have identified a mutation located at aa 451 of HCV E2 glycoprotein, which displays an accelerated spreading of the virus. This G451A mutation is located between HVR1 and HVR2 of E2 in a region that has been reported to modulate the accessibility of the CD81-binding site (Roccasecca *et al.*, 2003).

In the present study, the substitution of amino acids FP→CS at positions 172 and 173 of the capsid protein leads to an increased viral production. The amino acids Cys and Ser appear to be important determinants for the spreading of the virus and they are probably involved in the morphogenesis and/or the release of the viral particles. The C172 and S173 residues are highly conserved among the HCV isolates. In a former *in vitro* study, we showed that several amino acids located at the C terminus of the capsid protein were important for the mature p21 protein, and FP→CS mutations showed a higher level of the immature capsid protein (p23) (Kato *et al.*, 2003b). The hydrophobic sequence at the C terminus of the capsid protein was described as the signal sequence necessary for the translocation of E1 glycoprotein into the ER lumen (Hijikata *et al.*, 1991). Recently, this signal sequence was also described as a substrate for signal peptide peptidase (SPP) (McLauchlan *et al.*, 2002). During the assembly of the virus, two consecutive membrane-dependent cleavages are responsible for the production of p23 and p21 forms of the capsid protein (Liu *et al.*, 1997). Detected in particles isolated from the blood of infected patients (Yasui *et al.*, 1998), the p21 mature capsid protein is produced by cleavage of the p23 immature capsid protein by a cellular protease identified as SPP (Hussy *et al.*, 1996; Lemberg & Martoglio, 2002; McLauchlan *et al.*, 2002). It may be hypothesized that the production of p23 and then p21 is important for the morphogenesis of the virus and the production of infectious viral particles. In this hypothesis, the immature capsid protein p23 would be necessary for an initial step of the particle formation, for example its accumulation and its oligomerization at the ER membrane where a progressive maturation would be introduced by SPP cleavage. The completion of the maturation of the viral particle could then occur after the budding process. However, immature capsid protein (p23) has not been detected in an *in vivo* study using JFH-1/CS probably due to the low production or a completion of the cleavage during the preparation of cell extracts. Additional experiments have to be conducted to understand the function of these amino acids in the morphogenesis of the viral particle.

In conclusion, the data presented in this study show that few modifications are sufficient for a more efficient production of HCVcc in Huh-7 cells. These mutations are located in the structural proteins and likely affect the recognition of a cellular receptor and/or the morphogenesis of the viral particle. Extensive modifications introduced in the C terminus of the capsid protein and analyses of the resulting viruses will help the understanding of the role of individual amino acids in particle assembly.

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Original Article

An infectious and selectable full-length replicon system with hepatitis C virus JFH-1 strain

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Aim: The hepatitis C virus (HCV) strain JFH-1 was cloned from a patient with fulminant hepatitis. A JFH-1 subgenomic replicon and full-length JFH-1 RNA efficiently replicate in cultured cells. In this study, an infectious, selectable HCV replicon containing full-length JFH-1 cDNA was constructed.

Methods: The full-genome replicon was constructed using the neomycin-resistant gene, EMCV IRES and wild-type JFH-1 cDNA. Huh7 cells were transfected with RNA synthesized *in vitro*, and then cultured with G418. Independent colonies were cloned to establish cell lines that replicate the full-length HCV replicon.

Results: HCV RNA replication was detected in each isolated cell line. HCV proteins and HCV RNA were secreted into culture medium, and exhibited identical density profiles. Interestingly, culture supernatants of the replicon cells were infectious for naïve Huh7 cells. Long-term culture did not affect replication of replicon RNA in the replicon cells, but it reduced core protein secretion and infectivity of culture supernatant. Culture supernatant obtained after serial passage of replicon virus was infectious for Huh7 cells.

Conclusions: Selectable infection was established using HCV replicon containing full-length genotype 2a JFH-1 cDNA. This system might be useful for HCV research.

Key words: hepatitis C virus, infectious virus, replicon, RNA replication

INTRODUCTION

HEPATITIS C VIRUS (HCV) is a plus-strand RNA virus and is the principal cause of post-transfusion hepatitis and sporadic acute hepatitis.[1,2] Infection with HCV causes chronic liver diseases, including cirrhosis and hepatocellular carcinoma.[3] Although HCV belongs to the *Flaviviridae* family, and has a genome structure similar to other flaviviruses, it has been difficult to develop an efficient cell culture system for HCV.[4] A subgenomic HCV RNA replicon system has been developed,[5] enabling assessment of HCV replication in cultured cells. Although that system is a powerful tool for studies of HCV replication mechanisms and development of antiviral agents, its replicon cells do not produce infectious viral particles, even when the replicons contain structural genes.[6,7] Studies conducted using the above replicon system indicate that wild-type HCV genomes have low replication capacities.[7,8]

Adaptive mutations can substantially increase replication of HCV, but introduction of these adaptive mutations into full-length genomes causes loss of infectivity *in vivo*.[8] The JFH-1 strain was cloned from a patient with fulminant hepatitis, and its sequence differs from those of chronic hepatitis isolates.[9] Using JFH-1 cDNA, we previously established subgenomic replicon constructs that replicate in Huh7 cells with greater efficiency than other HCV strains, and that also replicate in other cell lines.[10,11] In a previous study, when we transfected Huh7 cells with *in-vitro*-transcribed full-length JFH-1 HCV RNA, the JFH-1 RNA efficiently replicated and the cells produced viral particles that were infectious for cultured cells and a chimpanzee.[12] In the present study, we established a full-length HCV replicon using the JFH-1 strain.

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Replicon virus particles were secreted from the replicon cells, and the replicon virus was infectious for naïve Huh7 cells.

METHODS

Cell culture system

HUH7 CELLS WERE donated by Dr Tetsuro Suzuki (National Institute of Infectious Diseases, Tokyo, Japan), and were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (DMEM-10), as previously described.[10]

Construction of the full-genome HCV replicon

A full-genome replicon construct of JFH-1 (pFGR-JFH1; Fig. 1a) was assembled based on the consensus sequence of JFH-1, as follows. The gene for the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) was amplified from a subgenomic replicon construct of JFH-1 (pSGR-JFH1; Fig. 1a)[10] using the primers Pm/EI-S (5'-AGC TTT GTT TAA ACC CTC TCC CTC CCC CCC CCC TAA CGT T-3'; the underlined segment is the *PmeI* site) and EI/FH/Core-R (5'-TGA GGT TTA GGA TTT GTG CTC ATG GTA TCA TCG TGT TTT T-3'). The core region was amplified from pJFH1[12] using the primers EI/FH/Core-S (5'- TTG AAA AAC ACG ATG ATA CCA TGA GCA CAA ATC CTA AAC C-3') and FH/1592-R (5'- CGG TTG ATG TGC CAA CTG CC-3'). These two polymerase chain reaction (PCR) fragments were purified, mixed and reamplified using the primers Pm/EI-S and FH/1592-R. Reamplified PCR product was digested with *PmeI* and *BsiWI*. Another DNA fragment containing a 5' untranslated region (5' UTR) and neomycin-resistant gene was digested from pSGR-JFH1 using *AgeI* and *PmeI* (Fig. 1a). These two DNA fragments were cloned into the vector pJFH1 at sites for *AgeI* and *BsiWI* to produce the pFGR-JFH1 construct (accession number: AB237837, Fig. 1a).

As a control, we also created the mutant construct pFGR-JFH1/GND that includes a point mutation that changes a GDD motif to GND, which abolishes the RNA polymerase activity of non-structural protein (NS) 5B.[10]

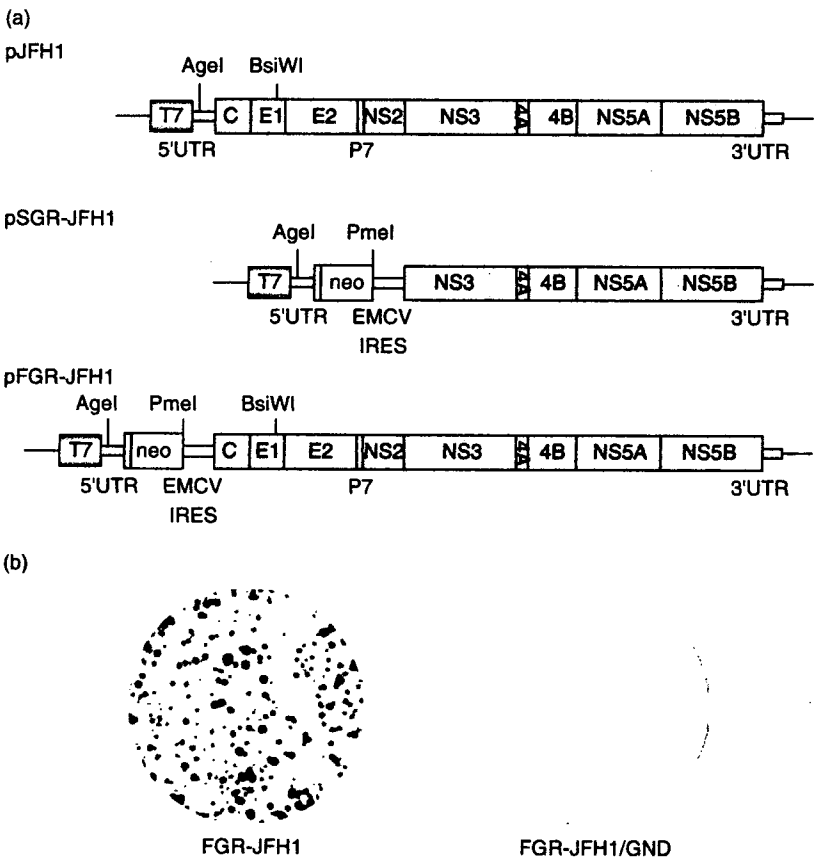

Figure 1 Structure of the full-genome hepatitis C virus (HCV) RNA replicon constructed from genotype 2a JFH-1 and colony formation of replicon RNA-transfected Huh7 cells. (a) Organization of the full-length JFH-1 genome (top), subgenomic replicon construct pSGR-JFH1 (middle) and full-genome replicon construct pFGR-JFH1 (bottom). Open reading frames (thick boxes) are flanked by untranslated regions (thin boxes). '*AgeI*', '*BsiWI*' and '*PmeI*' indicate positions of restriction sites. A T7 RNA promoter is located upstream from the 5' end of the replicon construct. (b) Colony formation of JFH-1 HCV full-genome replicon. Huh7 cells were transfected with transcribed RNA (1 μg), and transfected cells were cultured in medium supplemented with G418 (1 mg/mL) for 3 weeks before staining with crystal violet.

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All plasmid DNA was transformed using DH5 α-competent cells. Amplified plasmid DNA was purified by performing ultra-centrifugation twice.

RNA synthesis

Replicon RNA was synthesized as described previously.[10,12] Briefly, the plasmid pFGR-JFH1 was digested with *Xba*I and treated with Mung Bean nuclease (New England Biolabs, Beverly, MA, USA). Digested plasmid DNA fragments were purified and used as templates for RNA synthesis. HCV RNA was synthesized *in vitro* using a MEGAscript T7 kit (Ambion, Austin, TX, USA). Synthesized RNA was treated with DNaseI, followed by acid phenol extraction to remove any remaining template DNA.

RNA transfection and colony formation experiment

Synthesized replicon RNA was used for transfection via electroporation. Synthesized RNA (0.1 ng to 10 μg) was adjusted to 10 μg with cellular RNA isolated from untransfected Huh7 cells. Naïve Huh7 cells were transfected with transcribed replicon RNA from pFGR-JFH1, or were transfected with control RNA transcribed from pFGR-JFH1/GND, in which the catalytic domain of the RNA polymerase NS5B is mutated. Trypsinized Huh7 cells were washed with Opti-MEM I reduced-serum medium (Invitrogen, Carlsbad, CA, USA) and resuspended at 7.5×10^6 cells/mL with Cytomix buffer.[10] RNA (10 μg) was mixed with 400 μL of cell suspension and transferred to an electroporation cuvette (Precision Universal Cuvettes, Thermo Hybrid, Middlesex, UK). The cells were then pulsed at 260 V and 950 μF with the Gene Pulser II apparatus (Bio-Rad, Hercules, CA, USA). Transfected cells were immediately transferred to 10-cm culture dishes, each containing 8 mL of culture medium. G418 (1.0 mg/mL) (Nacalai Tesque, Kyoto, Japan) was added to the culture medium at 16–24 h after transfection. Culture medium supplemented with G418 was replaced twice per week.

Three weeks after transfection, cells were fixed with buffered formalin and stained with crystal violet. Colony formation efficiency of the transfected cells was determined by counting the number of colonies that formed.

Analysis of G418-resistant cells

Sparsely grown G418-resistant colonies were independently isolated using a cloning cylinder (Asahi Techno Glass, Tokyo, Japan), and were expanded until they were 80–90% confluent in 10-cm dishes. Expanded cells were harvested for nucleic acid and protein analyses. Total RNA and genomic DNA were simultaneously isolated from expanded cells using the Isogen reagent (Nippon Gene, Tokyo, Japan). Another portion of each cell pellet was dissolved with radioimmune precipitation assay (RIPA) buffer containing 0.1% SDS. Eight cloned cell lines were selected for further analysis.

Northern blot analysis

Isolated RNA fragments (4 μg) were separated in a 1% agarose gel containing formaldehyde, transferred to a positively charged nylon membrane (Hybond-N+, Amersham Pharmacia, Buckinghamshire, UK), and immobilized by Stratalinker UV crosslinker (Stratagene, La Jolla, CA, USA). Replicon RNA was detected using probes specific for certain positions. Hybridization was performed using a [α-^{32}P]dCTP-labeled DNA probe and Rapid-Hyb buffer (Amersham Pharmacia). The DNA probe was synthesized from the genes *neo*^{r} and EMCV IRES, using the Megaprime DNA labeling system (Amersham Pharmacia).

Western blotting and immunofluorescence analysis

We analyzed protein expression in replicon cells by performing western blotting and immunofluorescence. Cells were lysed using a RIPA buffer containing 0.1% SDS, 0.5% NP-40, 10 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), and 150 mM NaCl. Protein samples were separated on 10% or 12% polyacrylamide gels, and were subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Tokyo, Japan). Transferred proteins were incubated with blocking buffer containing 5% non-fat dried milk in phosphate-buffered saline (PBS). HCV proteins were detected using anticore monoclonal antibodies (25, clone 2H9), anti-E1 and anti-E2 polyclonal antibodies,[12] anti-NS3 polyclonal antibodies,[10] anti-NS5A polyclonal antibodies,[11] peroxidase-labeled goat antirabbit IgG (Biosource), and peroxidase-labeled sheep antimouse IgG (Amersham Pharmacia). Signals were detected using a chemiluminescence system (Amersham Pharmacia).

Cells containing the HCV replicon were grown on a cover glass, and were then fixed in acetone-methanol (1:1 v/v) for 10 min at −20°C. Cells were then incubated in immunofluorescence assay buffer (PBS, 1% bovine serum albumin, 2.5 mM EDTA). Anti-core monoclonal antibodies or anti-NS3 and anti-NS5a polyclonal antibodies were added at 50 μg/mL or a dilution of 1:50, respectively, in immunofluorescence buffer. After incubation for 1 h at room temperature, cells were washed,

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followed by incubation with fluorescein isothiocyanate-conjugated antimouse IgG (Cappel, Durham, NC, USA) in immunofluorescence assay buffer. Cover slips were washed and mounted on glass slides using Shandon PermaFluor mounting solution (Thermo Electron, Pittsburgh, PA, USA). Cells were examined by fluorescence microscopy (Carl Zeiss, Oberkochen, Germany).

To assay secretion of viral protein and virus particles into culture medium from cells replicating replicon RNA, we measured levels of core protein in culture medium using a sensitive immunoassay. Culture supernatants from all eight replicon cell lines were used in this immunoassay.

Genomic DNA PCR

To detect integration of the *neo*^{r} gene into the genomic DNA, isolated cellular genomic DNA was amplified by PCR using *neo*^{r}-specific primers (NEO-S3; 5′-AACAA GATGGATTGCACGCA-3′, NEO-R; 5′-CGTCAAGAAG GCGATAGAAG-3′).

RT-PCR and sequencing analysis

We sequenced the replicating HCV RNA in each of the eight selected clones. The cDNAs of the HCV RNA replicon were synthesized from total RNA isolated from cells using a reverse primer for the 3′× region. These cDNAs were subsequently amplified with DNA polymerase (TaKaRa LA *Taq*, Takara Bio, Shiga, Japan). Six separate PCR primer sets were used to amplify the following sections of the pFGR-JFH1 replicon construct, to cover the entire open reading frame: nt 151–2043, nt 1913–3778, nt 3597–6046, nt 5997–8685, nt 8649–10782, and nt 10713–11017. The sequence of each amplified DNA was determined.

Quantification of HCV core protein and RNA

To estimate levels of HCV core protein in culture supernatant, concentrations of HCV core protein were measured. Aliquots (250 μL) of samples were assayed using a new immunoassay technique described elsewhere.[13] Total RNA was isolated from harvested cells or culture media using Isogen. Copy numbers of HCV RNA were determined by real-time detection reverse transcription (RT)-PCR, using an ABI Prism 7700 sequence detector system (Applied Biosystems, Tokyo, Japan).[14]

Density gradient analysis

To determine whether secreted viral core protein was incorporated in viral particles, we analyzed culture medium by sucrose density gradient centrifugation. Culture medium derived from replicon cells was harvested for density gradient analysis. Collected culture medium was cleared by low-speed centrifugation at 2000 r.p.m. for 10 min, and was then passed through a disk filter with a pore size of 0.45 μm (Millipore). Filtered culture medium was layered on a stepwise sucrose gradient (60% to 10%, wt/vol) and centrifuged for 16 h in a SW41 rotor (Beckman, Palo Alto, CA, USA) at 40 000 r.p.m. at 4°C. After centrifugation, 22 fractions were harvested from the bottoms of the tubes. The core protein concentration of each fraction was measured by performing an immunoassay using 100 μL of the fraction. The HCV RNA titer of each fraction was determined by real time detection RT-PCR using RNA isolated from 100 μL of the fraction.

Infectivity of secreted viral particles

To assess the infectivity of secreted viral particles, naïve Huh7 cells were inoculated with culture supernatant from replicon cell lines. Culture supernatant used for inoculation was centrifuged and filtered to remove cell debris. Cleared culture supernatant was concentrated by ultrafiltration as described previously,[12] and G418 was removed from the concentrated culture supernatant during ultrafiltration. Naïve Huh7 cells were inoculated with concentrated culture medium. Inoculated cells were cultured for 3 weeks in medium supplemented with G418 (0.3 mg/mL).

Long-term culture of replicon cells

To examine replicon RNA replication and virus secretion and infectivity after long-term culture, the eight replicon clones were serially passaged for more than 7 months. HCV RNA levels in replicon cells were measured by real time detection RT-PCR. Infectivity of culture supernatants was determined by measuring colony formation efficiency.

Serial passage of replicon virus

To examine long-term replicon virus passage, replicon virus was serially passaged for approximately 6 months. Culture supernatants harvested from the replicon-RNA-transfected Huh7 cells were used to inoculate naïve Huh7 cells. Inoculated cells formed colonies after 4 weeks of G418 selection culture. This infection and selection procedure was repeated seven times. Infectivity of culture supernatants was determined by measuring colony formation.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test or Student's *t*-test. *P*-values of <0.05 were considered to indicate significance.

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RESULTS

Construction of full-genome replicon using JFH-1, and colony formation

FIGURE 1A SHOWS the full-genome replicon construct pFGR-JFH1, which was produced from the full-length JFH-1 construct pJFH1 and which contains a neomycin-resistant gene.[12] Huh7 cells transfected with pFGR-JFH1 replicon RNA formed colonies efficiently (Fig. 1b). Huh7 cells transfected with control RNA transcribed from pFGR-JFH1/GND did not form colonies. Cells transfected with full-genome replicon RNA formed colonies 80.7-fold less efficiently than cells transfected with JFH-1 subgenomic replicon RNA (Table 1).[10]

Analysis of replicon cells

Figure 2a shows the results of northern blot analysis of replicon RNA replication in the cloned cell lines using cellular RNA extracted from each replicon cell. Intensities of replicon RNA signals differed among the eight clones. Replicon RNAs were clearly identified at the same position as in control RNA; however, additional specific signals were present higher on the gel as was also observed in subgenomic replicon cells.[10] These signals may represent replication intermediates or double-stranded RNA. The replicon RNA titer ranged from 1.14×10^7 to 7.09×10^7 copies/μg cellular RNA (Table 2). We also estimated the replicon RNA copy numbers per cell. Full-genome replicon cell clones were harvested and counted 2 days after passage. Mean HCV RNA titer of eight full-genomic replicon clones was 6.93×10^3 copy/cell. RNA replication levels in the full-genomic replicon cells were at the similar level with subgenomic JFH-1 replicon cells.[10]

Figure 2b shows the results of western blot analysis. In the cell lysate extracted from each replicon cell, we detected core, E1, E2, NS3 and NS5a proteins at the expected positions. Signals detected by anti-NS5a antibody exhibited doublet bands that may represent p58 and p56 with different degrees of phosphorylation.

Figure 2c shows the results of the immunofluorescence assay. In replicon clone 3 (Fig. 2c) and other clones (data not shown), core protein exhibited a perinuclear punctuate staining pattern, and NS3 and NS5a proteins exhibited a cytoplasmic diffuse staining pattern.

Table 2 shows the results of the immunoassay of core protein in culture medium. Figure 3a shows the results of sucrose density gradient centrifugation of the culture medium of clone 3. Core protein and HCV RNA exhibited identical peaks in a single fraction with a density of approximately 1.16 g/mL (Fig. 3a); this density is similar to that of wild-type JFH-1 virus particles.[12] This result indicates that viral particles were secreted from cells that replicated replicon RNA.

Cells inoculated with culture supernatant of full-genome replicon cell lines formed visible colonies by G418 (1 mg/mL) selection culture for 10–14 days after inoculation; these cells were fixed and stained. In the preliminary experiment, very few colonies formed (data not shown). Consequently, to increase colony formation efficiency after inoculation, the inoculated Huh7 cells were passed 1 day before seeding, culture supernatants from replicon cell lines were concentrated by ultrafiltration as described previously,[12] and inoculated cells were cultured with a lower concentration of G418 (0.3 mg/mL). These changes increased the number of colonies that formed, and colony formation by cells inoculated with supernatant of full-genome replicon cell lines occurred in a dose-dependent manner (Fig. 3b, FGR-JFH1). No colonies were formed by cells inoculated with culture supernatant of subgenomic replicon cells (Fig. 3b, SGR-JFH1).

When genomic DNA from each clone was isolated and amplified by PCR using *neo*^{r} -specific primers, we did not detect any signals (data not shown).

Long-term culture of replicon cells

Figure 4 shows the results of serial passaging of clones 3 and 5. Core protein titer of culture supernatant of clone 3 decreased rapidly at 50 days of culture. In contrast, core protein titer of culture medium of clone 5 gradually decreased throughout the observation period (Fig. 4a). HCV RNA replication levels in the cells ranged from 1.6×10^7 to 7.8×10^7 copies/μg RNA, with no significant differences between clones (Fig. 4a). Colony formation efficiency of clone 3 decreased significantly at 50 days of culture (Fig. 4b). Colony formation efficiency of clone 5 decreased gradually throughout the observation period (Fig. 4b).

Serial passages of replicon virus

The HCV RNA levels in the inoculated cells did not change significantly during the observation period

Table 1 Colony formation efficiency of JFH-1 replicon

Replicon	JFH-1 (c.f.u./μg RNA)
Subgenomic	$5.32 \times 10^4 \pm 5.02 \times 10^4$†
Full-genome	$6.59 \times 10^2 \pm 3.58 \times 10^2$

†Kato *et al.*[10] Values shown as mean ± SD.

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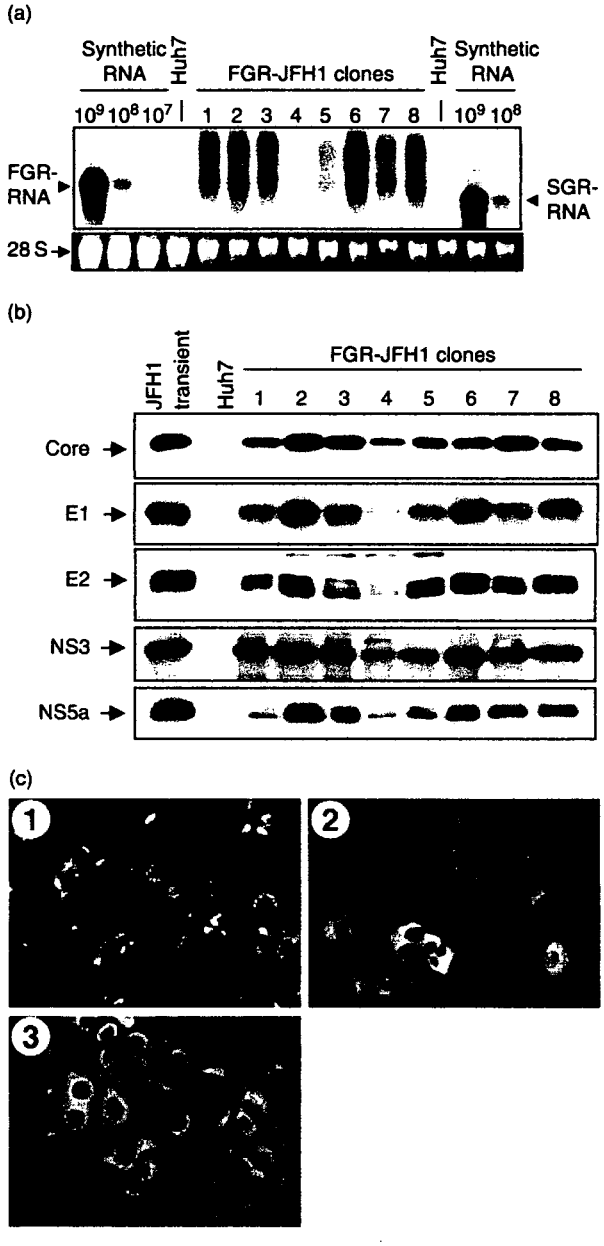

Figure 2 Analysis of isolated full-genome replicon cells. (a) Northern blot analysis of replicon RNA. Total RNA from eight isolated replicon cell clones (FGR-JFH1 clones 1–8) was analyzed by northern blotting with DNA probes of the *neo*^{r} – EMCV IRES and β-actin genes. We performed *in vitro* synthesis of 10^9, 10^8 and 10^7 copies of transcribed positive-strand full-genome replicon JFH-1 RNA (FGR-RNA) and 10^8 and 10^7 copies of positive-strand subgenomic JFH-1 replicon RNA (SGR-RNA). The synthesized RNA was loaded onto the gel as positive controls (left 3 lanes and right 2 lanes, respectively). Left and right arrowheads indicate target positions of full-genome and subgenomic replicon RNAs, respectively. Arrow indicates position of β-actin. 'Huh7' indicates cellular RNA of normal Huh7 cells, which was used as a negative control. (b) Western blot analysis. Cell lysates were prepared from clones of Huh7 cells transfected with FGR-JFH1 RNA (FGR-JFH1 clones 1–8). Huh7 cells transfected with full-length JFH-1 RNA were used as positive controls (JFH1 transient), and untransfected parental Huh7 cells (Huh7) were used as negative controls. Anti-core monoclonal antibodies and anti-E1, -E2, -NS3 and -NS5A polyclonal antibodies were used to detect HCV antigens. Target sizes of HCV proteins are indicated by arrows. (c) Subcellular localization of HCV antigens determined by immunofluorescence. Isolated FGR-JFH1 replicon cell clone 3 was cultured on cover slips. Cultured cells were fixed before being incubated with anti-Core (1, α-Core), anti-NS3 (2, α-NS3) and anti-NS5A (3, α-NS5A) antibodies. (Original magnification ×200).

(Fig. 5a). Core protein titers in culture supernatants gradually decreased in one series of the passages (Fig. 5a, Transfection 1). However, in another series (Fig. 5a, Transfection 2), core protein titer in culture supernatant increased beginning with the fourth inoculation. Colony formation of cells inoculated with culture supernatant of transfection 1 gradually decreased during the observation period. Colony formation of cells inoculated with culture supernatant of transfection 2 increased beginning with the fourth passage (Fig. 5b, P4/d118).

DISCUSSION

IN THE PRESENT study, we established a selectable, infectious full-length HCV replicon. Transcribed full-length replicon RNA was transfected into Huh7 cells. Cells transfected with the full-length replicon formed colonies at reduced efficiency, compared with cells transfected with the subgenomic replicon. However, expanded replicon cells supported efficient replicon RNA replication. Furthermore, although the replicon genome (approximately 11 kb) is longer than the wild-type genome of HCV (approximately 9.6 kb), culture supernatant from the replicon cells were infectious for naïve Huh7 cells. After long-term culture, replicon cells did not stop replicating replicon RNA, but they did stop secreting infectious viral particles. Viral adaptation might occur during repeated serial infection of the

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Table 2 Mutations and titers of JFH-1 replicon

Clone	Nucleotide†	Amino acid‡	Region	Replicon RNA titer§ (copies/μg RNA)	Core protein titer¶ (fmol/L)	Colony formation efficiency†† (c.f.u./mL)
1	3893 A > C	707 Y > S	E2	2.71×10^7	64	0.3
	5610 T > A	1279 N > K	NS3			
	7236 G > A	Synonymous‡‡	NS4b			
	10161 C > A	Synonymous	NS5b			
2	None			5.19×10^7	826	63.3
3	None			4.47×10^7	3450	133.3
4	6599 A > C	1609 D > A	NS3	1.14×10^7	33	1.0
	8902 T > A	2377 S > T	NS5a			
5	9653 C > A	2627 A > E	NS5b	1.60×10^7	2904	89.3
6	None			7.09×10^7	363	15.3
7	394 C > A	Synonymous	Core§§	1.51×10^7	571	41.0
	5295 C > A	Synonymous	NS3			
	7189 T > C	1806 S > P	NS4b			
	8076 G > A	Synonymous	NS5a			
8	6483 A > G	Synonymous	NS3	1.15×10^7	387	11.3
	8972 G > A	2400 G > E	NS5a			
	9216 T > C	Synonymous	NS5b			

†Position of mutated nucleotide within replicon; ‡position of mutated amino acid within complete open reading frame of full-length JFH-1; §HCV RNA copy titer in replicon cell; ¶core protein concentration in culture supernatant of replicon cells; ††naïve Huh7 cells were inoculated with concentrated culture supernatants from replicon cells, and inoculated cells were cultured for 3 weeks in medium supplemented with G418 (0.3 mg/mL); ‡‡synonymous mutation does not change amino acid sequence; §§sequential region from 5′-untranslated region upstream of *neo*^{r} gene.

replicon virus. Importantly, selectable infection was established using HCV replicon containing full-length genotype 2a JFH-1 cDNA. This system may be useful for HCV research.

Several full-length HCV cDNAs have been cloned, and their infectivity has been confirmed *in vivo* using chimpanzee models.[15,16] However, it has been difficult to produce recombinant viral particles and test their infectivity using cell culture systems,[4,7] and this limits the ability to perform detailed analyses of the HCV life cycle and pathogenesis in cell culture. The JFH-1 strain was isolated from a patient with fulminant hepatitis, and it efficiently replicates in Huh7 cells and other hepatic and non-hepatic cell lines in subgenomic replicon form.[10,11,17] Full-length wild-type JFH-1 RNA and chimeric JFH-1 RNA can replicate in Huh7 cells and produce infectious virus.[12,18,19] Sequence analysis has revealed that the JFH-1 strain clusters with genotype 2a HCV isolates, and exhibits 89–90% homology with other genotype 2a strains at the nucleic acid level and 91–92% homology at the amino acid level.[9] The relationship between the high levels of replication and virus production of JFH-1 in cell culture is unclear. Chimeric virus, which contains structural region of J6CF strain and non-structural region from JFH-1, replicates as well as wild-type JFH-1 and produces infectious virus in cell culture.[19,20] However, wild type J6CF strain or another chimeric virus containing structural region of JFH-1 and non-structural region from J6CF did not replicate in tissue culture (unpublished data).[12] It is thus clear that non-structural proteins or genome are important for the efficient replication of JFH-1 strain.

In the present study, the full-genome JFH-1 replicon produced infectious virus particles. Full-genome replicon clones have previously been developed using genotype 1a and 1b HCV clones, but none of those replicons produced viral particles from replicon cells.[6,7,21,22] This inability to produce virus particles may be related to adaptive mutations in the replicon genome, because adaptive mutations increase replication of replicons in cultured cells. However, H77-S strain was recently reported to produce infectious virus particles into culture medium from the transfected cells, although this strain contains at least five adaptive mutations.[23] The full-length JFH-1 replicon does not require adaptive mutations to efficiently replicate in cultured cells.[10,11,17] In the present study, the full-genome replicon cells with amino acid mutations had a lower HCV RNA

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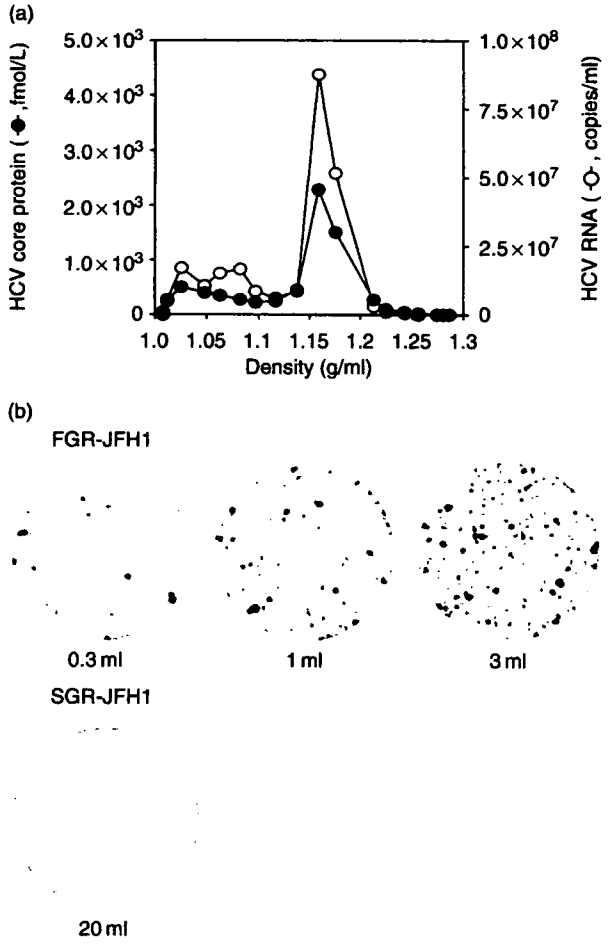

Figure 3 Analysis of culture supernatant from replicon cells. (a) Density gradient analysis. Culture supernatant from full-genome JFH-1 replicon cell clone 3 was filtered and layered on a stepwise sucrose gradient (60% to 10% wt/vol) in centrifugation tubes. After centrifugation, 22 fractions were collected from the bottom of the tubes. Core protein concentration (●) and HCV RNA titer (○) were measured in each fraction. (b) Colony formation by cells inoculated with culture supernatant from JFH-1 replicon cells. Culture supernatants from full-genome FGR-JFH1 replicon cell clone 3 and subgenomic SGR-JFH1 replicon cell clone 4–1 were cleared by centrifugation and filtration. Naïve Huh7 cells were inoculated with the cleared culture supernatant, and the inoculated cells were cultured in medium supplemented with G418 (0.3 mg/mL) for 3 weeks before staining with crystal violet. The figure shows representative staining of Huh7 cells inoculated with 0.3 mL, 1 mL and 3 mL of culture medium from FGR-JFH1 clone 3, and cells inoculated with 20 mL of medium from SGR-JFH1 clone 4–1. Before inoculation, culture media were concentrated by ultrafiltration.

titer than the full-genome replicon cells without mutations ($1.62 \times 10^7 \pm 6.43 \times 10^6$ *vs* $5.58 \times 10^7 \pm 1.35 \times 10^7$ copies/μg RNA, $P < 0.05$); however, when HCV RNA titer per cell was calculated, there was no significant difference ($5.75 \times 10^3 \pm 2.45 \times 10^3$ *vs* $8.90 \times 10^3 \pm 1.29 \times 10^3$ copies/cell, $P = 0.09$). We also determined the colony formation efficiency of replicon clones 1–8 by transfection of cellular RNA isolated from replicon cells as 1.66×10^{-6}, 1.48×10^{-6}, 3.67×10^{-7}, 8.98×10^{-7}, 5.60×10^{-7}, 1.23×10^{-6}, 1.16×10^{-6} and 7.28×10^{-7} c.f.u./RNA copy, respectively. Thus, the mutations that occurred in the full-genome of the JFH-1 replicon genome have no or slight effect of reducing RNA replication efficiency. Studies indicate that certain adaptive mutations in genotype 1 HCV replicon clones significantly increase RNA replication.[24–26] Many adaptive mutations alter the phosphorylation status of NS5A protein, and it has been reported that RNA replication efficiency is associated with NS5A phosphorylation status.[27,28] In the present study, p56 and p58 bands were observed in all the full-genome JFH-1 replicon cell clones (Fig. 2b). Thus, the high replication capacity of JFH-1 and its efficient production of infectious virus may be dependent on mechanisms other than phosphorylation of NS5A.

We previously reported incorporation of the luciferase reporter gene into a JFH-1 replicon construct and detected neutralizing antibody in chronically HCV infected patient sera.[12] In addition, Koutsoudakis *et al.* characterized the early steps of HCV infection using this luciferase reporter virus.[29] The wild-type JFH-1 genome has been shown to replicate efficiently in permissive cell lines.[18] However, an infectious, selectable full-length HCV replicon containing a neomycin-resistant gene is particularly useful for tests of the infectivity of HCV in cells with low permissiveness for HCV infection. It would be also interesting to test cell lines such as HepG2, IMY, HeLa and 293 cells, which support JFH-1 subgenomic replicon replication.[10] Recently, we also found that JFH-1 replicon can replicate in mouse cell lines.[30] These cells were not permissive for HCV infection;[12] however, they might support full-genomic replicon replication and infectious virus production. In particular, replicon cells using HeLa and 293 cells should be useful to analyze the host factors important for virus infection because these cell lines express CD81, SR-BI and LDL receptor, which are potentially important for HCV infection. In preliminary observations, full-genomic replicon could replicate in HepG2, IMY, HeLa and 293 cells, and replicon cells were established (unpublished data).

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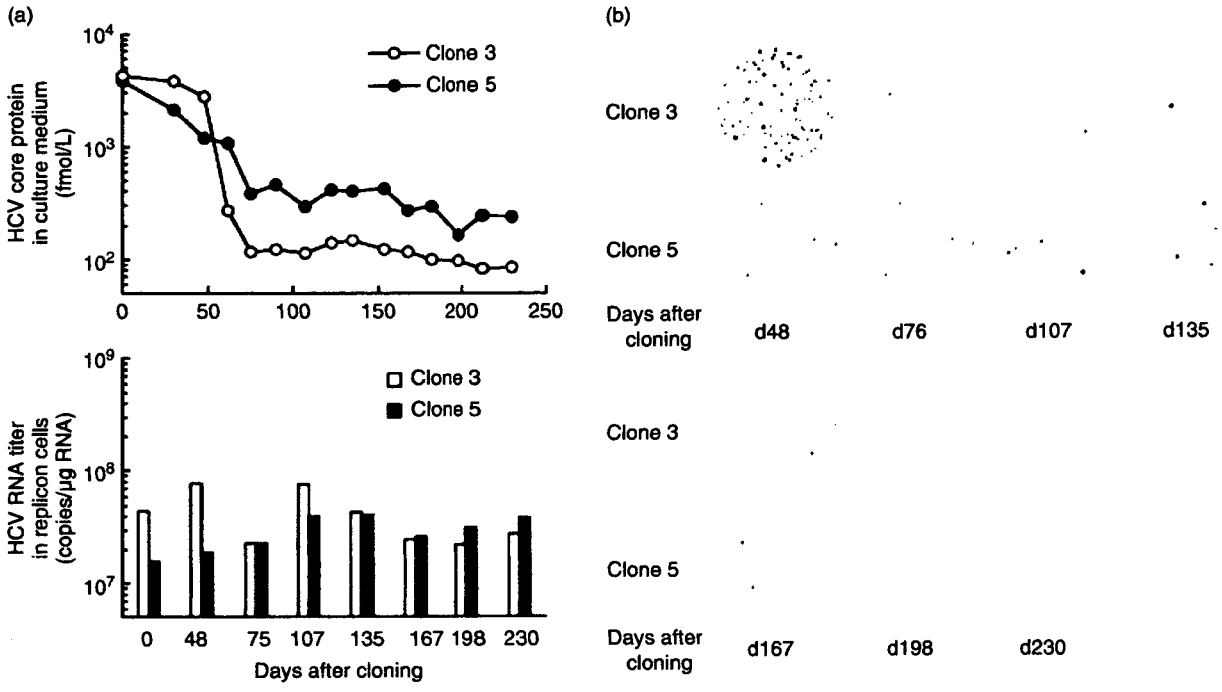

Figure 4 Long-term culture of full-genome replicon cell clones. FGR-JFH1 replicon cell clones 3 and 5 were cultured continuously for 230 days after the clones were transfected. (a) We measured the HCV core protein concentration in the culture supernatant and HCV RNA titer in replicon cells harvested at each passage. (b) We measured colony formation by naïve Huh7 cells inoculated with culture supernatant (harvested at each passage) and cultured in medium supplemented with G418 (0.3 mg/mL) for 3 weeks before staining with crystal violet.

Permissiveness for HCV infection has been shown to vary among Huh7 cell subtypes. Mutant cell lines such as Huh7.5 and Huh7.5.1 exhibit greater permissiveness than standard Huh7 cells,[18,19] whereas other Huh7 subtypes exhibit relatively low permissiveness.[12] In the present study, secretion of core protein into culture media and infectivity of culture supernatant were abolished by long-term culture of replicon cells (Fig. 4). However, long-term repeated infection of secreted replicon virus increased core protein secretion and infectivity of secreted virus, suggesting that some viruses become adapted to naïve Huh7 cells, resulting in increased secretion of infectious replicon virus (Fig. 5a, transfection 2). It is also interesting that virus replication levels were not significantly changed by repeated virus infection, which has been demonstrated to decrease the infectivity and virus secretion of some virus strains (Fig. 5a, transfection 1). Further study is needed to determine whether these differences are dependent on mutations in the virus genome or selection within infected cells. In future studies, we plan to examine mechanisms of virus adaptation to Huh7 cells and adaptive mechanisms of host cell lines.[31]

In the present study, colony formation efficiency after inoculation with culture supernatant was partly dependent on the core protein concentration of the supernatant. Colony formation efficiency for culture supernatant from clone 3 was 133.3 c.f.u./mL. The cells used in the present study were standard Huh7 cells, which are not highly permissive for HCV.[12,18] Use of cured cells such as Huh7.5 cells may increase the infection efficiency of replicon culture supernatants.[19] However, the present low infection efficiency of the replicon virus may also be due to its genomic length. The present replicon genome is about 1.5 kb longer than the wild-type HCV genome. The colony formation efficiency of the present full-genome replicon was significantly lower than that of the subgenomic replicon. The ability of viral particles to incorporate a longer genome than the wild-type genome may allow us to add other genes to the viral replicon genome, and to test expression of those genes in the infected cells.

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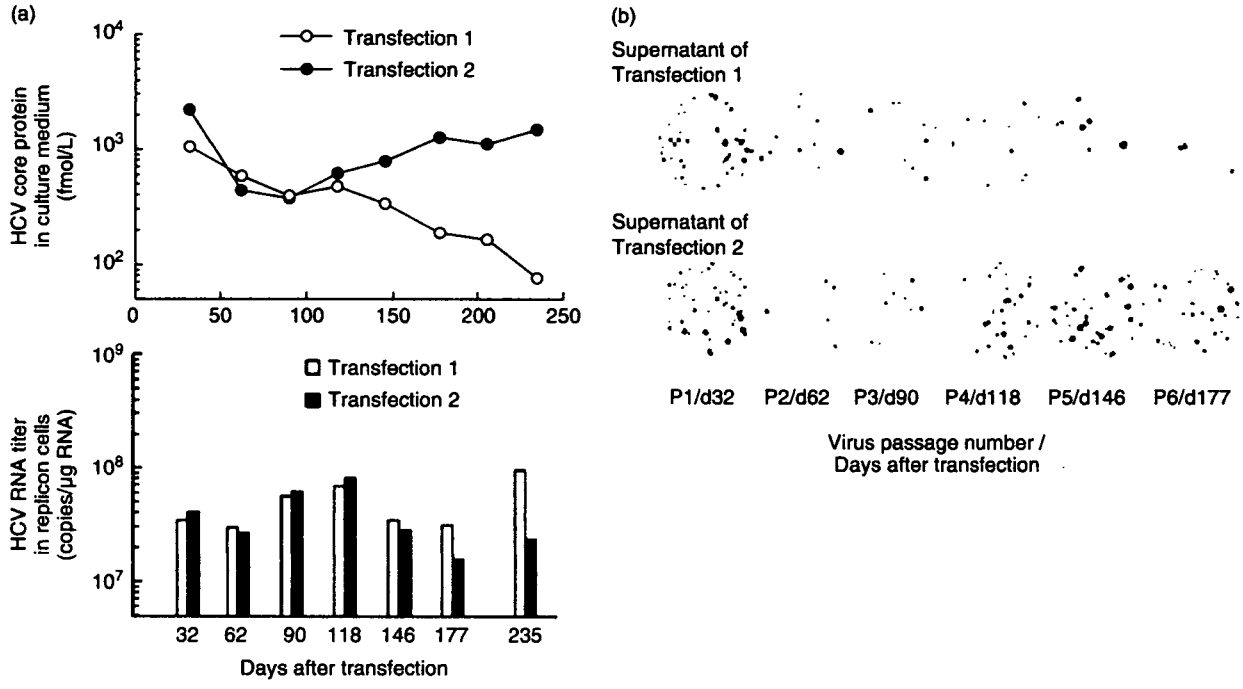

Figure 5 Repeated infection of cells with culture supernatant harvested from full-genome replicon cells. Naïve Huh7 cells were transfected with FGR-JFH1 RNA. Transfected cells were cultured for 4 weeks in medium supplemented with G418 (1 mg/mL), and culture supernatant was then harvested. New naïve Huh7 cells were inoculated with the harvested supernatant, and were then cultured in medium supplemented with G418 (0.3 mg/mL) for 4 weeks. Culture supernatant was harvested at the end of the 4 weeks, and was used to inoculate new Huh7 cells. This harvesting of supernatant, inoculation of new Huh7 cells, and incubation of the inoculated cells was repeated every 4 weeks for 235 days after transfection. Six independent experiments were performed and two representative results are shown. (a) We measured the HCV core protein concentration in culture supernatant (upper panel) and HCV RNA titer in infected cells (lower panel) harvested at each passage. (b) We measured colony formation by naïve Huh7 cells inoculated with culture supernatant (harvested at each passage) and cultured in medium supplemented with G418 (0.3 mg/mL) for 4 weeks before staining with crystal violet.

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The NS3 Helicase and NS5B-to-3′X Regions Are Important for Efficient Hepatitis C Virus Strain JFH-1 Replication in Huh7 Cells▽

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The JFH-1 strain of hepatitis C virus (HCV) is a genotype 2a strain that can replicate autonomously in Huh7 cells. The J6 strain is also a genotype 2a strain, but its full genomic RNA does not replicate in Huh7 cells. However, chimeric J6/JFH-1 RNA that has J6 structural-protein-coding regions and JFH-1 nonstructural-protein-coding regions can replicate autonomously and produce infectious HCV particles. In order to determine the mechanisms underlying JFH-1 RNA replication, we constructed various J6/JFH-1 chimeras and tested their RNA replication and virus particle production abilities in Huh7 cells. Via subgenomic-RNA-replication assays, we found that both the JFH-1 NS5B-to-3′X (N5BX) and the NS3 helicase (N3H) regions are important for the replication of the J6CF replicon. We applied these results to full-length genomic RNA replication and analyzed replication using Northern blotting. We found that a chimeric J6 clone with JFH-1 N3H and N5BX could replicate autonomously but that a chimeric J6 clone with only JFH-1 N5BX had no replication ability. Finally, we tested the virus production abilities of these clones and found that a chimeric J6 clone with JFH-1 N3H and N5BX could produce infectious HCV particles. In conclusion, the JFH-1 NS3 helicase and NS5B-to-3′X regions are important for efficient replication and virus particle formation of HCV genotype 2a strains.

Hepatitis C virus (HCV) is a major cause of chronic liver disease (7, 22). The lack of a robust cell culture system for producing virus particles has hampered the development of HCV research (2). Although the development of a subgenomic-replicon system enabled research into HCV RNA replication (32), infectious-virus-particle production remained impossible. Recently, an HCV cell culture system was developed using a JFH-1 genotype 2a strain of HCV cloned from a fulminant hepatitis patient (30, 48, 54), allowing investigation of the virus life cycle.

HCV is a positive-strand RNA virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family. The HCV genome comprises about 9,600 nucleotides that encode a single polyprotein of around 3,000 amino acids (8, 18, 44), which is processed by cellular and viral encoded proteases into at least 10 different structural and nonstructural proteins (11, 13, 14, 33).

The JFH-1 strain of HCV is a genotype 2a strain, and it is the first HCV strain that can produce HCV particles in Huh7 cells (48). Subgenomic replicons of JFH-1 replicate efficiently in Huh7 cells and do not require cell culture-adaptive mutations (19). The J6CF strain of HCV is also a genotype 2a strain and is known to be infectious in chimpanzees (49), but its entire genomic RNA does not replicate in Huh7 cells, despite the ~90% nucleotide sequence homology between JFH-1 and J6CF. However, J6/JFH-1 chimeric RNA that has J6 structural-protein-coding regions and JFH-1 nonstructural-protein-coding regions can replicate autonomously and produce infectious HCV particles (30, 39). Why only the JFH-1 clone can replicate efficiently in Huh7 cells remains unclear.

In this study, to investigate the mechanisms underlying efficient JFH-1 replication, we focused on the differences in replication between JFH-1 and J6CF strains by using intragenotypic JFH-1 and J6CF chimeras and compared their respective abilities to replicate RNA and produce virus particles in Huh7 cells.

MATERIALS AND METHODS

Cell culture. Huh7 cells (36) were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under 5% CO_2 conditions.

Subgenomic-replicon constructs. pSGR-JCH1 and pSGR-JCH4 were constructed based on pSGR-JFH1 (19, 21). pSGR-J6CF was also constructed from pJ6CF (a kind gift from Jens Bukh) (49), using the same method used to construct pSGR-JFH1. Plasmids used in luciferase assays were constructed based on pSGR-JFH1/Luc (20). Chimeric replicons were constructed by substitution of the corresponding regions. For convenience, several restriction enzyme recognition sites (ClaI [2275], EcoT22I [3639], and BsrGI [6127]) were introduced into the pSGR-J6CF sequence via nucleotide substitutions. The substitutions of the corresponding regions were achieved as follows, with the 5′ untranslated region (5′UTR) inserted between NotI and AgeI: NS3, PmeI-EcoT22I; NS3 protease, PmeI-ClaI; NS3 helicase, ClaI-EcoT22I; NS4, EcoT22I-MunI; NS5A, MunI-BsrGI; NS5B, BsrGI-StuI; and 3′UTR, StuI-XbaI (see Fig. 2A and 3A). pSGR-JCH1/Luc and pSGR-JCH4/Luc were also constructed using the same procedure as that for pSGR-JFH1/Luc (20, 21). The Con1 replicon (pSGR-Con1/Luc) was

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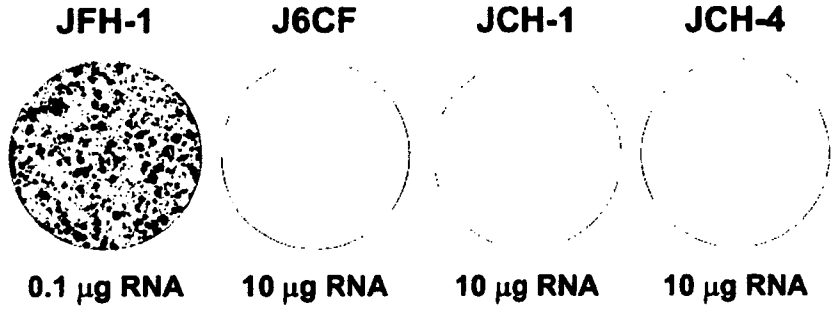

FIG. 1. G418-resistant colony formation of JFH-1, J6CF, JCH-1, and JCH-4. Subgenomic RNAs were synthesized in vitro, using pSGR-JFH1, pSGR-J6CF, pSGR-JCH1, and pSGR-JCH4 as templates. Transcribed subgenomic RNAs were electroporated into Huh7 cells, and cells were cultured with G418 for 3 weeks before staining with crystal violet as described in Materials and Methods. JFH-1 subgenomic RNA (0.1 μg) and 10 μg of J6CF, JCH-1, and JCH-4 subgenomic RNAs were transfected into Huh7 cells. Experiments were performed in triplicate, and representative staining examples are shown.

constructed from pFK-I389/neo/NS3-3'/wt (a kind gift from Ralf Bartenschlager) (32), and the H77c replicon (pSGR-H77c/Luc) was constructed from pCV-H77c (a kind gift from Robert H. Purcell) (50). For convenience, ClaI (2275) and BsrGI (6127) recognition sites were introduced into the pSGR-Con1/Luc and pSGR-H77c/Luc sequences via nucleotide substitutions. Substitutions of the NS3 helicase region and N5BX regions were performed as described above.

Full-length genomic HCV constructs. Plasmids used in the analysis of genomic RNA replication were constructed based on pJFH1 (48) and pJ6CF (49). For convenience, several restriction enzyme recognition sites (ClaI [3929], EcoT22I [5293], and BsrGI [7781]) were introduced into the J6CF sequence via nucleotide substitutions. Substitutions of the NS3 helicase regions were performed by replacement of the ClaI-EcoT22I fragment, substitutions of the N5BX regions were performed by replacement of the BsrGI-XbaI fragment, substitutions of the N5B regions were performed by replacement of the BsrGI-StuI fragment, and a substitution of the 3'UTR was performed by replacement of the StuI-XbaI fragment (see Fig. 5A).

RNA synthesis and transfection. RNA synthesis and transfection were performed as described previously (48). In brief, plasmids were linearized with XbaI, treated with mung bean nuclease (New England Biolabs, Ipswich, MA), and purified. Linearized, purified DNAs were used as templates for in vitro RNA synthesis using a MEGAscript T7 kit (Ambion, Austin, TX) in accordance with the manufacturer's instructions. Synthesized RNA was treated with DNase I (Ambion), followed by purification using ISOGEN-LS (Nippon Gene, Tokyo, Japan). The quality of synthesized RNA was examined by agarose gel electrophoresis. Ten micrograms of in vitro-synthesized RNA was used for each electroporation. Trypsinized Huh7 cells (3×10^6 cells) were washed with Opti-MEM I (Invitrogen, Carlsbad, CA) and resuspended in Cytomix buffer (47). RNA was mixed with 400 μl of cell suspension, and the mixture was then transferred to an electroporation cuvette (Precision Universal Cuvettes, Thermo Hybaid, Middlesex, United Kingdom). The cells were then pulsed at 260 V and 950 μF using a Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred to 10-cm culture dishes or six-well plates, each containing culture medium, and incubated at 37°C under 5% CO_2. Luciferase mRNA was synthesized from luciferase T7 control DNA (Promega, Madison, WI) by using a mMESSAGE mMACHINE T7 kit (Ambion). To monitor transfection efficiency, in vitro-synthesized luciferase RNA was cotransfected with HCV RNA and luciferase activity measured at 4 h after transfection.

G418-resistant colony formation assay. The G418-resistant colony formation assay was performed as described previously (19). In brief, 0.1 μg or 10 μg of transcribed RNAs was transfected into 3×10^6 Huh7 cells by electroporation. Transfected cells were immediately transferred to 10-cm culture dishes containing 10 ml of culture medium. G418 (1.0 mg/ml) (Nakalai Tesque, Kyoto, Japan) was added to the culture medium at 16 to 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.

Luciferase reporter assay. The luciferase activities of the JFH-1 subgenomic replicon and chimeras in Huh7 cells were measured as described previously (20). Briefly, 5 μg of transcribed RNAs was transfected into 3×10^6 Huh7 cells by electroporation. Transfected cells were immediately resuspended in culture medium and seeded into six-well culture plates. Cells were harvested serially at 4, 24, and 48 h after transfection and lysed with 200 μl of cell culture lysis reagent (Promega). Debris was then removed by centrifugation. Luciferase activity was quantified using a Lumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany) and a luciferase assay system (Promega). Assays were performed three times independently, with each value corrected for transfection efficiency as determined by measuring luciferase activity 4 h after transfection. The data are expressed as relative luciferase units (RLU).

Quantification of HCV core protein. To estimate the concentration of HCV core protein in the culture medium, we performed an HCV core enzyme-linked immunosorbent assay (Ortho-Clinical Diagnostics, Tokyo, Japan) in accordance with the manufacturer's instructions.

Northern blot analysis. Northern blot analysis was performed as described previously (48). In brief, total cellular RNA from HCV RNA-transfected cells was extracted using ISOGEN (Nippon Gene) in accordance with the manufacturer's instructions. Isolated RNA (2 μg) was separated on a 1% agarose gel containing formaldehyde, transferred to a Hybond N+ positively charged nylon membrane (GE Healthcare, Piscataway, NJ), and immobilized using a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). Hybridization was performed with [α-^{32}P]dCTP-labeled DNA by using Rapid-Hyb buffer (GE Healthcare). The DNA probe was synthesized using the NS5B-to-3'X fragment of JFH1 excised from pJFH1 by BsrGI and XbaI and labeled using the Megaprime DNA labeling system (GE Healthcare).

Infection of cells with secreted HCV and determination of infectivity. Culture medium from RNA-transfected cells was collected at 72 h posttransfection. Huh7 cells were seeded at a density of 1×10^4 cells per well in poly-D-lysine-coated 96-well plates (CORNING, Corning, NY). On the following day, the collected culture media were serially diluted and used for inoculation of the seeded cells, and the plates were incubated for another 3 days at 37°C. The cells were fixed in methanol for 15 min at −20°C, and the infected foci were visualized by immunofluorescence as described below.

Cells were blocked for 1 h with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) supplemented with 0.3% Triton X-100 and then washed with phosphate-buffered saline, followed by incubation with anti-core antibody at 50 μg/ml in BlockAce. After incubation for 1 h at room temperature, the cells were washed and incubated with a 1:400 dilution of AlexaFluor 488-conjugated anti-mouse immunoglobulin G (Molecular Probes, Eugene, OR) in BlockAce. The cells were then washed and examined using fluorescence microscopy (Olympus, Tokyo, Japan). Infectivity was quantified by counting the infected foci and expressed as numbers of focus-forming units per milliliter (FFU/ml).

RESULTS

G418-resistant colony formation of JFH-1, J6CF, and other genotype 2a subgenomic replicons. First, to compare the replication efficiencies of the JFH-1 and J6CF strains, we performed a G418-resistant colony formation assay with JFH-1 and J6CF RNAs by using subgenomic replicons. The JFH-1 subgenomic replicon formed many colonies with transfection of only 0.1 μg RNA, but the J6CF subgenomic replicon formed no colonies, even with transfection of 10 μg RNA (Fig. 1). We also tested

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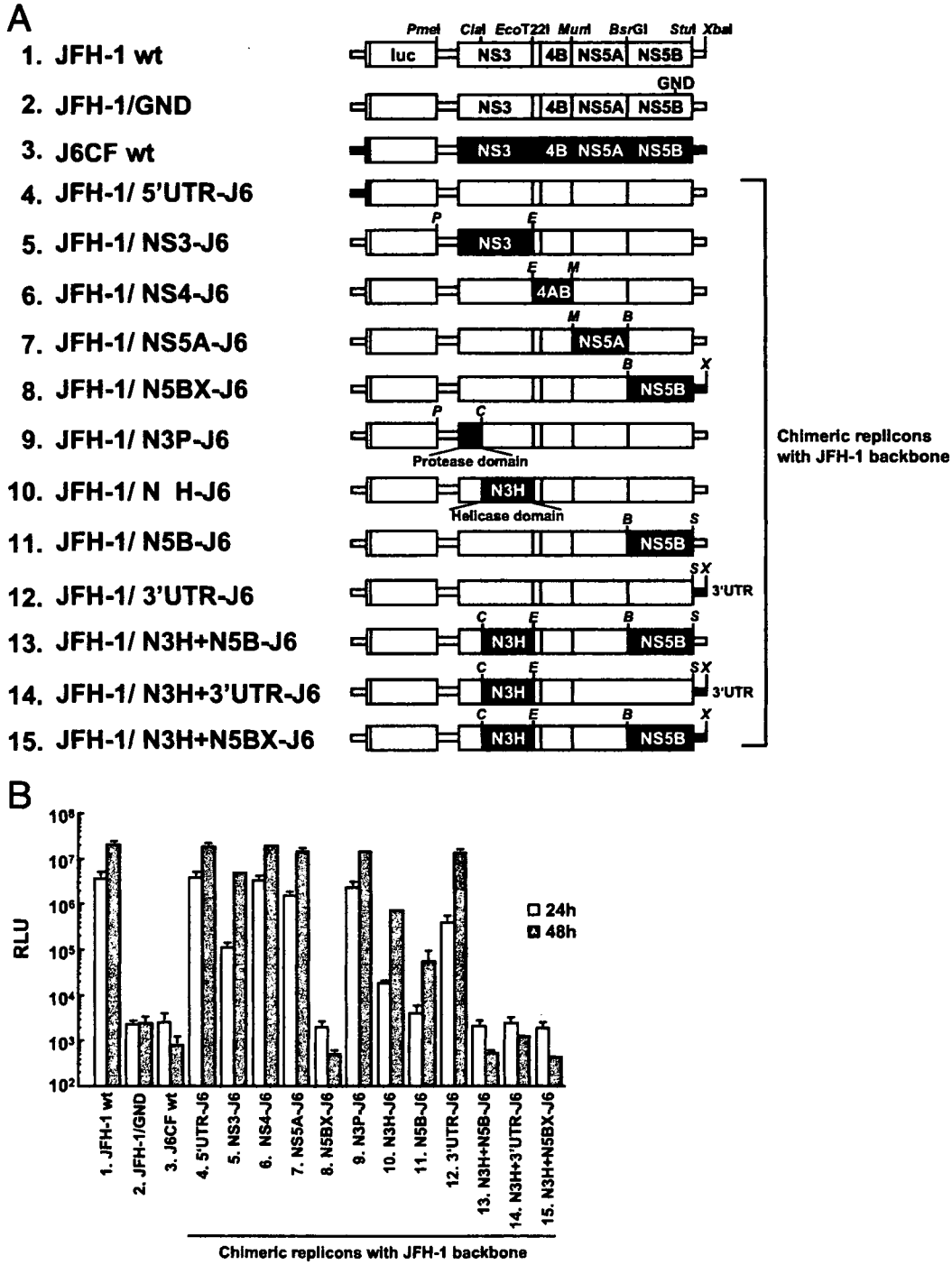

FIG. 2. Luciferase activities of chimeric replicons with a JFH-1 backbone. (A) Structures of chimeric subgenomic replicons with a JFH-1 backbone. The restriction enzyme recognition sites used for the construction of plasmids are indicated. *P*, PmeI; *C*, ClaI; *E*, EcoT22I; *M*, MunI; *B*, BsrGI; *S*, StuI; *X*, XbaI; wt, wild type. (B) Subgenomic RNAs were synthesized in vitro from wild-type or chimeric replicon constructs. Transcribed subgenomic RNAs (5 μg) were electroporated into Huh7 cells, and cells were harvested serially at 4, 24, and 48 h after transfection. The harvested cells were lysed, and then luciferase activities in the cell lysates were measured. The assays were performed three times independently and the results expressed as luciferase activities (RLU). Each value was corrected for transfection efficiency as determined by measuring the luciferase activity 4 h after transfection. Data are presented as means and standard deviations for luciferase activity at 24 h (white bars) and 48 h (gray bars) after transfection.

other genotype 2a clones (the JCH-1 and JCH-4 strains), which were isolated from patients with chronic hepatitis C (21). Their subgenomic replicons did not form colonies either. Given that chimeric J6/JFH-1 RNA that has the J6 structural-protein-coding regions and JFH-1 nonstructural-protein-coding regions reportedly replicates autonomously and produces infectious HCV particles (30, 39), we hypothesized that some of the JFH-1 nonstructural-protein-coding regions are important for JFH-1 replication.

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Regions of JFH-1 essential for replication. In order to determine which regions of JFH-1 are important for JFH-1 RNA replication, we constructed a series of chimeric JFH-1 subgenomic replicons replacing the 5'UTR, NS3, NS4AB, NS5A, and NS5B-to-3'X (N5BX) regions from the J6CF strain and tested their replication abilities. For this analysis, we adopted luciferase replicon systems (20) because colony formation assays are time-consuming to perform and it is difficult to evaluate precise replication levels using this method. Furthermore, efficient JFH-1 RNA replication may reduce cellular growth, thus affecting colony formation efficiency (34). We constructed JFH-1 chimeric subgenomic luciferase replicons with the J6CF clone because this clone was reportedly infectious in a chimpanzee (49). However, the JCH-1 and JCH-4 clones were not tested for infectivity. The 5'UTR, NS3, NS4AB, NS5A, or N5BX sequences of the JFH-1 replicon were replaced by J6CF sequences (5'UTR-J6, NS3-J6, NS4-J6, NS5A-J6, or N5BX-J6, respectively [Fig. 2A]). The luciferase activities of these replicons are shown in Fig. 2B. The JFH-1 subgenomic replicon replicated efficiently and had a luciferase activity of approximately 10^7 RLU (Fig. 2B, JFH-1 wt). GND, which was replication incompetent because of a mutation at the GDD motif in the NS5B region, had a luciferase activity of only 10^3 RLU (Fig. 2B, JFH-1/GND), which was taken as the background level. The J6CF subgenomic replicon did not replicate and had the same luciferase activity as GND (Fig. 2B, J6CF wt). Replacement of the 5'UTR, NS4AB, and NS5A sequences of JFH-1 by J6CF sequences (5'UTR-J6, NS4-J6, and NS5A-J6, respectively) did not reduce replication (Fig. 2B, 5'UTR-J6 and NS4-J6) or reduced it only slightly (Fig. 2B, NS5A-J6). However, there was no replication for the JFH-1 chimera with J6 N5BX (Fig. 2B, N5BX-J6). In addition, the JFH-1 chimera with the J6 NS3 region (NS3-J6) had a replication level that was more than 10-fold lower at 24 h and around 10-fold lower at 48 h than that of the wild-type JFH-1 replicon (Fig. 2B, JFH-1 wt and NS3-J6). These data show that the JFH-1 NS5B-to-3'X region is essential for JFH-1 RNA replication and indicate that the JFH-1 NS3 region is also important for JFH-1 RNA replication.

Involvement of the NS3 helicase region in efficient JFH-1 replication. The JFH-1 chimera with the J6 NS3 region (NS3-J6) reduced the replication level (Fig. 2B, NS3-J6). The NS3 protein is known to have two domains: a protease domain at the amino terminal one-third and a helicase domain at the carboxyl terminal two-thirds. To determine which region is important for replication, we compared the replication activity of a JFH-1 chimera with that of the NS3 protease-coding region of J6CF (N3P-J6) and that of a JFH-1 chimera with that of the NS3 helicase-coding region of J6CF (N3H-J6) (Fig. 2A, JFH-1/N3P-J6 and JFH-1/N3H-J6). Although N3P-J6 had the same luciferase activity as JFH-1, N3H-J6 had lower activity than JFH-1 (Fig. 2B, N3P-J6 and N3H-J6). These data show that the JFH-1 NS3 helicase-coding region has an important role in JFH-1 replication.

Importance of the JFH-1 NS5B-coding region and 3'UTR in replication. The JFH-1 chimera with J6 N5BX completely abolished replicon replication (Fig. 2B, N5BX-J6). The N5BX region contains two regions, the NS5B protein-coding region and the 3'UTR. The NS5B protein-coding region encodes RNA-dependent RNA polymerase. To analyze which region of N5BX is important for replication, we separated N5BX into two regions, that is, the NS5B-coding region and the 3'UTR. JFH-1 replicons with NS5B or with the 3'UTR of J6 were constructed (Fig. 2A, JFH-1/N5B-J6 and JFH-1/3'UTR-J6) and their replication abilities analyzed. The replication level of JFH-1/N5B-J6 was reduced more than 100-fold compared with that of the wild-type JFH-1 replicon at 48 h (Fig. 2B, N5B-J6). JFH-1/3'UTR-J6 replicated similarly to JFH-1 at 48 h, but the replication activity at 24 h was reduced more than 10-fold compared with that of the original JFH-1 replicon (Fig. 2B, 3'UTR-J6). These data indicate that the NS5B-coding region and the 3'UTR of JFH-1 are both involved in efficient JFH-1 replication.

Rescue of J6CF replicon replication by incorporation of the JFH-1 sequences. Because the JFH-1 N5BX region appeared to be essential for JFH-1 replication (Fig. 2B, N5BX-J6), we tested whether JFH-1 N5BX could restore the replication of J6CF RNA. We constructed a chimeric J6CF subgenomic replicon containing the JFH-1 N5BX region (Fig. 3A, J6/N5BX-JFH1) and tested its replication abilities. The luciferase activity of J6CF subgenomic RNA was recovered by inclusion of JFH-1 N5BX (Fig. 3B, N5BX-JFH1), but this chimeric replicon showed lower replication activity than the original JFH-1 replicon (Fig. 3B, JFH-1 wt). Furthermore, J6CF replication was not restored by only JFH-1 NS5B (J6/N5B-JFH1) or only the 3'UTR (J6/3'UTR-JFH1) (Fig. 3B, N5B-JFH1 or 3'UTR-JFH1, respectively). These observations clearly indicate that the JFH-1 NS5B-to-3'X region is essential, and the NS5B-coding region and 3'UTR are both important for efficient RNA replication in Huh7 cells. However, other JFH-1 regions are also involved in efficient replication.

The JFH-1 NS3 helicase-coding region was also important for efficient replication, and we thus tested whether the JFH-1 NS3 helicase region by itself could restore J6CF replication (as occurred for the JFH-1 N5BX region). Insertion of only the NS3 helicase region of JFH-1 into J6CF (Fig. 3A, J6/N3H-JFH1) did not restore replication (Fig. 3B, N3H-JFH1). However, replication of the J6 chimeric replicon seemed considerably restored by insertion of JFH-1 NS5B or the 3'UTR in addition to the NS3 helicase-coding region (Fig. 3B, N3H+N5B-JFH-1 or N3H+3'UTR-JFH-1, respectively) and fully restored by insertion of the JFH-1 NS3 helicase region and JFH-1 N5BX region (Fig. 3B, N3H+N5BX-JFH1). These results indicate that the JFH-1 N5BX region is essential for subgenomic-replicon replication and that the JFH-1 NS3 helicase-coding region has an additional role in replication. This was also confirmed by analysis of the replication abilities of JFH-1 replicons with double substitutions of J6CF (Fig. 2A, JFH-1/N3H+N5B-J6, JFH-1/N3H+3'UTR-J6, and JFH-1/N3H+N5BX-J6). Neither of these chimeric JFH-1 replicons replicated (Fig. 2B, N3H+N5B-J6, N3H+3'UTR-J6, and N3H+N5BX-J6).

The NS3 helicase and NS5B-3'X regions of JFH-1 can restore the replication of other genotype 2a replicons but not of genotype 1 replicons. To test whether the JFH-1 NS3 helicase and N5BX regions could restore other HCV replicon replication, chimeric replicon constructs N3H-JFH1, N5BX-JFH1, and N3H+N5BX-JFH1 were constructed using two genotype 2a replicons (JCH-1 and JCH-4), a genotype 1a replicon (H77c), and a genotype 1b replicon (Con1), respectively. The

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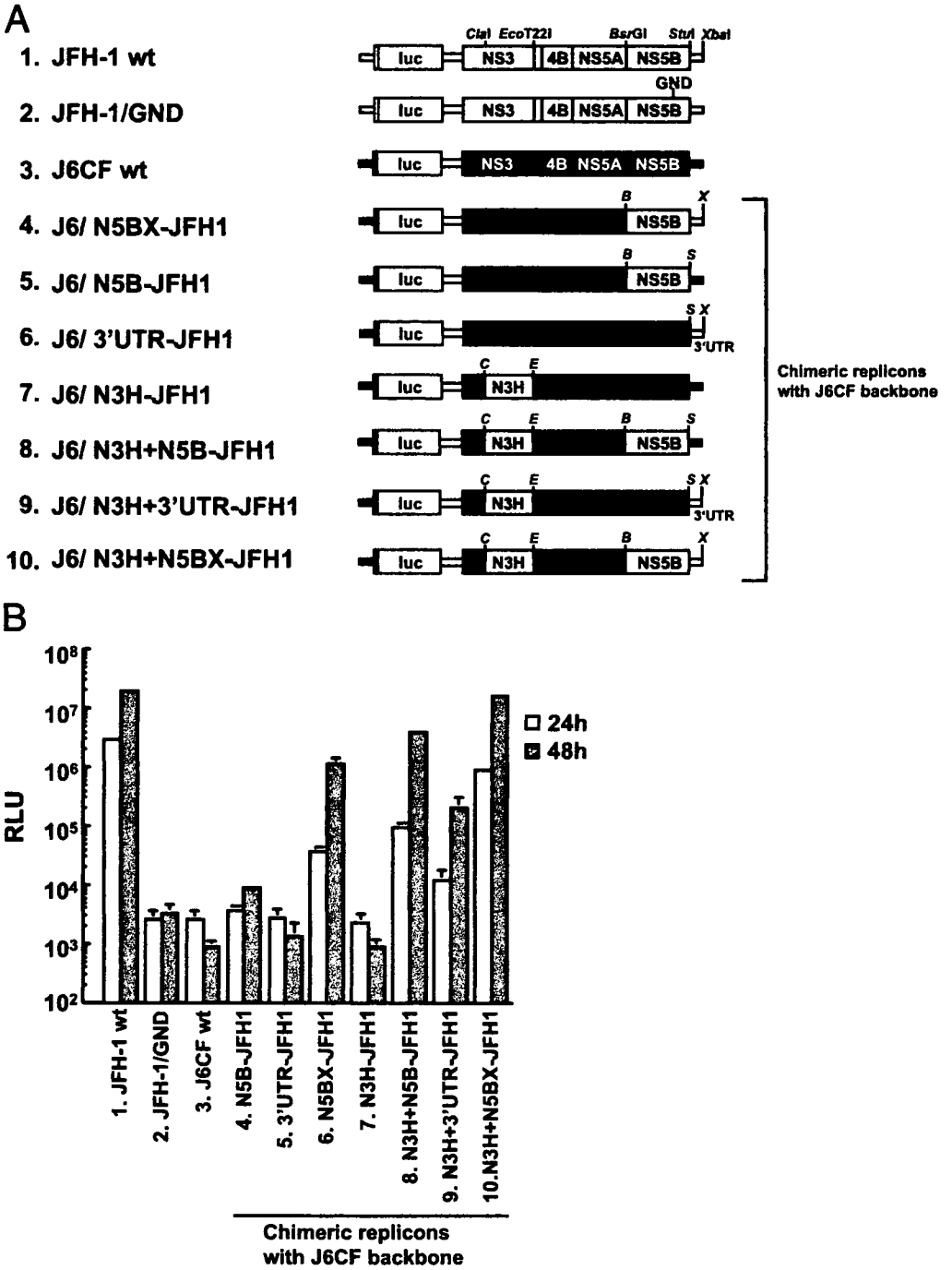

FIG. 3. Luciferase activities of chimeric replicons with a J6CF backbone. (A) Structures of chimeric subgenomic replicons with a J6CF backbone. The restriction enzyme recognition sites used for the construction of plasmids are indicated. *C*, ClaI; *E*, EcoT22I; *B*, BsrGI; *S*, StuI; *X*, XbaI; wt, wild type. (B) Wild-type or chimeric subgenomic RNAs were transfected into Huh7 cells, and the luciferase activities of the transfected cells were examined as described in the legend to Fig. 2B. Assays were performed three times independently, and data are presented as means and standard deviations for luciferase activity (RLU) at 24 h (white bars) and 48 h (gray bars) after transfection.

replication level of each wild-type and chimeric replicon was evaluated by luciferase activity measurement after transient transfection of replicon RNA. No replication of any of the wild-type replicons (Fig. 4, JCH-1 wt, JCH-4 wt, H77c wt, and Con1 wt) or of any of the replicons with insertion of the JFH-1 NS3 helicase region (Fig. 4, JCH-1/N3H-JFH1, JCH-4/N3H-JFH1, H77c/N3H-JFH1, and Con1/N3H-JFH1) was detected. However, genotype 2a replicons with insertion of the JFH-1 N5BX region increased their replication levels severalfold at 48 h (Fig. 4, JCH-1/N5BX-JFH1 and JCH-4/N5BX-JFH1). Furthermore, insertion of both the N3H and the N5BX regions increased the JCH-1 replication over 10-fold compared to that

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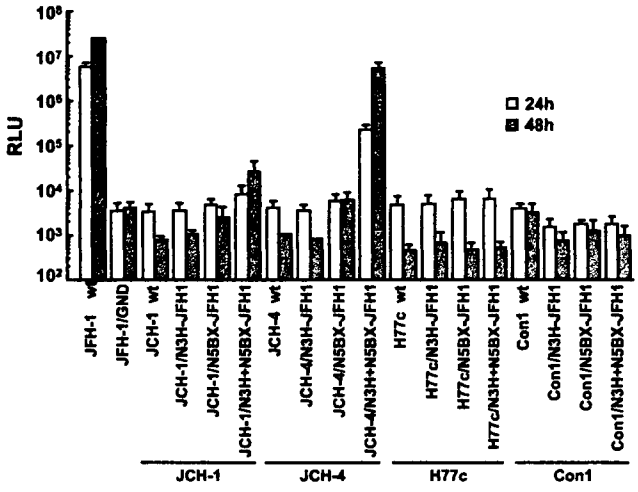

FIG. 4. Restoration of genotype 2a and genotype 1 replicon replication by the insertion of JFH-1 sequences. Two genotype 2a replicons, JCH-1 and JCH-4, a genotype 1a replicon, H77c, and a genotype 1b replicon, Con-1, were used in this assay. Three kinds of chimeric replicons, N3H-JFH-1, N5BX-JFH1, and N3H+N5BX-JFH-1, were prepared for all four HCV replicons. Wild-type (wt) or chimeric subgenomic RNAs were transfected into Huh7 cells and the luciferase activities of the transfected cells examined as described in the legend to Fig. 2B. The assays were performed three times independently, and data are presented as means and standard deviations for luciferase activity (RLU) at 24 h (white bars) and 48 h (gray bars) after transfection.

of wild-type JCH-1 at 48 h and recovered the JCH-4 replication to a level similar to that of wild-type JFH-1 at 48 h (Fig. 4, JCH-1/N3H+N5BX-JFH1 and JCH-4/N3H+N5BX-JFH1, respectively). On the other hand, insertion of the JFH-1 N5BX region or both the N3H and the N5BX regions did not restore H77c or Con1 replicon replication (Fig. 4, H77c/N5BX-JFH1, H77c/N3H+N5BX-JFH1, Con1/N5BX-JFH1, and Con1/N3H+N5BX-JFH1). HCV polyprotein processing is critically important for HCV RNA replication and virus production, and this processing may be affected by the chimeric RNA molecules between different isolates of genotype 2 as well as those between genotypes 1 and 2. However, our data indicated that HCV polyprotein processing did not differ among the chimeric constructs (data not shown). Thus, the JFH-1 N3H and N5BX regions can rescue the replication of genotype 2a replicons at different levels but not the replication of genotype 1 replicons.

The NS3 helicase and NS5B-3'X regions are both important for JFH-1 genomic RNA replication. Next, we applied the previously described results to genomic RNA replication. The structures of HCV, the template DNA for JFH-1, and the chimeric full-genomic RNAs are shown in Fig. 5A. Full-length HCV RNAs were synthesized as described above and their quality and integrity then confirmed by gel electrophoresis (data not shown). To analyze the transient RNA replication of these chimeric RNAs in Huh7 cells, the synthesized RNAs were transfected into Huh7 cells and total RNA was extracted from HCV RNA-transfected cells at various time points. Northern blot analysis was then performed. The equality of the transfection efficiencies was confirmed by the cotransfection of luciferase mRNA (data not shown). As shown in Fig. 5B, JFH-1 RNA decreased at 10 h after transfection but replicated efficiently at 24 to 48 h after transfection, as described previously (48). J6 chimeric RNA with the NS3 helicase and N5BX regions of JFH-1 (J6/N3H+N5BX-JFH1) replicated with similar kinetics but with lower efficiency. J6 chimeric RNA with JFH-1 N5BX (J6/N5BX-JFH1) showed no replication in this assay, like J6CF or JFH-1 GND, although this chimera replicated to a considerable extent in subgenomic-replicon assays. Taken together, these data indicate that the NS3 helicase-coding region and the NS5B-to-3'X region of JFH-1 are both essential for full-length genomic HCV RNA replication in Huh7 cells.

Core protein and infectious-chimeric-virus secretion from chimeric J6CF RNA-transfected cells. Finally, we tested whether chimeric RNA-transfected cells could secrete infectious virus particles. Figure 5C shows the core protein secretion into the culture medium from JFH-1, JFH-1/GND, J6CF, and chimeric-RNA-transfected cells. Core protein was efficiently secreted from cells transfected with JFH-1 RNA (Fig. 5C and Table 1) and those transfected with J6/N3H+N5BX-JFH1 RNA, but with efficiencies lower than that for JFH-1 (Fig. 5C and Table 1). J6/N5BX-JFH1, JFH-1/GND, and J6CF RNA-transfected cells, which showed no RNA replication by Northern blot analysis (Fig. 5B), did not secrete core proteins into the culture medium (Table 1). By the replicon assay, JFH-1/N5BX-J6 showed no replication in Huh7 cells (Fig. 2B, N5BX-J6), and full-length JFH-1/N5BX-J6 RNA-transfected cells did not secrete core protein into the culture medium (Table 1). On the other hand, JFH-1/N5B-J6 replicated to some extent in the replicon assay (Fig. 2B, N5B-J6), and full-length JFH-1/N5B-J6 RNA-transfected cells secreted a smaller amount of core protein than JFH-1 RNA-transfected cells (Fig. 5C and Table 1). Both JFH-1/N3H-J6 and JFH-1/3'UTR-J6 RNA-transfected cells secreted about half the amount of core protein that the JFH-1 RNA-transfected cells did (Fig. 5C and Table 1); however, the replication level of the JFH1/N3H-J6 replicon was markedly lower than those of the JFH-1 and JFH-1/3'UTR-J6 replicons (Fig. 2B, JFH-1 wt, N3H-J6, and 3'UTR-J6), and the replication level of full-length JFH-1/N3H-J6 RNA was also lower than those of the JFH-1 and JFH-1/3'UTR-J6 RNAs as determined by Northern blot analysis (data not shown). Transfection of the other two chimeric RNAs, JFH-1/N3H+N5B-J6 and JFH-1/N3H+N5BX-J6, did not induce core protein secretion (Table 1), and this is in agreement with the finding that neither chimeric replicon replicated (Fig. 2B, N3H+N5B-J6 and N3H+N5BX-J6).

Then, we tested the infectivity of the culture medium from the RNA-transfected cells by a focus formation assay. The infectivity of the culture medium from JFH-1 RNA-transfected cells was determined as $8.8 \times 10^3 \pm 5.7 \times 10^2$ FFU/ml (Table 1). The infectivity of the culture medium was also detected from cells transfected with J6/N3H+N5BX/JFH-1, JFH1/N3H-J6, JFH-1/N5B-J6, or JFH-1/3'UTR-J6 RNA but not with other chimeric RNAs (Table 1). This result thus indicates that efficient core protein secretion is at least indispensable for infectious-virus secretion. However, the levels of infectivity of culture medium did not correlate with core protein concentrations. In particular, JFH-1/N3H-J6 RNA-transfected cells secreted a rather higher level of core protein, but its infectious titer was low. The RNA replication capacity of JFH-1/N3H-J6 was lower than that of wild-type JFH-1 or JFH-1/3'UTR-J6

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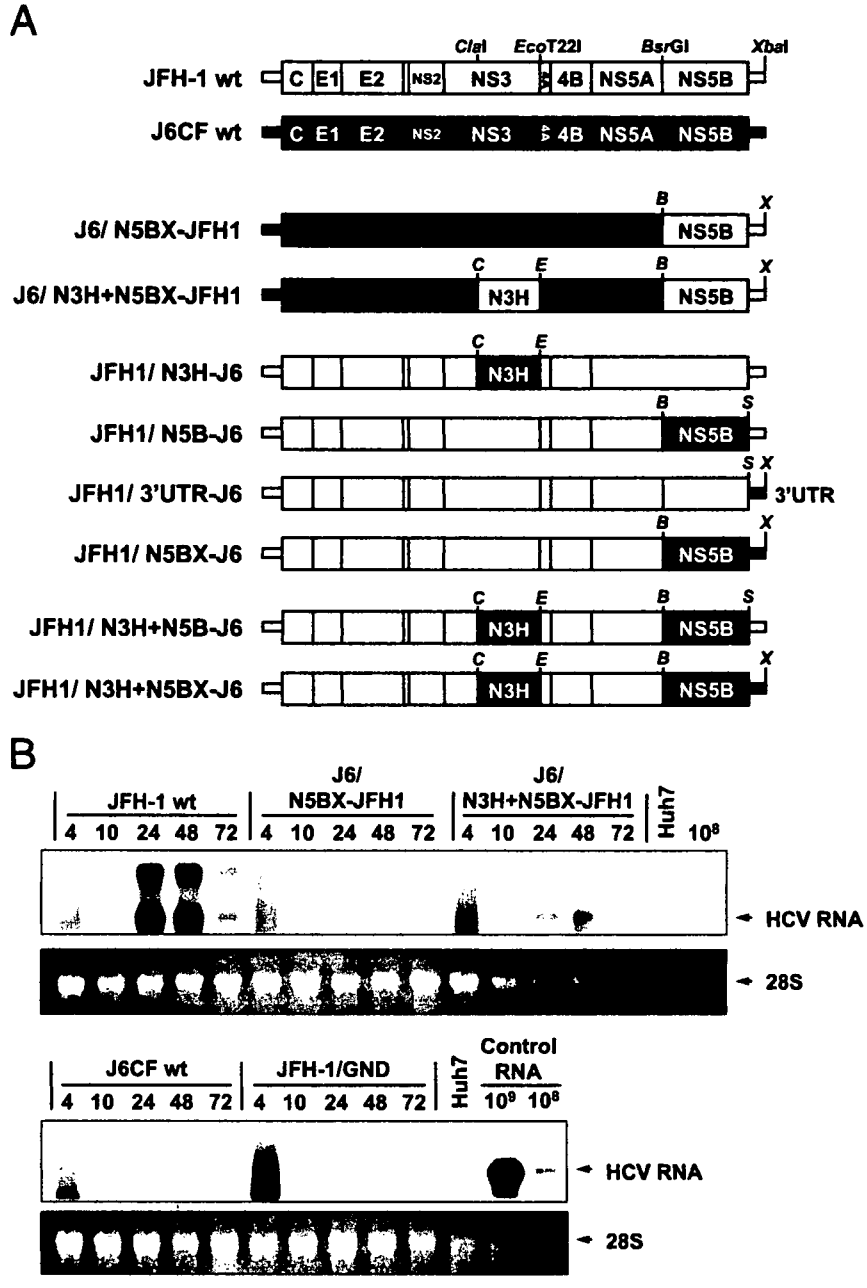

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