JFH-1　　　J6CF　　　JCH-1　　　JCH-4

0.1 μg RNA　　10 μg RNA　　10 μg RNA　　10 μg RNA

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-1389/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], EcoT221 [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-EcoT221 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 × 10⁶ 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% CO₂. 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 × 10⁶ 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 × 10⁶ 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-³²P]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 × 10⁴ 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
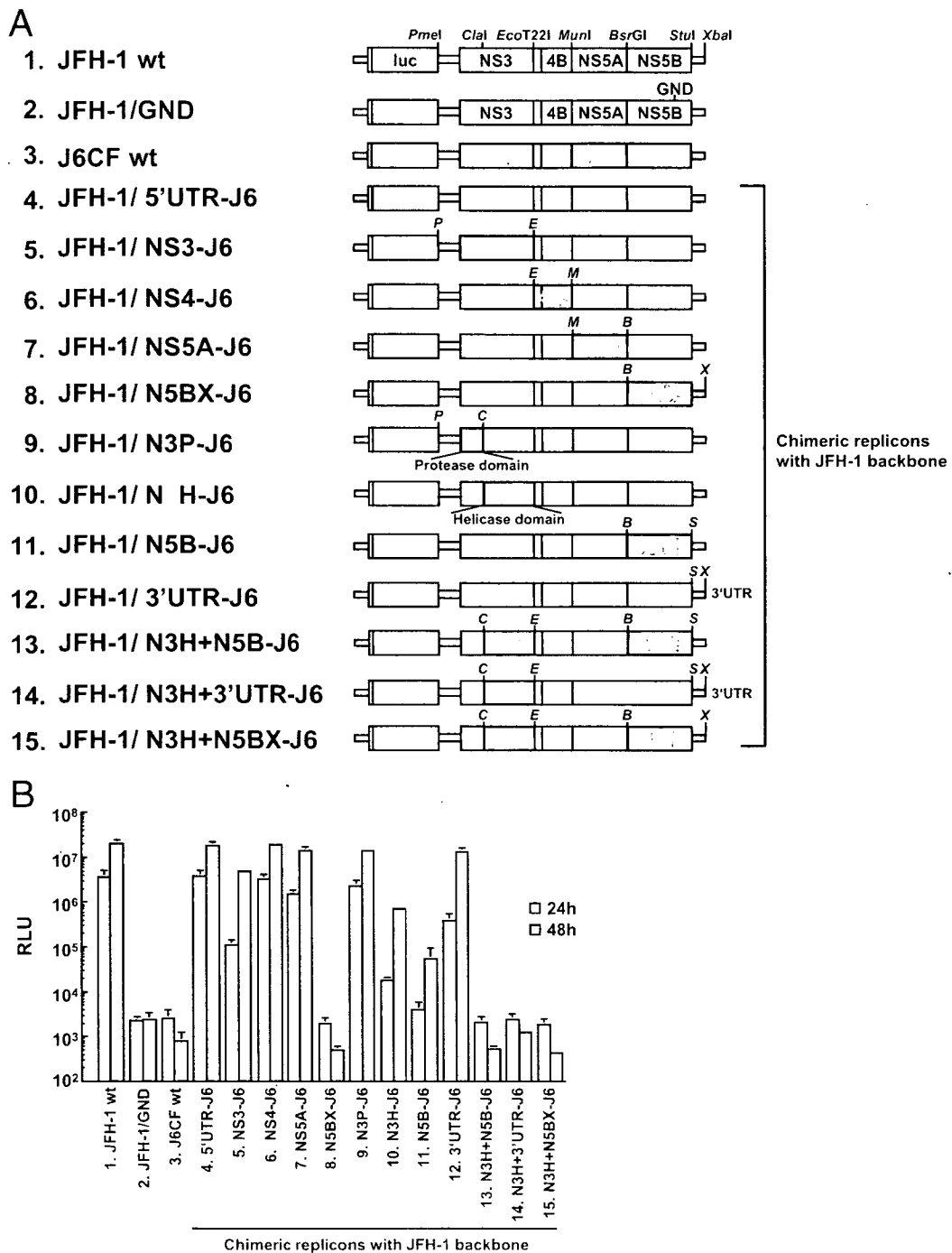
FIG. 2. Luciferase activities of chimeric replicons with a JFH-1 backbone. (A) Structures of chimeric subgenomic replicons with a JFH-1 backbone. The restriction enzyme recognition sites used for the construction of plasmids are indicated. *P*, PmeI; *C*, ClaI; *E*, EcoT22I; *M*, MunI; *B*, BsrGI; *S*, 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.

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

A

1. JFH-1 wt

2. JFH-1/GND

3. J6CF wt

4. J6/ N5BX-JFH1

5. J6/ N5B-JFH1

6. J6/ 3'UTR-JFH1

7. J6/ N3H-JFH1

8. J6/ N3H+N5B-JFH1

9. J6/ N3H+3'UTR-JFH1

10. J6/ N3H+N5BX-JFH1

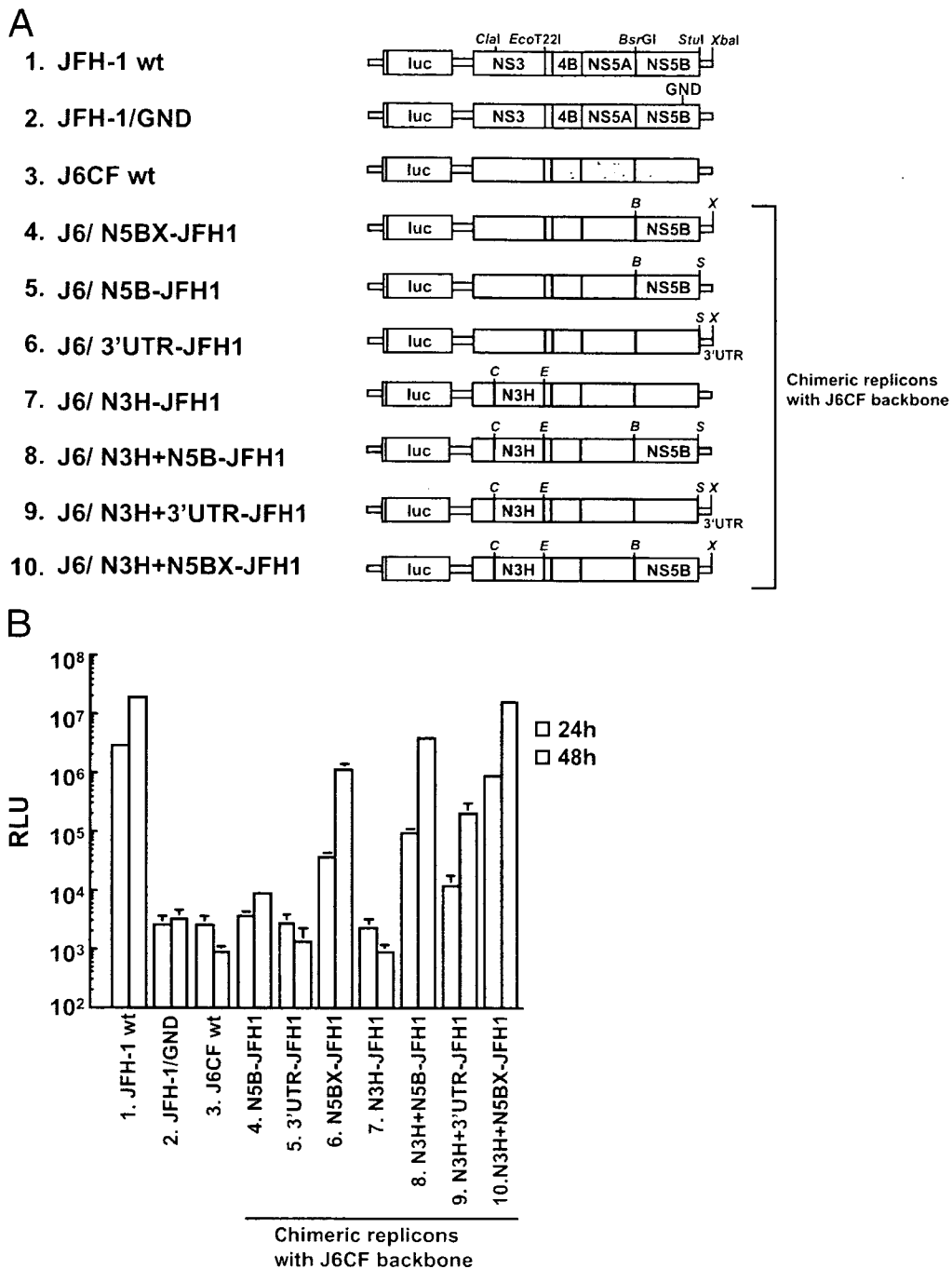Chimeric replicons
with J6CF backbone

B

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
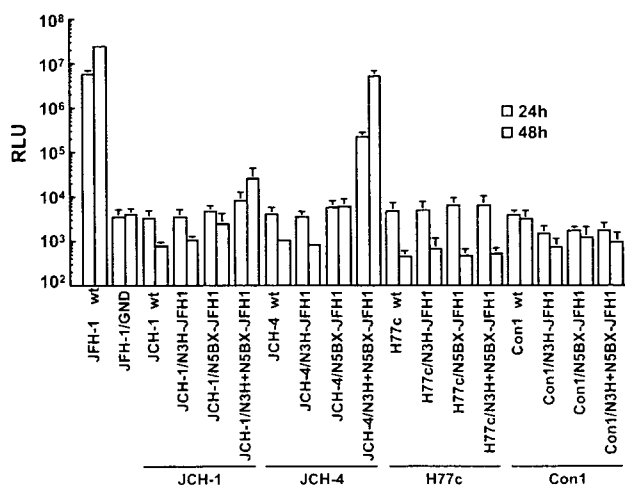
FIG. 4. Restoration of genotype 2a and genotype 1 replicon replication by the insertion of JFH-1 sequences. Two genotype 2a replicons, JCH-1 and JCH-4, a genotype 1a replicon, H77c, and a genotype 1b replicon, Con-1, were used in this assay. Three kinds of chimeric replicons, N3H-JFH-1, N5BX-JFH1, and 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.
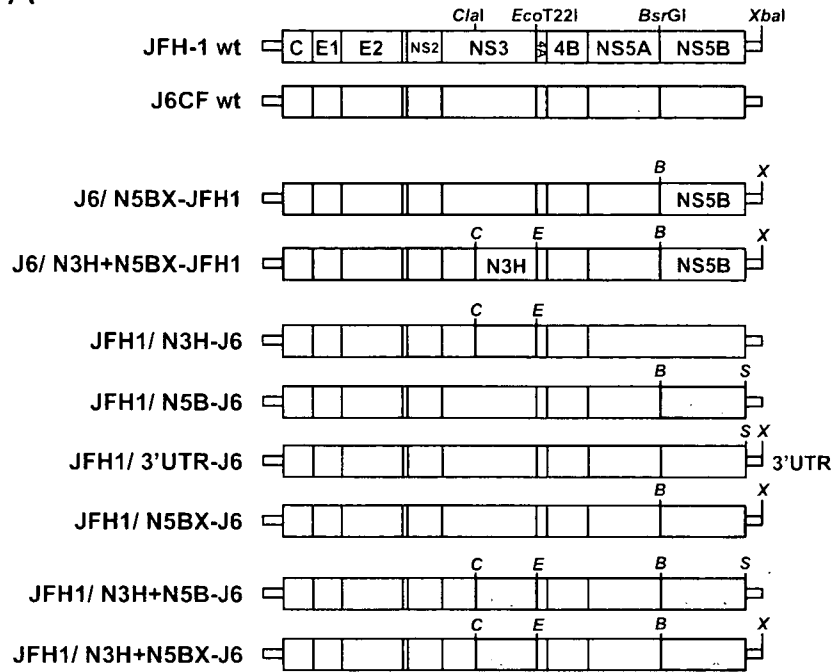
The NS3 helicase and NS5B-3'X regions are both important for JFH-1 genomic RNA replication. Next, we applied the previously described results to genomic RNA replication. The structures of HCV, the template DNA for JFH-1, and the chimeric full-genomic RNAs are shown in Fig. 5A. Full-length HCV RNAs were 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.
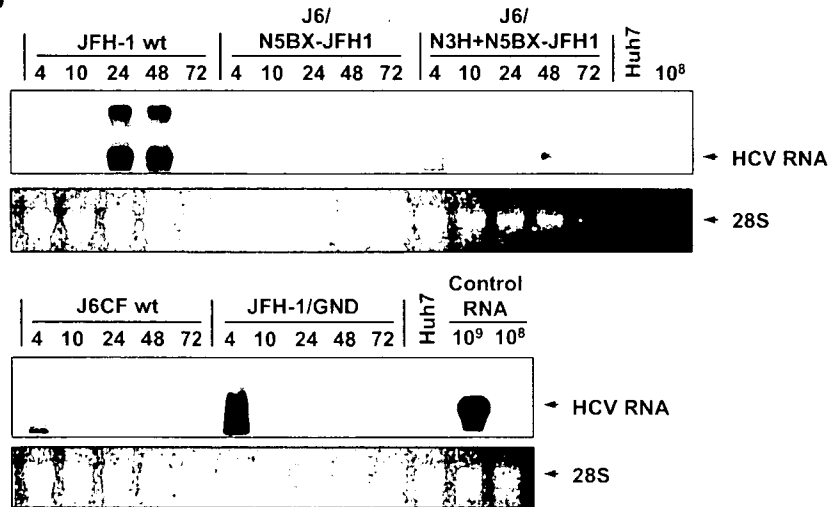
Core protein and infectious-chimeric-virus secretion from chimeric J6CF RNA-transfected cells. Finally, we tested whether chimeric RNA-transfected cells could secrete infectious virus particles. Figure 5C shows the core protein secretion into the culture medium from JFH-1, JFH-1/GND, J6CF, and chimeric-RNA-transfected 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
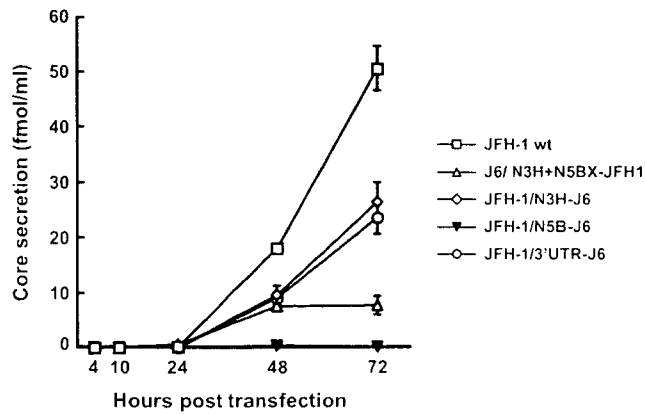
A

JFH-1 wt   ClaI EcoT22I   BsrGI   XbaI
C | E1 | E2 | NS2 | NS3 | | 4B | NS5A | NS5B

J6CF wt

J6/ N5BX-JFH1   B   X
                                          NS5B

J6/ N3H+N5BX-JFH1   C   E   B   X
                      N3H   NS5B

JFH1/ N3H-J6   C   E

JFH1/ N5B-J6   B   S

JFH1/ 3'UTR-J6   S X
                                          3'UTR

JFH1/ N5BX-J6   B   X

JFH1/ N3H+N5B-J6   C   E   B   S

JFH1/ N3H+N5BX-J6   C   E   B   X

B

| | JFH-1 wt | | J6/ N5BX-JFH1 | | J6/ N3H+N5BX-JFH1 | Huh7 | 10^8 |
| 4 10 24 48 72 | 4 10 24 48 72 | 4 10 24 48 72 | | |

→ HCV RNA

→ 28S

| | J6CF wt | | JFH-1/GND | | Huh7 | Control RNA |
| 4 10 24 48 72 | 4 10 24 48 72 | | 10^9 10^8 |

→ HCV RNA

→ 28S

C

Core secretion (fmol/ml)

60
50
40
30
20
10
0

4 10   24   48   72

Hours post transfection

—□— JFH-1 wt
—△— J6/ N3H+N5BX-JFH1
—◇— JFH-1/N3H-J6
—▼— JFH-1/N5B-J6
—○— JFH-1/3'UTR-J6

TABLE 1. Infectious titers of the media from chimeric HCV RNA-transfected cells

Construct[a]	Core protein level (fmol/ml)	Infectivity (FFU/ml)
JFH-1 (wild type)	50.7 ± 4.1	$8.8 \times 10^3 \pm 5.7 \times 10^2$
JFH-1/GND	0	0
J6CF (wild type)	0	0
J6/N5BX-JFH1	0	0
J6/N3H+N5BX-JFH1	7.7 ± 1.7	$9.1 \times 10^1 \pm 4.1 \times 10^1$
JFH-1/N3H-J6	26.3 ± 3.6	$1.7 \times 10^1 \pm 1.2 \times 10^1$
JFH-1/N5B-J6	0.1 ± 0.0	$6.7 \times 10^0 \pm 4.1 \times 10^0$
JFH-1/3'UTR-J6	23.6 ± 2.9	$2.6 \times 10^3 \pm 7.1 \times 10^2$
JFH-1/N5BX-J6	0	0
JFH-1/N3H+N5B-J6	0	0
JFH-1/N3H+N5BX-J6	0	0

[a] Culture media were collected from the RNA-transfected cells 72 h after transfection.

(Fig. 2B), and currently, there is no clear explanation for this discrepancy. This will be further examined in a future study.

Importantly, we found that the J6/N3H+N5BX-JFH1 chimera produced infectious virus. These results strongly indicate that the NS3 helicase and NS5B-to-3'X regions of JFH-1 are important for autonomous replication of the replication-incompetent J6CF strain and for secretion of infectious chimeric virus, although the virus secretion efficiency and the infection efficiency of the secreted virus were low.

DISCUSSION

In the present study, we identified the regions that are important for efficient JFH-1 replication in Huh7 cells by using chimeric constructs with other genotype 2a clones. Via transient replication assays of JFH-1 and J6CF chimeras, both the NS3 helicase-coding (N3H) region and the NS5B-to-3'X (N5BX) region of JFH-1 were found to be important for replication (Fig. 2 and 3). This was also confirmed by full-length genomic RNA replication, but the replication level of J6/N3H+N5BX-JFH1 was lower than that of wild-type JFH-1 (Fig. 5B). The N5BX region of JFH-1 was the minimum essential region for subgenomic-replicon replication (Fig. 3B, N5BX-JFH-1), but in full-length RNA replication, the NS3 helicase-coding region of JFH-1 was also necessary (Fig. 5B). This contradiction might be explained by differences in RNA length, because shorter RNAs such as subgenomic replicons are likely to replicate even with a less powerful replication engine. Alternatively, there could be some negative element for replication in the J6CF structural-protein-coding region or some positive element in the *neo* encephalomyocarditis virus

internal ribosome entry site region of the subgenomic replicon. Furthermore, J6 chimeric RNA with the minimum essential regions of JFH-1 (J6/N3H+N5BX-JFH1) caused Huh7 cells to secrete infectious chimeric virus particles. However, the infection efficiency of J6/N3H+N5BX-JFH1 was lower than that of wild-type JFH-1. First, this may be due to the low RNA replication level. With JFH-1 NS3 helicase and N5BX, J6CF was able to replicate, but the replication efficiency was lower than that of JFH-1 (Fig. 5B). Because J6CF replication could occur only with JFH-1 NS3 helicase and N5BX, more *cis*-acting replication elements (CREs) of JFH-1 may be needed for more efficient replication of J6CF. Second, the levels of virus assembly may be low. This chimera had only the NS3 helicase, NS5B, and 3'UTR regions of JFH-1, possibly omitting some regions important for efficient virus particle secretion. Given that the NS2 region of JFH-1 is reportedly important in virus assembly and release (39), the NS2 region may be a possible candidate. JFH-1/N3H-J6 RNA-transfected cells secreted a substantial amount of core protein; however, its infectivity was much lower (Table 1). The JFH-1 N3H region may be important for the infectivity of the secreted virus and/or for virus particle secretion itself. This will be determined in a future study.

Significance of JFH-1 N5BX for replication. We demonstrated the importance of both the NS5B-coding region and the 3'UTR in JFH-1 replication in the present study. There are several reports regarding CREs within the NS5B-coding region and 3'UTR of Con1 (9, 28, 52). The importance of the interaction between CREs in NS5B and the 3'UTR for replication has also been reported for the Con1 strain (9). The nucleotide sequences involved in the kissing-loop interaction were conserved between JFH-1, J6CF, and Con-1. However, mutations in other regions may affect this interaction by disrupting the RNA secondary structures. On the other hand, given that the NS5B-coding region encodes an RNA-dependent RNA polymerase, the enzymatic activities of the polymerase may differ among the tested strains. The sequence similarities of the JFH-1 and J6CF NS5B regions are 92.2% for the nucleotide sequence and 95.1% for the amino acid sequence. Out of 591 amino acids, only 29 amino acids differ, and the GDD motif that is highly conserved among RdRps is conserved. There are many reports regarding the interaction between NS5B and other viral or cellular proteins, and some of the interactions have been reported to play a role in replication (6, 10, 12, 15, 17, 27, 41–43, 45, 46). Furthermore, the importance of the membrane localization of NS5B with respect to replication has also been reported (29, 35). Mutations in J6CF NS5B may affect these roles. It is thus important to examine the RdRp activities of JFH-1 and J6CF NS5B proteins in vitro.

FIG. 5. Analysis of transient replication of genomic chimeric HCV RNA. (A) Structures of full-length chimeric HCV RNAs. Each chimeric full-length construct was prepared by the insertion of the restricted fragments as indicated. The restriction enzyme recognition sites used for the plasmid constructions are indicated. C, ClaI; E, EcoT22I; B, BsrGI; S, StuI; X, XbaI; wt, wild type. (B) Northern blot analysis of total RNA prepared from cells transfected with transcribed genomic HCV RNA. Numbers of synthetic JFH-1 RNA (control RNA), RNA isolated from naïve cells (Huh7), and hours after transfection (4, 10, 24, 48, and 72) are indicated. Arrowheads indicate full-length HCV RNA (HCV RNA) and 28S rRNA (28S). A representative autoradiogram (6-h exposure) of three independent experiments is presented. (C) HCV core protein secretion from the RNA-transfected cells. Transcribed wild-type or chimeric full-length HCV RNAs (10 μg) were transfected into Huh7 cells. Culture medium was harvested at 4, 10, 24, 48, and 72 h after transfection. The amounts of core proteins in the harvested culture medium were measured using an HCV core enzyme-linked immunosorbent assay. The assays were performed five times independently, and data are presented as means and standard deviations.

On the other hand, the effect of the 3'UTR is very surprising, especially since the nucleotide sequences of this region are very similar between JFH-1 and J6CF. In this study, the 3'UTR includes four parts: 22 nucleotides at the 3'-end NS5B region (as a result of the cloning strategy), 39 nucleotides of variable region, the poly(U/UC) region, and a 98-nucleotide 3'X region. There are a single synonymous nucleotide mutation in the 3'-end NS5B region and three nucleotide mutations in the variable region. The poly(U/UC) regions are 99 and 132 nucleotides in JFH-1 and J6CF, respectively. There are no mutations in the 3'X region in either strain. It is thus quite interesting to pursue the mechanisms of these mutations in the 3'UTR that affect the HCV RNA replication levels. Further studies are important for precise elucidation of the efficient replication mechanisms of JFH-1.

Significance of the JFH-1 NS3 helicase region for replication. In the present study, we demonstrated the importance of the JFH-1 NS3 helicase region, especially in full-length genomic RNA replication. It has been reported that an active NS3 helicase is required for replication of subgenomic replicons (25). The NS3 helicase domain possesses helicase activity and ATPase activity, and it has been reported that the characters of these enzymes differ among the genotypes and the strains (26). NS3 has also been reported to interact with positive- and negative-strand RNA 3'UTRs (1). One possible model of the role of NS3 in RNA replication is that NS3 helicase unwinds RNA secondary structures and/or a double-stranded RNA intermediate before RNA synthesis by NS5B (37). The sequence similarity of the NS3 helicase regions of JFH-1 and J6CF is rather high, 89.5% for the nucleotide sequence and 93.8% for the amino acid sequence, and out of 487 amino acids, only 30 amino acids differ. These mutations may affect the enzymatic activities of NS3 helicase.

Furthermore, it has been reported that NS3 can stimulate NS5B RdRp activity (38). It has also been reported that the NS3 protease domain and NS5B stimulate NS3 helicase activity (53). Taken together, these findings show that not only the enzymatic activities themselves but also the combination or interaction of the NS3 and NS5B proteins could be important. However, it is still important to examine and compare the NS3 helicase enzymatic activities in vitro of JFH-1 and other HCV strains in a further study.

Replication in vitro and in vivo. We previously reported that JFH-1 RNA could replicate efficiently in Huh7 cells. Cell-cultured JFH-1 virus was also found to be infectious in chimpanzees; however, the virus was cleared immediately after transient viremia (48). In contrast, J6CF does not replicate in Huh7 cells, but it is infectious in chimpanzees (49). J6/JFH-1 chimeric RNA replicated efficiently in Huh7 cells (39) and Huh7-derived cell lines (30), and cell-cultured chimeric J6/JFH-1 virus was infectious in chimpanzees and in chimeric uPA-SCID mice (31). Replication efficiency in vitro may not necessarily correlate with that in vivo. The H77, Con-1, and HCV-N strains were infectious in chimpanzees (3, 5, 23, 50). However, the H77 and Con-1 strains need adaptive mutations for efficient replication in cultured cells (4, 24) and HCV-N replicates relatively efficiently in cultured cells (16). On the other hand, H77-S containing five adaptive mutations can produce infectious virus particles (51), but the Con-1 and HCV-N strains do not produce virus particles (16, 40). It is still unclear

what viral or host factors are important for efficient replication and infectious-virus production in vitro and in vivo. However, understanding HCV replication mechanisms by using cell culture models is still important for elucidation of the HCV life cycle.

Significance of the regions responsible for JFH-1 replication. Using two HCV strains, JFH-1 and J6CF, which are very closely related but have different characteristics, we were able to determine which regions are important for replication in cultured cells. Replication of two other genotype 2a strains, JCH-1 and JCH-4, was also recovered by replacement of the N3H and N5BX regions of JFH-1 at the lower levels compared to replication of the J6 replicon (Fig. 3B and 4). This may be because J6CF is an infectious clone in chimpanzees, but the JCH-1 and JCH-4 strains are clinical isolates from chronic-hepatitis patients (21) and may include critical mutations in other important regions. Furthermore, replication of genotype 1 HCV replicons was not restored by the same procedure as that for genotype 2a replicons (Fig. 4). Functional complementation in the nonstructural region and 3'UTR may be difficult beyond the genotypes.

Obtaining virus particles is an important step in antiviral research. Although infection efficiency is improved in permissive cell lines, most HCV strains still cannot replicate or produce virus particles in cultured cells. Therefore, chimeric virus particles with the JFH-1 replication engine may be suitable substitutes. Furthermore, analyses using chimeric viruses that have structural proteins and other regions from various strains may give us new information regarding strain-specific effects on HCV life cycles. Consequently, applying the findings of the present study to replication-incompetent strains may be useful not only for analyses of virus strain specificity and precise analyses of the HCV life cycle but also for antiviral studies.

In conclusion, we analyzed the mechanism underlying efficient JFH-1 replication by using intragenotypic chimeras of JFH-1 and J6CF and clearly showed the importance of the JFH-1 NS3 helicase region and the NS5B-to-3'X region for efficient replication of HCV genotype 2a strains.

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Original Article

Transcriptomic Comparison of Human Hepatoma Huh-7 Cell Clones with Different Hepatitis C Virus Replication Efficiencies

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SUMMARY: Hepatitis C virus (HCV) infection represents a major public health problem throughout the world. The establishment of viral replicons has enhanced our understanding of the mechanism underlying HCV replication. However, the specific virus-host cell interactions involved in HCV RNA replication are not well understood. In the present study, we isolated several human hepatoma Huh-7-derived subclones with a range of HCV RNA replication efficiencies by end-point dilution. Of these, the clones HuhTe4 and HuhTe6 were observed to proliferate at the same rate; however, HuhTe6 supported a significantly greater degree of viral RNA replication. Using cDNA microarray analysis, a total of 36 genes (0.4%) demonstrated variable expression, with a $\geq$2-fold difference in expression noted between HuhTe4 and HuhTe6. Among genes that are implicated in a variety of functional categories, a subset of these differentially-expressed genes has a role in signal transduction and cell communication, including thioredoxin-interacting protein, Rab6B, sorting nexin 16 and UDP-galactose:ceramide glycosyltransferase. The genes identified in this study should be examined further to determine their roles in HCV RNA replication. The Huh-7 subclones identified in this study provide a tool for identifying novel host factors involved in viral replication.

INTRODUCTION

Hepatitis C virus (HCV) is a positive-strand RNA virus that belongs to the family *Flaviviridae*, which causes acute and chronic hepatitis, as well as hepatocellular carcinoma (1,2). The HCV genome encodes a long polyprotein precursor of approximately 3,000 amino acids that is processed into at least 10 proteins: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (3, 4). The nonstructural proteins are processed by two viral proteases; the junction of NS2 and NS3 is cleaved by NS2-3 protease, which spans NS2 and the N-terminal domain of NS3, while four other junctions are cleaved by a serine protease located at the N-terminal 180 residues of NS3. Helicase and nucleotide triphosphatase, which are essential for HCV replication, reside in the C-terminal 500 residues of NS3 in addition to N-terminal protease. NS4A functions as a cofactor for NS3 serine protease and is required for efficient polyprotein processing. Although the replicative functions of NS4B and NS5A have yet to be identified, both are thought to play a role in viral replication. NS4B, a hydrophobic and membrane-associated protein, may contribute to the formation of the HCV RNA replication complex. NS5A is a phosphorylated protein, and most cell culture-adaptive mutations are located within the NS5A region. NS5B is a RNA-dependent RNA polymerase of HCV. A recently established system, which uses a JFH-1 clone isolated from a Japanese patient with fulminant hepatitis C in order to produce infectious HCV particles in cell culture (5-7), is very useful for examining the

HCV life cycle. Our understanding of HCV RNA replication has also been enhanced by the establishment of a HCV replicon system (8). Subgenomic and genome-length HCV RNA replicates efficiently and stably under selective pressure in the human hepatoma cell line, Huh-7. The nonstructural proteins NS3 through NS5B are necessary and sufficient for HCV RNA replication (8,9). A relationship between viral replication and physiological status of the host cell, in particular its stage of cell proliferation, is known to exist. HCV RNA replicates efficiently in the early logarithmic growth phase, while RNA levels promptly decline when cells reach the stationary phase (10-12). A number of studies have identified host-cell factors involved in HCV RNA replication (13-21). However, the molecular mechanisms underlying the regulation of viral RNA replication through virus-host interactions remain unclear.

To gain further insight into the various host factors involved in the regulation of HCV replication, we established Huh-7 subclones with different HCV replication efficiencies by limiting the dilution and used cDNA microarray analysis to identify differentially-expressing genes among cell clones with different rates of viral RNA replication.

MATERIALS AND METHODS

Cell culture and single cell cloning: Human hepatoma Huh-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich. St. Louis. Mo., USA) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin (Invitrogen, Carlsbad, Calif., USA), and 10% (vol/vol) fetal bovine serum (FBS) (JRH Biosciences, Lenexa, Kans., USA). To obtain Huh7-derived subclones, the cells were diluted to 0.5 cells/well in 96-well plates and grown in complete DMEM as above. Stable cells were selected and four

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clones identified: HuhTe3, HuhTe4, HuhTe6, and HuhTe7.

In vitro transcription, RNA transfection, and selection of G418-resistant cells: The replicon constructs used in this study were kindly provided by Ralf Bartenschlager, University of Heidelberg, Germany. Linearized pFKI389neo/NS3-3'/NK5.1 (22,23) and pFKI389Luci/NS3-3'/NK5.1 (22) with ScaI were used as the template DNA for in vitro RNA transcription (AmpliScribe™ T7 High Yield Transcription Kits; EPICENTRE Biotechnologies, Madison, Wis., USA). The concentrations were determined by measuring the optical density at 260 nm, and RNA integrity was confirmed by agarose gel electrophoresis. Parental and subcloned Huh-7 cells (10^7) were electroporated with 50 μg of RNA in K-PBS (30 mM NaCl, 120 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 5 mM $MgCl_2$, pH 7.9). The electroporation conditions were 975 μFD and 290 mV using a Gene Pulser system (Bio-Rad Laboratories, Hercules, Calif., USA) and a cuvette with a gap width of 0.4 cm (Bio-Rad Laboratories). For the selection of G418-resistant cells, the transfectants were immediately transferred to 10 ml of complete DMEM and seeded into a 10 cm-diameter cell culture dish. After 24 to 48 h, the medium was replaced by complete DMEM supplemented with 0.5 mg/ml of G418.

Luciferase reporter assay: After Huh-7 cells were transfected with the luciferase-replicon, DMEM with 10% FBS was added, and the cell suspension was seeded into 24-well plates. At the time-points specified in the Results section, the cells were washed once with phosphate-buffered saline and then lysed with 400 μl of cell culture lysis reagent (Promega, Madison, Wis., USA). Aliquots of the lysate samples were mixed with luciferase assay reagent (Promega), after which measurements were performed with a luminometer, LUMAT LB9501 (Berthold Technologies, Bad Wilbad, Germany). Assays were performed at least in triplicate.

Determination of cell growth: To examine cell growth, 10^4 cells per well were seeded into 24-well culture plates and harvested daily. Cells from triplicate wells were lysed with 100 μl of cell culture lysis reagent, and viable cell numbers were measured using Celltitre Glo Luminescent Cell Viability Assay (Promega).

Quantitation of HCV RNA: Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Real-time RT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, Calif., USA), as described previously (24-26).

Analysis of gene expression by microarray: Total RNA was isolated from cells using TRIzol reagent and purified using the RNeasy mini kit (Qiagen, Valencia. Calif., USA). The integrity of the RNA was assessed qualitatively by electrophoresis and spectrophotometry using a ratio of A260/A280. Antisense biotinylated cRNA target probes were synthesized from total RNA using GeneChip One-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, Calif., USA), according to the manufacturer's instructions. Probes were individually hybridized to the Human Genome Focus Array (Affymetrix) representing ~8,500 characterized human genes. Arrays were washed and stained with streptavidin-phycoerytrin in Fluidic Station 400 (Affymetrix). and scanned for fluorescence using the GeneChip Scanner 3000 (Affymetrix). Microarray data were processed using GeneChip Operating Software (GCOS; Affymetrix).

RESULTS AND DISCUSSION

Most experiments to date have used Huh-7 cells to examine the expression of HCV replicons. However, it is accepted that the Huh-7 cell line is not homogeneous but rather heterogeneous, as it is inconsistent with regard to the level of HCV RNA replication among the cells maintained in laboratories. To determine whether the Huh-7 cells used in the present experiment also exhibit heterogeneity in terms of HCV replication, a pFKI389neo/NS3-3'/NK5.1 transcript, which is a Con1-derived subgenomic HCV replicon with adaptive mutations, was introduced into Huh-7 cells, after which the transfected cells were grown for 2 weeks under G418 selection. We then isolated 20 of the drug-resistant colonies and quantified HCV RNA in each clone by real-time RT-PCR. As shown in Fig. 1A, the levels of viral RNA varied over a wide range, from 1.7×10^4 to 2.2×10^7 copies/μg total RNA, in cells supporting HCV replication, suggesting that the Huh-7 cells comprised a variety of cell populations with different HCV replication efficiencies. Adaptive mutations of the replicon RNA were unlikely to occur during culture since a highly adapted replicon was used.

To further characterize the diversity of HCV permissiveness among cells, we isolated Huh-7 cell clones using an endpoint dilution technique and stabilized four clones: HuhTe3, HuhTe4, HuhTe6, and HuhTe7. These clones were transfected with the replicon RNA as described above and cultured in the presence of G418. As shown in Fig. 1B, HCV RNA levels were comparable among the four clones one day after transfection, indicating that similar amounts of viral RNA were introduced into each cell clone. Fourteen days after transfection, approximately 10^6 copies of HCV RNA/μg total RNA were found among the HuhTe6 and HuhTe7 transfectants, while less than 10^5 copies of HCV RNA/μg total RNA were observed among HuhTe3- and HuhTe4-derived cells, a relatively small amount compared to parental
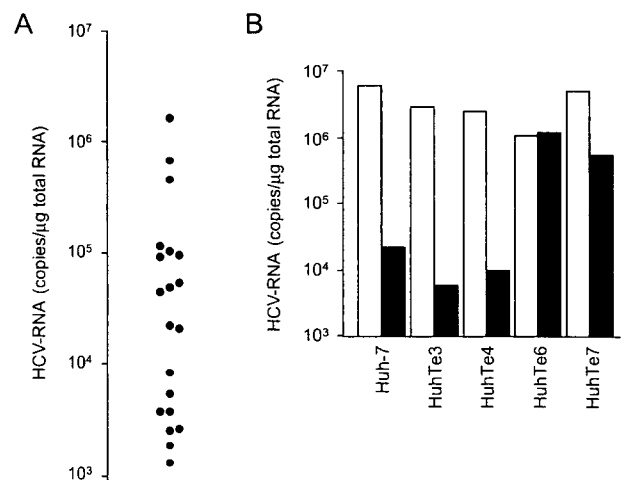

Fig. 1. Copy numbers of HCV RNA after transfection of Huh-7 cells and Huh-7 subclones with subgenomic HCV replicon RNA derived from pFKI389neo/NS3-3'/NK5.1. (A) Transfected Huh-7 cells were subjected to G418 selection. Eighteen colonies were isolated after transfection and cultured in a 96-well plate for 1 week, followed by extraction of total cellular RNA. Dots indicate the copy numbers of HCV RNA per total cellular RNA among individual cell clones, as quantified by real-time RT-PCR. (B) HCV RNA levels in transfected cells (HuhTe3, HuhTe4, HuhTe6, HuhTe8 and parental Huh-7 cells) were determined as described above on day 1 (open bars) and day 14 (closed bars) post-transfection.

Huh-7 cells.

Because the cell growth conditions of HuhTe4 and HuhTe6 were more stable than those of the other two clones after many passages and reproducible results were obtained using HuhTe4 and HuhTe6 cells in the above experiments, the differences in HCV RNA replication efficiencies among HuhTe4 and HuhTe6 cells were further assessed using a luciferase reporter-based transient replication assay enabling sensitive and precise quantification of HCV replication (Fig. 2A). HuhTe4 and HuhTe6 cells were transfected with replicon RNA encoding firefly luciferase as a reporter gene (22) by electroporation. The cells were harvested daily for up to 72 h post-transfection, and luciferase activity was monitored to examine the replication kinetics of replicon RNA in HuhTe4 and HuhTe6 cells. Replicon RNA replicated at low levels in HuhTe4 cells during this time. Conversely, a steady increase in replication was observed in HuhTe6 cells with ~10-fold greater luciferase activity than observed in HuhTe4 cells at 72 h post-transfection. Luciferase activity measured 4 h after transfection was used to verify transfection efficiencies. Thus, two

different replication assays were used to demonstrate that the efficiency of HCV RNA replication in HuhTe6 cells is greater than that in HuhTe4 cells.

HCV replication is related to the physiological state of the host cells, specifically the stage of cell growth (10-12). For example, Huh-7 cells containing the HCV genome carry numerous copies of viral RNA in the early logarithmic growth phase, while RNA levels decline significantly when cells reach the stationary phase. Flow cytometry has shown that synthesis of HCV RNA is specifically stimulated during the S phase of the cell cycle in replicon cells (11). Therefore, HCV replication efficiency might be influenced by the stage of cell growth of Huh7 subclones. We therefore compared the cell growth properties of HuhTe4 and HuhTe6 cells using a cell-based luminescence assay, as described in the Materials and Methods section. As shown in Fig. 2B, both cell clones exhibited similar growth curves with a doubling time of 37- 40 h during the exponential growth phase, as well as similar saturation densities under the specified culture conditions, indicating little to no difference between the cell growth of HuhTe4 and HuhTe6 cells. Thus, the differences in their ability to support HCV RNA replication cannot be due to differences in their cell growth conditions.

To further understand the inherent diversity of these clones and to identify any factor(s) which might play a role in the permissiveness of HCV replication, we looked for cellular genes with different transcript levels in HuhTe6 and HuhTe4 cells using a cDNA microarray system of approximately 8,500 known genes. Hybridization image analysis showed enhanced expression of 17 genes (0.2%) and reduced expression of 19 genes (0.2%) in HuhTe6 cells compared with HuhTe4 cells, when the 2-fold change in the signal intensity with statistical significance is considered to be a difference limit for RNA expression. The genes that are up-regulated in HuhTe6 cells are listed in Table 1. Among the genes implicated in a variety of functional categories, some of the genes related to signal transduction and/or cell communication may be fascinating. Thioredoxin-interacting protein is an endogeneous inhibitor of thioredoxin. The thioredoxin system is a ubiquitous thiol oxidoreductase system that regulates cellular reduction/ oxidation status. Thioredoxin-interacting protein negatively regulates thioredoxin activity and affects cellular redox status (27). A complex relationship between HCV replication and redox signaling has been revealed. HCV infection is associated with elevated circulating reactive oxygen species (ROS) in patients (28,29), while viral gene expression in cultured cells increases ROS levels through calcium signaling (30). Conversely, biologically relevant concentrations of ROS may suppress HCV RNA replication in Huh-7 cells (31). Rab6B is a member of the Rab subfamily of small GTPases, which plays an important role in the regulation of intracellular transport routes. Rab6B, which mainly localizes at the Golgi apparatus and at ERGIC-53-positive vesicles, may enable retrograde membrane traffic at the level of the Golgi complex (32). Golgi complex-derived lipid raft or membranous webs are known to contain the HCV replication complex (33-35). This raises the possibility that Rab6B-associated intracellular transport might be involved in the assembly and formation of the HCV replication complex. Transglutaminase 2 is a multifunctional protein involved in a range of cellular processes. It has two well-characterized activities: GTP-mediated receptor-stimulated signaling, and calcium-activated transamidation or cross-linking, which is inhibited by GTP (36). Transglutaminase influences the HCV life cycle through
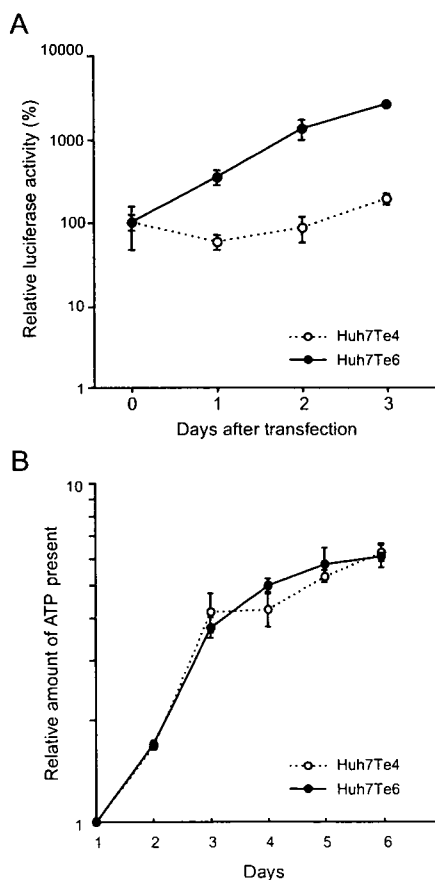

Fig. 2. Cell growth of HuhTe4 and HuhTe6 cells, and transient replication of the HCV replicon. (A) Transient replication of the HCV replicon carrying a luciferase gene in HuhTe4 (open circles) and HuhTe6 (closed circles) cells. Replicon RNA was transfected into the cells by electroporation. Luciferase activity within the cell lysate samples was determined and expressed as a percentage of the relative light units measured at 24, 48, and 72 h, compared to 4 h post-transfection. Each data point indicates the mean and standard deviation of triplicate results. (B) Cell growth of HuhTe4 (open circles) and HuhTe6 (closed circles) cells. Cell growth rates were determined at the indicated time points and are presented as relative values compared to day 1. Each data point indicates the mean and standard deviation of triplicate results.

Table 1. Genes with increased levels of expression in HuhTe6 cells compared to HuhTe4 cells

Functional category/Gene name	Accession no.	Fold change
Signal transduction/cell communication		
sema domain, immunoglobulin domain, short basic domain, secreted, 3G	NM_020163	8.6
thioredoxin interacting protein	NM_006472	8.0
stathmin-like 4	NM_030795	5.7
RAB6B, member RAS oncogene family	AW118072	4.0
erythropoietin receptor	X97671	2.6
Metabolism		
sulfotransferase family 1E, estrogen-preferring, member 1	NM_005420	13.9
NADPH oxidase 1	NM_007052	6.1
transglutaminase 2	AL031651	2.1
Transcription		
v-rel reticuloendotheliosis viral oncogene homolog	NM_002908	9.8
Zic family member 3 heterotaxy 1	NM_003413	9.2
neurogenic differentiation 2	AB021742	4.3
Transport		
solute carrier family 10, member 1	NM_003049	2.0
solute carrier organic anion transporter family, member 1C1	NM_017435	2.0
Apoptosis		
phorbol-12-myristate-13-acetate-induced protein 1	NM_021127	16.0
Immune response		
triggering receptor expressed on myeloid cells 1	NM_018643	2.1
Unknown		
similar to RNA binding motif protein, Y chromosome, family 2 member B	NM_005405	9.2
similar to chymotrypsinogen B precursor	NM_001906	4.0

Table 2. Genes with decreased levels of expression in HuhTe6 cells compared to HuhTe4 cells

Functional category/Gene name	Accession no.	Fold change
Signal transduction/cell comminication		
purinergic receptor P2Y, G-protein coupled, 2	BC012104	7.5
activin A receptor type II-like 1	BC042637	4.9
RAB3B, member RAS oncogene family	NM_002867	3.2
adenomatosis polyposis coli	BC111591	2.6
HUS1 checkpoint homolog (S. pombe)	BT019482	2.3
progesterone receptor membrane component 2	DQ496105	2.0
Metabolism		
UDP glycosyltransferase 8 (UDP-galactose ceramide galactosyltransferase)	NM_003360	4.9
bone morphogenetic protein 1	BC101765	2.3
Transcription		
general transcription factor IIH, polypeptide 3, 34 kDa	NM_001516	2.8
Translation		
eukaryotic translation initiation factor 5A2	NM_020390	4.0
Transport		
Rh-associated glycoprotein	NM_000324	7.5
sorting nexin 16	BC0336301	7.5
solute carrier family 7, member 2	BC10490	2.0
Immune response		
GLI pathogenesis-related 1	NM_006851	7.5
Unkown		
neuronal thread protein AD7c-NTP	AF10144	7.5
elastase 2B	BC069412	6.5
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	NM_020142	3.5
ring finger protein 17	BC064847	2.8
hypothetical protein LOC440345	XR_015786	2.3

post-translational modification of the viral core protein (37), and induction of hepatic fibrosis as a result of HCV infection (38).

Genes down-regulated in HuhTe6 cells, compared to HuhTe4 cells, are listed in Table 2. Of interest is the differential expression of sorting nexin 16 and UDP-galactose:ceramide glycosyltransferase among the two cell lines. Sorting nexins are a family of cytoplasmic and membrane-associated pro-

teins that mediate the intracellular trafficking of plasma membrane receptors, such as the epidermal growth factor (EGF) receptor. Sorting nexin 16 is associated with the EGF receptor and accelerates EGF-induced EGF receptor down-regulation (39). It has been shown that HCV NS5A protein, as well as the viral replicon, inhibit EGF-stimulated activation of the Ras-ERK activated protein kinase pathway (40-42). Since signaling pathways downstream from the EGF receptor are known to regulate a variety of cellular processes, thereby influencing cell survival, cytoskeletal rearrangement, vesicular trafficking, and protein synthesis, any perturbation of the events prior to the activation of the EGF receptor may influence the cellular environment to favor HCV replication and persistence. UDP-galactose:ceramide glycosyltransferase mediates galactosylation of glycosphingolipids associated with the Golgi UDP-galactose transporter. Recent studies based on the chemical biological approach have demonstrated the physiological significance of the lipid raft and sphingolipid biosynthesis pathways in HCV RNA replication (14,24,43,44). Altered processing of glycosphingolipids may directly or indirectly affect the HCV life cycle, presumably through modulation of lipid-rich microdomains containing the viral replication complex.

In the present study, we isolated four subclones derived from parental Huh-7 cells with a range of HCV RNA replication efficiencies. Among the four subclones, HuhTe4 and HuhTe6 demonstrated similar cell growth, however the efficiency of HCV RNA replication in HuhTe6 cells was significantly greater than that in HuhTe4 cells. The fact that these subclones share a common origin enables us to explore the differences that result in their different HCV replication efficiencies. cDNA microarray analysis showed significant differences in the transcript levels of 36 genes among HuhTe4 and HuhTe6 cells. Detailed analysis of the correlation between the expression of the candidate genes identified and HCV replication as well as studies to determine the role of these genes in HCV RNA replication are underway. This approach will enable us to expand our research, thereby improving our understanding of the regulatory mechanisms underlying HCV replication.

ACKNOWLEDGMENTS

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Dynamic behavior of hepatitis C virus quasispecies in a long-term culture of the three-dimensional radial-flow bioreactor system

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Abstract

Hepatitis C virus (HCV) exists in infected individuals as quasispecies, usually consisting of a dominant viral isolate and a variable mixture of related, yet genetically distinct, variants. A prior HCV infection system was developed using human hepatocellular carcinoma cells cultured in the three-dimensional radial-flow bioreactor (RFB), in which the cells retain morphological appearance and their differentiated hepatocyte functions for an extended period of time. This report studies the selection and alteration of the viral quasispecies in the RFB system inoculated with pooled serum derived from HCV carriers. Monitoring the viral RNA and core protein in the culture supernatants, together with nucleotide sequencing of hypervariable region 1 of the HCV genome, demonstrated that (1) the virus production intermittently fluctuated in the cultures, (2) the viral genetic diversity was markedly reduced 3 days post-infection (p.i.), and (3) dominant species changed on days 19–33 p.i., suggesting that the virus populations can be selected according to susceptibility to the viral infection and replication. A therapeutic effect of interferon-α also demonstrated the inhibition of HCV expression. Thus, this HCV infection model in the RFB system should be useful for investigating the dynamic behavior of HCV quasispecies in cultured cells and evaluating anti-HCV compounds.
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1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990) and has been estimated to infect more than 170 million people throughout the world (Poynard et al., 2003). Symptoms of persistent HCV infection extend from chronic hepatitis to cirrhosis and ultimately hepatocellular carcinoma (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990). HCV belongs to the genus *Hepacivirus*, included in the family of Flaviviridae, and possesses a viral genome of a single, positive-stranded RNA with a nucleotide (nt) length of approximately 9.6 kb (Choo et al., 1991; Grakoui et al., 1993; Hijikata et al., 1991). It has been shown that HCV, like many other RNA viruses, circulates within infected individuals as a diverse population and closely related variants are referred to as quasispecies (Martell et al., 1992). This quasispecies model of mixed virus populations may imply a significant survival advantage because the simultaneous presence of multiple variant genomes and/or high rate of generation of new variants allow rapid selection of the mutants are better suited to new environmental conditions (Pawlotsky, 2006).

Studies on HCV replication and development of selective antiviral drugs have been hampered primarily by the lack of efficient cell culture systems. Establishment of selectable dicistronic HCV RNAs that are capable of autonomous replication to high levels in human hepatoma Huh-7 cells was a

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significant breakthrough in HCV research; however, virus production has not been observed in the conventional monolayer cultures (Blight et al., 2000; Lohmann et al., 1999). Recently, it has been described that infectious HCV particles are efficiently produced from a genotype 2a isolate JFH-1 in Huh-7 cells (Blight et al., 2000; Wakita et al., 2005; Zhong et al., 2005). This JFH-1 based HCV culture system is an invaluable achievement permitting a variety of studies on the complete HCV life cycle. However, HCV infection systems with human sera or plasmas containing intact virions are still limited because of low levels of propagation in the cultures. Reverse transcription (RT)-PCR was typically used to detect the viral RNA in cell extracts; however, synthesized viral proteins were not observed in these systems (Ikeda et al., 1998; Tagawa et al., 1995).

There are reports of differentiated human hepatoma FLC4 (functional liver cell 4) cells grown in a three-dimensional (3D) radial-flow bioreactor (RFB) that can be infected by HCV-positive serum and support viral replication (Aizaki et al., 2003). Furthermore, production and release of infectious HCV has been observed in the RFB system following transfection of FLC4 cells with *in vitro* transcribed HCV genomic RNA, as well as in a 3D system using Huh-7 cells harboring genome-length dicistronic RNAs (Murakami et al., 2006). The RFB system, in which the bioreactor column consists of a cylindrical matrix with porous bead microcarriers extended vertically, was aimed initially at developing artificial liver tissues and allows liver-derived cells to maintain morphological appearance as well as their physiological functions, such as the ability to synthesize albumin and drug-metabolizing activity mediated by cytochrome P450 (Iwahori et al., 2003). The radial-flow configuration permits full contact between culture medium and cells at a physiologic perfusion flow rate, and prevents excessive shear stresses and buildup of waste products, thus ensuring the long-term viability of 3D cell culture.

The aim of the present study was to characterize HCV dynamics in the RFB system during long-term cultures inoculated with pooled serum obtained from HCV carriers, and to examine the therapeutic effects of interferon-alpha (IFN-α) in this HCV infection model.

2. Materials and methods

2.1. Cell cultures

FLC4 cells (Aoki et al., 1998), which were derived from human hepatocellular carcinoma cells and negative for HCV RNA and HBV DNA, were maintained in serum-free ASF104 medium (Ajinomoto, Japan) supplemented with 4 g/L D-glucose on the collagen-coated dishes before inoculating into the RFB column. The RFB system (ABLE, Japan) was manipulated as described previously (Aizaki et al., 2003) with minor modifications. Briefly, RFB columns, which have bed volumes of 30 or 4 mL and are filled with porous glass microcarriers (diameter 0.6 mm, vacant capacity 50%, pore size <120 μm) (Hongo et al., 2005), were seeded with FLC4 cells, which subsequently attached to the surface and inside of porous glass beads. ASF104 medium containing 2% fetal calf serum was added at a flow rate

of 50 mL/day, and the culture condition was automatically controlled by monitoring temperature, pH value and oxygen levels in the vessel throughout the duration of the study.

2.2. Infection of HCV-positive sera

HCV antibody-positive sera used in this study were blood donor samples supplied by The Japanese Red Cross Center, Tokyo, Japan. HCV RNA loads in the sera were as follows: serum A, 2.4×10^6 copies/mL; serum B, 8.6×10^6 copies/mL; serum C, 5.9×10^6 copies/mL; serum D, 2.5×10^6 copies/mL; serum E, 1.0×10^7 copies/mL; serum F, 1.4×10^7 copies/mL (Table 1). In the first experiment (Fig. 3), aliquots of each serum containing 2×10^6 copies of HCV RNA were mixed and pooled serum sample with 1.2×10^7 copies was prepared as an inoculum. The pooled serum (2.5 mL) was added to the 3D cultured-FLC4 cells in the 30-mL RFB column, and the culture medium was changed after 12 h of incubation. At various times during the culture period, culture medium (50 mL) was collected to determine HCV RNA and the core protein. Collected culture media were passed through a 0.20-μm filter to remove the debris, and stored at −80 °C. In the second experiment to evaluate a therapeutic effect of anti-HCV drug (Fig. 4), 4-mL RFB columns were used. IFN-α (Sumiferon 300; Sumitomo Pharmaceuticals, Japan) was added to one of two columns at a final concentration of 100 IU/mL after the infection. Culture medium was periodically collected for determination of HCV RNA, the core protein and transaminases, and was replaced with the same volume of fresh medium with or without IFN-α.

2.3. Quantitation of HCV RNA and core protein

HCV RNA was extracted from 140 μL of each serum or culture medium using QIAamp Viral RNA Mini spin column (QIAGEN); RNA was eluted in 60 μL of water and stored at −80 °C. Real-time RT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems), as described previously (Aizaki et al., 2003; Suzuki et al., 2005). The viral core antigen in the culture medium was quantified by immunoassay (Ortho HCV-Core ELISA Kit; Ortho-Clinical Diagnotics), according to the manufacturer's instruction (Murakami et al., 2006).

2.4. PCR amplification and nucleotide sequencing of HVR1 domain and its flanking region

Five microliters of RNA samples prepared as above were reverse transcribed using SuperScript II (Invitrogen) and a specific primer 5′-CATCCATGTGCAGCCGAACC-3′ (corresponding to nucleotides [nt] 2006–1987 of HCV NIHJ1) (Aizaki et al., 1998). For the nested PCR, a genotype-independent set of primers specific for hypervariable region 1 (HVR1). The first round of PCR was performed with the outer sense primer 5′-GCATGGCTTGGGATATGATG-3′ (nt 1291–1310) and with the reverse transcription primer described above as the outer antisense primer. After the initial 3.5-min denaturation step at 94 °C, 35 PCR cycles, with each cycle

Table 1
HCV-positive sera used in this study

Serum	Clone	HCV HVR1 sequence	% in the serum	genotype
A	A1	KVLI VMLS FAGV DGSTRTIG GRTA HTTQGSAS LFS SGPAQKIQLINTNGS	75	1
	A2	-------L------N-H-V--AV-SS---FT---KL----------S---	12.5	
	A3	-------L------N-YAS---AGLL-R-V--I-TA---------S---	12.5	
B	B1	KVVV ILLLAAGV DAGTNTIG GSAAQTTS GFTG LFR SGARQNIQLINTNGS	50	2
	B2	--R	12.5	
	B3	--S---------	12.5	
	B4	--L-V---F-----E-HVT--N-GR--A-LV--LTP--K----------	12.5	
	B5	--I---	12.5	
C	C1	KVLI VMLL FAGV DGDT HVSGGTQGRAAY GLAS LFALGPTQKIQLVNTNGS	83.3	1
	C2	-------------------------------------A----------	16.7	
D	D1	KVLI VMLL FAGV DGVT HTSG AAAG HNAR SLSG LFS LGSAQKLQLINTNGS	40	1
	D2	--------------A-Y---GT--Y-TKTFT-F--R-PS--I--------	20	
	D3	------------------T--Y---T-T----P--------V-----	10	
	D4	--------------------V---T----P--------V-----	10	
	D5	--V-----	10	
	D6	---------------------Y-T--FT----S-----I--V-----	10	
E	E1	KVLI VMLL FAGV DGSTRVSG GQAG RVTK SLAS FFS PGPQQKIQLVNSNGS	40	1
	E2	------------------------------HGFT-L--A-S-----------	30	
	E3	-------------------------------QGFT-L--A-S-----------	10	
	E4	--------------------------------S-FT-L-TV------------	10	
	E5	---------------N-Y---------AH--T-L--A-S-----------	10	
F	F1	KVLI VMLL FAGV DGETNVMGGRAG HTTN TFTS LFS VGPAQKIQLVNSNGS	37	1
	F2	--------------D-K----------S-L----N---S-----------	27	
	F3	----------------K----Q-----S-L----N---S-----------	18	
	F4	--------------------A-------A--TK--------D----	9	
	F5	--------------G--------A--A--L----TR--S----------	9	

consisting of 1 min at 94 °C, 2 min at 45 °C, and 3 min at 72 °C, were carried out, followed by a 10-min extension step at 72 °C. The second round was performed with the inner sense primer 5'-GGTAAGCTTTCCATGGTGGGGAACTGGGC-3' (nt 1419–1447) and the inner antisense primer 5'-CTGGAATTCGCAGTCCTGTTGATGTGCCA-3' (nt 1627–1599). The amplified products were cloned into the pGEM-T vector (Promega), and at least 8 independent clones were sequenced with an automatic DNA sequencer (ABI PRISM 310, PE Applied Biosystems).

3. Results

3.1. The outline of the RFB system

The RFB system was initially aimed at developing artificial liver tissues and allows liver-derived cells to maintain morphological appearance as well as their physiological functions, such as the ability to synthesize albumin and drug-metabolizing activity mediated by cytochrome P450 (Iwahori et al., 2003). Fig. 1 shows the outline of the RFB system. The bioreactor column consists of a vertically extended cylindrical matrix with porous glass microcarriers, which were most suitable for FLC4 culture as described in Section 2. The conditioning vessel is connected to a circulation system including tanks either for supplying fresh medium or for recovering sample aliquots. Oxygen consump-

tion, temperature and pH of the culture medium are monitored continuously and conditioned in the vessel by computer and mass flow controller throughout the culture. Thus, the radial-flow configuration permits full contact between culture medium and cells at a physiologic perfusion flow rate, and prevents excessive shear stresses and a buildup of waste products, thus ensuring the long-term viability of 3D culture. For the long-term culture up to 110 days, temperature in the vessel gradually decreased from 37 to 30 °C as shown in Fig. 2A. The oxygen consumption, which indicates the cell growth condition, increased slowly from days 0 to 80 post-inoculation of the cells, and maintained a constant level afterwards. Under this condition, the production rate of albumin was found to be stable from days 15 to 105. The following experiments of HCV infection were done in such a stable phase of the cell condition after 3 weeks of pre-culture. Cell grown in the RFB column reached confluence at the end of culture (day 110) since the cells were observed outside the matrix bed (Fig. 2B).

3.2. Infection of HCV-positive sera to RFB cultured FLC4 cells

Previously, HCV RNA could be detected in FLC4 cells grown in the RFB up to 4 weeks of culture following inoculation with an HCV carrier plasmid (Aizaki et al., 2003). Establishment of a long-term stable culture system of human liver-derived cells
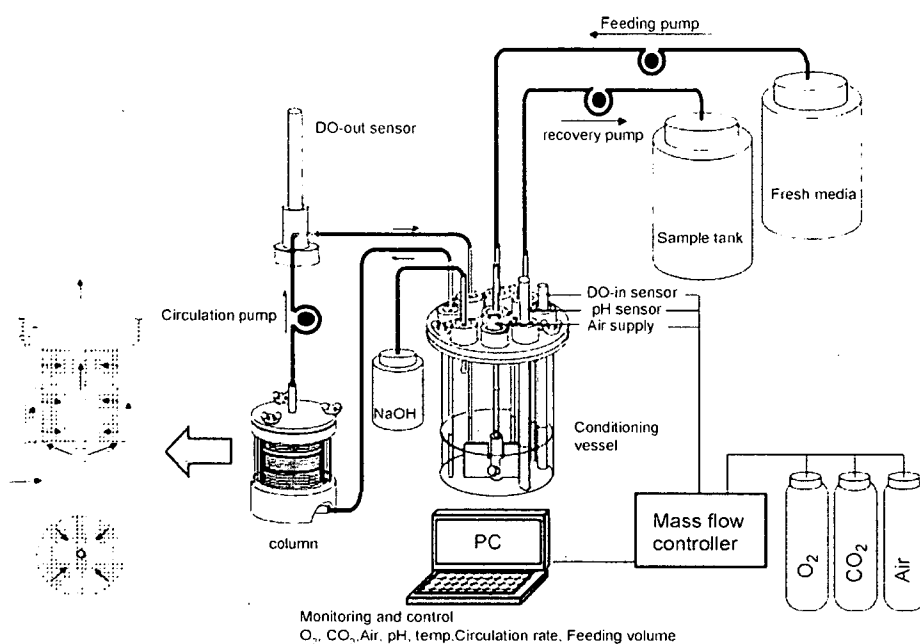
Fig. 1. Outline of the RFB system. RFB system consists of vessel, column and PC monitoring system. Culture condition was automatically controlled: oxgen concentration, temperature, pH, and oxgen level in the conditioning vessel are continuously monitored by PC and conditioned by mass flow controller.

retaining their differentiated hepatocyte function, as described above, enables evaluations of dynamic analysis of HCV replication and selection of viral variability and quasispecies. The potential of this culture system for screening HCV-positive sera was well suited for the viral infection.

Table 1 shows the serum samples (A–F) from six HCV carriers. The nucleotide complexity of HCV in serum samples was determined by sequencing the 1449–1598 nt region of the HCV genome, which includes HVR1 located at the N-terminal region of E2. Each serum was a mixture of a dominant HCV clone and related but distinct viral populations. The dominant species in

sera A, C, D, E, and F were found to be genotype 1, and that in serum B was genotype 2. Viral loads in A–F, respectively, were 2.4×10^6, 8.6×10^6, 5.9×10^6, 2.5×10^6, 1.0×10^7 and 1.4×10^7 copies/mL, which were determined by real-time RT-PCR, as previously described (Aizaki et al., 2003; Suzuki et al., 2005). HCV loads of 2×10^6 copies from each serum sample were mixed to prepare a pooled serum sample containing 1.2×10^7 copies of HCV RNA. After FLC4 cells were inoculated into the RFB and subjected to 2 weeks of pre-culture for the preparation of 3D culture, the cells were infected with the pooled serum. Cell number at infection was about 10^8 in the 30-
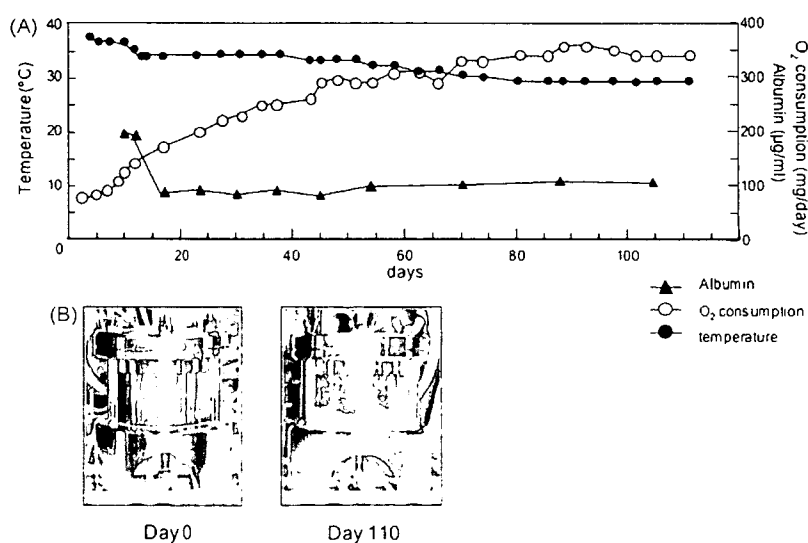

Fig. 2. Long-term culture of FLC4 cells in the RFB system. (A) Long-term culture of FLC4 cells in the RFB system. Temperature (closed circles) was gradually decreased from 37 to 30 °C. Oxygen consumption (open circles) was gradually increased from days 0 to 80 and reached the steady-state level. Albumin concentration (closed triangles) was constant from days 15 to 105. (B) The appearance of the RFB column at the beginning (day 0) and at the end (day 110) of culture.