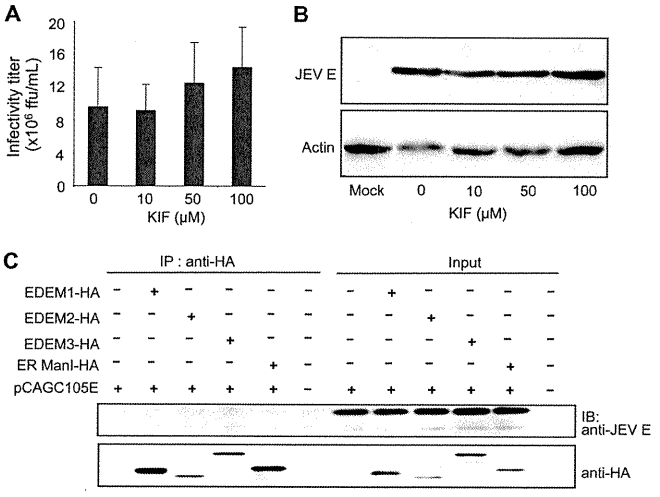

FIGURE 6. **Binding of JEV envelope glycoprotein with EDEMs and effect of KIF on JEV production.** *A*, JEV production in HuH-7 cells treated with KIF. The mean ± S.D. (*error bars*) of three independent experiments are shown. *B*, effect of KIF on the steady-state level of JEV envelope protein. *C*, binding of EDEMs with the JEV envelope.

tion of an HCV clone, JFH-1, capable of replicating and assembling infectious virus particles in cultured hepatocytes (15). In the present study, we used JFH-1 to examine the effect of HCV infection on activation of the ERAD pathway and its role in the virus life cycle. Our results show that the ERAD pathway is activated in HCV-infected cells, as evidenced by the maturation of XBP1 mRNA to its active form and up-regulation of EDEM1 (Fig. 1, *A–D*). Knocking down IRE1 reversed the induction of EDEM1, indicating that HCV infection-induced activation of the ERAD pathway is mediated through IRE1 (Fig. 1*F*). Loss- and gain-of-function analyses indicated that EDEM1 and EDEM3, particularly EDEM1, are involved in the post-translational control of HCV glycoproteins by which viral production is down-regulated (Figs. 3, *D* and *E*, and 4*A*). Our results suggest that EDEM1 and EDEM3 play a role in delivery of viral glycoproteins to the SEL1L-containing ubiquitin-ligase complex. It has recently been reported that coronavirus infection causes an accumulation of EDEM1 in membrane vesicles which are sites of viral replication, but that EDEM1 is not required for coronavirus replication (23). To our knowledge, the present study is the first to demonstrate regulation of the viral life cycle by ERAD machinery through interaction of EDEMs with viral glycoproteins.

We propose that the mechanisms described here are important during the early stages of establishing persistent HCV infection. ER stress caused by high levels of HCV infection during the acute phase presumably results in activation of the ERAD pathway. Induced EDEMs enhance the degradation of HCV envelope proteins, thereby reducing virus production. Maintenance of moderately low levels of HCV in the infected liver may contribute to the persistence of HCV infection, often associated with a lengthy asymptomatic phase that can last for decades. A range of viruses, including flaviviruses such as JEV, dengue virus, and West Nile virus, have been reported to induce XBP1 mRNA splicing triggered by ER stress (2, 3, 24). However, we demonstrate here that, in contrast to HCV, the envelope protein of JEV, which causes acute encephalitis, is not recognized by EDEMs, and the ERAD pathway does not control JEV production.

N-Linked glycoproteins displaying the glycan precursor Glc1Man9GlcNAc2 bind ER chaperones, such as calnexin or calreticulin, which facilitates protein folding. Removal of the terminal Glc from glycans disrupts this interaction with chaperones leading to Man trimming and delivery to ERAD machinery. A glucosyltransferase can transfer the terminal Man-linked Glc back to glycans, thereby allowing the "calnexin cycle" to continue until the glycoproteins are properly folded (for review, see Ref. 25). During this cycle, the decision of when to abandon additional folding attempts for immature polypeptides and to direct them instead toward the degradation pathway appears to be a crucial element of protein quality control. The basis by which this occurs, however, is not fully understood. Here, we demonstrate that stabilization of HCV envelope proteins and increased virus production occurs with KIF treatment (Fig. 5, *A* and *B*) and with gene silencing of either EDEM1 or EDEM3 (Figs. 3, *D* and *E*, and 4*A*). It is generally accepted that ERAD functions to eliminate proteins that are unable to adopt their native structure after translocation into the ER. From our results, however, one could argue that, during the HCV life cycle, at least a fraction of the competently folded viral glycoprotein intermediates may be released from the calnexin cycle before maturation and thereby be recognized as ERAD substrates. As suggested previously, the processes of protein folding and ERAD compete to some extent for newly synthesized polypeptides (26, 27). Under conditions in which high concentrations of ERAD-related factors are found in the ER due to induction of ER stress by viral infection, activated ERAD machinery may efficiently capture protein intermediates with folding/refolding capacity and cause premature termination of chaperone-assisted protein folding.

EDEM1 has recently been found to bind SEL1L, which is involved in the translocation of ERAD substrates from the ER to the cytoplasm (20). Our results demonstrate efficient binding of EDEM1 and EDEM3 to SEL1L, whereas EDEM2 exhibits only residual binding. In agreement with these results, increased ubiquitylation of HCV E2 protein was observed in cells overexpressing EDEM1 and EDEM3, but not in cells overexpressing the EDEM2 ortholog (Fig. 3*B*). Furthermore, KIF inhibited the binding of EDEM1 and EDEM3 with SEL1L, thus abrogating the ubiquitylation and enhancing the stability of HCV E2 protein (Fig. 5, *B* and *D*). It has been reported that KIF inhibits the interaction between EDEM1 and SEL1L, thus stabilizing ERAD substrates (4). Therefore, our results confirm previous findings and show that, along with EDEM1, KIF inhibits the binding of SEL1L to EDEM3. Furthermore, we have been the first to show that HCV E2 is a virus-derived ERAD substrate that can be used to analyze the mechanisms of this pathway. Taken together, our results indicate that EDEM1 and EDEM3, but not EDEM2, might be involved in targeting ERAD substrates to the translocation machinery, which may partly explain the different roles of the three EDEMs in HCV production. Although both EDEM1 and EDEM3 bind SEL1L and HCV envelope proteins, EDEM1 appears to have a larger role in regulation of HCV production than EDEM3. This is supported further by the finding that enhanced ubiquitylation of HCV E2 occurs in the presence

of EDEM1 overexpression (Figs. 3*B* and 5*D*). In EDEM3-knockdown cells, EDEM1 may take over the function of delivering ERAD substrates to the translocation machinery. We also speculate that EDEM1 may function as a helper for EDEM3. This is supported by the observation that EDEM1 and EDEM3 synergistically increase HCV production when knocked down together (data not shown). HCV glycoproteins are a suitable means by which to investigate differences and redundancies pertaining to the role of EDEMs in the ERAD pathway.

HCV-infected and TM-treated cells demonstrated the greatest activation of EDEM1 transcript production among EDEMs (Fig. 1, *C* and *D*, and supplemental Fig. S1). Although it is known that XBP1 binds to specific ER stress-responsive *cis*-acting elements to induce EDEMs (28, 29), the exact mechanism of transcriptional regulation is not fully understood. It will be interesting to examine regulatory mechanism(s) specific to individual EDEM homologs in an ER stress-dependent or -independent manner.

These findings highlight the crucial role of the ERAD pathway in the HCV life cycle. Further studies are needed to clarify the details of this complex pathway. The data generated in this work, however, further contribute to our understanding of the mechanisms that govern the maturation and fate of viral glycoproteins in the ER.

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In Vivo Adaptation of Hepatitis C Virus in Chimpanzees for Efficient Virus Production and Evasion of Apoptosis

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Hepatitis C virus (HCV) employs various strategies to establish persistent infection that can cause chronic liver disease. Our previous study showed that both the original patient serum from which the HCV JFH-1 strain was isolated and the cell culture–generated JFH-1 virus (JFH-1cc) established infection in chimpanzees, and that infected JFH-1 strains accumulated mutations after passage through chimpanzees. The aim of this study was to compare the *in vitro* characteristics of JFH-1 strains emerged in each chimpanzee at early and late stages of infection, as it could provide an insight into the phenomenon of viral persistence. We generated full-genome JFH-1 constructs with the mutations detected in patient serum-infected (JFH-1/S1 and S2) and JFH-1cc–infected (JFH-1/C) chimpanzees, and assessed their effect on replication, infectious virus production, and regulation of apoptosis in cell culture. The extracellular HCV core antigen secreted from JFH-1/S1-, S2-, and C-transfected HuH-7 cells was 2.5, 8.9, and 2.1 times higher than that from JFH-1 wild-type (JFH-1/wt) transfected cells, respectively. Single cycle virus production assay with a CD81-negative cell line revealed that the strain JFH-1/S2, isolated from the patient serum-infected chimpanzee at a later time point of infection, showed lower replication and higher capacity to assemble infectious virus particles. This strain also showed productive infection in human hepatocyte–transplanted mice. Furthermore, the cells harboring this strain displayed lower susceptibility to the apoptosis induced by tumor necrosis factor α or Fas ligand compared with the cells replicating JFH-1/wt. *Conclusion:* The ability of lower replication, higher virus production, and less susceptibility to cytokine-induced apoptosis may be important for prolonged infection *in vivo*. Such control of viral functions by specific mutations may be a key strategy for establishing persistent infection. (HEPATOLOGY 2011;00:000–000)

Currently, approximately 200 million people are infected with hepatitis C virus (HCV) and are at continuous risk of developing chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.[1,2] Although acute HCV infection elicits innate and adaptive immune responses, the virus successfully evades clearance in approximately 75% of infected individuals.[3,4] The mechanisms by

Abbreviations: Ag, antigen; CTL, cytotoxic T lymphocytes; FasL, Fas ligand; HCV, hepatitis C virus; JFH-1cc, cell culture–generated JFH-1 virus; JFH-1/wt, JFH-1 wild-type; MFI, mean fluorescence intensity; NK, natural killer, NS, nonstructural; PARP, poly(adenosine diphosphate ribose) polymerase; TNF-α, tumor necrosis factor α; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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which HCV leads to persistent infection at a high frequency are not yet fully understood. Lack of appropriate animal models, except chimpanzees, has rendered such studies difficult. Human hepatocyte-transplanted mice,[5,6] a useful small animal model to study HCV infection, are unsuitable to study the mechanisms of virus persistence because of a lack of B and T cell–mediated immunity.

HCV is a noncytopathic positive-stranded RNA virus of the *Flaviviridae* family. It primarily infects hepatocytes of humans and chimpanzees, where, thanks to error-prone RNA-dependent RNA polymerase, the infected virus accumulates a high number of mutations rapidly, thus providing opportunity for selection of viruses that have the ability to escape the immune system and establish persistent infection. Deciphering the strategies employed by HCV to establish persistence can be helpful in the development of new strategies to eradicate the virus and to stop disease progression. Until recently, the lack of an HCV strain having the ability to establish infection *in vivo* and *in vitro* was a substantial hindrance in studying the molecular mechanisms of virus persistence. This problem was solved by the identification of an HCV strain, JFH-1, that was isolated from a fulminant hepatitis patient and found to be capable of replicating and assembling infectious virus particles in chimpanzees as well as in cell culture.[7-10] This clone can be used to study the molecular mechanisms by which HCV evades the host immune system and causes chronic infection.

In a previous report, we inoculated patient serum from which the JFH-1 strain was originally isolated and cell culture–generated JFH-1 virus (JFH-1cc) into two different chimpanzees.[11] HCV established infection in both animals within 3 days of inoculation. In the JFH-1cc–infected chimpanzee, genome sequence of predominant infecting virus at week 2 was identical to JFH-1 wild-type (JFH-1/wt [in this study, this abbreviation was used instead of JFH-1 to distinguish it from other variant strains]), and the infecting virus has four synonymous and seven nonsynonymous mutations at week 7. In the JFH-1 patient serum–infected chimpanzee, 19 synonymous and six nonsynonymous mutations were observed in predominantly circulating virus at week 2, and this number increased to 35 synonymous and 17 nonsynonymous mutations at the later stage of infection course (week 23).[11] From these observations, we presumed that the isolates evolved in each chimpanzee at later stages of infection might have some advantage over the viruses isolated at earlier time points for survival in infected animals. Thus, in this study, we generated JFH-1 variants containing the mutations observed in these animals and assessed their effect on replication and infectious virus production in cell culture. Furthermore, we examined the effects of infection of these strains to tumor necrosis factor α (TNF-α)– or Fas ligand (FasL)–mediated apoptosis.

Materials and Methods

The complete Materials and Methods are provided in the Supporting Information.

Results

Effects of Mutations Identified in Chimpanzees. To investigate the effect of mutations on virus phenotype, we generated constructs containing the mutations observed in JFH-1 patient serum–infected chimpanzee and JFH-1cc–infected chimpanzee at various time points. The JFH-1 variants JFH-1/S1 and JFH-1/S2 contain the mutations observed in the patient serum–infected chimpanzee at week 2 and week 23, respectively, and JFH-1/C contains the mutations observed in the JFH-1cc–infected chimpanzee at week 7 (Supporting Table 1). The replication and virus production capacity of these variants in HuH-7 cells was compared with that of JFH-1/wt. After electroporation of *in vitro*–synthesized full-genome RNA of JFH-1/wt and variant strains, extracellular and intracellular HCV RNA and core antigen (Ag) were measured (Fig. 1). At day 5 posttransfection, all constructs displayed similar intracellular HCV RNA levels. However, extracellular HCV RNA level of JFH-1/C was 1.6 times higher than that of JFH-1/wt. Likewise, extracellular HCV RNA level of JFH-1/S2 was 3.4 times higher than that of JFH-1/S1 (Fig. 1A). Intracellular HCV core Ag levels of JFH-1/S2 and C were 240.9 ± 58.2 and 189.8 ± 42.1 fmol/mg protein, respectively, and were significantly lower ($P < 0.005$) than that of JFH-1/S1 (526.1 ± 58.2 fmol/mg protein) and JFH-1/wt (511.7 ± 32.9 fmol/mg protein) at day 1, but reached comparable levels at day 5 posttransfection. On the other hand, extracellular HCV core Ag level of JFH-1/C was 2.2 times higher than that of JFH-1/wt, and that of JFH-1/S2 was 3.6 times higher than that of JFH-1/S1 at day 5 posttransfection (Fig. 1B). Transfection efficiency of these strains, indicated by intracellular HCV core Ag levels at 4 hours posttransfection, was almost identical (data not shown).

Single Cycle Virus Production Assay. For detailed analysis of the effects of these mutations on different stages of the virus lifecycle, we used a Huh7-25 cell

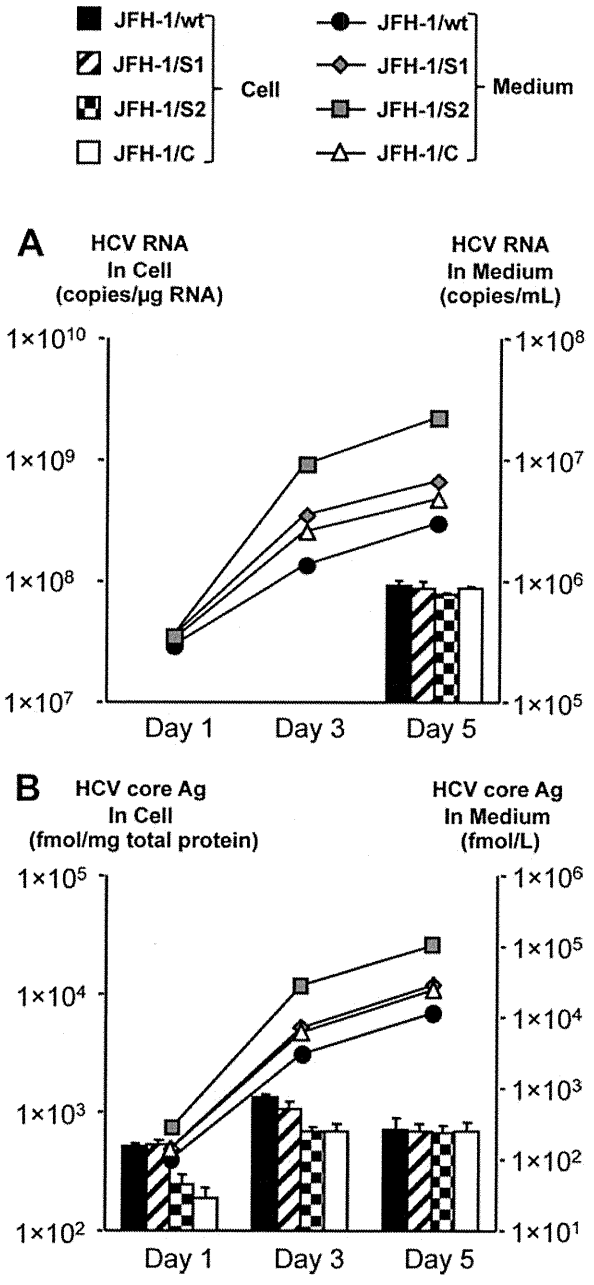

Fig. 1. Effects of *in vivo* adaptive mutations on virus production in HuH-7 cells. One million cells were transfected with 10 μg *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C. (A) HCV RNA and (B) core Ag levels in cell lysates and medium were measured at the indicated time points. Assays were performed in triplicate, and data are presented as the mean ± SD.

line that lacks the surface expression of CD81, one of the cellular receptors for HCV entry. Three days after transfection with full-genome RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C, HCV RNA levels and infectivity titer were measured, and the specific infectivity was calculated (Table 1). Intracellular HCV RNA levels of JFH-1/C and JFH-1/S2 were lower than those of JFH-1/wt and S1, suggesting lower replication efficiency of these strains. However, the intracellular infectivity titers of JFH-1/C and JFH-1/S2 were 2.03 and 11.0 times higher than those of JFH-1/wt and JFH-1/S1, respectively ($P < 0.005$). Intracellular-specific infectivities (infectivity titer/HCV RNA copy number) of JFH-1/C and JFH-1/S2 showed more pronounced difference from those of JFH-1/wt and JFH-1/S1 (3.92 times and 12.9 times higher, respectively; $P < 0.005$). The infectious virus secretion rate (extracellular infectivity titer/intracellular infectivity titer) was not significantly different between JFH-1/wt and variant strains. These data indicate that mutations identified in chimpanzees at the later time point of infection led to reduced viral replication and increased assembly of infectious virus particles without any effect on viral release in cell culture.

Subgenomic Replicon Assay. To further confirm the replication efficiencies of strains observed in chimpanzees, we generated subgenomic replicons of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C carrying the firefly luciferase reporter gene (SGR-JFH-1/Luc/wt, SGR-JFH-1/Luc/S1, SGR-JFH-1/Luc/S2, and SGR-JFH-1/Luc/C). *In vitro*–transcribed RNAs of these constructs were transfected into HuH-7 cells, and luciferase activity was measured to assess their replication capacity. The luciferase activities of SGR-JFH-1/Luc/C and SGR-JFH-1/Luc/S2 replicons were 7.30 and 7.33 times lower than those of SGR-JFH-1/Luc/wt and SGR-JFH-1/Luc/S1, respectively, at day 1 ($P < 0.00005$), suggesting attenuated replication capacities of variant replicons isolated from each animal at later time points of infection (Supporting Fig. 1A). The luciferase activity 4 hours after transfection was comparable, indicating similar levels of transfection efficiency (data not shown). Based on these data, we found that the mutations that emerged in nonstructural (NS)3-NS5B of JFH-1/S2 and JFH-1/C reduced the replication efficiency in cell culture.

Genomic Regions Responsible for Lower Replication and Higher Assembly of JFH-1/S2. To further clarify the genomic region responsible for lower replication efficiency and higher assembly rate of JFH-1/S2, we generated the chimeric constructs JFH-1/S2-wt and JFH-1/wt-S2 as described in the Supporting Materials and Methods. *In vitro*–transcribed RNAs of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2 were introduced into HuH-7 cells by electroporation and intracellular and extracellular HCV RNA and core Ag were measured. At day 5 posttransfection, all constructs displayed comparable intracellular HCV RNA levels (Fig. 2). However, extracellular HCV RNA levels of JFH-1/S2 and JFH-1/S2-wt were significantly

Table 1. Infectious Virus Production and Release of JFH-1/wt and Variants in Huh7-25 Cells

Strain	Intracellular			Extracellular	Secretion Ratio (Extracellular/ Intracellular)
	HCV RNA (copies/μg RNA)	Infectivity Titer (ffu/well)	Specific Infectivity (ffu/copies)	Infectivity Titer (ffu/well)	
JFH-1/wt	$7.75 \times 10^8 \pm 1.04 \times 10^8$	$4.21 \times 10^2 \pm 4.32 \times 10^1$	$2.09 \times 10^{-7} \pm 7.06 \times 10^{-8}$	$1.94 \times 10^3 \pm 3.76 \times 10^1$	4.6 ± 1.3
JFH-1/S1	$7.04 \times 10^8 \pm 8.49 \times 10^7$	$4.72 \times 10^2 \pm 5.63 \times 10^1$	$2.91 \times 10^{-7} \pm 6.00 \times 10^{-8}$	$3.02 \times 10^3 \pm 2.77 \times 10^2$	5.4 ± 2.0
JFH-1/S2	$4.16 \times 10^{8}** \pm 7.47 \times 10^6$	$5.19 \times 10^{3}** \pm 8.24 \times 10^1$	$3.76 \times 10^{-6}** \pm 7.01 \times 10^{-7}$	$3.23 \times 10^{4}** \pm 3.52 \times 10^3$	6.2 ± 3.0
JFH-1/C	$3.15 \times 10^{8}* \pm 5.02 \times 10^7$	$8.59 \times 10^{2}* \pm 4.81 \times 10^1$	$8.19 \times 10^{-7}* \pm 5.68 \times 10^{-8}$	$3.68 \times 10^3 \pm 3.02 \times 10^3$	4.3 ± 1.4
JFH-1/S2-wt	$7.07 \times 10^8 \pm 8.43 \times 10^7$	$4.40 \times 10^{3}* \pm 9.5 \times 10^1$	$2.73 \times 10^{-6}* \pm 2.35 \times 10^{-7}$	$3.0 \times 10^{4}* \pm 1.1 \times 10^3$	6.7 ± 0.7
JFH-1/wt-S2	$4.21 \times 10^{8}* \pm 1.97 \times 10^7$	$2.7 \times 10^2 \pm 2.9 \times 10^1$	$2.02 \times 10^{-7} \pm 4.0 \times 10^{-8}$	$1.7 \times 10^3 \pm 1.3 \times 10^2$	4.5 ± 0.4

Abbreviation: ffu, focus-forming units.
*$P < 0.005$ versus JFH-1/wt.
**$P < 0.005$ versus JFH-1/S1.

higher ($P < 0.0005$) than that of JFH-1/wt. On the other hand, extracellular RNA level of JFH-1/wt-S2 chimeric construct was lower than that of JFH-1/S2 and JFH-1/S2-wt and similar to that of JFH-1/wt. Likewise, extracellular core Ag levels of JFH-1/S2 and JFH-1/S2-wt were also significantly higher than that of JFH-1/wt. Intracellular HCV core Ag levels of JFH-1/S2 and JFH-1/wt-S2 on day 1 posttransfection were 240.9 $\pm$ 58.2 and 134.3 $\pm$ 17.1 fmol/mg protein, respectively, and were significantly lower ($P < 0.005$) than that of JFH-1/wt (526.1 $\pm$ 58.2 fmol/mg protein), whereas intracellular HCV core Ag level of JFH-1/S2-wt was comparable to that of JFH-1/wt. Transfection efficiency of these strains, indicated by intracellular HCV core Ag levels at 4 hours posttransfection, was almost identical (data not shown).

To further elucidate, we transfected Huh7-25 cells with *in vitro*–transcribed RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2 and measured HCV RNA, core Ag, and infectivity titer in the cells and culture medium. Intracellular HCV RNA levels of JFH-1/S2 and JFH-1/wt-S2 were similar and lower than those of JFH-1/wt and JFH-1/S2-wt, suggesting mutations in NS3-NS5B were responsible for lower replication efficiency of JFH-1/S2 (Table 1). Intracellular infectivity titer of JFH-1/S2 and JFH-1/S2-wt was 12.3 and 10.4 times higher, respectively, than that of JFH-1/wt ($P < 0.005$) on day 3 posttransfection. The intracellular specific infectivities of JFH-1/S2 and JFH-1/S2-wt were significantly higher than that of JFH-1/wt (18 times and 13.1 times higher, respectively; $P < 0.005$). On the other hand, intracellular specific infectivity of JFH-1/wt-S2 was comparable to that of JFH-1/wt. The infectious virus secretion rate was not significantly different among all the constructs (Table 1). These data indicate that mutations emerged in the core-NS2 region of JFH-1/S2 are responsible for the enhanced assembly of infectious virus particles compared with JFH-1/wt.

Mapping Study for JFH-1/S2 Strain. Because our experiments with JFH-1/S2 subgenomic replicon and JFH-1/wt-S2 chimeric construct showed that mutations emerged in the NS3-NS5B region are responsible for reduced replication efficiency of JFH-1/S2, we performed mapping studies by generating various JFH-1 subgenomic replicons, each containing the mutations observed in individual nonstructural protein. Although mutations in NS4B and NS5A were associated with attenuated replication capacity of JFH-1, the most significant decrease in replication was observed with NS5B mutations (Supporting Fig. 1B).

For detailed analysis of mutations responsible for higher assembly, *in vitro*–transcribed RNAs of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, JFH-1/N397S, JFH-1/L752V, JFH-1/S2-NS2 (containing mutations G838R, A878V, and V881A), JFH-1/G838R, and JFH-1/A878V were transfected into Huh7-25 cells, and intracellular-specific infectivities were compared (Supporting Table 2). As reported previously, JFH1/G838R showed higher intracellular specific infectivity than that of JFH-1/wt, but could not reach the level of JFH-1/S2 or JFH-1/S2-wt. Among the mutants, intracellular specific infectivities of JFH1/L752V, JFH1/NS2, and JFH1/G838R were 4.02, 5.42, and 3.07 times higher than that of JFH-1/wt, but those of JFH1/N397S and JFH1/A878V were similar to that of JFH-1/wt. Thus, the combination of mutations in P7 and NS2 was found to contribute to the higher assembly of the JFH-1/S2 strain.

Human Hepatocyte–Transplanted Mouse Assay. To assess the *in vivo* infectivity of these strains, we inoculated culture medium containing 10^7 copies (HCV RNA titer measured by RTD-PCR) of JFH-1/wt, JFH-1/S1, JFH-1/S2, and C viruses into human

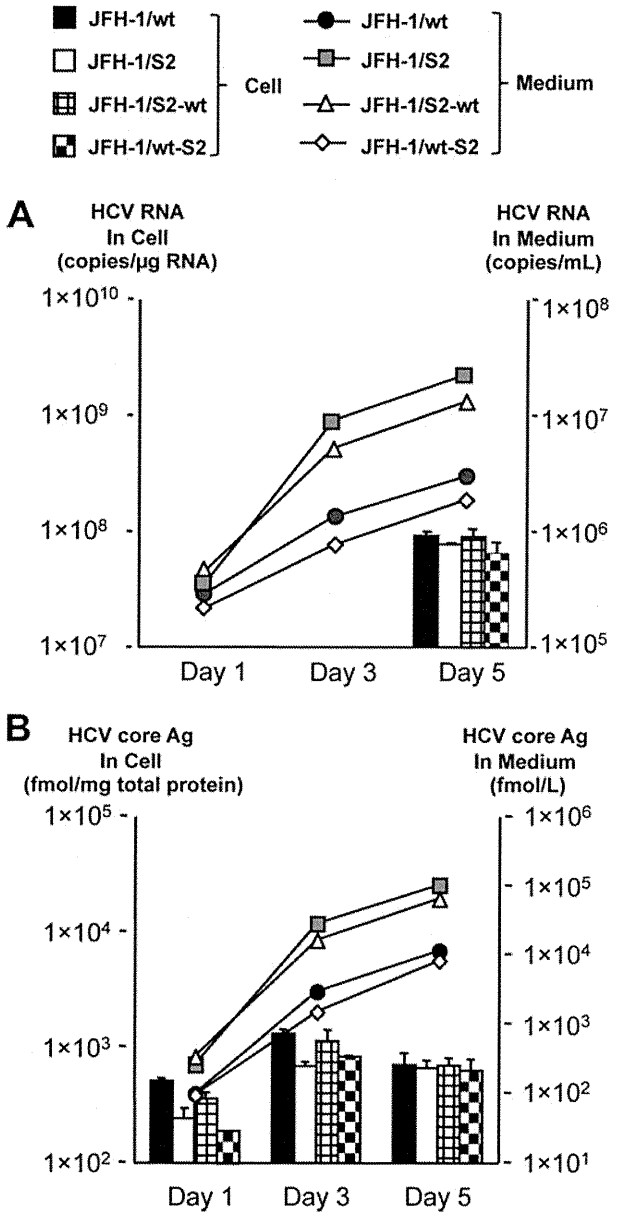

Fig. 2. Virus production of JFH-1/S2 chimeric constructs in HuH-7 cells. One million cells were transfected with 10 μg *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2. (A) HCV RNA and (B) core Ag levels in cell lysates and medium were measured at the indicated time points. Assays were performed in triplicate, and data are presented as the mean ± SD.

hepatocyte-transplanted mice. Two mice were used for each virus. Two weeks after intravascular inoculation, all mice but one became HCV RNA–positive (Fig. 3). Two mice died 3 weeks after inoculation; one was inoculated with JFH-1/wt and had developed infection, and the other was inoculated with JFH-1/C and died without developing infection. HCV RNA levels in infected mice fluctuated, ranging from 10^6 to 10^9 copies/mL. We could not observe much difference of infected HCV RNA titer among these inoculated mice. Sequence analyses of the complete open reading frames revealed that infecting JFH-1/wt virus and variant strains had no nonsynonymous mutations at the time of development of infection. From these data, we concluded that not only JFH-1/wt virus but also JFH-1/S1, JFH-1/S2, and JFH-1/C viruses were able to establish productive infection in human hepatocyte-transplanted mice.

Apoptosis Induction Assay. To investigate the survival strategy against the host defense system, we examined the susceptibility of JFH-1/wt and variant strains to TNF-α–mediated apoptosis induction. After transfection with *in vitro*–transcribed RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C, Huh-7.5.1 cells were exposed to TNF-α plus actinomycin D. Without exposure, apoptosis was observed in a limited number of HCV-positive cells (Supporting Fig. 2A). Forty-eight hours later, cells were harvested, fixed, and

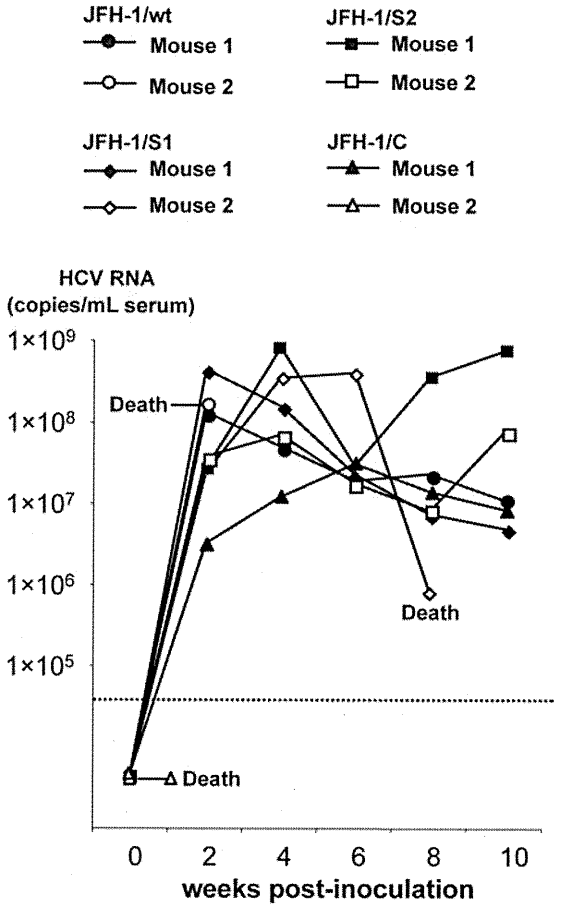

Fig. 3. *In vivo* infection study of JFH-1/wt and its variants in human hepatocyte-transplanted mice. Cell culture medium containing 1 × 10^7 HCV RNA copies of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C were inoculated into human hepatocyte-transplanted mice, and HCV RNA levels in mice serum were monitored.

subjected to terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay and anti-HCV NS5A staining. The effects of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C transfection on apoptosis induction were determined by calculating the ratio of apoptosis between HCV-positive and HCV-negative populations and expressed as an apoptosis induction index. After treatment of JFH-1/wt–transfected cells with TNF-α, apoptosis was observed in 36.8% of the HCV-positive population and in 19.3% of the HCV-negative population, and the apoptosis induction index was 1.85 ± 0.06 (Fig. 4). The apoptosis induction indexes of JFH-1/S1–transfected and JFH-1/C– transfected cells were 1.23 ± 0.06 and 1.16 ± 0.10, respectively, suggesting lower susceptibility to apoptosis induction compared with JFH-1/wt. On the other hand, the apoptosis induction index of JFH-1/S2 was 0.74 ± 0.17, which was substantially lower than that of JFH-1/wt, demonstrating the more reduced apoptosis in the cells harboring this strain. Similar results were obtained by treatment with FasL plus actinomycin D (Supporting Fig. 2B). To confirm the lower susceptibility of JFH-1/S2–transfected cells, apoptosis was also detected by staining with anticleaved poly(adenosine diphosphate ribose) polymerase (PARP) antibody. The apoptosis induction indexes of JFH-1/wt and JFH-1/S2–transfected cells were 2.28 ± 0.24 and 1.15 ± 0.14, respectively, and were consistent with TUNEL assay (Fig. 5). Although the HCV NS5A-positive rate in JFH-1/S2–transfected cells was higher than that in JFH-1/wt, the mean fluorescence intensity of the NS5A-positive population in JFH-1/S2–transfected cells was significantly lower (185.0 ± 8.7) than that in JFH-1/wt–transfected cells (395.0 ± 98.0), corresponding to the observed phenotype of the JFH-1/S2 strain in the single cycle virus production assay (i.e., lower replication efficiency and rapid spread to surrounding cells).

To clarify the genomic region responsible for lower susceptibility of JFH-1/S2 to cytokine-induced apoptosis, we examined the effect of TNF-α on the cells carrying subgenomic reporter replicons. The apoptosis induction index of SGR-JFH1/Luc/S2–transfected cells was lower than that of SGR-JFH1/Luc/wt–transfected cells (Supporting Fig. 2C); however, the difference was not as pronounced as with full-genome constructs, indicating that mutations in the NS3-NS5B region contribute to lower susceptibility of JFH-1/S2 to cytokine-induced apoptosis, but they are not sufficient to explain the difference between JFH-1/wt and JFH-1/S2. We confirmed these results by use of the chimeric

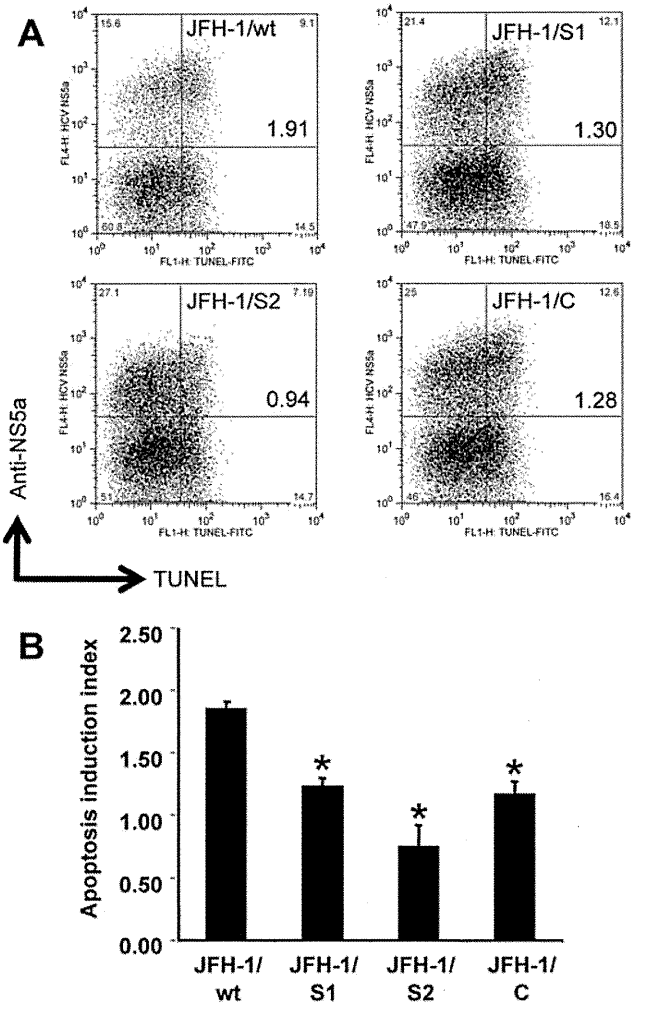

Fig. 4. Apoptosis induction in Huh-7.5.1 cells transfected with JFH-1/wt and its variants. (A) Three million cells were transfected with 3 μg *in vitro*-transcribed full-genome RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C. Forty-eight hours later, apoptosis was induced by exposing cells to 20 ng/mL TNF-α plus 50 ng/mL actinomycin D. Cells were harvested after 48 hours of treatment and subjected to TUNEL and anti-HCV NS5A staining. Dot plots show HCV replication and apoptosis at the single cell level. Quadrant gates were determined using unstained and a terminal deoxynucleotidyltrasferase-untreated control in each culture condition. The clone names and apoptosis induction indexes are indicated in the upper right box. (B) Apoptosis induction indexes of JFH-1/wt–, JFH-1/S1–, JFH-1/S2–, and JFH-1/C– transfected cells. The mean ± SD of three independent experiments is shown. *$P < 0.005$ versus JFH-1/wt.

constructs JFH-1/S2-wt and JFH-1/wt-S2. The apoptosis induction indexes of JFH-1/S2-wt–transfected and JFH-1/wt-S2–transfected cells were 1.42 ± 0.13 and 1.71 ± 0.08, respectively (Fig. 5). These data indicate that both structural and nonstructural regions of JFH-1/S2 were associated with lower susceptibility to cytokine-induced apoptosis, although mutations in core-NS2 seemed to have higher contribution toward this phenotype. Together, these results indicate that the JFH-1/S2 strain, which was selected after passage in

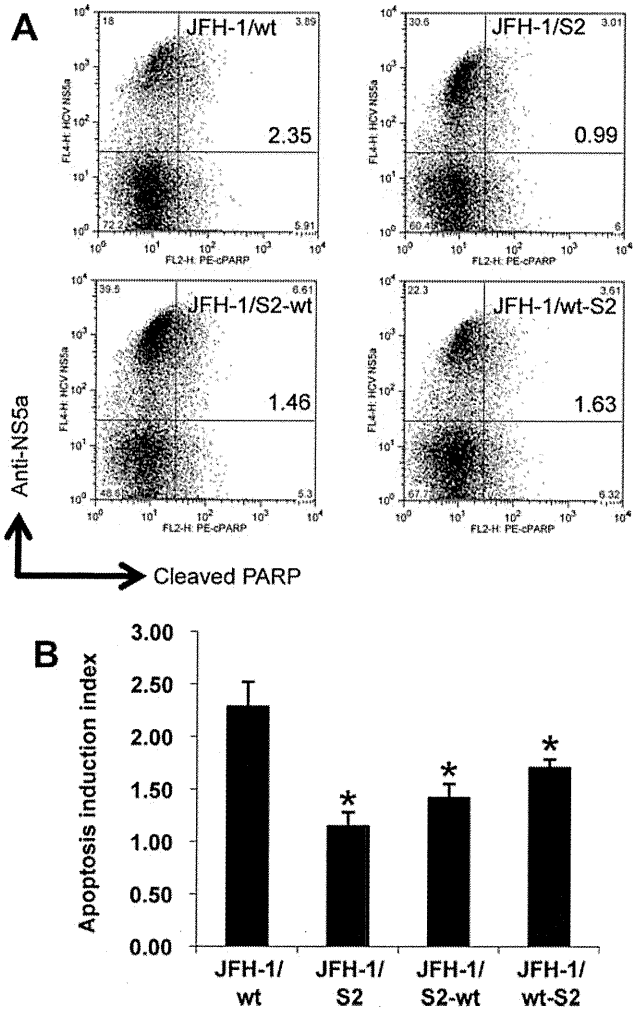

Fig. 5. Apoptosis induction in Huh-7.5.1 cells transfected with JFH-1/wt, JFH-1/S2, and their chimeric constructs. (A) Three million cells were transfected with 3 μg *in vitro*-transcribed full-genome RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2. Apoptosis was induced by exposing cells to 20 ng/mL TNF-α plus 50 ng/mL actinomycin D and detected by anticleaved PARP staining. The clone names and apoptosis induction indexes are indicated in the upper right box. (B) Apoptosis induction indexes of JFH-1/wt-, JFH-1/S2-, JFH-1/S2-wt-, and JFH-1/wt-S2-transfected cells. The mean $\pm$ SD of three independent experiments is shown. *$P < 0.05$ versus JFH-1/wt.

the patient serum–infected chimpanzee, acquired less susceptibility to the cytokine-induced apoptosis.

Discussion

HCV develops chronic infection in the vast majority of infected patients[1]; however, the mechanisms of its persistence are still under investigation. Many viruses have evolved different strategies to cope with host immune systems, thus causing the development of persistent infection. For example, some viruses interfere with the major histocompatibility complex class I presentation of viral antigens, whereas others modulate lymphocyte and macrophage functions, including cytokine production.[12-16] In our previous study, we detected an increasing number of mutations in the HCV genome isolated from JFH-1 patient serum–infected chimpanzees. Thus, we reasoned that these detected mutations might have imparted some advantage to this virus for long-time survival. To examine this hypothesis, we compared the phenotypes of JFH-1 variant strains emerged at early and late stages of infection in JFH-1 patient serum–infected and JFH-1cc–infected chimpanzees and found that the JFH-1/S2 strain isolated from the patient serum–infected chimpanzee at a later time point of infection replicated slowly, produced more infectious viruses, and displayed reduced susceptibility to cytokine-induced apoptosis.

The JFH-1 variant strain JFH-1/C, which contains seven nonsynonymous mutations identified in the JFH-1cc–infected chimpanzee at week 7, showed comparatively slower replication kinetics and slightly enhanced infectious virus production in cell culture. The intracellular specific infectivity of this strain in Huh7-25 cells was 3.9 times higher than that of JFH-1/wt (Table 1). These characteristics might have imparted some advantage to this strain for establishing productive infection in the chimpanzee. The other JFH-1 variant strains, JFH-1/S1 and JFH-1/S2, contain 6 and 17 nonsynonymous mutations identified in the JFH-1 patient serum–infected chimpanzee at weeks 2 and 23 postinfection, respectively. Replication kinetics and infectious virus production of the JFH-1/S1 strain were comparable to that of JFH-1/wt in cultured cells (Fig. 1, Table 1). In contrast, the JFH-1/S2 strain showed lower replication efficiency. Although the intracellular HCV RNA level of this strain in Huh7-25 cells was lower than that of JFH-1/wt and JFH-1/S1, and almost the same as that of JFH-1/C (Table 1), intracellular specific infectivity was 18.0 and 12.9 times higher than that of JFH-1/wt and JFH-1/S1, respectively, suggesting a significant increase in the assembly of infectious virus particles ($P < 0.005$, Table 1). The enhanced capacity of this strain to assemble infectious virus particles resulted in a higher extracellular infectivity titer that contributed to the rapid spread of virus to surrounding cells. Flow cytometry analyses of cells transfected with JFH-1/wt and variant strains revealed that the percentage of the HCV NS5A-positive population in JFH-1/S2–transfected cells was higher, but the mean fluorescence intensity of the anti-NS5A signal was lower than that in JFH-1/wt–transfected cells, thus confirming higher spread and lower replication of this strain. Taken together, both JFH-1/C and JFH-1/S2 exhibited a tendency toward

decreased replication and increased infectious virus production. However, the extent of enhanced virus production was substantially lower in JFH-1/C than in JFH-1/S2, which might have led to the earlier elimination of infection in the JFH-1cc–infected chimpanzee. In other words, the potency of infectious virus production and spread seems to correspond to the duration of infection in infected animals.

The association between a lower replication efficiency and persistent infection is still unclear. It has been reported that an escape mutant with an amino acid substitution at the cytotoxic T lymphocyte (CTL) epitope in the NS3 region exhibits lower NS3/4 protease activity and replication capacity *in vitro*.[17,18] The JFH-1/S2 strain contains the T1077A mutation in the NS3 region (Supporting Table 1), and this mutation is located close to mutations reported to be associated with immune evasion and lower replication.[17] Thus, the lower replication efficiency of the JFH-1/S2 strain may be a result of an immune escape mutation at the expense of viral fitness. Meanwhile, we cannot deny the advantage of lower replication in establishing persistent infection. Lower replication may contribute to the avoidance of major histocompatibility class I–mediated antigen presentation and to escape from the host immune system. Either way, by acquiring the ability to produce more viral particles, the JFH-1/S2 strain could rapidly spread to surrounding cells, irrespective of its lower replication efficiency. Importantly, these emerged mutations did not attenuate *in vivo* infectivity, unlike cell culture adaptive mutations reported to cause attenuated infection *in vivo*.[19] Upon inoculation into human hepatocyte–transplanted mice, JFH-1/S1, JFH-1/S2, and JFH-1/C strains could establish infection without any mutations, produced levels of viremia similar to JFH-1/wt, and persisted for a similar observed period of infection (Fig. 2). This observation is different from that in chimpanzees, where JFH-1/wt and JFH-1/C strains were eliminated earlier than JFH-1/S2. In contrast to chimpanzees, human hepatocyte–transplanted mice lack a CTL and natural killer (NK) cell–mediated immune system, which could be responsible for this difference.[6] Taken together, our results suggest that along with efficient infectious virus production, the JFH-1/S2 strain might have acquired an advantage that helps it evade the CTL and NK cell–mediated immune system.

Apoptosis of virus-infected cells by the immune system is crucial as a general mechanism of clearing infections.[20,21] The J6/JFH-1 chimeric virus has been reported to exhibit proapoptotic characteristics in cell culture.[22] However, because HCV needs to escape the host immune system in order to establish chronic infection, immune cell–mediated apoptosis may be inhibited in infected hepatocytes. In the liver, HCV-infected hepatocytes are eliminated by targeted apoptosis induced by NK cells, macrophages, and CTLs with ligand-mediated and receptor-mediated signals such as TNF-α, FasL, and TNF-related apoptosis–inducing ligand.[23-26] Thus, we used TNF-α to mimic natural immunomediated apoptosis and found that the JFH-1/S2–replicating cells have lower susceptibility to the apoptosis induced by these cytokines. In JFH-1/S2–transfected cells, TNF-α–induced apoptosis detected by TUNEL assay was substantially lower than that of JFH-1/wt–transfected cells (Fig. 4). We confirmed it by staining with anticleaved PARP. In complete agreement with the results produced by way of TUNEL assay, the number of anticleaved PARP stained cells among JFH-1/S2–infected cells was significantly lower than that among JFH-1/wt–infected cells (Fig. 5). In our previous study, we reported that HCV-specific immune responses with T cell proliferation and interferon-γ production were maintained until the disappearance of viremia in the patient serum–infected chimpanzee.[11] This finding indicates that continuous selection pressure in the infected chimpanzee might have contributed to the emergence of a clone with an ability to escape the cytokine-induced apoptosis. We are not sure whether this phenotype of JFH-1/S2 is due to its lower replication efficiency and thus lower production of HCV proteins. The accumulation of viral proteins might predispose cells to the apoptosis induced by TNF-α. To answer this question, it will be necessary to investigate the genomic regions of JFH-1/S2 and cellular host factors responsible for the ability of this strain to escape the apoptosis.

By way of mapping analysis for JFH-1/S2, we could determine responsible regions; NS5B was for lower replication efficiency (Supporting Fig. 1B), and P7 and NS2 were for enhanced viral particle assembly (Supporting Table 2). For the evasion of apoptosis, we could not specify the responsible region, because both chimeric constructs, JFH-1/S2-wt and JFH-1/wt-S2, showed less susceptibility to cytokine-induced apoptosis to a certain extent. These data indicate that both structural and nonstructural regions might have contributed to the acquisition of this phenotype. Previously, a potent antiapoptotic effect of the HCV NS5A protein was described.[27] NS5A interacts with Bin1, which is a nucleocytoplasmic c-Myc–interacting protein with tumor suppressor and apoptotic properties, thus inhibiting Bin1-

associated apoptosis. Because JFH-1/S2 contains several mutations in the NS5A region (Supporting Table 1), one or more mutations in this protein may be associated with antiapoptotic effects.

In conclusion, we demonstrated that the JHF-1/S2 strain acquired phenotypes of lower replication, higher virus production, and less susceptibility to cytokine-induced apoptosis. These phenotypes were associated with mutations that emerged 23 weeks after infection in a chimpanzee, and might have contributed to long-term infection *in vivo*. Such control of viral functions by specific mutations may be a key viral strategy to establish persistent infection.

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Development of recombinant hepatitis C virus with NS5A from strains of genotypes 1 and 2

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ABSTRACT

Nonstructural protein 5A (NS5A) of hepatitis C virus (HCV) plays multiple and diverse roles in the viral lifecycle, and is currently recognized as a novel target for anti-viral therapy. To establish an HCV cell culture system with NS5A of various strains, recombinant viruses were generated by replacing NS5A of strain JFH-1 with those of strains of genotypes 1 (H77; 1a and Con1; 1b) and 2 (J6CF; 2a and MA; 2b). All these recombinant viruses were capable of replication and infectious virus production. The replacement of JFH-1 NS5A with those of genotype 1 strains resulted in similar or slightly reduced virus production, whereas replacement with those of genotype 2 strains enhanced virus production as compared with JFH-1 wild-type. A single cycle virus production assay with a CD81-negative cell line revealed that the efficient virus production elicited by replacement with genotype 2 strains depended on enhanced viral assembly, and that substitutions in the C-terminus of NS5A were responsible for this phenotype. Pulse-chase assays revealed that these substitutions in the C-terminus of NS5A were possibly associated with accelerated cleavage kinetics at the NS5A–NS5B site. Using this cell culture system with NS5A-substituted recombinant viruses, the anti-viral effects of an NS5A inhibitor were then examined. A 300- to 1000-fold difference in susceptibility to the inhibitor was found between strains of genotypes 1 and 2. This system will facilitate not only a better understanding of strain-specific roles of NS5A in the HCV lifecycle, but also enable the evaluation of genotype and strain dependency of NS5A inhibitors.

1. Introduction

Approximately 3% of the world's population is persistently infected with hepatitis C virus (HCV) and at increased risk of fatal chronic liver diseases such as decompensated liver cirrhosis and hepatocellular carcinoma. HCV have significant diversity in their genome and are grouped into six major genotypes. Among these genotypes, genotypes 1 and 2 are distributed worldwide and are predominant in Japan. The genotype is an important viral factor to predict the outcome of interferon (IFN)-based therapy. Because the efficacy of current therapy with pegylated IFN and ribavirin is insufficient, there is great interest in the development of novel HCV-specific inhibitors. The development of an HCV cell culture system with strain JFH-1 has enabled the study of the viral lifecycle and research into anti-viral compounds [1]. However, the available strains used in the HCV cell culture system are still limited to JFH-1 (genotype 2a) and H77S (genotype 1a) [2]. Thus, JFH-1 based recombinant viruses harboring specific regions of other strains would be useful to assess the genotype or strain-specific sensitivity to novel anti-HCV compounds.

Although NS5A is an essential and involved in HCV RNA replication and virus assembly [3,4], it has been reported to be tolerable for trans-complementation in replication-defective mutants due to critical mutations in NS5A [5]. We hypothesized that the NS5A of strain JFH-1 could be replaced with those of other strains. In the present study, we developed a cell culture system with JFH-1 based intra- and inter-genotypic recombinant HCV harboring NS5A of strains H77 (genotype 1a) [6], Con1 (genotype 1b) [7], J6CF (genotype 2a) [8], and MA (genotype 2b) [9]. Through the use of these recombinant viruses, we evaluated the effects of NS5A replacement on the HCV lifecycle and susceptibility to the NS5A inhibitor BMS-790052.

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2. Materials and methods

2.1. Cell culture

The human hepatoma cell line, HuH-7, and derivative cell lines, Huh7.5.1 [10] and Huh7-25 [11], were cultured in complete growth medium as described previously [1,11].

2.2. Plasmid construction

Plasmids containing the full-genome of HCV strain JFH-1 (pJFH1) and of a replication defective mutant (pJFH1/GND) have been described previously [1]. The construction of the NS5A replaced recombinant viruses and subgenomic reporter replicons was described in Supplementary materials.

2.3. In vitro RNA synthesis and RNA transfection

In vitro synthesis of HCV RNA and RNA transfection were performed as described elsewhere [1].

2.4. Quantification of HCV core protein, luciferase activity, and extra- and intra-cellular infectivity

Quantification of these values was described in Supplementary materials.

2.5. Inhibition of HCV production by a specific NS5A inhibitor

Huh7.5.1 cells (3×10^6) were electroporated with 3 μg of synthetic HCV RNA, suspended in 15 mL complete growth medium, and seeded into 24-well plates. At 4 h after electroporation, the culture medium was replaced with medium containing 0.1% dimethyl sulfoxide (DMSO) with or without various concentrations of the specific NS5A inhibitor BMS-790052 (provided from Bristol-Myers Squibb Company, Plainsboro, NJ) [12]. After 44 h incubation, cells were harvested and HCV core protein was quantified.

2.6. Statistical analysis

Unpaired 2-tailed *t*-test was performed to evaluate the significance of results, and $p < 0.05$ was considered significant.

3. Results

3.1. Development of recombinant HCV with NS5A of genotypes 1 and 2

To establish an HCV cell culture system with NS5A of various strains, we generated recombinant viruses by replacing NS5A of strain JFH-1 with those of genotypes 1 and 2 strains. By transfection of *in vitro* transcribed RNA, efficient production of HCV core protein was detected in JFH-1 wild-type (JFH1/wt) and other recombinant viruses, but not in the replication defective mutant JFH1/GND (Fig. 1A). When compared between JFH1/wt and other recombinant viruses, intracellular core protein levels were comparable at days 2 and 3 after transfection, while extracellular core protein levels were very different. The extracellular core protein level of JFH1/wt-transfected cells increased exponentially up to 23,515 ± 1790 fmol/L at day 3. Similar kinetics was observed in JFH1/5A-H77-transfected cells. However, the extracellular core protein level of JFH1/5A-Con1-transfected cells was approximately 2.5-fold lower than that of JFH1/wt at days 2 and 3. Interestingly, the extracellular core protein levels of intra-genotypic recombinant viruses, JFH1/5A-J6CF and 5A-MA, were 2.5- to 3.5-fold higher than that of JFH1/wt at days 2 and 3. To evaluate the effect of these NS5A replacements on HCV replication, we used recombinant subgenomic reporter replicons, SGR-JFH1/RLuc/wt, 5A-H77, 5A-Con1, 5A-J6CF, and 5A-MA. The *Renilla* luciferase activities of these recombinant subgenomic replicons were comparable to that of SGR-JFH1/Rluc/wt, suggesting similar levels of replication efficiency (Fig. 1B).

To further assess whether NS5A replacement affected other steps of the viral lifecycle, we used a single cycle virus production assay with Huh7-25 cells, a HuH-7-derived cell line lacking CD81 expression on the cell surface [11]. This cell line can support replication and infectious virus production upon transfection of HCV genomic RNA, but cannot be reinfected by produced HCV, therefore allowing the observation of a single cycle of infectious viral production without the confounding effects of reinfection [13]. As shown in Fig. 1C, JFH1/wt yielded an extracellular infectivity titer of 1585 ± 436 FFU/well at day 2 after transfection. JFH1/5A-H77 and 5A-Con1 showed significantly lower titers, while JFH1/5A-J6CF and 5A-MA showed significantly higher intracellular infectivity titers compared to JFH1/wt ($p < 0.05$). These data were consistent with the extracellular core protein levels of JFH1/wt and recombinant viruses (Fig. 1A). A similar tendency was observed in the intracellular infectivity titers of JFH1/wt and recombinant viruses (Fig. 1C). To estimate the efficiency of viral particle assembly, we determined the intracellular specific infectivity by calculating the ratio of the intracellular infectivity titer over the intracellular HCV core protein level. The intracellular specific infectivities of JFH1/5A-H77 and 5A-Con1 were 2.5- and 8-fold lower than that of JFH1/wt, respectively, while JFH1/5A-J6CF and 5A-MA showed 12- and 4-fold higher infectivities compared to JFH1/wt, respectively, suggesting a low assembly efficiency of JFH1/5A-H77 and 5A-Con1, and a high assembly efficiency of JFH1/5A-J6CF and 5A-MA (Fig. 1D). Taken together, all recombinant viruses could replicate and yielded infectious virus. Intra-genotypic recombinant viruses, JFH1/5A-J6CF and 5A-MA, had a higher ability to produce infectious virus than JFH1/wt in cultured cells.

3.2. The C-terminus of NS5A is responsible for enhanced viral assembly

The efficient infectious virus production of intra-genotypic recombinant viruses was unexpected. This prompted us to search for causes of the enhancement. To analyze the enhanced virus assembly of JFH1/5A-J6CF and 5A-MA, we focused on the C-terminus of NS5A of these strains, because this region influence the cleavage between NS5A and NS5B, and the cleavage is reported to be involved in virus assembly [14]. We generated recombinant JFH-1 viruses harboring 10 amino acids of the C-terminus of NS5A of J6CF and MA (JFH1/5AcJ6 and 5AcMA, respectively), and investigated replication and infectious virus production. In these 10 amino acids of the C-terminus of NS5A, JFH1/5AcJ6 and 5AcMA contain 2 and 6 substitutions, respectively, as compared with JFH1/wt, and 2 of them, T2438S and T2439V, are common (Fig. 2A). As shown in Fig. 2B, the extracellular core protein level of JFH1/5AcJ6-transfected cells was higher than those of JFH1/wt- and 5A-J6CF-transfected cells at the examined time points. A similar tendency was observed between JFH1/5AcMA and JFH1/wt or 5A-MA (Fig. 2C). In contrast to the extracellular core protein levels, the intracellular core protein levels were comparable for all NS5A recombinants at the examined time points.

We next assessed the replication of recombinant subgenomic luciferase reporter replicons on the basis of JFH1/5AcJ6 and 5AcMA (Fig. 2D). JFH1/5AcJ6 and 5AcMA showed similar levels of replication to JFH1/wt at day 2 after transfection. To investigate the effects of substitutions at the C-terminus of NS5A on infectious viral particle assembly, we determined the extra- and intracellular infectivity with the single cycle virus production assay with Huh7-25 cells. As shown in Fig. 2E, extra- and intracellular infectivities of

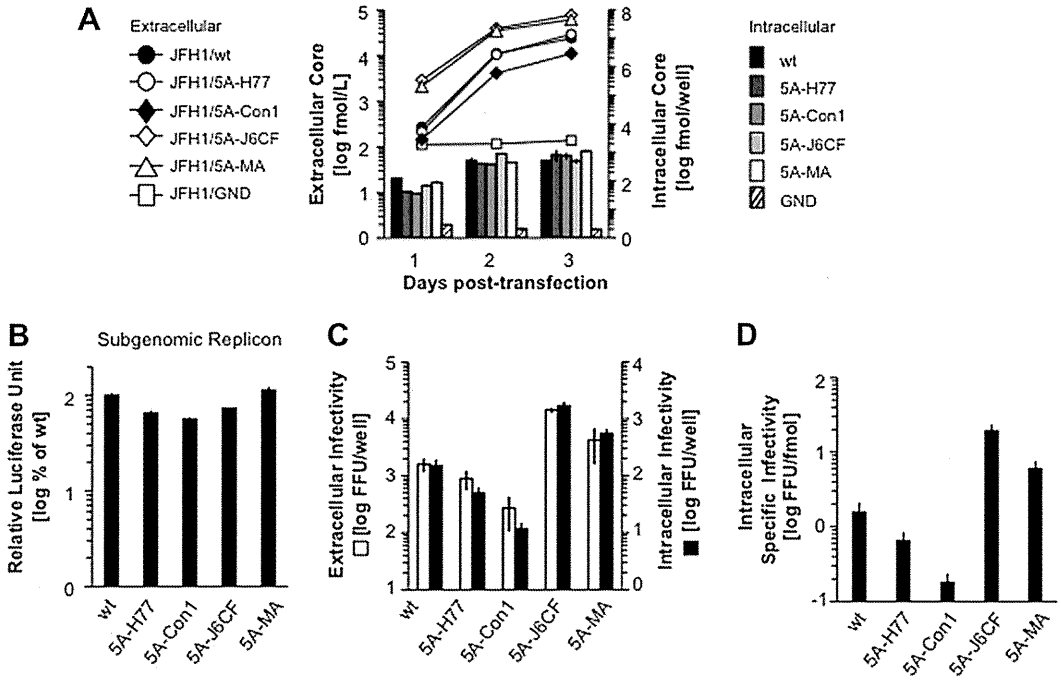

Fig. 1. Production and replication of recombinant viruses with NS5A of strains of genotypes 1 and 2. (A) Huh7.5.1 cells were transfected with *in vitro* synthesized RNA of JFH1/wt and indicated recombinants. The amount of extracellular (line graph) and intracellular (bar graph) HCV core protein was determined at the indicated time points. Assays were performed in triplicate, and means ± standard deviation are plotted. (B) Huh7.5.1 cells were transfected with subgenomic replicon RNA of JFH1/wt and indicated recombinants. Luciferase activity at day 2 was measured. Replication levels of JFH1/wt and indicated recombinants were calculated as fold increases at 4 h and are expressed as percentages of JFH1/wt. (C) Huh7-25 cells were transfected with RNA of JFH1/wt and recombinants. Forty-eight hours after transfection, extra- and intracellular infectivities were determined by inoculating into naïve Huh7.5.1 cells. (D) Intracellular specific infectivity of JFH1/wt and indicated recombinants.

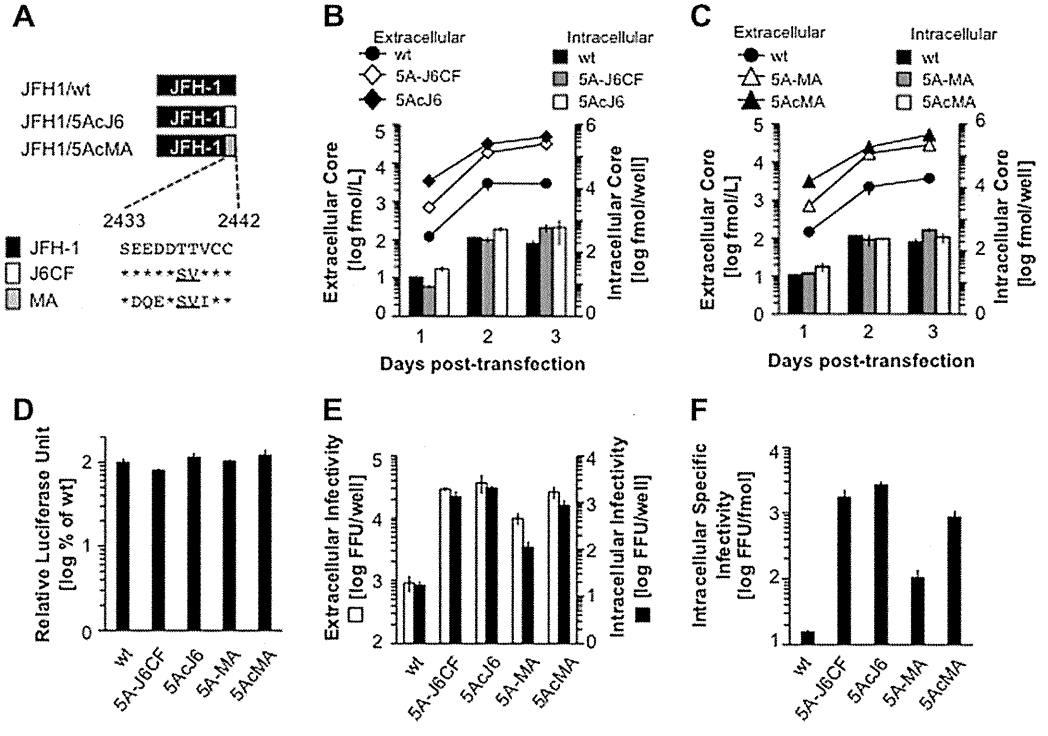

Fig. 2. C-terminal amino acids in NS5A were responsible for the enhanced virus production of recombinant viruses with NS5A of genotype 2 strains. (A) Alignment of C-terminal amino acids in NS5A of JFH-1, J6CF, and MA. Identical amino acids are indicated by asterisks. The indicated number represents the position of the amino acid in the entire polyprotein of JFH-1. (B) Huh7.5.1 cells were transfected with RNA of JFH1/wt, 5A-J6CF, and 5AcJ6. The amount of extracellular (line graph) and intracellular (bar graph) core proteins were quantified at the indicated time points. (C) Huh7.5.1 cells were transfected with RNA of JFH1/wt, 5A-MA, and 5AcMA. The amount of extracellular (line graph) and intracellular (bar graph) core proteins were quantified at the indicated time points. (D) Huh7.5.1 cells were transfected with subgenomic replicon RNA of JFH1/wt and indicated recombinants. Luciferase activity at day 2 was measured. Replication levels of JFH1/wt and indicated recombinants were calculated as the fold increase at 4 h and are expressed as percentages of JFH1/wt. (E) Huh7-25 cells were transfected with RNA of JFH1/wt and recombinant viruses. Forty-eight hours after transfection, extra- and intracellular infectivities were determined by inoculating into naïve Huh7.5.1 cells. (F) Intracellular specific infectivities of JFH1/wt and indicated recombinants.

JFH1/5AcJ6 and 5AcMA were approximately 20-fold higher than that of the JFH1/wt ($p < 0.05$), and were slightly higher than those of JFH1/5A-J6CF and 5A-MA. We also determined the specific intracellular infectivity of each recombinant virus to assess virus assembly (Fig. 2F). As with extra- and intracellular infectivities, the specific intracellular infectivities of JFH1/5AcJ6 and 5AcMA were more than 10-fold higher than that of the JFH1/wt ($p < 0.05$), and were slightly higher than those of JFH1/5A-J6CF and 5A-MA. These results suggest that these C-terminal amino acids of NS5A are responsible for enhanced assembly of intra-genotypic recombinant viruses JFH1/5A-J6CF and 5A-MA.

3.3. Amino acid substitutions at the C-terminus of NS5A accelerate cleavage kinetics between NS5A and NS5B

To investigate whether substitution of the C-terminus of NS5A affects the cleavage kinetics between NS5A and NS5B, we performed pulse-chase assays using a T7-based expression system. Immunoprecipitations were performed with an NS5B-specific antibody and immunocomplexes were analyzed on a 7.5% SDS–PAGE (Supplementary Fig. A). Fully processed NS5B and an uncleaved NS5A–NS5B precursor with a size of approximately 130 kDa could be detected for JFH1/wt and recombinant constructs JFH1/5AcJ6 and 5AcMA. In the case of JFH1/wt, the amount of uncleaved precursor was gradually decreased but still detectable at 4 h of the chase period. On the other hand, in the case of the recombinant constructs, JFH1/5AcJ6 and 5AcMA, the amounts of precursor were reduced more rapidly and were undetectable by 4 h of chase. To assess the kinetics of the cleavage, the percentages of uncleaved NS5A–NS5B precursor at the examined time points were plotted and analyzed using nonlinear regression (Supplementary Fig. B). Rapid cleavage kinetics was observed in JFH1/5AcJ6 and 5AcMA transfected cells as compared with JFH1/wt. These observations suggest that substitutions at the C-terminus of NS5A of these recombinant viruses are responsible for the accelerated cleavage kinetics between NS5A and NS5B, and might be associated with enhanced infectious viral particle assembly.

3.4. Susceptibility of recombinant HCV to the NS5A inhibitor BMS-790052

Using developed JFH-1 based inter- and intra-genotypic recombinant viruses, we assessed their susceptibility to the NS5A inhibitor BMS-790052 [12]. After transfection with synthesized HCV RNA, cells were treated with different concentrations of BMS-790052 for 2 days and intracellular HCV core protein levels were determined. No cytopathic effects were observed at the concentrations used (data not shown). As shown in Fig. 3, the intracellular core protein levels of JFH1/wt and recombinant viruses were inhibited to different extents. Recombinant viruses with NS5A of genotype 1, JFH1/5A-H77 and 5A-Con1, showed higher susceptibility to BMS-790052 as compared with JFH1/wt, while JFH1/5A-J6CF and 5A-MA showed much lower susceptibility. To compare the susceptibilities, the effective concentrations required to inhibit 50% of intracellular core protein level (EC_{50}) were determined, because the intracellular core protein levels of these recombinant viruses were at almost the same level at day 2 after transfection (Fig. 1A). The EC_{50} of JFH1/wt and recombinant viruses with NS5A of genotype 1, JFH1/5A-H77 and 5A-Con1, were 6.4, 3.1, and 1.4 pM, respectively, and do not conflict with results using replicon systems reported previously [12]. In contrast, recombinant viruses with NS5A of genotype 2, JFH1/5A-J6CF and 5A-MA, were more resistant to BMS-790052, and EC_{50} values were 1.5 and >5 nM, respectively. Collectively, the anti-HCV effect of the specific NS5A inhibitor BMS-790052 showed strain and genotype dependency. In particular, the NS5A of genotype 2 strains, J6CF and MA, excepting JFH-1, showed 300- to 1000-fold lower susceptibility to BMS-790052 compared with the NS5A of genotype 1 strains, H77 and Con1.

4. Discussion

HCV NS5A is essential for replication and infectious virus production, similar to other nonstructural proteins possessing enzymatic activities, including NS3 (a serine protease) and NS5B (an RNA-dependent RNA polymerase). Currently, these nonstructural proteins are being targeted to establish anti-viral compounds to improve the outcome of therapy for chronic HCV infection, and several inhibitors for these proteins are entering into clinical trials. A great deal of interest has also been shown in the development of NS5A inhibitors, and one potent inhibitor, BMS-790052, has recently been described [12]. In this study, to assess strain and genotype dependent susceptibility for this inhibitor, we generated recombinant HCV with NS5A from strains other than JFH-1, because a limited number of strains are available in the HCV cell culture system. We replaced NS5A of JFH-1 with those of genotype 1 and 2 strains, and observed efficient replication and infectious virus production in cell culture.

The replication efficiencies of these NS5A recombinant viruses were almost the same, whereas virus production levels into the culture medium were very different from JFH1/wt (Fig. 1A and

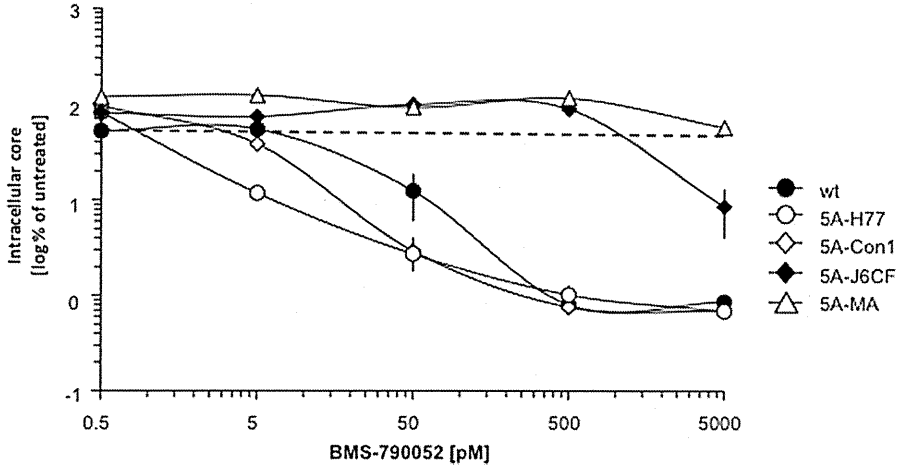

Fig. 3. Susceptibility of JFH1/wt and recombinant viruses to the NS5A inhibitor. Huh7.5.1 cells were transfected with RNA of JFH1/wt and recombinant viruses and treated with serially diluted BMS-790052 for 48 h. The amounts of intracellular HCV core protein were quantified and normalized against untreated control set to 100%.

Table 1
Amino acid substitutions in NS5A of strains used, and reported resistant mutations to BMS-790052.

AA[a]		Strains used in this study					Reported resistant mutations[b]			Ref.
Entire	NS5A	JFH1	H77	Con1	J6CF	MA	1a	1b	2a	
2004	28	F	M	L	F	L	T (683)	T (20)	–	[20]
2006	30	K	Q	R	K	K	E (24,933) K (24,317) H (1450) R (1217)	E (6)	–	[20]
2007	31	L	L	L	M	M	M (350) V (3350)	F (5) M (3) V (23)	M (170)	[12,20]
2008	32	P	P	P	P	P	L (233)	L (17)	–	[20]
2068	93	A	A	C	C	C	–	–	E (150)	[12]
2069	93	Y	Y	Y	Y	Y	C (1850) H (5367) N (47,017)	H (19) N (28)	H (130–1400)	[12,20]

[a] AA, amino acid position which are according to entire polyprotein (Entire) and NS5A of JFH1.
[b] Fold resistance as compared with parental amino acid is indicated in parentheses.

B). Enhanced virus production was detected in recombinant viruses replaced with NS5A of genotype 2 strains, while reduced virus production was observed in recombinant viruses replaced with NS5A of genotype 1 strains (Fig. 1A). The single cycle virus production assay revealed that this enhanced virus production with NS5A of genotype 2 was due to efficient viral particle assembly (Fig. 1D). To analyze the mechanism of efficient virus assembly by NS5A of strains J6CF and MA, we focused on the cleavage between NS5A and NS5B. Several reports have shown that amino acids in the C-terminus of NS5A influence the cleavage [15,16]. Thus, we used recombinant JFH-1 viruses harboring amino acids of J6CF and MA in the C-terminus of JFH-1 NS5A, and assessed replication and infectious virus production. We found that both of these recombinant viruses, JFH1/5AcJ6 and 5AcMA, showed more enhanced virus assembly (Fig. 2F), and reasoned that the amino acid substitutions T2438S and T2439V were responsible for the enhanced infectious virus production. In pulse-chase assays for the cleavage of NS5A and NS5B, accelerated cleavage was observed in recombinant viruses JFH1/5AcJ6 and 5AcMA. Uncleaved NS5A–NS5B disappeared earlier in JFH1/5AcJ6- and 5AcMA-transfected cells than in JFH1/wt-transfected cells (Supplementary Fig. A). Taken together, the enhanced virus assembly observed with JFH1/5A-J6CF and 5A-MA depended on the C-terminal amino acid substitutions in NS5A, possibly through accelerated cleavage kinetics between NS5A and NS5B. The reason for the correlation between accelerated cleavage and enhanced virus assembly is still unknown. Accelerated cleavage may lead to an increased amount of mature NS5A used for virus assembly or affect the interaction with the core protein, which has been reported to be important for infectious virus assembly [17]. Another possibility is the interaction between the C-terminus of NS5A and some host factor(s) involved in virus particle assembly, such as apolipoprotein E [18]. Amino acid substitutions at this region may alter the potency of this interaction directly. A previous report has also shown that another mutation in this region, V2440L, is associated with delayed cleavage kinetics between NS5A and NS5B, but enhanced virus assembly [14]. Further investigation will be necessary to clarify this mechanism and to solve the discrepancy.

Using this cell culture system with NS5A recombinant viruses, we assessed strain and genotype dependent susceptibility to the novel NS5A inhibitor, BMS-790052. This potent inhibitor successfully inhibited replication of JFH1/wt and recombinant viruses with NS5A of genotype 1 strains (Fig. 3). However, it showed limited effectiveness on recombinant viruses with NS5A of genotype 2 strains. This high efficacy for genotype 1 strains makes sense because this inhibitor and its lead compound were identified using genotype 1a and 1b subgenomic replicons [19].

During preparation of this paper, another study was published describing an HCV cell culture system with NS5A-substituted recombinant viruses [20]. That study used a J6/JFH-1 chimeric virus that is known to have high virus production efficiency, but not natural viruses, and established nine recombinant viruses with NS5A from strains of eight different subtypes. They found that recombinant viruses with NS5A of strains of genotypes 1a, 1b, 4a, 5a, and 6a were sensitive, and strains of genotypes 2a and 3a were resistant to the NS5A inhibitor, data that are consistent with our own observations. In addition, we found that recombinant virus with NS5A of genotype 2b, which is the one of the predominant genotypes in Japan, was also resistant to the compound. Resistant mutations to BMS-790052 have been reported and are frequently observed in the N-terminus of NS5A, suggesting inhibition of membrane localization and dimerization of NS5A (Table 1) [12,20]. Among these reported mutations, one of the most potent, 2006E/K/H/R (amino acid position (AA) 30 in NS5A), is found in all strains but H77, and another potent resistant mutation, 2007F/M/V (AA 31 in NS5A), is also found in J6CF and MA. Thus, the lower susceptibilities of recombinant viruses JFH1/5A-J6CF and 5A-MA, as compared with JFH1/wt, might be due to the latter mutation. Based on an analysis of the database of submitted strains (Hepatitis Virus Database; http://s2as02.genes.nig.ac.jp/index.html), this resistant mutation, 2007M, is detected in 84.2% and 79.0% of genotype 2a and 2b strains, respectively, whereas it is observed in only 0.2% of genotype 1a and 3.8% of genotype 1b strains [20,21]. From these observations, most of genotype 2a and 2b strains may be resistant to BMS-790052, although these are known to be sensitive to interferon [22].

In conclusion, we established JFH-1 based recombinant viruses by replacement of NS5A with those from strains of genotypes 1 and 2. All the generated recombinant viruses could replicate and produce infectious viruses in cell culture, and were useful to assess the genotype and strain dependency to a novel NS5A inhibitor. The strategy of using recombinant virus will facilitate not only a better understanding of the strain-specific roles of NS5A in the HCV lifecycle, but also aid in developing and testing specific inhibitors against NS5A from different genotypes and strains.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.144.

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Production of Infectious Chimeric Hepatitis C Virus Genotype 2b Harboring Minimal Regions of JFH-1

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To establish a cell culture system for chimeric hepatitis C virus (HCV) genotype 2b, we prepared a chimeric construct harboring the 5′ untranslated region (UTR) to the E2 region of the MA strain (genotype 2b) and the region of p7 to the 3′ UTR of the JFH-1 strain (genotype 2a). This chimeric RNA (MA/JFH-1.1) replicated and produced infectious virus in Huh7.5.1 cells. Replacement of the 5′ UTR of this chimera with that from JFH-1 (MA/JFH-1.2) enhanced virus production, but infectivity remained low. In a long-term follow-up study, we identified a cell culture-adaptive mutation in the core region (R167G) and found that it enhanced virus assembly. We previously reported that the NS3 helicase (N3H) and the region of NS5B to 3′ X (N5BX) of JFH-1 enabled replication of the J6CF strain (genotype 2a), which could not replicate in cells. To reduce JFH-1 content in MA/JFH-1.2, we produced a chimeric viral genome for MA harboring the N3H and N5BX regions of JFH-1, combined with a JFH-1 5′ UTR replacement and the R167G mutation (MA/N3H+N5BX-JFH1/R167G). This chimeric RNA replicated efficiently, but virus production was low. After the introduction of four additional cell culture-adaptive mutations, MA/N3H+N5BX-JFH1/5am produced infectious virus efficiently. Using this chimeric virus harboring minimal regions of JFH-1, we analyzed interferon sensitivity and found that this chimeric virus was more sensitive to interferon than JFH-1 and another chimeric virus containing more regions from JFH-1 (MA/JFH-1.2/R167G). In conclusion, we established an HCV genotype 2b cell culture system using a chimeric genome harboring minimal regions of JFH-1. This cell culture system may be useful for characterizing genotype 2b viruses and developing antiviral strategies.

Hepatitis C virus (HCV) is a major cause of chronic liver disease (5, 13), but the lack of a robust cell culture system to produce virus particles has hampered the progress of HCV research (2). Although the development of a subgenomic replicon system has enabled research into HCV RNA replication (15), infectious virus particle production has not been possible. Recently, an HCV cell culture system was developed using a genotype 2a strain, JFH-1, cloned from a fulminant hepatitis patient (14, 29, 32), thereby allowing investigation of the entire life cycle of this virus. However, several groups of investigators have reported genotype- and/or strain-dependent effects of some antiviral reagents (6, 17) and neutralizing antibodies (7, 25). Therefore, efficient virus production systems using various genotypes and strains are indispensable for HCV research and the development of antiviral strategies.

The JFH-1 strain is the first HCV strain that can efficiently produce HCV particles in HuH-7 cells (29). Other strains can replicate and produce infectious virus by HCV RNA transfection, but the efficiency is far lower than that of JFH-1 (24, 31). In the case of replication-incompetent strains, chimeric virus containing the JFH-1 nonstructural protein coding region is useful for analyses of viral characteristics (6, 9, 14, 23, 30, 31).

In this study, we developed a genotype 2b chimeric infectious virus production system using the MA strain (accession number AB030907) (19) harboring minimal regions of JFH-1 and cell culture-adaptive mutations that enhance infectious virus production.

MATERIALS AND METHODS

Cell culture. Huh7.5.1 cells (a kind gift from Francis V. Chisari) (32) and Huh7-25 cells (1) were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under 5% CO_2 conditions. For follow-up study, RNA-transfected cells were passaged every 2 to 5 days depending on cell status.

Full-length genomic HCV constructs. Plasmids used in the analysis of genomic RNA replication were constructed based on pJFH1 (29) and pMA (19). For convenience, an EcoRI recognition site was introduced upstream of the T7 promoter region of pMA by PCR, and an XbaI recognition site was introduced at the end of the 3′ untranslated region (UTR). To construct MA/JFH-1, the EcoRI-BsaBI (nucleotides [nt] 1 to 2570; 5′ UTR to E2) fragment of pMA was substituted into pJFH1 (Fig. 1A). Replacement of the 5′ UTR was performed by exchanging the EcoRI-AgeI (nt 1 to 159) fragment. A point mutation in the core region (R167G) was introduced into MA chimeric constructs by PCR using the following primers: sense, 5′-TTA TGC AAC GGG GAA TTT ACC CGG TTG CTC T-3′; antisense, 5′-GGT AAA TTC CCC GTT GCA TAA TTT ATC CCG TC-3′. G167R substitution in the JFH-1 construct was performed by PCR using the following primers: sense, 5′-ATT ATG CAA CAA GGA ACC TAC CCG GTT TCC C-3′; antisense, 5′-GGT AGG TTC CTT GTT GCA TAA TTA ACC CCG TC-3′. Point mutations (L814S, R1012G, T1106A, and V1951A) were introduced into MA chimeric constructs by PCR using the following primers: L814S, 5′-GCT TAC GCC TCG GAC GCC GCT GAA CAA GGG G-3′ (sense) and 5′-AGC GGC GTC CGA GGC GTA AGC CTG CTG CGG C-3′ (antisense); R1012G, 5′-GAG GCT AGG TGG

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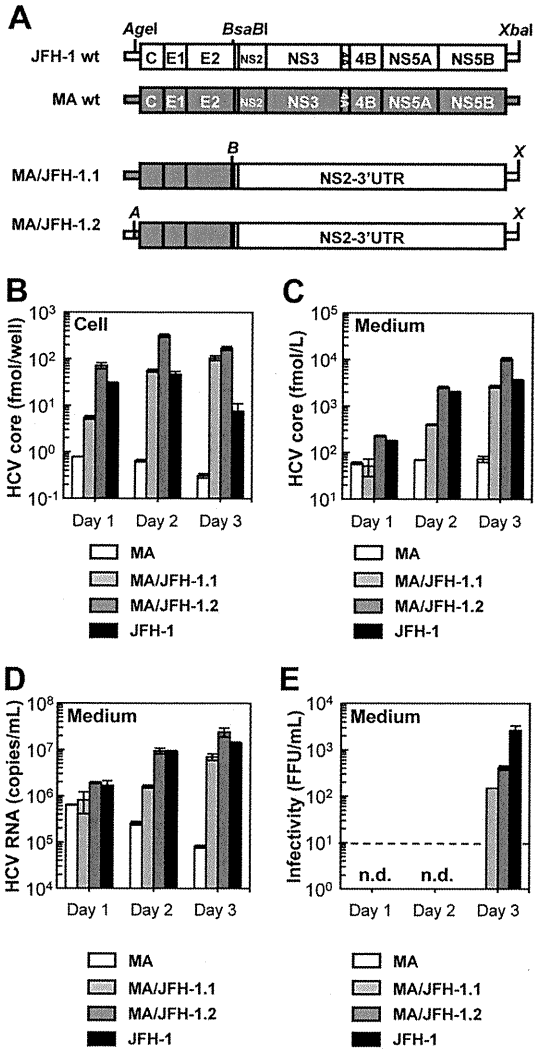

FIG 1 Replication and virus production by MA/JFH-1 chimeras in Huh7.5.1 cells. (A) Schematic structures of JFH-1, MA, and two MA/JFH-1 chimeras (MA/JFH-1.1 and MA/JFH-1.2). The junction of JFH-1 and MA in the 5′ UTR is an AgeI site, and the junction of MA and JFH-1 in the NS2 region is a BsaBI site. A, AgeI; B, BsaBI; X, XbaI. (B to E) Chimeric HCV RNA replication in Huh7.5.1 cells. HCV core protein level in cells (B) and culture medium (C) and HCV RNA levels in medium (D) and infectivity of culture medium (E) from HCV RNA-transfected Huh7.5.1 cells are shown. Ten micrograms of HCV RNA was transfected into Huh7.5.1 cells, and cells and culture medium were harvested on days 1, 2, and 3. n.d., not determined. Assays were performed three times independently, and data are presented as means ± standard deviation. Dashed line indicates detection limit. wt, wild type.

GGA AGT TCT GCT CGG CCC T-3′ (sense) and 5′-AGA ACT TCC CCA CCT AGC CTC GCG GAA ACC G-3′ (antisense); T1106A, 5′-CAG ATG TAC GCC AGC GCA GAG GGG GAC CTC-3′ (sense) and 5′-CTG CGC TGG CGT ACA TCT GGG TGA CTG GTC-3′ (antisense); and V1951A, 5′-GTG ACG CAG GCG TTA AGC TCA CTC ACA ATT ACC-3′ (sense) and 5′-TGA GCT TAA CGC CTG CGT CAC GCG CAG CGA G-3′ (antisense). To construct the MA chimeric virus harboring minimal regions of JFH-1 (MA/N3H+N5BX-JFH1), ClaI (nt 3930), EcoT22I (nt 5294), and BsrGI (nt 7782) recognition sites were introduced into pMA by site-directed mutagenesis. The 5′ UTR (EcoRI-AgeI), the region of the NS3 helicase (N3H; ClaI-EcoT22I), and the region of NS5B to 3′ X (N5BX; BsrGI-XbaI) were then replaced with the corresponding regions from JFH-1.

RNA synthesis, transfection, and determination of infectivity. RNA synthesis and transfection were performed as described previously (12, 22). Determination of infectivity was also performed as described previously, with infectivity expressed as the number of focus-forming units per milliliter (FFU/ml) (12, 22). When necessary, culture medium was concentrated 20-fold in Amicon Ultra-15 spin columns (100-kDa molecular-weight-cutoff; Millipore, Bedford, MA) in order to determine infectivity.

Quantification of HCV core protein and HCV RNA. In order to estimate the concentration of HCV core protein in culture medium, we performed a chemiluminescence enzyme immunoassay (Lumipulse II HCV core assay; Fujirebio, Tokyo, Japan) in accordance with the manufacturer's instructions. HCV RNA from harvested cells or culture medium was isolated using an RNeasy Mini RNA kit (Qiagen, Tokyo, Japan) or QiaAmp Viral RNA Minikit (Qiagen), respectively. Copy number of HCV RNA was determined by real-time quantitative reverse transcription-PCR (qRT-PCR), as described previously (28).

HCV sequencing. Total RNA in culture supernatant was extracted with Isogen-LS (Nippon Gene Co., Ltd., Tokyo, Japan). cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). cDNA was subsequently amplified with LA *Taq* DNA polymerase (TaKaRa, Shiga, Japan). Four separate PCR primer sets were used to amplify the fragments of nt 130 to 2909, 2558 to 5142, 4784 to 7279, and 7081 to 9634 covering the entire open reading frame and part of the 5′ UTR and 3′ UTR of the MA strain. Sequences of amplified fragments were determined directly.

Immunostaining. Infected cells were cultured on Multitest Slides (MP Biomedicals, Aurora, OH) and were fixed in acetone-methanol (1:1, vol/vol) for 15 min at −20°C. After a blocking step, infected cells were visualized with anti-core protein antibody (clone 2H9) (29) and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen), and nuclei were visualized with 4′,6′-diamidino-2-phenylindole (DAPI).

Assessment of interferon sensitivity. Two micrograms of *in vitro* transcribed RNA was transfected into 3×10^6 Huh7.5.1 cells. Four hours after transfection, cells were placed in fresh medium or medium containing 0.1, 1, 10, 100, and 1,000 IU/ml of interferon α-2b (Intron A; Schering-Plough Corporation, Osaka, Japan). Culture medium was then harvested on day 3, and HCV core levels in the cells and in the medium were measured.

Statistical analysis. Significant differences were evaluated by Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Transient replication and production of 2b/2a chimeric virus. We first tested whether the MA strain (genotype 2b) (19) was able to replicate and produce infectious virus in cultured cells. When the *in vitro* transcribed RNA of MA was transfected into Huh7.5.1 cells, a highly HCV-permissive cell line, replication and virus production were not observed (Fig. 1A to C). We then tested whether 2b/2a chimeric RNA harboring the structural region (5′ UTR to E2) of the MA strain and the nonstructural region (p7 to 3′ UTR) of JFH-1 (Fig. 1A, MA/JFH-1.1) was able to replicate in the cells. After MA/JFH-1.1 RNA transfection, time-dependent accumulation of core protein in the cells (Fig. 1B) and culture medium (Fig. 1C) was observed, indicating that MA/JFH-1.1 RNA was able to replicate in the cells autonomously. HCV RNA levels in the medium were determined by qRT-PCR, and time-dependent increases in HCV RNA level were also observed (Fig. 1D). Infectious virus production was observed on day 3, but infectivity was 17.6-fold lower than that of JFH-1 (Fig. 1E).

In order to improve the level of infectious virus production, we tested another chimeric construct, MA/JFH-1.2, which contained an additional MA-to-JFH-1 replacement of the 5′ UTR (Fig. 1A),

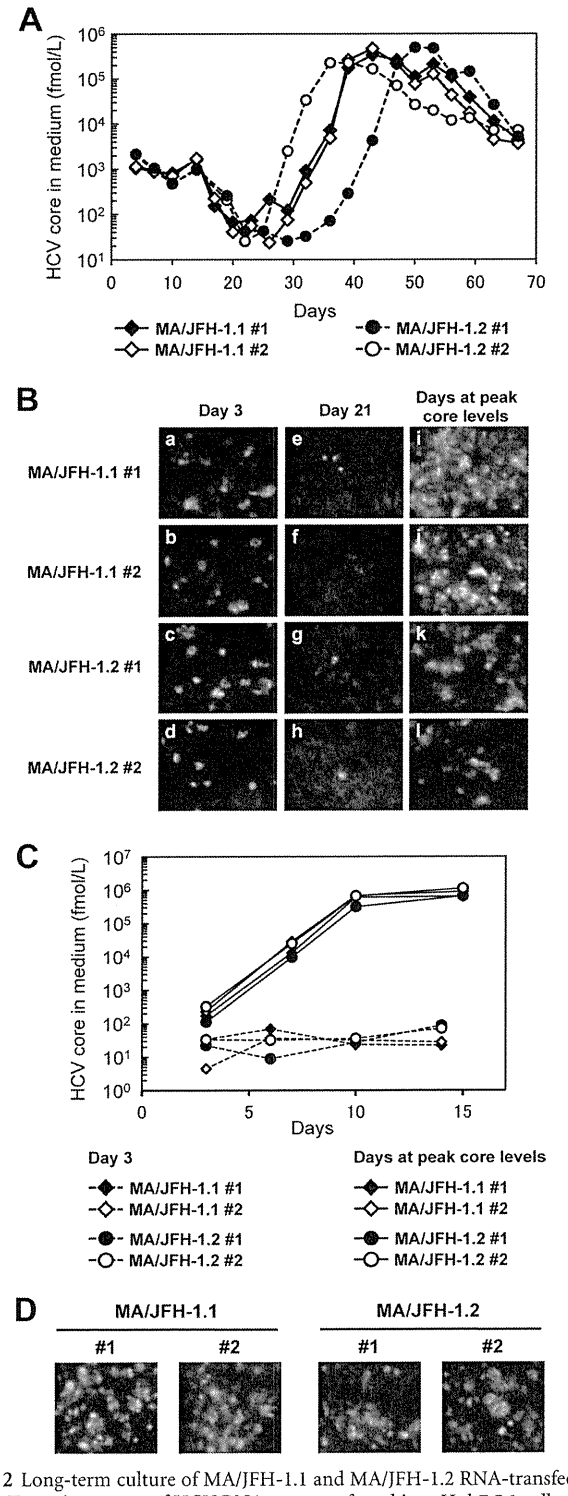

FIG 2 Long-term culture of MA/JFH-1.1 and MA/JFH-1.2 RNA-transfected cells. Ten micrograms of HCV RNA was transfected into Huh7.5.1 cells, and cells were passaged every 2 to 5 days, depending on cell status. Culture medium was collected after every passage, and HCV core protein levels were measured. Transfection was performed twice for each chimeric RNA (1 and 2 for each construct). (A) HCV core protein levels in culture medium from MA/JFH-1.1 and MA/JFH-1.2 RNA-transfected cells. (B) Immunostained cells at 3 days after transfection (a to d), at 21 days after transfection (e to h), and at the time of peak core levels (days 42 to 49). Infected cells were visualized with anti-core protein antibody (green), and nuclei were visualized with DAPI (blue). (C) Infection of naïve cells by culture medium at an MOI of 0.001. (D) Immunostained cells at 15 days after infection with medium at peak core protein levels (Fig. 2A) at an MOI of 0.001. Infected cells were visualized with anti-core antibody (green), and nuclei were visualized with DAPI (blue).

TABLE 1 HCV core protein levels and infectivity in culture medium immediately after RNA transfection (day 3) and after long-term culture (days 35 to 49)

Sample period and virus	Sample no.	Day no.[a]	HCV core (fmol/liter)	Infectivity (FFU/ml)
After transfection				
MA/JFH-1.1	1	3	1.06×10^3	5.00×10^1
	2	3	1.14×10^3	5.70×10^1
MA/JFH-1.2	1	3	2.14×10^3	7.30×10^1
	2	3	2.15×10^3	9.30×10^1
After long-term culture				
MA/JFH-1.1	1	42	3.38×10^5	1.62×10^5
	2	42	4.70×10^5	3.23×10^5
MA/JFH-1.2	1	35	2.27×10^5	1.61×10^5
	2	49	4.93×10^5	3.27×10^5

[a] For the long-term culture, the days are those of peak core protein levels.

as a 5′ UTR replacement from J6CF (genotype 2a) to JFH-1 enhanced virus production of chimeric J6CF virus harboring the region of NS2 to 3′ X of JFH-1 (J6/JFH-1) (A. Murayama et al., unpublished data). The core protein accumulation levels with MA/JFH-1.2 RNA-transfected cells were higher than those with MA/JFH-1.1 ($P < 0.05$) (Fig. 1B). Similarly, core protein and HCV RNA levels in the medium of MA/JFH-1.2 RNA-transfected cells were higher than those of MA/JFH-1.1 ($P < 0.05$) (Fig. 1C and D). Infectivity on day 3 was also higher than with MA/JFH-1.1 ($P < 0.05$) (Fig. 1E), indicating that the 5′ UTR of JFH-1 enhanced virus production. However, infectivity of medium from MA/JFH-1.2 RNA-transfected cells on day 3 remained 6.4-fold lower than that of JFH-1 although HCV RNA levels in the medium were similar to those of JFH-1 (Fig. 1D and E).

These results indicate that 2b/2a chimeric RNA is able to replicate autonomously in Huh7.5.1 cells and produce infectious virus although infectivity remains lower than that of JFH-1.

Assembly-enhancing mutation in core region introduced during long-term culture. Because MA/JFH-1.1 and MA/JFH-1.2 replicated efficiently but produced small amounts of infectious virus, we performed long-term culture of these RNA-transfected cells in order to examine whether these chimeric RNAs would continue replicating and producing infectious virus over the long term. We prepared two RNA-transfected cell lines for each construct (MA/JFH-1.1 and MA/JFH-1.2) as both of these replicated and produced infectious virus at different levels.

Immediately after transfection, core protein levels and infectivity in culture medium were low (1.06×10^3 to 2.15×10^3 fmol/liter and 5.00×10^1 to 9.30×10^1 FFU/ml, respectively) (Fig. 2A and Table 1) although a considerable number of core protein-positive cells were observed by immunostaining (Fig. 2B, frames a to d). Subsequently, core protein levels in the culture medium decreased gradually (Fig. 2A), and core protein-positive cells were rare (Fig. 2B, frames e to h). However, at 30 to 40 days