

**Fig. 6.** Comparison of infectivity of each mutant by immunofluorescence. (a) Huh-7 cells were electroporated with RNA transcripts of JFH-1, JFH-1/N6, JFH-1/CS and JFH-1/CS-N6. Transfected cells were grown on coverslips. After 3 days, the cells were fixed and processed for double-label immunofluorescence for capsid protein (green) and nuclei (blue, Hoescht). (b) Naïve Huh-7 cells seeded on coverslips were infected with the supernatant obtained at 3 days post-transfection, and then fixed and processed at 3 days post-infection for double-label immunofluorescence for capsid protein (green) and nuclei (blue, Hoescht).

cells (Fig. 6b). The profile of E2 produced by each virus was also analysed before and after treatment with PNGase F and was consistent with predicted results (Fig. 3b). Our results suggest that the JFH-1/CS-N6 virus expands more rapidly and reaches higher titres than the JFH-1, JFH-1/N6 and JFH-1/CS viruses (Fig. 6b and Table 1). As for JFH-1/CS, no differences were observed in the intracellular distribution of the capsid protein and E2 glycoprotein of JFH-1/CS-N6 (data not shown). Furthermore, we also observed that after a single round of amplification the JFH-1/CS-N6 virus displayed an accelerated cytopathic effect, which was faster than what we observed for the JFH-1/CS virus (data not shown). These data suggested that the combined mutations located in the capsid- and E2-coding sequences resulted in an enhanced virus infectivity.

## DISCUSSION

Although a low efficiency of infection has been detected by transfection of Huh-7 cells with the RNA generated from a genomic JFH-1 viral clone, this discovery has been a major breakthrough in HCV research (Wakita *et al.*, 2005). Different groups have developed robust cell culture systems for the propagation of HCV, and the data led to the conclusions that the Huh-7 cell culture is important for the propagation of the virus and that each Huh-7 cell line can display different permissiveness to the virus. In this study, we established a strong production of infectious HCV particles by using successive infections of naïve Huh-7 cells or by introducing specific mutations into the JFH-1 genome.

Contrary to what was observed in other groups (Lindenbach *et al.*, 2005; Zhong *et al.*, 2005), the robust production of HCVcc in our Huh-7 cell line was only obtained after several successive infections. Initially, the JFH-1 virus released into the culture medium after the first

transfection was low, which was consistent with a previous report (Wakita *et al.*, 2005). It is worth noting that several passages of transfected cells did not change the viral titre. However, after repeated infections of naïve Huh-7 cells, analyses of HCV RNA in the medium of infected cells revealed an increase in the particle release, which was correlated with a higher titre of infectious virus. The direct sequencing of HCV RNA genome was used to determine the major modifications appearing in JFH-1. Surprisingly, the only coding mutation identified was an Asn to Lys mutation located at aa 534 (N534K) in E2. This modification, which is characterized as preventing the addition of an *N*-glycan at the E2 glycosylation site 6, may favour the interaction between HCV E2 glycoprotein and a cellular receptor. Indeed, the introduction of this mutation in JFH-1 leads to a higher infectious titre after only two successive infections. This suggests that the particles produced by JFH-1/N6 are more effective than those produced by JFH-1 for the reinfection of Huh-7 cells. This is also true when the mutation N534K is combined with the modifications FP→CS at positions 172 and 173 in JFH-1 (Fig. 6b). Consequently these mutations might lead to a better viral expansion on Huh-7 cells. There is some evidence that CD81 is essential for the entry of HCVpp (Hsu *et al.*, 2003; Lavillette *et al.*, 2005) or HCVcc (Lindenbach *et al.*, 2005; Zhong *et al.*, 2005) into hepatoma cells via an interaction with the HCV E2 glycoprotein (Pileri *et al.*, 1998). Residues critical for the CD81 binding have been identified in the HCV glycoprotein E2. These residues are located at positions 420, 437, 438, 441, 442, 529, 530 and 535 of E2 glycoprotein (Drummer *et al.*, 2006; Owsianka *et al.*, 2006). Moreover, replacement of Thr at the E2 glycosylation site 6 results in moderately increased CD81 binding (Owsianka *et al.*, 2006). This is consistent with the hypothesis that the loss of the *N*-linked E2 glycosylation site 6 favours a better exposure of E2 to the E2-binding site of CD81 and then the JFH-1 reinfection. In a similar way, Zhong *et al.*, (2006)

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 HVRI 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.

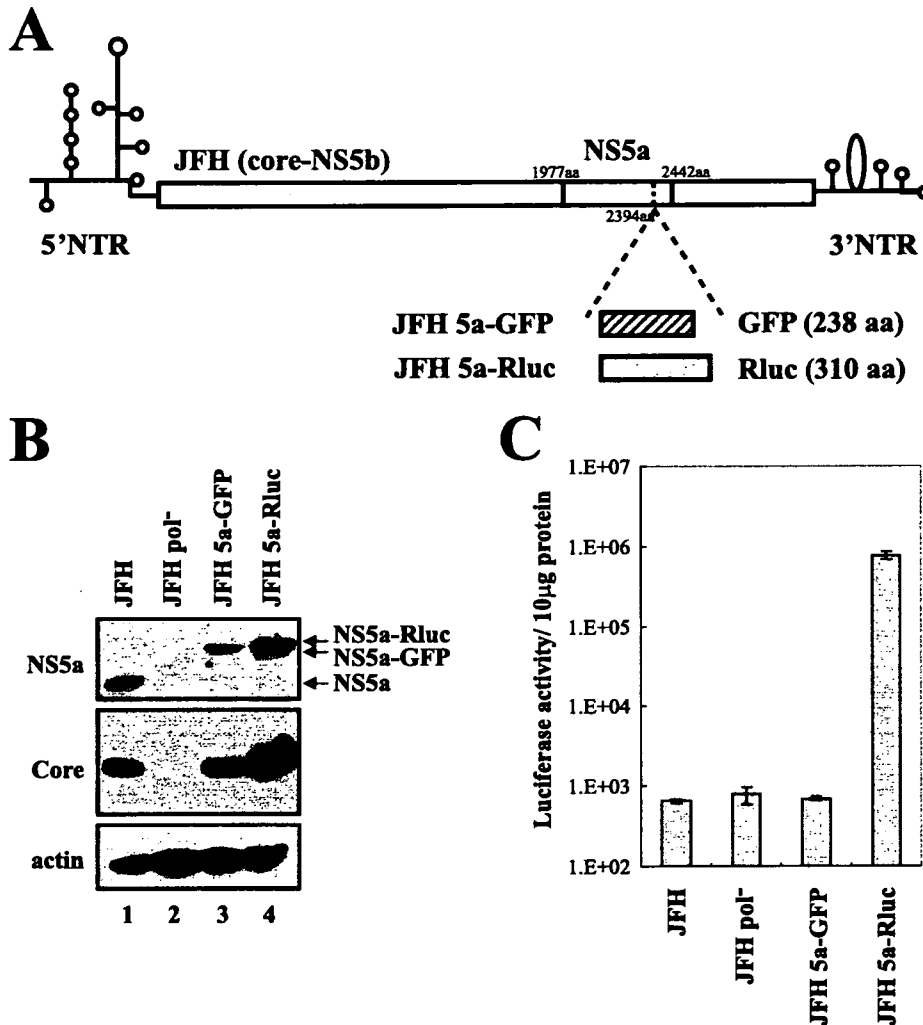
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**FIG. 1.** Generation of infectious HCV containing GFP or Rluc fused with NS5a. (A) Schematic diagrams of JFH and its derivatives containing the GFP or Rluc gene within the NS5a gene. NTR, nontranslated region; aa, amino acids. (B) Western blot analysis of Huh 7.5.1 cells transfected with JFH, JFH Pol<sup>-</sup>, JFH 5a-GFP, or JFH 5a-Rluc RNA. Protein levels were analyzed by Western blotting with anti-NS5a, anticore, or antiactin antibodies. (C) The expression of NS5a-Rluc was monitored by measuring luciferase activity in the cells transfected with JFH, JFH Pol<sup>-</sup>, JFH 5a-GFP, or JFH 5a-Rluc RNA. The luciferase activities in the cells were normalized by the protein concentrations determined by the Bradford assay. Experiments were performed three times. Means and standard deviations are shown.

GFP, or JFH 5a-Rluc RNAs (Fig. 2A). Three days after transfection, cells were fixed and fluorescence microscopy was performed as described previously (8). Core protein was visible in a ring-like pattern around lipid droplets in the JFH RNA-transfected cells (Fig. 2A, panel a; see Fig. S1A at [http://www.postech.ac.kr/dept/life/mv1/figure\\_s1.pdf](http://www.postech.ac.kr/dept/life/mv1/figure_s1.pdf)), as previously reported (14). The same patterns of localization of core protein were observed in the cells transfected with JFH 5a-GFP and JFH 5a-Rluc RNAs (compare panels d and g with panel a in Fig. 2A). No signal was detected by the antibody against core protein in cells transfected with JFH Pol<sup>-</sup> RNA (data not shown). Huh 7.5.1 cells transfected with JFH 5a-GFP RNA displayed bright punctate patterns in the cytoplasm (Fig. 2A, panel e). This 5a-GFP fluorescence was perfectly colocalized with the signal of NS5a displayed by an anti-NS5 monoclonal antibody (AUSTRAL Biologicals) (Fig. 2B). Similar cytoplas-

mic punctate patterns of NS5a protein were observed in the cells transfected with JFH and JFH 5a-Rluc RNAs (compare panels a and b in Fig. S1B at [http://www.postech.ac.kr/dept/life/mv1/figure\\_s1.pdf](http://www.postech.ac.kr/dept/life/mv1/figure_s1.pdf) with panel a in Fig. 2B). GFP fluorescence was not detected in cells transfected with other constructs (Fig. 2A, panels b and h). A partial colocalization of core and NS5a-GFP, shown as yellow signals, was observed when the core and NS5a-GFP images were merged (Fig. 2A, panel f). The pattern of colocalization of core and NS5a proteins was similar to that of core and NS3 proteins reported by Rouille et al. (14).

Next, we assessed the ability of the reporter constructs to release infectious virion particles and whether it was possible to quantify their infectivity using fluorescence microscopy and a luciferase assay. We transfected the JFH, JFH Pol<sup>-</sup>, JFH 5a-GFP, and JFH 5a-Rluc RNAs into Huh 7.5.1 cells and then harvested the culture supernatants at 8 days after transfection.

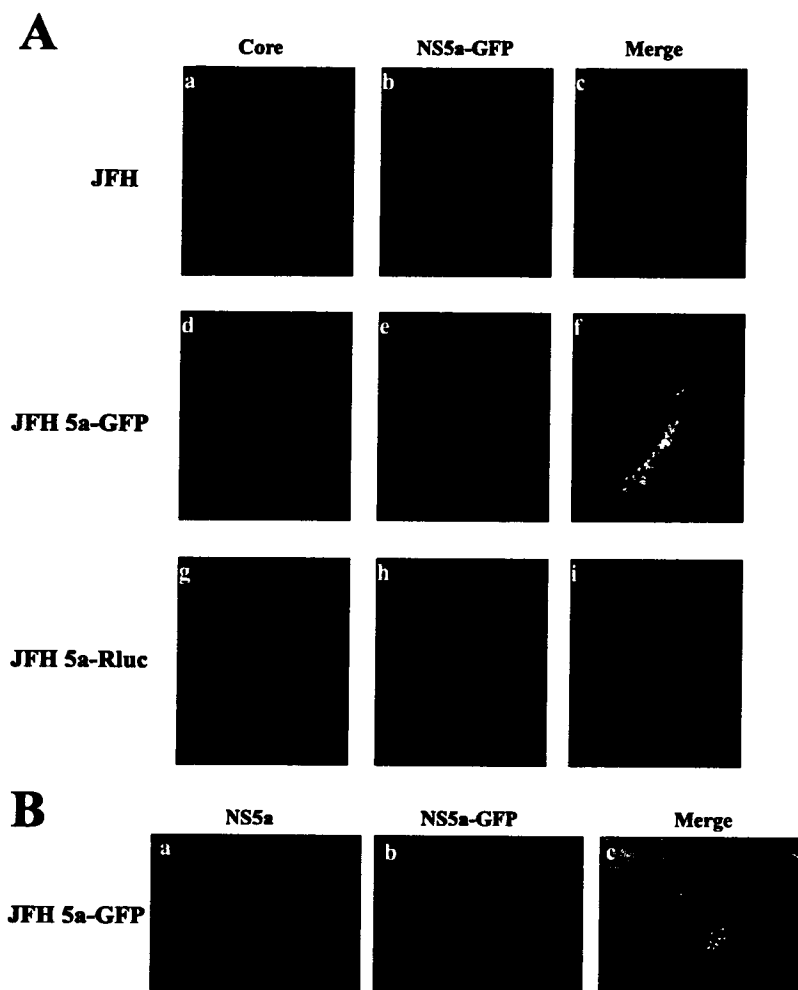


FIG. 2. Subcellular localizations of core and 5a-GFP proteins. (A) Huh7.5.1 cells transfected with JFH, JFH 5a-GFP, and JFH 5a-Rluc RNAs were grown on coverslips for 3 days. Cells were fixed, permeabilized, and treated with anticore monoclonal antibody (Affinity Bioreagents) and secondary antibody (tetramethyl rhodamine isocyanate-conjugated donkey anti-mouse immunoglobulin G). The localization patterns of core and NS5a-GFP are shown in red and green, respectively. The merged images are also shown. (B) Following transfection of Huh 7.5.1 cells with JFH 5a-GFP RNA, immunocytochemistry was performed with an anti-NS5 antibody (AUSTRAL Biologicals) and a tetramethyl rhodamine isocyanate-conjugated donkey anti-mouse immunoglobulin G. The NS5a signal is shown in red, and the NS5a-GFP signal is shown in green. The merged image is also shown.

Infection was performed as described previously by using medium (100  $\mu$ l) of the transfected cell culture (16). At 3 days after infection, total cellular RNA was isolated from infected cells and the level of HCV RNA was measured by quantitative real-time PCR (Fig. 3A). Similar levels of HCV RNAs were detected in cells infected with JFH, JFH 5a-GFP, and JFH 5a-Rluc viruses (Fig. 3A, lanes 1, 3, and 4, respectively). By contrast, HCV RNA was not detectable in cells infected with culture supernatant obtained from cells transfected with JFH Pol<sup>-</sup> RNA (Fig. 3A, lane 2). Luciferase activity was detected in cells inoculated with culture supernatant containing JFH 5a-Rluc virus and increased for up to 3 days postinfection (Fig. 3B).

Infectivity of HCV derivatives was also shown by fluorescence microscopy. Naïve Huh 7.5.1 cells were inoculated with culture supernatants, and productive infections were demonstrated by an immunocytochemical method using an antibody

against HCV core (Fig. 3C). As shown in Fig. 3C, infection was readily detectable with JFH, JFH 5a-GFP, and JFH 5a-Rluc viruses (Fig. 3C, panels a, c, and d), whereas no core-expressing cells were found after inoculation with the polymerase mutant (JFH Pol<sup>-</sup>) (Fig. 3C, panel b). Moreover, in the same core-expressing cells, 5a-GFP fluorescence was observed after inoculation with JFH 5a-GFP virus (Fig. 3C, panel g).

To determine the infectivity of the JFH, JFH 5a-GFP, and JFH 5a-Rluc viruses, plaque assays were performed by the method used in determination of the infectivity of bovine viral diarrhea virus (4). After incubation of viral stocks at 37°C for 3 h, the Huh 7.5.1 cells were overlaid with semisolid medium containing gum tragacanth (Sigma). At 5 days after infection, virus-infected cells were visualized by immunostaining with NS5a-specific antibody (AUSTRAL Biologicals) as described previously (11) (Fig. 3D). Viral titers in the medium on Huh 7.5.1 cells transfected with RNAs of HCV variants are depicted

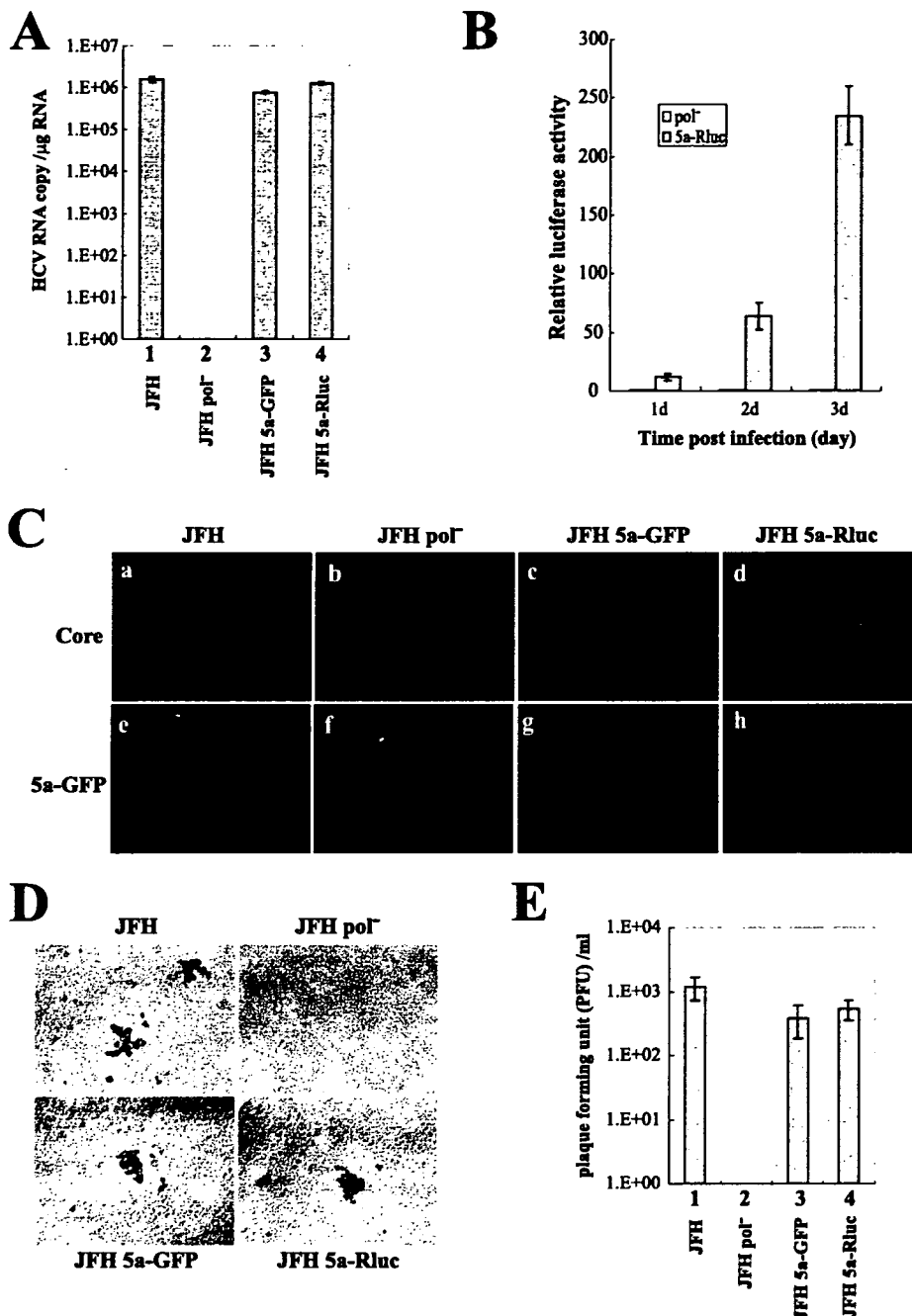


FIG. 3. Infectivity of assayable viruses. (A) Cell-free culture fluids were collected 8 days after transfection. Supernatants were used to inoculate naïve Huh 7.5.1 cells. Total RNAs were isolated from infected cells, and the levels of HCV RNA were measured by real-time reverse transcription-PCR. The levels of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA were used as internal mRNA controls. The HCV RNA levels shown are the copy number per 1  $\mu$ g of cellular RNA. Experiments were performed three times, and the values are depicted as described for Fig. 1. (B) Huh 7.5.1 cells were infected with JFH 5a-Rluc virus for 3 days. Each day, cells were harvested and luciferase activities were measured. Luciferase activities were normalized to those obtained from cells inoculated with culture supernatants of cells transfected with JFH Pol<sup>-</sup> RNA, which were set to 1. Experiments were performed three times, and the values are shown as described for Fig. 1. (C) Huh 7.5.1 cells were fixed at 3 days postinfection with JFH, JFH Pol<sup>-</sup>, JFH 5a-GFP, or JFH 5a-Rluc virus, and the core-expressing cells are shown in red as in Fig. 2A (panels a to d). The NS5a-GFP signal was directly visualized by fluorescence microscopy (green) of the same cells (panels e to h). (D) Plaques generated by infection of JFH, JFH 5a-GFP, and JFH 5a-Rluc viruses were observed by phase-contrast microscopy (magnification,  $\times 100$ ). (E) Plaques were counted, and the viral titers are depicted as PFU per milliliter of medium. Experiments were performed four times, and the values are shown as described in the legend to Fig. 1C.

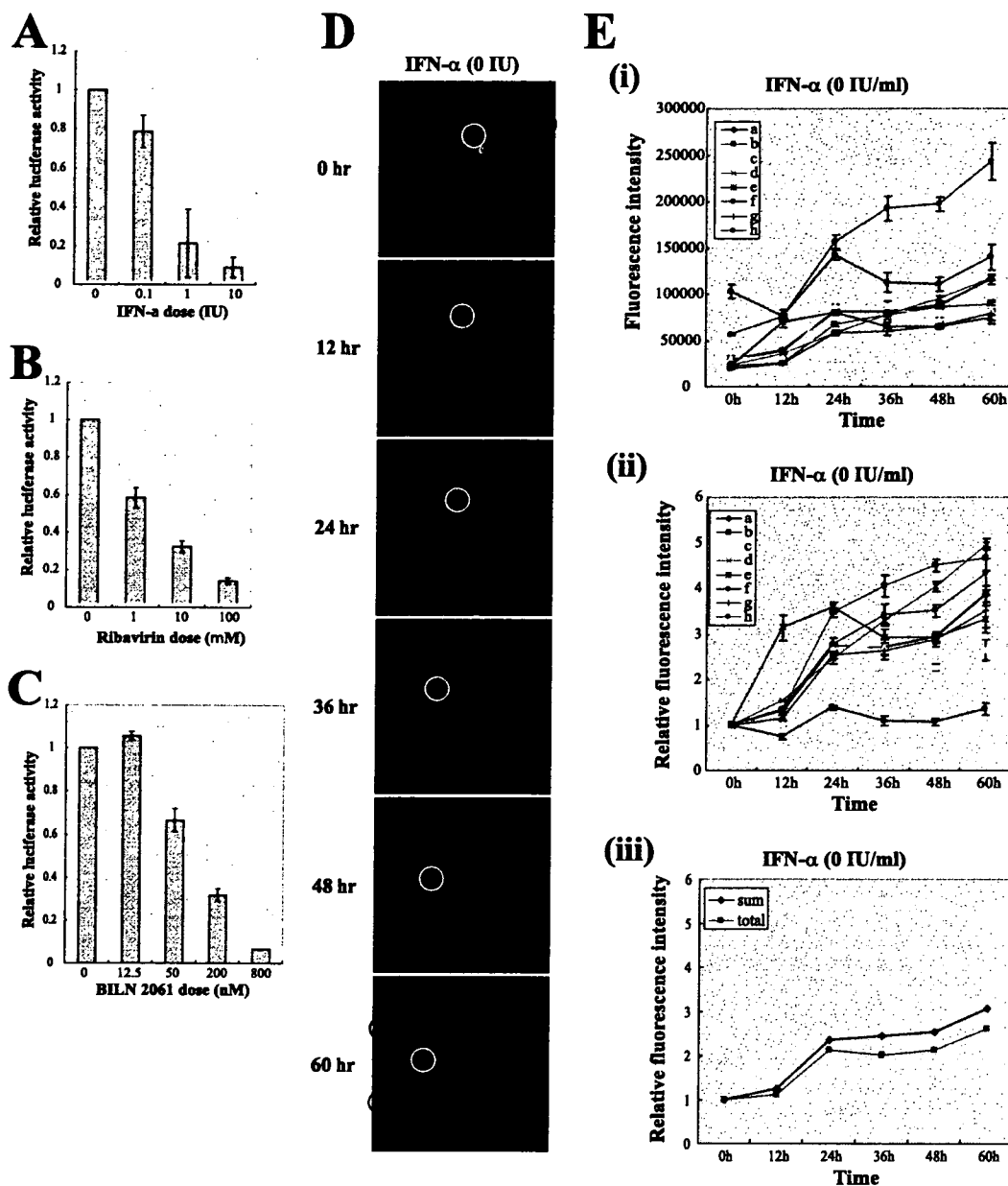


FIG. 4. Monitoring the antiviral effects of antiviral agents with infectious HCV containing reporter genes. (A, B, and C) Huh 7.5.1 cells were incubated with the indicated concentration of IFN- $\alpha$  (A), ribavirin (B), or BILN 2061 (C) for 8 h before infection with JFH 5a-Rluc virus. After inoculation with culture supernatant, the same concentrations of drugs were maintained for 3 days. Three days after inoculation, cells were harvested and luciferase activities were measured. Luciferase activities were normalized to those obtained from mock-treated cells, which were set to 1. Cytotoxic effects of drugs were monitored with the protein concentration as determined by the Bradford assay. Experiments were performed three times, and the values are shown as described for Fig. 1. (D) Huh 7.5.1 cells that were transfected with JFH 5a-GFP RNA without treatment with IFN- $\alpha$  were monitored every 12 h up to 60 h by time-lapse confocal microscopy (Zeiss LSM 5 Live). Eight cells (circled and labeled a, b, c, d, e, f, g, and h) were selected for quantitative analyses of the 5a-GFP fluorescence. Five z-stack images were taken at the indicated times, and representative images are shown. (E) Fluorescence intensities of the eight cells circled in panel D are plotted as arbitrary fluorescence units versus time in panel i and as relative fluorescence intensities versus time in panel ii. The relative fluorescence intensities were obtained by dividing the intensity at each time point by that at the starting time point. Mean values of five z-stack images with standard deviations are plotted. The fluorescence intensities obtained from the whole area of images reflecting whole cells in the panels (total) and the average intensities of eight cells (sum) are plotted in panel iii. All image analyses were performed using MetaMorph software. (F) Huh 7.5.1 cells transfected with JFH 5a-GFP RNA were treated with IFN- $\alpha$  (1000 IU/ml) and monitored every 12 h up to 60 h by time-lapse confocal microscopy (Zeiss LSM 5 Live). Eight cells (circled and labeled a, b, c, d, e, f, g, and h) were selected for quantitative analyses of the 5a-GFP fluorescence. Five z-stack images were taken at the indicated times, and representative images are shown. (G) Fluorescence intensities of eight cells circled in panel F are plotted as arbitrary fluorescence units versus time in panel i and as relative fluorescence intensities versus time in panel ii. The relative fluorescence intensities were obtained by dividing the intensity at each time point by that at the starting time point. Mean values of five z-stack images with standard deviations are plotted. The fluorescence intensities obtained from whole area of images reflecting whole cells in the panels (total) and the average intensities of eight cells (sum) are plotted in panel iii. All image analyses were performed using MetaMorph software.

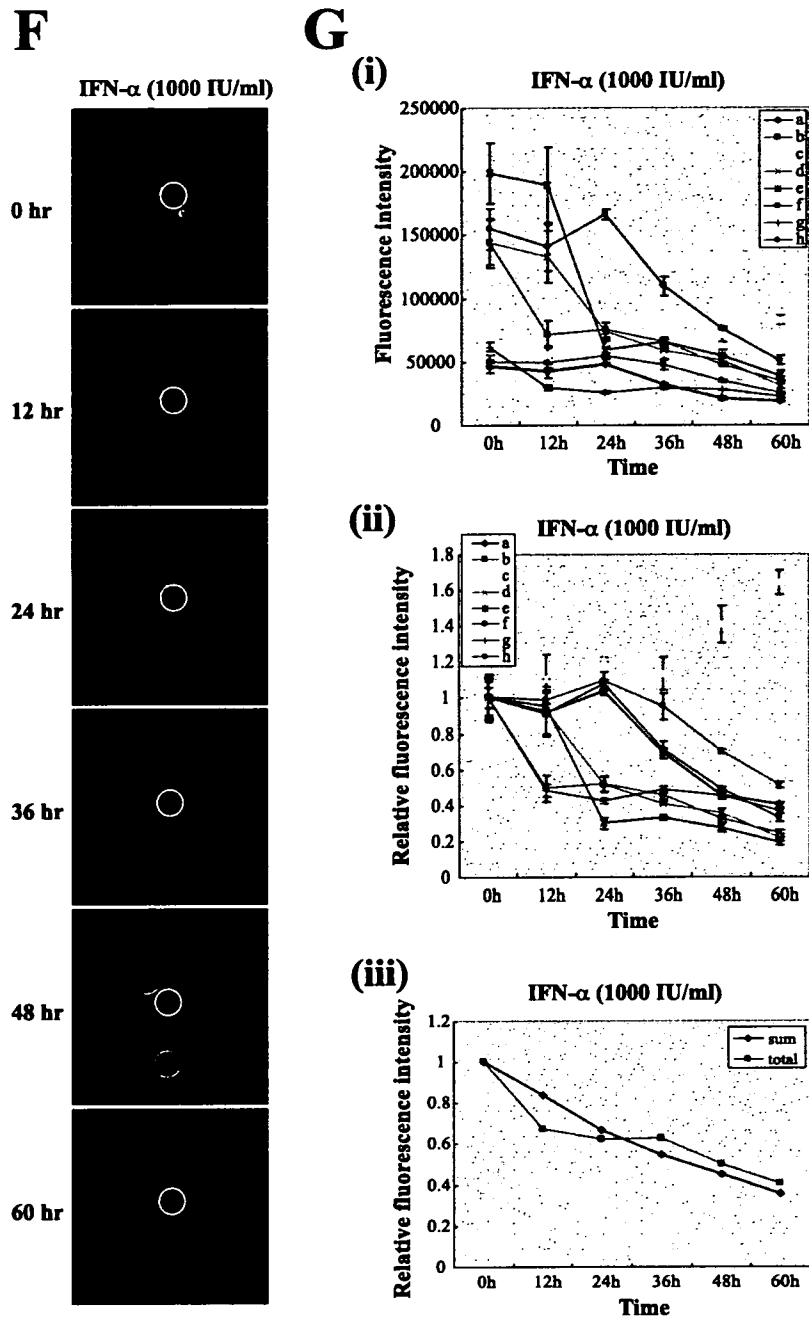


FIG. 4—Continued.

in Fig. 3E. The titers of JFH 5a-GFP and JFH 5a-Rluc virus were lower than that of JFH by 30% and 50%, respectively. These data indicate that the insertions of GFP and Rluc into NS5a moderately impaired the viral infectivity of JFH. Taking together the immunocytochemical data and viral infectivity tests, we concluded that the HCV derivatives containing GFP or Rluc produce functional proteins and replicate properly. Therefore, 5a-GFP fluorescence and 5a-Rluc activity can be used to visualize HCV-infected cells (JFH 5a-GFP) and quantify the level of virus infection (JFH 5a-Rluc).

Taking advantage of the ability to quantify the JFH 5a-Rluc virus, we examined the antiviral activities of IFN- $\alpha$ , ribavirin, and BILN 2061 (10) (Fig. 4). Dose-response experiments showed that IFN- $\alpha$ , ribavirin, and BILN 2061 inhibited proliferation of JFH 5a-Rluc virus in the infected cells (Fig. 4A, B, and C, respectively). The median effective concentrations of IFN- $\alpha$  and BILN 2061 against JFH 5a-Rluc virus were similar to those against J6/JFH virus as previously reported by Lindenbach et al. (11). This indicates that the modified virus JFH 5a-Rluc, which contains a heterologous polypeptide,



responds to antiviral agents in a similar manner as the JFH virus.

Even though IFN- $\alpha$  is used as therapy for HCV infections (6), many patients do not respond to IFN- $\alpha$  treatment (12). The mechanism of this IFN- $\alpha$  resistance is poorly understood. Moreover, the antiviral activity of IFN- $\alpha$  against HCV in individual HCV-infected cells has not yet been investigated due to technical limitations. We tried to monitor the anti-HCV effect of IFN- $\alpha$  in individual cells by using a derivative of HCV, JFH 5a-GFP, the replication of which can be microscopically monitored in individual cells in real time. Huh 7.5.1 cells transfected with JFH 5a-GFP RNA were treated with IFN- $\alpha$  or mock treated, and GFP fluorescence was monitored every 12 h up to 60 h by time-lapse confocal microscopy (Zeiss LSM 5 Live). For time-lapse imaging, coverslips were mounted onto the microscope stage, which was equipped with a temperature- and gas-controlled chamber (Chamlide IC; Live Cell Instrument, Korea). Quantitative analyses of the fluorescence images were performed using MetaMorph software. In cells that were not treated with IFN- $\alpha$ , the total intensity of 5a-GFP fluorescence increased with increasing cultivation time (Fig. 4E, panel iii). Analyses of the fluorescence intensities of eight cells (circled in Fig. 4D) showed that the fluorescence intensity of each cell increased by various amounts as the cultivation time increased (Fig. 4E, panels i and ii). The fluorescence intensities of eight cells were averaged (Fig. 4E, panel iii) and showed increases over time similar to that of the total intensity of whole images (Fig. 4E, panel iii). These results indicate that the selected eight cells represent the viral replication pattern of all cells on the coverslip. In cells treated with IFN- $\alpha$ , the total fluorescence intensity decreased in a time-dependent manner (Fig. 4G, panel iii). Analyses of fluorescence intensities of the eight cells circled in Fig. 4F showed that the antiviral effect of IFN- $\alpha$  varied in each cell (Fig. 4G, panels i and ii). 5a-GFP fluorescence intensities in seven cells (circles a, b, d, e, f, g, and h in Fig. 4F) gradually reduced even though the actual kinetics of the intensity reductions differed among individual cells (Fig. 4G, panels i and ii). However, the GFP signal in one cell (circle c in Fig. 4D) increased in the presence of IFN- $\alpha$  (Fig. 4G, panels i and ii). The averaged intensities of eight cells reduced in a manner similar to that for the total intensities of whole images (Fig. 4G, panel iii). These results indicate that the selected eight cells represent the IFN- $\alpha$  sensitivity of all cells on the coverslip. Taken together, the data indicate that the rates of replication of HCV RNA in HCV-infected cells and the IFN- $\alpha$  sensitivity of HCV-infected cells vary markedly and that HCV-infected cells showing IFN- $\alpha$ -resistance are present at the early stages of viral infection. The IFN- $\alpha$  resistance may be due to a putative variation in the host cell or a putative mutation in the viral genome. The molecular basis for these variations remains to be determined.

In this work, we generated novel reporter viruses that exhibited 5a-GFP fluorescence and 5a-Rluc activity in infected cells without the addition of a heterologous controlling element such as the internal ribosome entry site element of encephalomyocarditis virus or the foot-and-mouth disease virus 2A protease. Therefore, these systems reflect the HCV infection cycle and will be useful in investigating the viral life cycle and in development of new anti-HCV drugs.

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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<sup>∇</sup>

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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 (NSBX) 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 NSBX could replicate autonomously but that a chimeric J6 clone with only JFH-1 NSBX 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 NSBX 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<sub>2</sub> 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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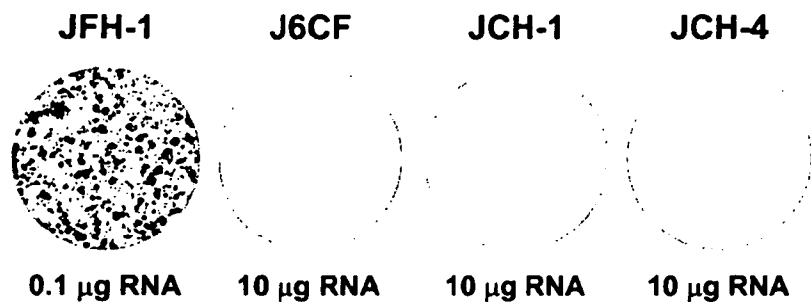


FIG. 1. G418-resistant colony formation of JFH-1, J6CF, JCH-1, and JCH-4. Subgenomic RNAs were synthesized *in vitro*, using pSGR-JFH1, pSGR-J6CF, pSGR-JCH1, and pSGR-JCH4 as templates. Transcribed subgenomic RNAs were electroporated into Huh7 cells, and cells were cultured with G418 for 3 weeks before staining with crystal violet as described in Materials and Methods. JFH-1 subgenomic RNA (0.1  $\mu$ g) and 10  $\mu$ g 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 NS5X 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 NS5X regions were performed by replacement of the BsrGI-XbaI fragment, substitutions of the NS5 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 ( $3 \times 10^6$  cells) were washed with Opti-MEM I (Invitrogen, Carlsbad, CA) and resuspended in Cytomix buffer (47). RNA was mixed with 400  $\mu$ l 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  $\mu$ F 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<sub>2</sub>. 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  $\mu$ g or 10  $\mu$ g of transcribed RNAs was transfected into  $3 \times 10^6$  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  $\mu$ g of transcribed RNAs was transfected into  $3 \times 10^6$  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  $\mu$ l 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  $\mu$ g) 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 [ $\alpha$ -<sup>32</sup>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  $1 \times 10^4$  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  $\mu$ g/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  $\mu$ g RNA, but the J6CF subgenomic replicon formed no colonies, even with transfection of 10  $\mu$ g RNA (Fig. 1). We also tested

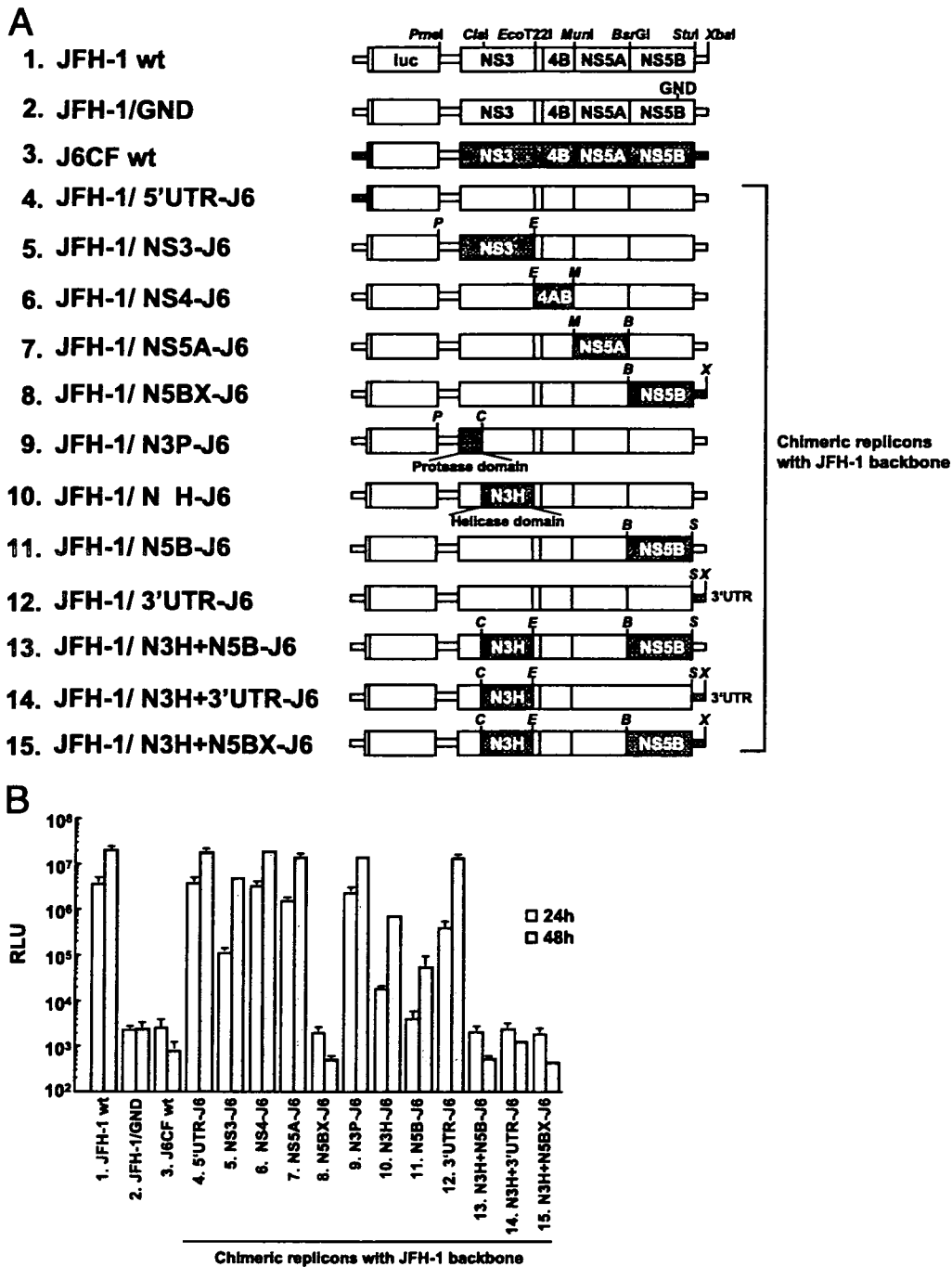


FIG. 2. Luciferase activities of chimeric replicons with a JFH-1 backbone. (A) Structures of chimeric subgenomic replicons with a JFH-1 backbone. The restriction enzyme recognition sites used for the construction of plasmids are indicated. *P*, PmeI; *C*, ClaI; *E*, EcoT221; *M*, MunI; *B*, BsrGI; *S*, StuI; *X*, XbaI; wt, wild type. (B) Subgenomic RNAs were synthesized in vitro from wild-type or chimeric replicon constructs. Transcribed subgenomic RNAs (5  $\mu$ g) 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/NS5B-J6 and JFH-1/3'UTR-J6) and their replication abilities analyzed. The replication level of JFH-1/NS5B-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

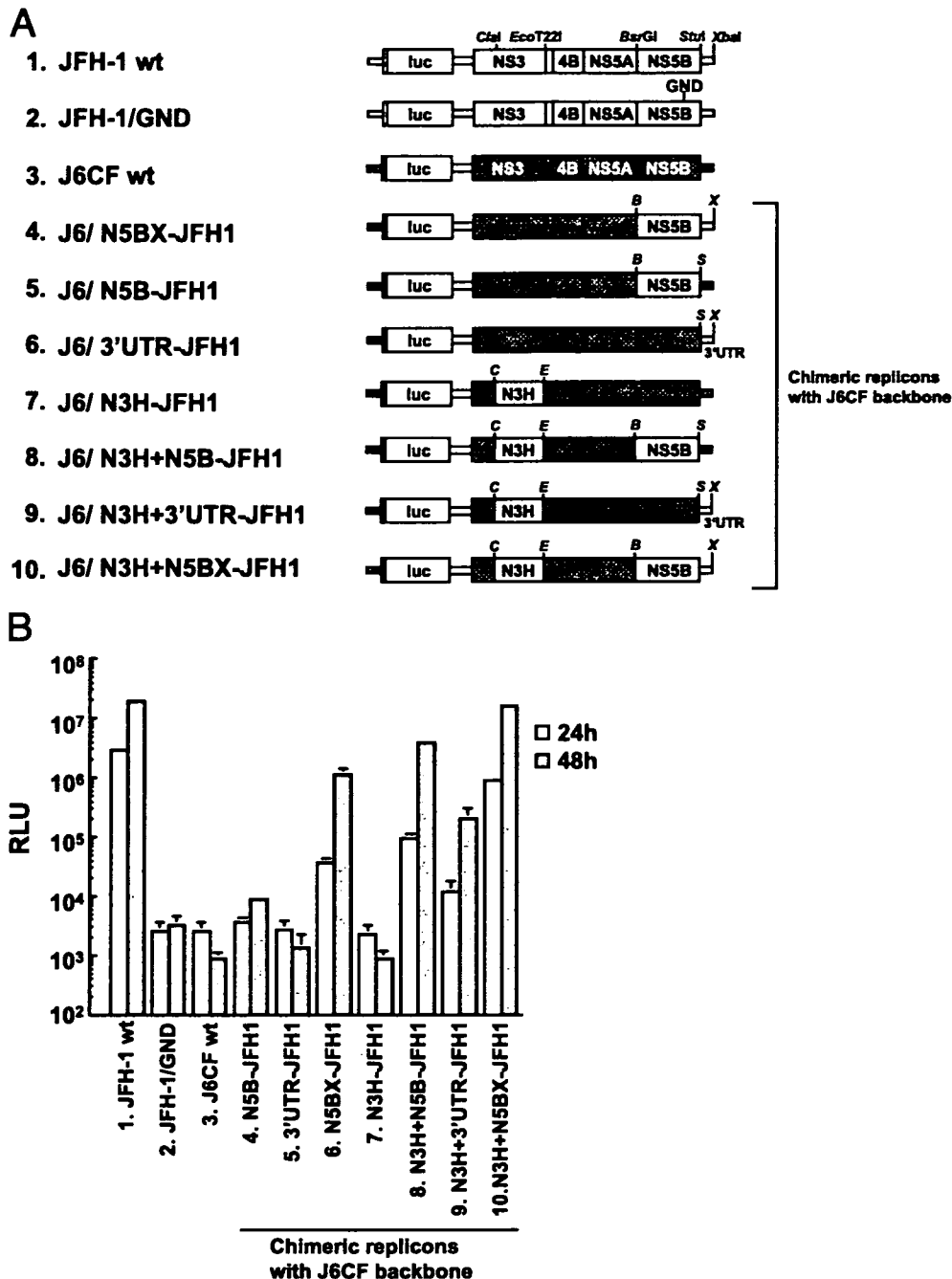


FIG. 3. Luciferase activities of chimeric replicons with a J6CF backbone. (A) Structures of chimeric subgenomic replicons with a J6CF backbone. The restriction enzyme recognition sites used for the construction of plasmids are indicated. C, ClaI; E, EcoT22I; B, BsrGI; S, StuI; X, XbaI; wt, wild type. (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

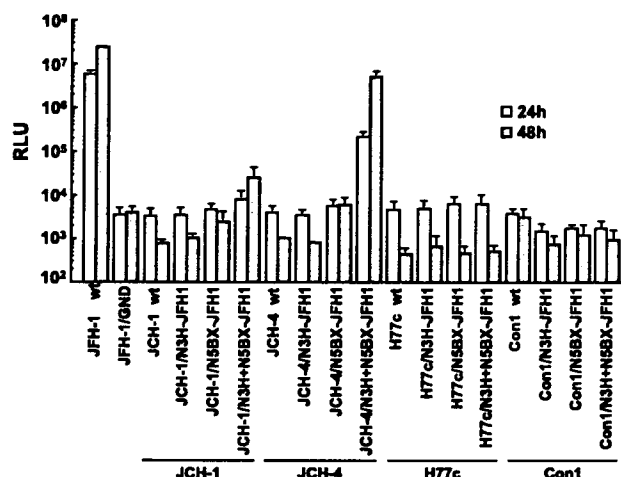


FIG. 4. Restoration of genotype 2a and genotype 1 replicon replication by the insertion of JFH-1 sequences. Two genotype 2a replicons, JCH-1 and JCH-4, a genotype 1a replicon, H77c, and a genotype 1b replicon, Con-1, were used in this assay. Three kinds of chimeric replicons, N3H-JFH-1, N5BX-JFH1, and 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

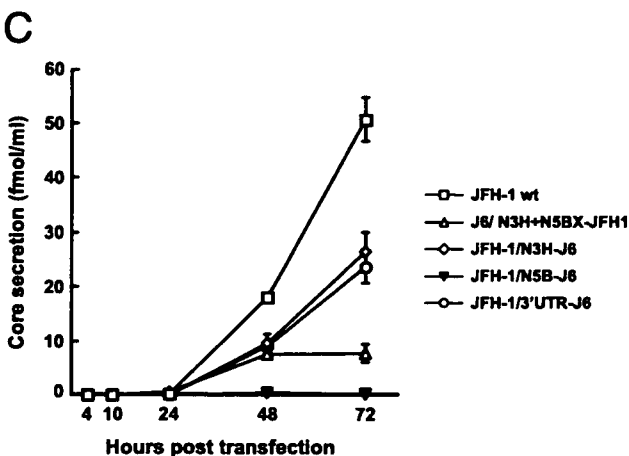
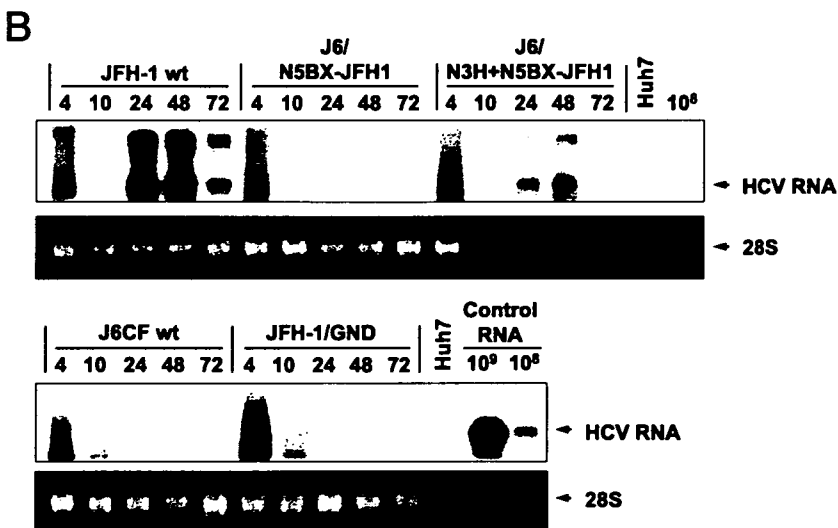
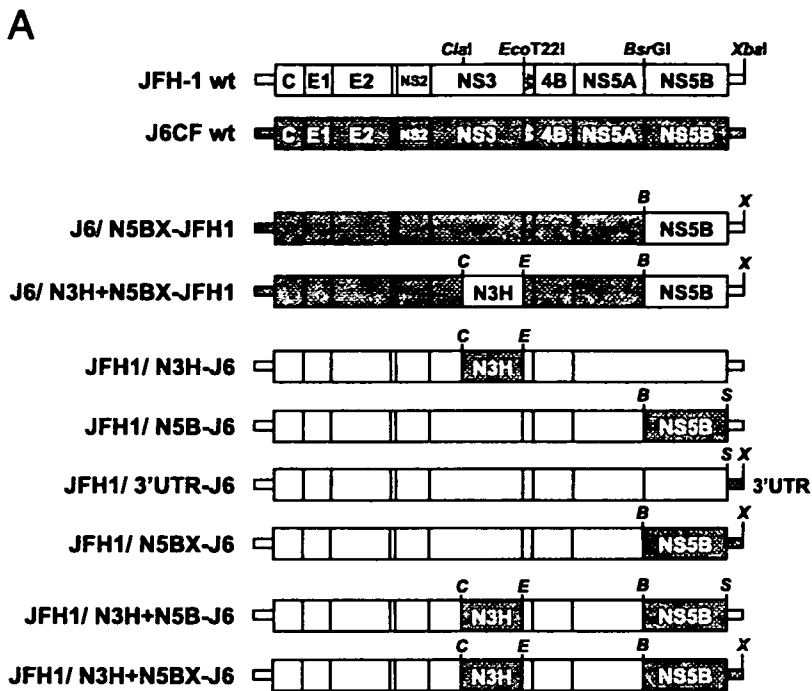




TABLE 1. Infectious titers of the media from chimeric HCV RNA-transfected cells

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

<sup>a</sup> 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 NSSB 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 NSSB 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 NSBX 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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