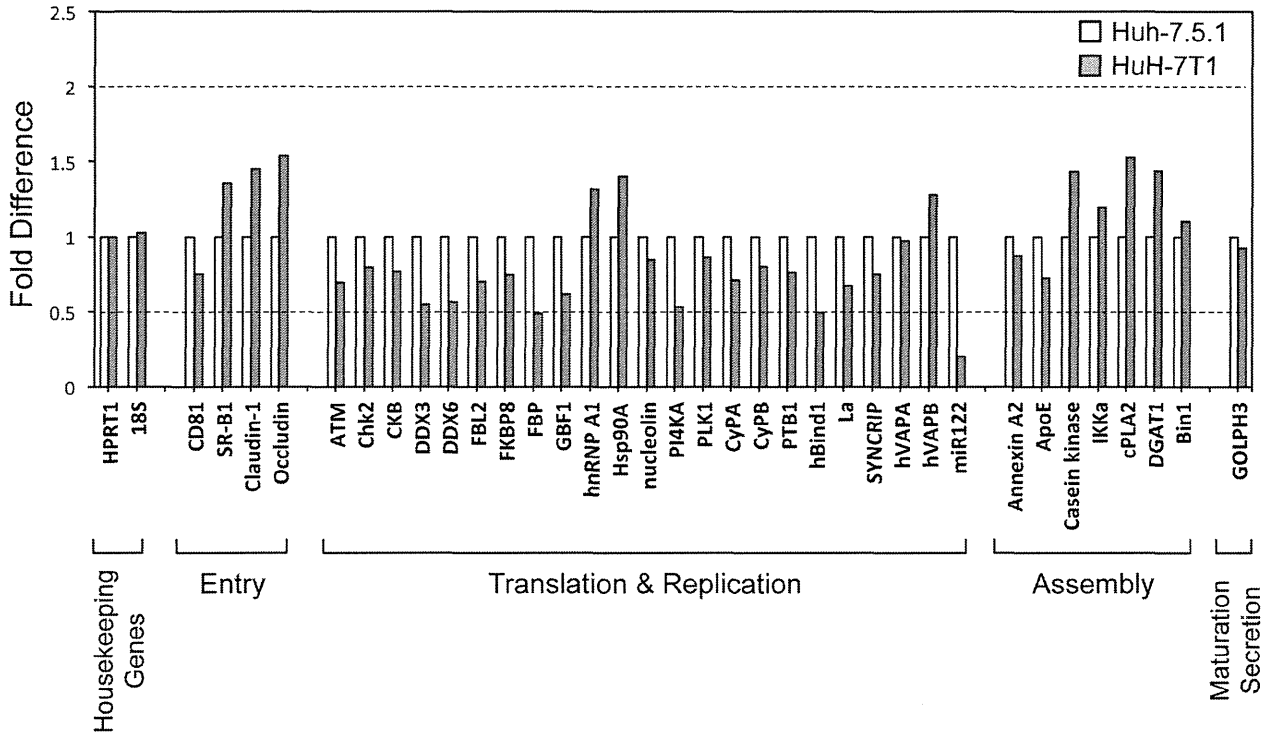
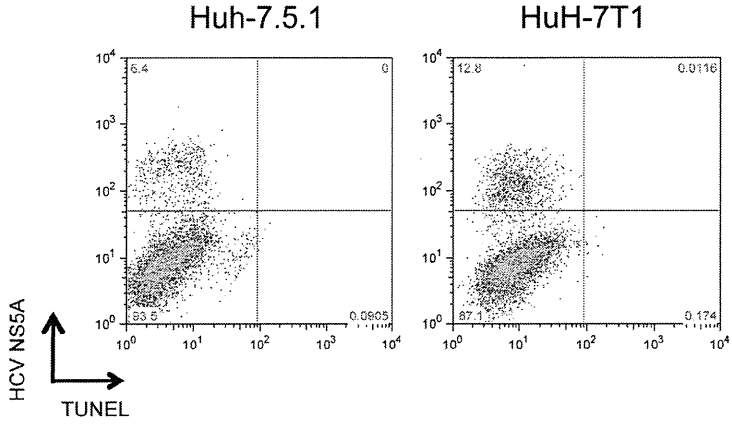

Huh-7.5.1
HuH-7T1
Fold Difference
2.5
2
1.5
1
0.5
0
HPRT1
18S
CD81
SR-B1
Claudin-1
Occludin
ATM
Chk2
CKB
DDX3
DDX6
FBL2
FKBP8
FBP
GBF1
hnRNP A1
Hsp90A
nucleolin
PI4KA
PLK1
CyPA
CyPB
PTB1
hBind1
La
SYNCRIP
hVAPA
hVAPB
miR122
Annexin A2
ApoE
Casein kinase
IKKa
cPLA2
DGAT1
Bin1
GOLPH3
Housekeeping Genes
Entry
Translation & Replication
Assembly
Maturation Secretion

Figure S5


Huh-7.5.1
HuH-7T1
6.4
0
93.5
0.0805
12.8
0.0116
87.1
0.174
HCV NS5A
TUNEL

Supporting Materials and Methods

JFH-1 infection study

Target cells were seeded into 12-well plates at a density of 2×10^5 cells/well. On the following day, the cells were infected with JFH-1 virus at a multiplicity of infection of 0.1 and incubated for 4 h. The supernatants were replaced with fresh medium, and the cells were incubated for 72 h at 37°C. The concentration of HCV core protein in the culture medium and cell lysate was measured.

CD81 expression analysis

CD81 expressed at the cell surface was detected by staining cells with anti-CD81 antibody (clone JS-81, BD) and Alexa Fluor 488 Goat Anti-mouse IgG, followed by single-cell sorting using a FACS Calibur flow cytometer (Beckman Coulter, Inc., Brea, CA).

17 **Gene expression analysis**

18 Total cellular RNA was extracted from Huh-7.5.1 and HuH-7T1 using
19 the RNeasy Mini RNA kit (QIAGEN). cDNA was synthesized from total cellular
20 RNA with random primer (TaKaRa, Shiga, Japan) by using Superscript III
21 reverse transcriptase (Invitrogen). Quantitative PCR was performed using
22 TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA) and
23 gene-specific primer and probe sets (TaqMan Gene Expression Assay; Applied
24 Biosystems) in accordance with the manufacturer's instructions. We evaluated
25 expression levels for genes encoding 36 host factors, including 4 infection and
26 entry-related factors (CD81 [1,2,3,4], SR-BI [1,2,5,6], Claudin-1 [7] and Occludin
27 [8,9]), 22 translation and replication-related factors (ATM [10], Chk2 [10], CKB
28 [11], DDX3 [12], DDX6 [13], FBL2 [14], FKBP8 [15,16], FBP [17], GBF1 [18],
29 hnRNP A1 [19], Hsp90A [15], nucleolin [20,21], PI4KA [22], PLK1 [23], CyPA
30 [24], CyPB [25], PTB1 [26,27], hBind1 [28], La [29], SYNCRIP [30], hVAPA [31]
31 and hVAPB [32]), 7 assembly related factors (Annexin A2 [33], ApoE [34],
32 casein kinase II [35], IKKα [36], cPLA2 [37], DGAT1 [38] and Bin1 [39]), and 1

maturation and secretion-related factor (GOLPH3 [40]). Detailed information regarding gene-specific primer and probe sets is available upon request. The expression levels of host factor-encoding genes were normalized to the expression level of the HPRT1-encoding gene in the respective cell line. Data were expressed as the fold-difference of expression level of each host factor-encoding gene relative to that in Huh-7.5.1.

Total cellular RNA including miRNA was extracted from Huh-7.5.1 and HuH-7T1 using the miRNeasy Mini RNA kit (QIAGEN). cDNA of miR-122 and U6 was synthesized from total cellular RNA with gene-specific primer (TaqMan MicroRNA Assay; Applied Biosystems) by using TaqMan MicroRNA RT Kit (Applied Biosystems). Quantitative PCR was performed using TaqMan Universal PCR Master Mix II (Applied Biosystems) and gene-specific primer and probe sets (TaqMan MicroRNA Assay) in accordance with the manufacturer's instructions. The expression level of miR-122 was normalized to the expression level of U6 in the respective cell line. Data were expressed as the fold-difference of expression level of miR-122 relative to that in Huh-7.5.1.

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Novel Cell Culture-Adapted Genotype 2a Hepatitis C Virus Infectious Clone

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Although the recently developed infectious hepatitis C virus system that uses the JFH-1 clone enables the study of whole HCV viral life cycles, limited particular HCV strains have been available with the system. In this study, we isolated another genotype 2a HCV cDNA, the JFH-2 strain, from a patient with fulminant hepatitis. JFH-2 subgenomic replicons were constructed. HuH-7 cells transfected with *in vitro* transcribed replicon RNAs were cultured with G418, and selected colonies were isolated and expanded. From sequencing analysis of the replicon genome, several mutations were found. Some of the mutations enhanced JFH-2 replication; the 2217AS mutation in the NS5A interferon sensitivity-determining region exhibited the strongest adaptive effect. Interestingly, a full-length chimeric or wild-type JFH-2 genome with the adaptive mutation could replicate in Huh-7.5.1 cells and produce infectious virus after extensive passages of the virus genome-replicating cells. Virus infection efficiency was sufficient for autonomous virus propagation in cultured cells. Additional mutations were identified in the infectious virus genome. Interestingly, full-length viral RNA synthesized from the cDNA clone with these adaptive mutations was infectious for cultured cells. This approach may be applicable for the establishment of new infectious HCV clones.

Hepatitis C virus (HCV) is a principal agent in posttransfusion and sporadic acute hepatitis (6, 19). HCV belongs to the *Flaviviridae* family and *Hepacivirus* genus. Infection with HCV leads to chronic liver diseases, including cirrhosis and hepatocellular carcinoma (16). HCV is a major public health problem, infecting an estimated 170 million people worldwide (6, 16, 19). Current standard therapy for HCV-related chronic hepatitis is based on the combination of interferon (IFN) and ribavirin although virus eradication rates are limited to around 50% (7, 24, 30). Telaprevir and boceprevir were approved by the U.S. Food and Drug Administration in 2011 in combination with pegylated alpha interferon and ribavirin for the treatment of genotype 1 chronic hepatitis C (34, 35). Both agents inhibit the NS3-NS4A serine protease essential for replication of HCV (25, 36). It is important to develop more anti-HCV drugs with different modes of action to achieve greater efficacy and to avoid the emergence of drug-resistant viruses. To that end, a detailed understanding of the viral replication mechanism is needed to discover novel antiviral targets. An efficient virus culture system is indispensable for detailed analysis of HCV life cycles. In an important development, a subgenomic HCV RNA replicon system has been developed (22) to assess HCV replication in cultured cells. Furthermore, an efficient HCV culture system was established by using a JFH-1 strain virus isolated from a fulminant hepatitis patient (20, 38, 41). By transfection of *in vitro* transcribed full-length JFH-1 HCV RNA into HuH-7 cells, efficient JFH-1 RNA replication and infectious viral particle production were detected. However, this efficient virus production was not reproduced by other HCV strains, even when adaptive mutations were introduced to enhance the replication efficiency in cultured cells (29). Thus, other HCV strains that can replicate in cultured cells and produce infectious virus particles are needed. The J6CF strain is infectious to chimpanzees but does not replicate in cultured cells (26, 27, 40). We constructed chimeric replicon and virus constructs of the J6CF and JFH-1 strains to elucidate the difference in their molecular mechanisms (26, 27). We determined that the NS3 helicase and the NS5B to 3′X regions are important for the efficient replication of the JFH-1 strain and that several amino acid mutations in the C terminus of NS5B are pivotal for replication. However, we could not rescue the replication of other virus strains, such as Con1, with these mutations. This result indicates that different approaches are needed to create replication-competent virus strains in cultured cells.

In the present study, we isolated HCV cDNA, named JFH-2, from a fulminant hepatitis patient. The replication efficiency of the JFH-2 clone in the subgenomic replicon assay was lower than that of JFH-1 although the introduction of adaptive mutations enhanced JFH-2 replication. Interestingly, the full-length chimeric or wild-type JFH-2 genome with adaptive mutations could replicate and produce infectious virus particles. The virus infection efficiency was sufficient for autonomous virus propagation in cultured cells.

MATERIALS AND METHODS

Cell culture system. HuH-7, Huh-7.5.1 (a generous gift from Francis V. Chisari), and Huh7-25 cells were cultured in 5% CO_2 at 37°C in Dulbec-

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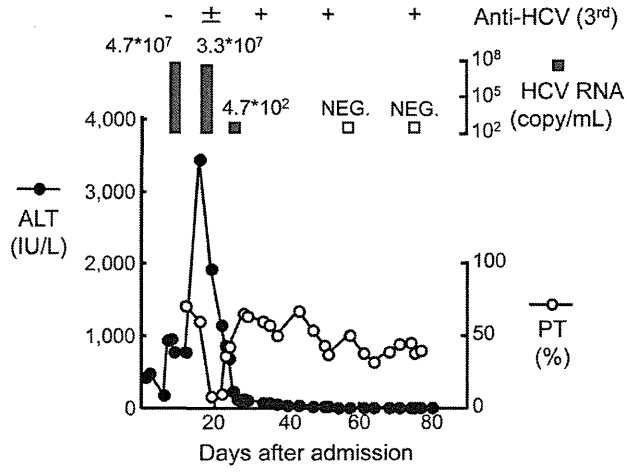

FIG 1 Clinical course of second fulminant hepatitis patient infected with JFH-2. The patient was admitted by reason of acute liver failure. Alanine aminotransferase (ALT) levels, prothrombin time (PT), HCV RNA, and anti-HCV antibodies were determined and followed in his serum.

co's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (DMEM-10) (3, 41).

HCV clones. The genotype 2a clone JFH-2 was isolated from a patient with fulminant hepatitis (15). Briefly, HCV cDNA was cloned from a fulminant hepatitis patient, a 62-year-old man who had a history of coronary artery bypass surgery without blood transfusion. One year after the surgery, he developed an acute auditory disorder and received a course of betamethasone therapy. After withdrawal of betamethasone, the patient developed fulminant hepatitis as diagnosed by acute liver failure associated with stage II encephalopathy and low prothrombin time. He experienced prolonged liver failure and died after 80 days. HCV RNA was detected in his serum only during the acute phase (Fig. 1). Total RNA was extracted from serum during the acute phase, and HCV cDNA covering the entire genome was amplified by reverse transcription-PCR (RT-PCR). All amplified products were purified and then cloned into pGEM-T EASY vectors (Promega, Madison, WI). PCR products and plasmids were sequenced by using specific primer sets (Table 1), BigDye Terminator Mix, and an automated DNA sequencer (models 310 and 377; PE Biosystems, Foster City, CA). The JFH-2 subgenomic replicon (SGR) clones, pSGR-JFH2.1 and pSGR-JFH2.2 (DDBJ/EMBL/GenBank accession numbers AB690456 and AB690457, respectively), were constructed according to the method for pSGR-JFH1 construction (11). Several mutations were introduced into the pSGR-JFH2.1 replicon construct, as reported previously (11). The reporter replicon constructs, pSGR-JFH2.1/Luc and pSGR-JFH2.1/Luc, were developed by rearrangement with pSGR-JFH2.1 and pSGR-JFH2.2 (accession numbers AB690458 and AB690459, respectively) as described previously (12). pJ6/JFH1 was previously obtained from pJFH1 by replacement with the 5′ untranslated region (UTR) to the p7 region (EcoRI-BclI) of the J6CF strain (a kind gift from Jens Bukh) (3, 40). A full-length HCV cDNA was constructed by using the 5′ end to NS2 of pJ6/JFH1 and NS3 to the 3′ end of pSGR-JFH2.1, and the resulting construct was named pJ6/JFH2 (accession number AB690460). Another full-length HCV construct, pJFH2 containing the full-length JFH-2 cDNA downstream of the T7 RNA promoter sequence, was also constructed by replacing the 5′ UTR to NS2 of pJ6/JFH2 with JFH2 sequences, as described previously (accession number AB690461) (1, 37, 38).

Subgenomic replicon assay. Subgenomic replicon RNA was synthesized as reported previously (11). Synthesized replicon RNA was adjusted to 10 μg with cellular RNA isolated from untransfected HuH-7 cells and then electroporated into naive HuH-7 cells as reported previously (11). G418 (1.0 mg/ml) was added to the culture medium, and the drug-resistant colonies were fixed with buffered formalin and stained with crystal violet or cloned and expanded for further analysis. Total RNA was extracted from the cloned G418-resistant cells by using Isogen reagent (Nip-

TABLE 1 Primer list used for cloning and sequencing of JFH-2 clone

Forward primer		Reverse primer	
Name	Sequence (5′→3′)	Name	Sequence (5′→3′)
44S	CTGTGAGGAACTACTGTCTT	1323R	GGTGACCAGTTCATCATCAT
317S	GGGAGGTCTCGTAGACCGTG	1440R	GCTCCCTGCATAGAGAAGTA
844S	GGGTTAATTATGCAACAGGGAAC	2367R	CATTCCGTGGTAGAGTGCA
1141S	TGTCCGCCACGCTCTGCT	2445R	TCCACGATGTTTTGGTGGAG
1361S	CCCGAGGTCATCATAGACAT	3568R	TGTTCCGAGGAAGGACTGAG
2106S	CTGTTGTGCCCCACGGACTG	3765R	TCAGCGTTCCGCGTGACCA
2285S	AACTTCACTCGTGGGGATCG	4706R	TTGCAGTCGATCACGGAGTC
3211S	GGCACTTACATCTATGACCACCTC	5331R	GAGGTCATGACCAGCACGTG
3471S	TGGGCACCATAGTGGTGAG	5563R	CTGCAGCAAGCCTTGGATCT
3930S	TCGATTTCATCCCCGTTGAG	5970R	TTCTCGCCAGACATGATCTT
4278S	CCTATGACATCATCATATGCGATGAATGCC	6152R	AGTGAGTAGGGGCGACGTGGTTTCCTCTGG
4301S	CCTATGACATCATCATATGCGATG	6505R	CCTGCCAGGTGTTCATGCAG
4547S	AAGTGTGACGAGCTCGCGG	6605R	GCATACTCTGAGGCCGCCAC
5021S	TTTTGGGAGGCAGTTTTCAC	6897R	GTGATGTGGGGCGGATCTGTTAGCATGGAC
6383S	TGTCAAAAGGGGTACAAGGG	7648R	TCCTCCTCGGAGCAAGTAGA
6774S	TCCGGGATGAGGTCTCGTTC	8913R	GCGTACTGGATGATGTTTCC
6881S	ATTGATGTCCATGCTAACAG	3X-54R	GCGGCTCACGGACCTTTCAC
7198S	GGCTTGGGCACGGCCTGA	3X-75R	TACGGCACTCTCTGCAGTCA
7244S	ACCGCTTGTGGAATCGTGGA		
7657S	CGTGTGCTGCTCCATGTCAT		
7993S	CAGCTTGTCCGGGAGGGC		
8337S	TTTCGTATGATACCCGATGCTT		
8704S	CGCCCCTCCGGGTGACCCCCCCAGACCGGA		
9123S	CACGAACTGACGCGGGTGGC		

pon Gene, Tokyo, Japan), and the replicon RNA was quantitated by Northern blotting and real-time detection RT-PCR as reported previously (11, 37). The cDNAs of the HCV RNA replicon were synthesized and then amplified by PCR. The sequence of each replicon was determined.

Luciferase reporter replicons were analyzed as follows. Five micrograms of synthesized replicon RNA was transfected into HuH-7 cells by electroporation. Transfected cells were harvested serially at 4, 24, 48, 72, and 96 h after transfection. Luciferase activities were quantified by a Lumat LB9507 instrument (EG&G Berthold, Bad Wildbad, Germany) and a luciferase assay system (Promega). Assays were performed at least in triplicate, and the results were expressed as relative luciferase activity.

Analysis of G418-resistant cells. In RNA-transfected dishes, G418-resistant colonies were isolated by using a cloning cylinder (Asahi Techno Glass Co., Tokyo, Japan) and expanded until 80% to 90% confluence in 10-cm diameter dishes. Expanded cells were analyzed as described previously (11).

Northern blot analysis. Four micrograms of isolated RNA samples was electrophoretically separated in a 1% agarose gel containing formaldehyde and transferred to a positively charged nylon membrane (Hybond-N+; GE Healthcare UK, Ltd., Buckinghamshire, England) and immobilized by a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). Hybridization was performed with a [α-^{32}P]dCTP-labeled DNA probe by using Rapid-Hyb Buffer (GE Healthcare UK, Ltd.). The NS3 to 3′X region of the JFH-1 sequence was used as a template of DNA probe synthesis with a Megaprime DNA Labeling System (GE Healthcare UK, Ltd.) (37).

Western blot analysis of HCV proteins. The protein samples were separated on a 10% polyacrylamide gel. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Bedford, MA) with a semidry blotting apparatus (Biocraft, Tokyo, Japan). Transferred proteins were incubated with blocking buffer containing 5% nonfat dry milk (Snow brand, Sapporo, Japan) in phosphate-buffered saline. Anti-NS3 rabbit polyclonal antibody raised against recombinant NS3 protein and horseradish peroxidase-labeled goat anti-rabbit Ig (BioSource, Camarillo, CA) were used to detect HCV NS3 protein. The signals were detected with a chemiluminescence system (ECL Plus; GE Healthcare UK, Ltd.). The quantity and quality of the loaded samples were confirmed to be similar by Coomassie brilliant blue staining of the gel.

RT-PCR and sequencing analysis. The cDNAs of HCV RNA were synthesized from total cellular RNA isolated from replicon RNA-transfected cells or from the culture medium of full-length HCV RNA-transfected cells with antisense primer in the 3′X tail region. These cDNAs were subsequently amplified with DNA polymerase (TaKaRa LA Taq; TaKaRa Bio Inc.). The sequence of each amplified DNA was determined directly as described above.

Full-length HCV RNA transfection. Full-length HCV RNA was synthesized from pJ6/JFH2, pJFH2, and the derivatives of these constructs with adaptive mutations, as described previously (13, 37, 38). Synthesized HCV RNA (10 μg) was transfected into Huh-7.5.1 or Huh7-25 cells. HCV core protein levels in the culture medium were measured by immunoassay (31). HCV RNA levels in the culture medium were quantified as described above. Infectivity of culture supernatants was determined by measuring the focus formation efficiency (13, 41). In some experiments, HCV core protein levels in the transfected cells were determined as described previously (37, 38). To examine virus secretion and infectivity after long-term culture, the transfected cells were serially passaged. Virus infection was neutralized by using mouse anti-CD81 monoclonal antibody (clone JS-81; BD Pharmingen, Franklin Lakes, NJ) and anti-HCV human IgG purified from HCV carrier serum (a gift from H. Yoshizawa and J. Tanaka, Hiroshima University).

Density gradient analysis. Culture medium derived from the transfected or infected cells was harvested for density gradient analysis. Cleared culture medium was layered onto a stepwise sucrose gradient (60% [wt/vol] to 10%) and centrifuged for 16 h in an SW41 rotor (Beckman, Palo Alto, CA) at 200,000 $\times$ *g* at 4°C. After centrifugation, 18 fractions were harvested from the bottom of the tubes. The HCV core protein, HCV RNA levels, and infectivity in each fraction were determined as described above.

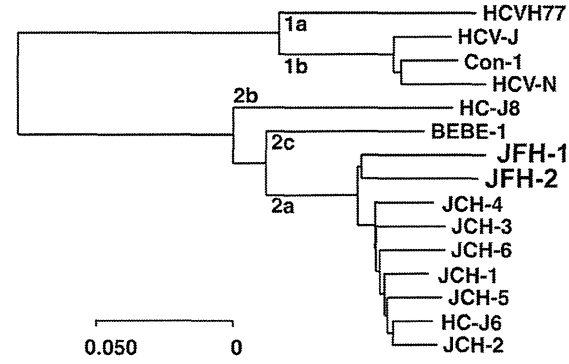

FIG 2 Phylogenetic analysis of JFH-2. Phylogenetic tree of the NS3 to NS5B amino acid sequences of HCV including the JFH-2 strain and genotype 2 strains for which the entire genome has been reported (JFH-1, accession number AB047639; HC-J6, D00944; HC-J8, D10988; and BEBE1, D50409) and representative genotype 1 strains for which the entire genome has been reported (H77, AF009606; HCV-Con1, AJ238799; HCV-J, D90208, and HCV-N, AF139594). This phylogenetic tree was drawn by using Kimura's two-parameter method.

Electron microscopy. To visualize HCV particles, we adsorbed the density gradient-purified virus samples onto carbon-coated grids for 1 min. Then, the grids were stained with 1% uranyl acetate for 1 min and examined under an H-7650 transmission electron microscope (Hitachi High-Technologies Co., Tokyo, Japan) (32). Immunogold labeling was performed with an antibody directed against E2 (AP33; a kind gift from Genentech, South San Francisco, CA) diluted 1:50 in blocking solution and secondary antibody coupled to 10-nm gold particles.

Human hepatocyte chimeric mouse experiments. Human hepatocytes were transplanted into urokinase-type plasminogen activator-transgenic SCID mice(uPA$^{+/+}$ SCID$^{+/+}$) as described previously (33). All mice received hepatocyte transplants from the same donor. Human hepatocyte chimeric mice, in which liver cells were largely (>90%) replaced with human hepatocytes, were used to reduce the potential influence by mouse-derived mRNA. Human albumin levels in the sera of mice were monitored to evaluate the replacement ratio of the human hepatocytes in the mouse liver. The mice were obtained from Phoenix Bio Co., Ltd. (Hiroshima, Japan). Four mice were divided into two groups. Each group of mice was inoculated with 1×10^6 RNA copies of either purified J6/JFH2/AS HCV particles or JFH-2 patient serum. The HCV RNA titer in inoculated mouse serum was monitored by real-time detection RT-PCR each week after inoculation.

RESULTS

HCV clone from a fulminant hepatitis patient. HCV cDNA was isolated from a fulminant hepatitis patient as described in Materials and Methods (clone JFH-2) (15). HCV RNA was detected by RT-PCR in the patient's serum during the acute phase (Fig. 1). All viral markers of the other hepatitis viruses were negative. By the phylogenetic analysis, the JFH-2 clone was clustered into genotype 2a (Fig. 2). JFH-2 exhibits 87.6%, 89.0%, and 88.9% nucleotide homology with JFH-1, J6CF, and JCH-1, respectively, and 90.6%, 91.8%, and 91.8% amino acid homology with JFH-1, J6CF, and JCH-1, respectively (Table 2). The JFH-1 strain is cell culture replication-competent, but the J6CF and JCH-1 strains are incompetent. However, the homology data for nucleotide and amino acid sequences are very similar in both the structural and nonstructural regions. We also mapped the

TABLE 2 Percent homology between JFH-2 and other genotype 2a strains

Region	JFH-2 nucleotide profile				JFH-2 amino acid profie			
	Lenght (nt)[a]	% Identity vs strain:			Length (aa)[b]	% Amino acid identity vs strain:		
		JFH-1	J6CF	JCH-1		JFH-1	J6CF	JCH-1
Entire genome	9683	87.60	88.98	88.88	3033	90.64	91.79	91.79
UTR[c]	576	96.35	98.61	96.88	NA[d]			
Structural	2439	86.14	87.90	86.51	813	89.30	89.54	88.56
Nonstructural	6663	87.44	88.59	89.12	2220	91.13	92.61	92.97
5′ UTR	340	98.82	99.71	99.71	NA			
Core	573	91.80	93.02	91.97	191	92.15	95.29	92.15
E1	576	87.50	88.89	89.06	192	90.10	92.19	89.58
E2-p7	1290	83.02	85.19	82.95	430	87.67	85.81	86.51
NS2	651	84.18	85.87	89.09	217	87.56	88.02	91.24
NS3	1893	87.64	88.54	89.33	631	92.87	94.61	94.45
NS4A	162	88.27	88.27	88.27	54	96.30	92.59	94.44
NS4B	783	89.91	90.04	89.14	261	96.93	97.32	96.55
NS5A	1398	83.48	85.98	85.48	466	82.83	86.70	86.48
NS5B	1776	90.37	91.10	91.84	591	94.08	94.75	95.43
3′ UTR	236	92.80	97.03	92.80	NA			

[a] nt, nucleotides.
[b] aa, amino acids.
[c] UTR, 5′ UTR plus 3′ UTR.
[d] NA, not applicable.

positions of different amino acid sequences of each strain (Fig. 3A). The E2 and NS5A regions are more variable than other regions (Fig. 3A and Table 2); however, it is difficult to find particular mutation positions or regions specific for the JFH-2 strain.

Subgenomic replicon analysis of the JFH-2 clone. Interestingly, some parts of the viral cDNA sequences in the JFH-2 viral genome were a mixture of different sequences, especially in the NS3 region. By the cloning analysis, we found two major se-

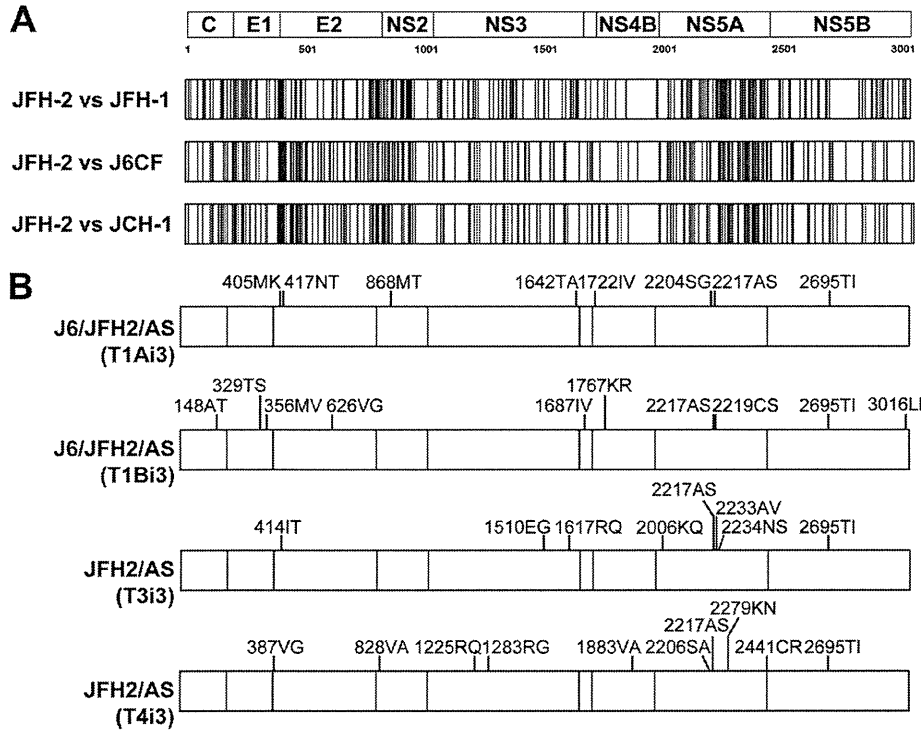

FIG 3 Maps of amino acid sequences among genotype 2a HCV strains and mutations found in the cell culture-adapted viruses. (A) Amino acid sequences of the entire open reading frame (3,033 amino acids) of JFH-1, JFH-2, J6CF (accession numbers AB047639, AB690461, and AF177036, respectively), and JCH-1 strains were compared. The positions of different sequences are indicated by vertical lines. (B) Virus genome sequences were determined in the T1Ai3 and T1Bi3 culture media of the J6/JFH2/AS virus-inoculated cells or T3i3 and T4i3 culture media of the JFH2/AS virus-inoculated cells, as described in the text. Amino acid mutations are indicated with their positions and residues.

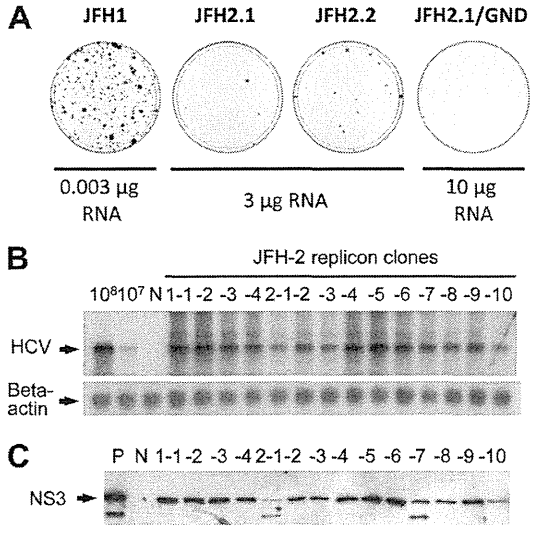

FIG 4 G418-resistant colony formation of JFH-1 and JFH-2 replicons and analysis of JFH-2 replicon cells. (A) Subgenomic RNAs were synthesized *in vitro* by using pSGR-JFH1, pSGR-JFH2.1, pSGR-JFH2.2, and pSGR-JFH2.1/GND as templates. Transcribed subgenomic RNAs were electroporated into HuH-7 cells, and cells were cultured with G418 for 3 weeks before being stained with crystal violet. JFH-1 subgenomic RNA (0.003 μg), 3 μg of JFH-2.1 and JFH-2.2 subgenomic RNA, and 10 μg of JFH-2.1/GND subgenomic RNA were transfected into HuH-7 cells. Experiments were performed in triplicate, and representative staining examples are shown. (B) Northern blot analysis. Total cellular RNA isolated from each of four SGR-JFH2.1 clones (1-1 to 1-4) and 10 SGR-JFH2.2 clones (2-1 to 2-10) was analyzed by using a random-primed DNA probe to detect replicon RNA. Isolated total cellular RNA (4 μg) was separated by denatured agarose gel electrophoresis. After electrophoresis, HCV- and beta-actin-specific RNAs were detected by Northern blot analysis with random-primed DNA probes specific to HCV and beta-actin sequences. Arrows indicate replicon RNA or beta-actin mRNA. (C) Western blot analysis. Cell lysates were prepared from four SGR-JFH2.1 clones (1-1 to 1-4) and 10 SGR-JFH2.2 clones (2-1 to 2-10). The NS3 proteins were detected with rabbit anti-HCV NS3 antibody. Positive-control (P) and negative-control (N) cell lysates were obtained from JFH-1 replicon cells and naive HuH-7 cells.

quences in the JFH-2 viral genome. One sequence contained alanine and isoleucine (AI) at amino acid positions 1204 and 1205, and the other contained methionine and leucine (ML) at the same positions. We referred to these viral genomes containing AI or ML as JFH-2.1 or JFH-2.2, respectively. From the cloning analysis of PCR products, JFH-2.1 populated 19 of 32 clones (59%), and JFH-2.2 populated 13 of 32 clones (41%). To analyze the replication efficiency of the JFH-2 clone, we thus constructed two subgenomic replicon constructs, pSGR-JFH2.1 and pSGR-JFH2.2, as pSGR-JFH1 (11). Synthesized replicon RNAs of JFH-2.1 and JFH-2.2 were independently transfected by electroporation into HuH-7 cells. The transfected cells were then grown for 3 weeks in selection culture that contained 1 mg/ml of G418. Several colonies survived the selection culture, as illustrated by crystal violet staining (Fig. 4A). The JFH2.1/GND replication-incompetent control RNA-transfected cells did not form any colonies, even when 10 μg of RNA was transfected. The colony formation efficiencies of the JFH-2.1 and JFH-2.2 replicons were 0.94 ± 0.54 and 6.43 ± 3.39 CFU/μg RNA, respectively, which were substantially lower than the colony formation efficiency of the JFH-1 subgenomic replicon ($5.32 \times 10^4 \pm 5.02 \times 10^4$ CFU/μg RNA) (11). Four colonies of the JFH-2.1 replicon and 10 colonies of the JFH-2.2 replicon were cloned and expanded for further analysis. Replicon RNA was isolated from each replicon cell clone, and the HCV RNA titer and sequence of the replicon genome were determined (Table 3). The average HCV RNA titer in replicon cell clones was determined by real-time RT-PCR detection as $(8.70 \pm 4.94) \times 10^7$ copies/μg of RNA. The size and amount of the replicon RNA in the replicon cells were confirmed by Northern blot analysis (Fig. 4B). We also detected NS3 protein in each clone of replicon cells by Western blot analysis (Fig. 4C). NS3 proteins were mainly found at approximately 70-kDa by polyclonal anti-NS3 antibody; however, an additional signal was also detected at a smaller molecular size in some replicon cells, including the positive-control JFH-1 replicon cells.

Next, we determined the sequences of replicating RNA in each replicon cell clone. Most of the clones, except replicon clone 2.2-8, had at least one nonsynonymous mutation (Table 3). We found nonsynonymous mutations in the NS3, NS5A, and NS5B regions, and three mutations were common among the different replicon genomes. Among the mutations found in the NS3 region, both 1547FL and 1614CW were found in two different replicon cells, and the 1651TN mutation was found in five replicon cells. The 2280QR mutation in NS5A was found in three replicon cells. 2217AS and 2222HQ, which are located in the interferon sensitivity-determining region (ISDR), were each found in a single replicon cell (8). To determine the adaptive effect of these mutations (Fig. 5A), we inserted these mutations (listed in Table 3), except for 1204MK, into pSGR-JFH2.1 and tested the colony formation efficiency of the mutant replicons. The 1204MK mutation was not tested since methionine at amino acid position 1204 was specific for the JFH2.2 sequence. As shown in Fig. 5B, 1547FL, 1614CW, 1651TN, 2222HQ, and 2280QR had weak to moderate adaptive effects for colony formation. Interestingly, the 2217AS mutation in the ISDR strongly enhanced the colony formation to approximately 3×10^4 times that of the parental JFH2.1 replicon (Fig. 5B). We further tested these adaptive mutations in the luciferase reporter replicon format, as described previously (12). SGR-JFH2.1 with the 2217AS construct exhibited significant replication compared to JFH2.1/GND, which is the replication-incompetent negative control. However, other constructs showed no evidence of replication in the transient replication assay (Fig. 5C).

Full-length HCV replication. The 2217AS mutation substantially enhanced RNA replication of the JFH-2.1 subgenomic replicon compared with other mutations. We examined whether a full-length JFH-2 HCV clone with the 2217AS mutation could produce infectious virus. In our previous study, we constructed the J6/JFH1 chimeric construct by replacement of the 5′ untranslated region to the p7 region (EcoRI-BclI) of J6 (1), and we found that J6/JFH1 produces a larger amount of infectious virus in the culture medium (3). We thus used the structural region of the J6CF clone and the NS2 region of the JFH-1 clone from a J6/JFH-1 chimeric virus construct and fused it to the NS3 to 3′X regions of JFH-2.1 with the 2217AS mutation (plasmid pJ6/JFH2/AS) since it was not clear if the structural and NS2 regions of the JFH-2 strain were functionally intact (Fig. 6A). Full-length viral RNA was synthesized from linearized pJ6/JFH2/AS and electroporated into Huh-7.5.1 cells. After two independent transfections, the transfected cells were divided into sub-cell lines to form a total of four sub-cell lines (T1A, T1B, T2A, and T2B). All four sub-cell lines were serially passaged, and HCV core protein, RNA, and infectivity levels in the culture supernatant were monitored (Fig.

TABLE 3 Mutations and RNA titer of the JFH-2 replicon cell clones

Replicon clone	Nucleotide		Amino acid		Region	Replicon titer (no. of copies/μg of RNA)
	Mutation	Position	Mutation	Position		
2.1-1	A→G	2012	E→G	1109	NS3	1.30E+8
	C→A	3638	T→N	1651	NS3	
2.1-2	T→C	3325	F→L	1547	NS3	1.52E+8
	C→A	3638	T→N	1651	NS3	
2.1-3	A→G	5525	Q→R	2280	NS5A	1.09E+8
	A→G	7155	None		NS5B	
2.1-4	C→A	3638	T→N	1651	NS3	1.41E+8
	A→G	7795	None		3′ UTR	
2.2-1	C→G	3528	C→W	1614	NS3	2.33E+7
2.2-2	G→T	5335	A→S	2217	NS5A (ISDR)	3.57E+7
2.2-3	C→G	919	None		*neo*	3.35E+7
	C→A	5352	H→Q	2222	NS5A (ISDR)	
2.2-4	C→A	1223	None		EMCV-IRES[a]	1.05E+8
	C→A	2115	None		NS3	
	G→T	6243	K→N	2519	NS5B	
2.2-5	C→A	3327	F→L	1547	NS3	1.67E+8
2.2-6	T→C	625	None		*neo*	1.09E+8
	C→A	3638	T→N	1651	NS3	
	A→G	5525	Q→R	2280	NS5A	
	T→A	5754	None		NS5A	
	G→A	5803	G→S	2373	NS5A	
2.2-7	C→G	3528	C→W	1614	NS3	6.25E+7
2.2-8	None		None			5.31E+7
2.2-9	C→G	3638	T→N	1651	NS3	6.71E+7
	G→A	5269	A→T	2195	NS5A	
	A→G	5525	Q→R	2280	NS5A	
2.2-10	T→A	2297	M→K	1204	NS3	2.95E+7
	A→G	7815	None		3′ UTR	

[a] EMCV-IRES, encephalomyocarditis virus internal ribosome entry site.

6B and C and Table 4). At the first cell passage, the HCV core protein levels were approximately 300 fmol/liter, and the infectivities were very low. Secreted HCV core protein levels decreased in all of the passaged cells until 25 days after the transfection. However, HCV core protein secretion of passaged T1A cells began to increase from 30 days after transfection. Subsequently, increased core protein secretion was also observed in other passaged cells although at different time points (Fig. 6B and Table 4). The maximum core protein levels in the medium were up to 9,241 fmol/liter in T1B cells at day 75 posttransfection. Infectivity detected in the culture medium was also first increased in T1A, and similar increases were observed with other passaged cells at later time points. Furthermore, specific infectivity (infectivity/HCV RNA or infectivity/HCV core protein) was also higher than in the initial culture medium (Table 4). The passaged cells were immunostained with anti-core monoclonal antibody (Fig. 6D). At 4 weeks after transfection, only a few cells were positive in all four sub-cell lines. However, the number of positive cells increased from 8, 12, 18, or 14 weeks after transfection in T1A, T1B, T2A, or T2B cells, respectively. These results indicate that phenotypic change occurred in the replicating virus after the serial passages of the transfected cells. Before this phenotypic change, the replicating viruses were not able to secrete significant amounts of infectious virus particles due to an unknown defect in infectious virus particle formation or secretion. After the phenotypic change, the robust core protein secretion might have been caused by changes in the efficiency of infectious virus production or secretion. To compare the virus characteristics before and after the phenotypic change, we analyzed T1A culture medium from 5 days, 8 weeks, and 11 weeks posttransfection by density gradient assay (Fig. 6E). The day 5 medium showed a broad density profile both of core protein and HCV RNA, and infectivity was not detected. Interestingly, the peaks of HCV core protein and RNA at around 1.15 mg/ml density became higher at 8 weeks and had a further increase at 11 weeks. Broader minor peaks at the lighter density remained small at week 11. The infectivity peak also became higher at 8 and 11

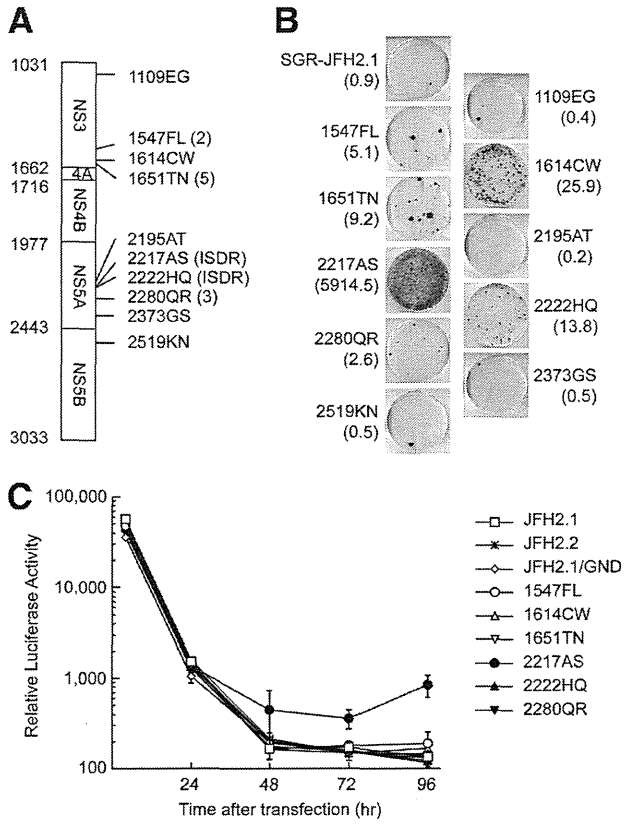

FIG 5 Analysis of the effect on colony formation and transient replication efficiency of mutations detected in replicon cell clones. (A) The box indicates the open reading frame of JFH-2 replicon and amino acid sequences at positions 1031 and 3033 (numbering by full-length JFH-2). The numbers on the left side of the box show the starting position of each protein, with the exception of 3033, which is the end position of NS5B. The numbers with the lines on the right side indicate the mutations introduced in the replicon constructs. 1547FL, 1651TN, and 2280QR mutations were found in 2, 5, and 3 replicon clones, respectively (Table 3). 2217AS and 2222HQ mutations were found in the ISDR (Table 3). (B) Each amino acid mutation found in the replicon genome was introduced into the pSGR-JFH2.1 replicon construct, and colony formation of the JFH2.1 replicon with the wild-type sequence (SGR-JFH2.1) and other mutations was tested. Briefly, transcribed RNA (5 μg) was transfected into HuH-7 cells, and cells were cultured for 3 weeks before being stained with crystal violet. The numbers in the parentheses show the colony formation efficiency (CFU/μg of RNA) of the replicon constructs. (C) Transient replication of JFH-2 subgenomic replicon. HuH-7 cells were transfected with the transcribed RNA from pSGR-JFH2.1/Luc, pSGR-JFH2.2/Luc, pSGR-JFH2.1/Luc/GND (replication-incompetent control), and pSGR-JFH2.1/Luc constructs with adaptive mutations (1547FL, 1614CW, 1651TN, 2217AS, 2222HQ, and 2280QR). Transfected cells were harvested at the indicated time points and at 4 h posttransfection. Relative luciferase activity (arbitrary units) was measured in the cell lysate. Assays were performed in triplicate, and data are presented as means ± standard deviations. The background signal of the luciferase measurement was 129.4 ± 27.4 units.

weeks after transfection. Interestingly, this density profile at 11 weeks posttransfection was quite similar to that of JFH-1 or the J6/JFH1 chimera, as previously described (21, 38). Furthermore, virus-like particles were visualized in the concentrated culture medium by electron microscopic analysis, whereas only unstructured aggregates were found with the mock-transfected control (Fig. 7, left panel; also data not shown). An aliquot of the culture medium was used for immunoelectron microscopy with an E2-specific antibody (AP33), and gold-labeled spherical structures were detected (Fig. 7, middle panel). The overall diameter of the structures (50 to 65 nm) is compatible with the predicted size of HCV.

Characterization of cell culture-adapted J6/JFH2/AS virus. During the serial passages of the transfected cells, the J6/JFH2/AS virus adapted to produce more infectious viruses in the cell culture. We next compared the adapted J6/JFH2/AS virus (T1B cells at day 75 posttransfection) with the J6/JFH1 virus. Huh-7.5.1 cells were inoculated with the viruses at a multiplicity of infection (MOI) of 0.03. The core protein production levels in both the infected cells and the culture medium were increased with similar kinetics after the virus infection, although at lower levels for J6/JFH2/AS virus than J6/JFH1 virus (Fig. 8A). We also tested the neutralization of the infection of these viruses by using mouse anti-CD81 monoclonal antibody and anti-HCV human IgG purified from HCV carrier serum (Fig. 8B). Both antibodies clearly inhibited the infectivity of inoculated virus to Huh-7.5.1 cells. Thus, the J6/JFH2/AS and J6/JFH1 viruses appeared to share similar infection pathways.

Adaptive mutations in the cell culture-adapted J6/JFH2.2/AS virus. We determined the full-length sequence of the HCV genome in the culture medium of T1A and T1B sub-cell lines at 75 days posttransfection by directly sequencing the amplified virus cDNA. We found the following nonsynonymous mutations, in addition to 2217AS, in the viral genomes: 1342ST in NS3 and 2219CR in NS5A of T1A and 148AT in the core protein, 2219CS in NS5A, and 2695TI and 3016LP in NS5B of T1B. These mutations were introduced into the J6/JFH2.2/AS cDNA, and synthetic RNA was transfected into Huh-7.5.1 cells. However, robust virus production was not observed at an early time point after transfection (data not shown). Because the important adaptive mutations might still not be detected in the virus population, we decided to concentrate on the dominant virus population and fix the important mutations in T1A and T1B virus by serial virus passages. We thus repeatedly inoculated naive Huh-7.5.1 cells three times with J6/JFH2/AS virus at a low MOI and harvested the virus when the virus titer plateaued. We sequenced the full-length genome of virus in the culture medium after the third inoculation (T1Ai3 or T1Bi3) and found the following nonsynonymous mutations: 405MK and 417NT in E2, 868MT in NS2, 1642TA in NS3, 1722IV in NS4B, 2204SG in NS5A, and 2695TI in NS5B of T1Ai3; and 148AT in the core protein, 329TS and 356MV in E1, 626VG in E2, 1678IV in NS4A, 1767KR in NS4B, 2219CS in NS5A, and 2695TI and 3016LP in NS5B of T1Bi3 (Fig. 3B). We then introduced these mutations into pJ6/JFH2/AS to construct pJ6/JFH2/AS/mtT1A and pJ6/JFH2/AS/mtT1B. Synthetic RNAs produced from both of the mutation-containing plasmids and control plasmids were transfected into Huh-7.5.1 cells. After the transfection, core proteins were secreted into the culture medium at levels similar to those of JFH-1 RNA-transfected cells but at lower levels than J6/JFH1 RNA-transfected cells (Fig. 9A). HCV RNA levels in the culture medium of J6/JFH2/AS/mtT1A (mtT1A) and J6/JFH2/AS/mtT1B (mtT1B) RNA-transfected cells were less than those in cells transfected with either or JFH-1 J6/JFH1 RNA (Fig. 9B). This discrepancy may be due to the lower detection efficiency of the JFH-1 core protein in the immunoassay, as reported previously (31). Infectivity in the culture medium was also determined. Interestingly, higher infectious titers were detected in the culture medium of the J6/JFH2/AS/mtT1A and J6/JFH2/AS/mtT1B