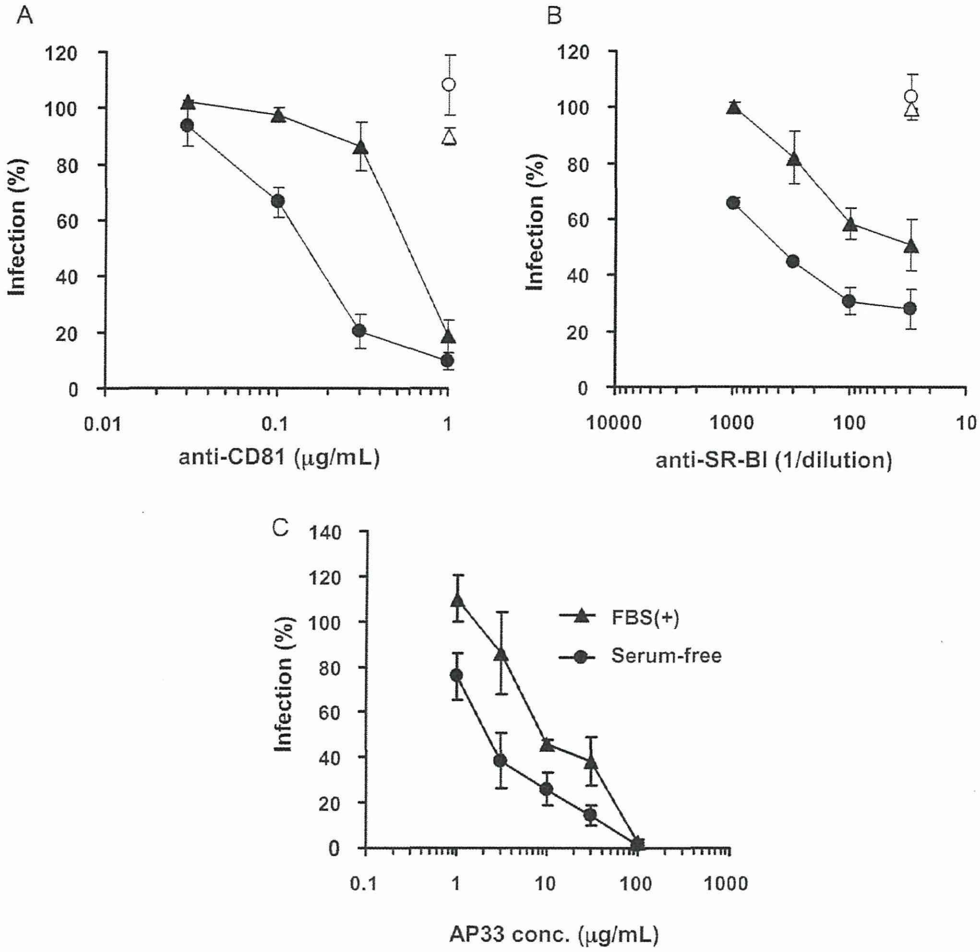

Fig. 5. Inhibition of serum-free cultured HCV infection by anti-CD81, anti-SR-BI and anti-E2 antibodies. Huh7.5.1 cells were pre-incubated with serially diluted anti-CD81 (JS-81, panel A) or anti-SR-BI (panel B) antibody for 1 h, and infectious J6/JFH-1 virus (MOI 0.1) obtained from FBS-supplemented (triangles) or serum-free (circles) culture was inoculated into each well for 72 h. Cells were lysed and HCV-core protein was measured. As controls, 1 μg/mL of mouse IgG (open triangles) and 30-fold diluted rat serum (open circles) were used. For incubation of virus particles with antibody, infectious J6/JFH-1 virus obtained from FBS-supplemented (closed triangle) or serum-free (closed circle) cultures was mixed with the indicated concentration of anti-E2 (AP33, panel C) antibody, and was then inoculated into naïve Huh7.5.1 cells. Infectivity was calculated by titration.

lost from fractions of lower density. Since exosome-like multivesicular particles have been reported to be present in lower density fractions [10], production of multivesicular particles may be different between serum-free and serum-supplemented cultures. When culture supernatants were used, the specific infectivity of serum-free cultured HCV was almost identical to that of FBS-supplemented HCV. However, infectivity of fractions separated on sucrose density gradients was lower for serum-free cultured HCV than for FBS-supplemented HCV cultures. These data may indicate that host factors that contribute to HCV infection differed under different culture conditions. Following infection and subsequent long-term culture under either serum-free or serum-supplemented conditions, HCV-core and RNA levels were identical for the first 40 days, but after this time period the ratio of HCV-core protein:RNA increased in both cultures. This result suggested that long-term culture may induce HCV-core protein which did not have HCV-RNA. As shown in Fig. 3, virus production under serum-free conditions seems to be more stable over 100 days than production under 10% FBS conditions. Fluctuation of viral titer during continuous cultivation of HCV-infected cells has been previously reported [24]. This fluctuation is dependent on the appearance of non-permissive cells with low or no CD81 expression. It is thus likely that these non-permissive cells appear more rapidly under 10% FBS culture conditions than under serum-free conditions. This result may be due to the higher cellular growth rate of these cells in serum-supplemented culture as shown in Fig. 1 and Supplementary Fig. S1.

Our study highlighted some differences between HCV that was produced under serum-free and serum-supplemented conditions. Thus, interestingly, serum-free cultured HCV showed a higher susceptibility to antibody inhibition of viral infection than HCV from serum-supplemented cultures. The multiplicity of infection (MOI) of partially purified HCV was calculated and an MOI of 0.02 was used for HCV infection of naïve Huh7 cells. Although infection by HCV produced under both culture conditions was inhibited by anti-CD81, anti-SR-BI and anti-E2 antibodies in a dose-dependent manner, the degree of antibody inhibition at each dose was stronger for serum-free cultured HCV than for HCV from serum-supplemented cultures (Fig. 5). The anti-CD81 and anti-SR-BI antibody targets the host cells, since CD81 and SR-BI are candidate cellular receptors for HCV. In contrast, the anti-E2 antibody AP33 is a neutralizing antibody for HCV [20,21], and targets virus particles. The antibody inhibition result obtained therefore suggested that HCV infection was affected by contaminating pro-

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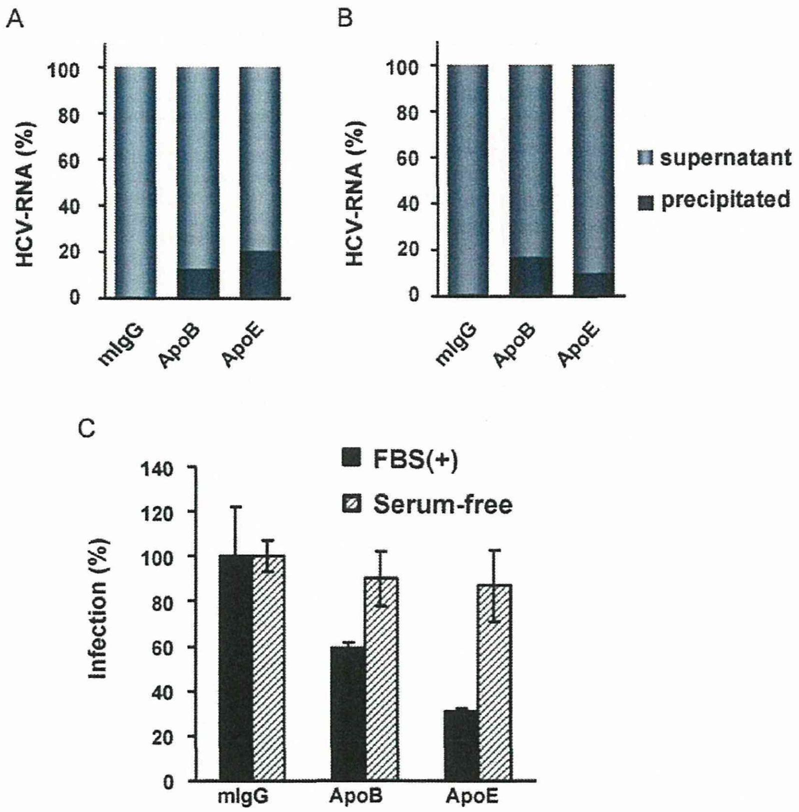

Fig. 6. Immunoprecipitation of cell-cultured HCV with anti-ApoB and ApoE antibodies. 1×10^7 HCV-RNA copies of the J6/JFH-1 virus were incubated with mouse IgG, anti-ApoB or anti-ApoE antibodies, and were then precipitated using protein G-Sepharose. Virus RNA was extracted from the supernatant and the precipitated resin, and HCV-RNA was analyzed using RTD-PCR. All experiments were performed in triplicate, and data are shown as percentages of the mean (A: serum-supplemented, B: serum-free cultured, HCV). Infectivity of the antibody-depleted virus (supernatant) was determined by inoculation into naïve Huh7.5.1 cells. All experiments were performed in triplicate, and data are shown as means ± SD. All data are indicated as percentage of infection, in which the infectivity (FFU/mL) of the supernatant from the mouse IgG (mIgG) precipitation was designated as 100% (C).

teins or by characteristics of HCV that arose due to the different culture conditions used for HCV production. Since CD81-negative Huh7 cells were also able to produce infectious HCV, and since anti-CD81 and anti-E2 antibodies similarly inhibited infection by HCV produced in serum-free culture (data not shown), it is highly unlikely that viral incorporation of CD81 that is produced by CD81-expressing virus-producing cells modulates anti-CD81-inhibition of viral infection. An alternative possibility was that the different antibody inhibition results may have been due to differences in the level, or type of apolipoprotein associated with HCV. HCV associates with lipoproteins in human sera [25], and it has been reported that the envelope proteins of HCV interact with lipoproteins [26]. Moreover, in a recent study, VLDL was reported to associate with HCV particles and affect viral infection and virus secretion [22,23]. Mature VLDL is composed of the apolipoproteins B, C-II and E, as well as lipid, and ApoB and ApoE have been reported to be important for the infectivity of HCV particles [22,23,27–29]. We found that the content of ApoB and ApoE in HCV from serum-supplemented and serum-free cultures was different (Fig. 6A). Thus, serum-free HCV particles had a greater amount of associated ApoB, and a lesser amount of ApoE, than HCV from serum-supplemented cultures. Since ApoE is usually only found in mature VLDL, the serum-free cultured HCV may associate with immature VLDL. A second difference related to associated apolipoprotein of HCV grown under different serum conditions is that the infectivity of HCV from serum-supplied culture, but not that of serum-free cultured HCV, was down-regulated by antibody depletion of ApoB and ApoE. This result suggested that the infection of HCV from serum-supplemented culture, but not infection of serum-free cultured HCV, depended on apolipoproteins. It is possible that production of HCV in serum-free culture results in the formation of HCV particles that differ in lipid and lipoprotein composition from particles produced under serum-supplemented conditions. Consequently, the affinity of serum-free cultured HCV for its cellular receptor might have been altered, and its susceptibility to antibody inhibition of infectivity may have been increased. However, further analysis of the lipid content of HCV grown under different serum conditions is necessary to confirm this point.

Production of HCV using a serum-free culture system has advantage for vaccine development because there is low protein contamination, it facilitates simple viral purification procedures, and it does not involve the use of animals. Furthermore, lipoproteins associated with virus particles may shield conserved epitopes. Purified virus particles produced under serum-free conditions may have these shielded epitopes exposed and thus potentially become stronger immunogens for the induction of neutralizing antibodies. In this study, serum-free cultured HCV was infectious *in vitro*, and the viral particles had a form similar to that of native enveloped viruses. However, we have not yet confirmed the antigenic or immunogenetic properties of serum-free cultured HCV, which would be important for its potential use as a vaccine. Nevertheless, we have recently designed a system for the purification of HCV particles (Morikawa, unpublished data) which will allow testing of these parameters. If these purified particles have high antigenicity, then a HCV vaccine that is derived from HCV produced in cell culture may be available in the future.

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5. Conclusions

Infectious HCV could be efficiently produced in serum-free culture. However, the serum-free cultured HCV was highly susceptible to anti-CD81, anti-SR-BI and anti-E2 inhibition of infectivity. The content of ApoB and ApoE in HCV from serum-supplemented and serum-free cultures was different and the infectivity of serum-free cultured HCV appeared to be independent of apolipoproteins. Therefore, lipids conjugated with HCV may affect virus infection and neutralization.

Acknowledgements

This work was partially supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science and from the Ministry of Health, Labor, and Welfare of Japan by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation. Huh7.5.1 was a kind gift from Dr. Francis V. Chisari. Anti-SR-BI antibody and rat serum were kind gifts from Dr. H. Barth. Antibody AP33 was a kind gift from Genentech, Inc.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.04.069.

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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

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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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Received November 26, 2010; accepted April 18, 2011.

Supported by grants-in-aid from the Japan Society for the Promotion of Science, the Ministry of Health, Labor, and Welfare of Japan, and the Ministry of Education, Culture, Sports, Science, and Technology, by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation, and in part by the Intramural Research Program of the NIDDK, NIH (T. J. L.).

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DOI 10.1002/hep.24399

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

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

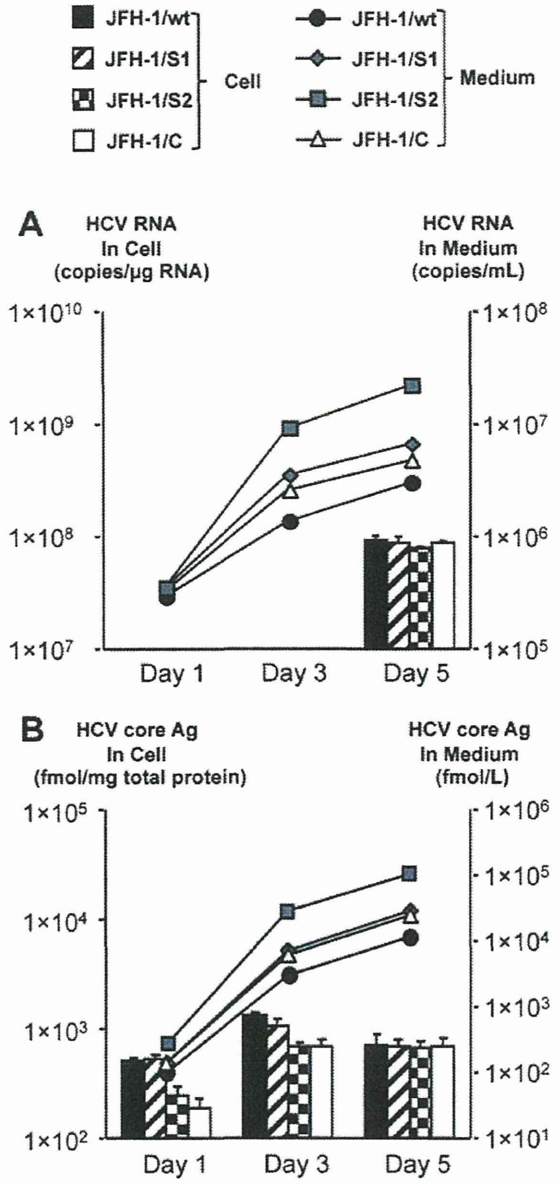

Fig. 1. Effects of *in vivo* adaptive mutations on virus production in HuH-7 cells. One million cells were transfected with 10 μg *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C. (A) HCV RNA and (B) core Ag levels in cell lysates and medium were measured at the indicated time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

	Intracellular			Extracellular	Secretion Ratio (Extracellular/ Intracellular)
Strain	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 ± 58.2 and 134.3 ± 17.1 fmol/mg protein, respectively, and were significantly lower ($P < 0.005$) than that of JFH-1/wt (526.1 ± 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

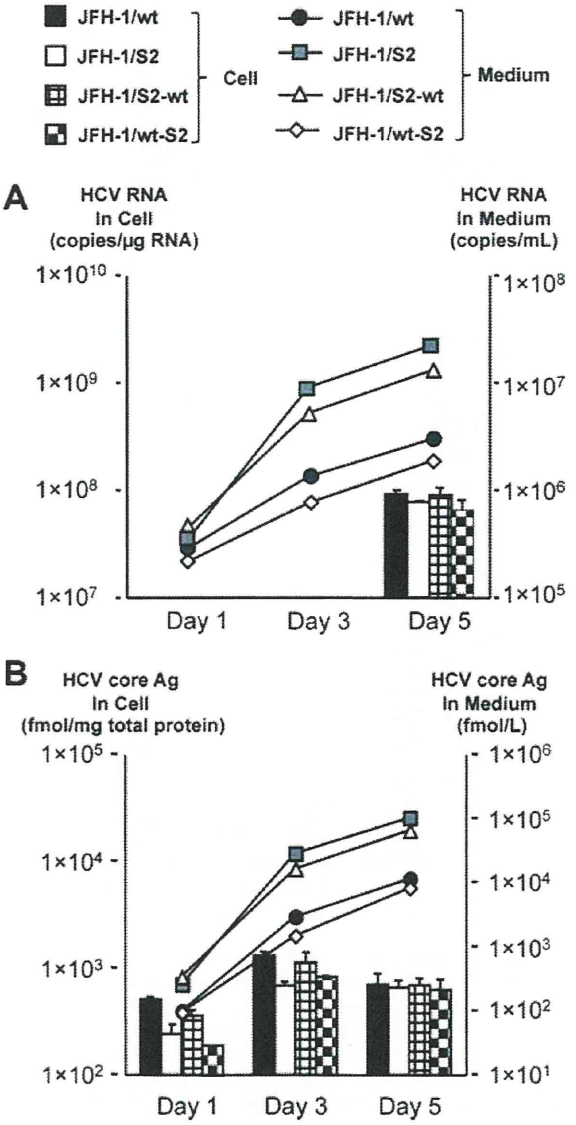

Fig. 2. Virus production of JFH-1/S2 chimeric constructs in HuH-7 cells. One million cells were transfected with 10 μg *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2. (A) HCV RNA and (B) core Ag levels in cell lysates and medium were measured at the indicated time points.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

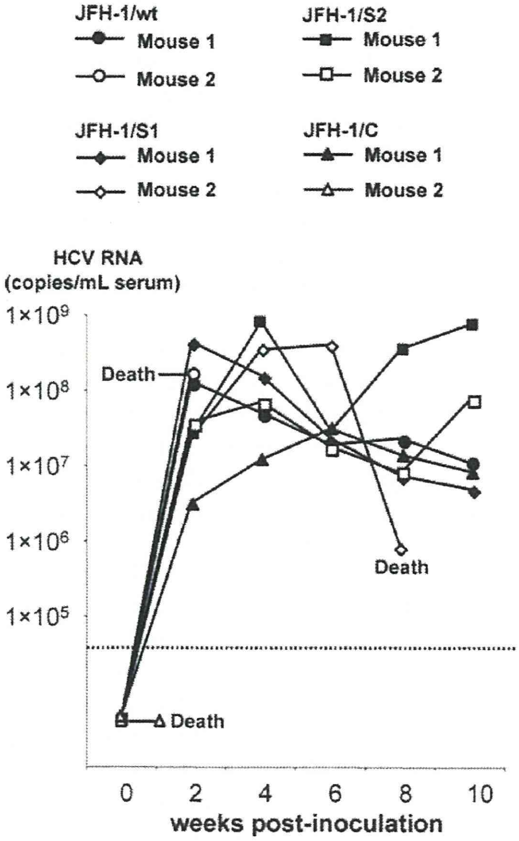

Fig. 3. *In vivo* infection study of JFH-1/wt and its variants in human hepatocyte-transplanted mice. Cell culture medium containing 1 × 10^7 HCV RNA copies of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C were inoculated into human hepatocyte-transplanted mice, and HCV RNA levels in mice serum were monitored.

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).

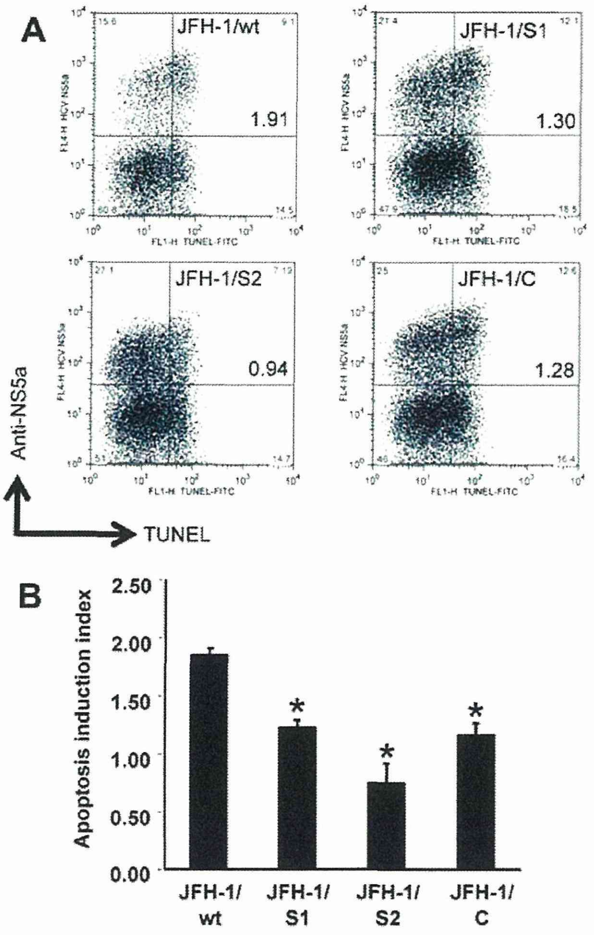

Fig. 4. Apoptosis induction in Huh-7.5.1 cells transfected with JFH-1/wt and its variants. (A) Three million cells were transfected with 3 μg *in vitro*-transcribed full-genome RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C. Forty-eight hours later, apoptosis was induced by exposing cells to 20 ng/mL 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.

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 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

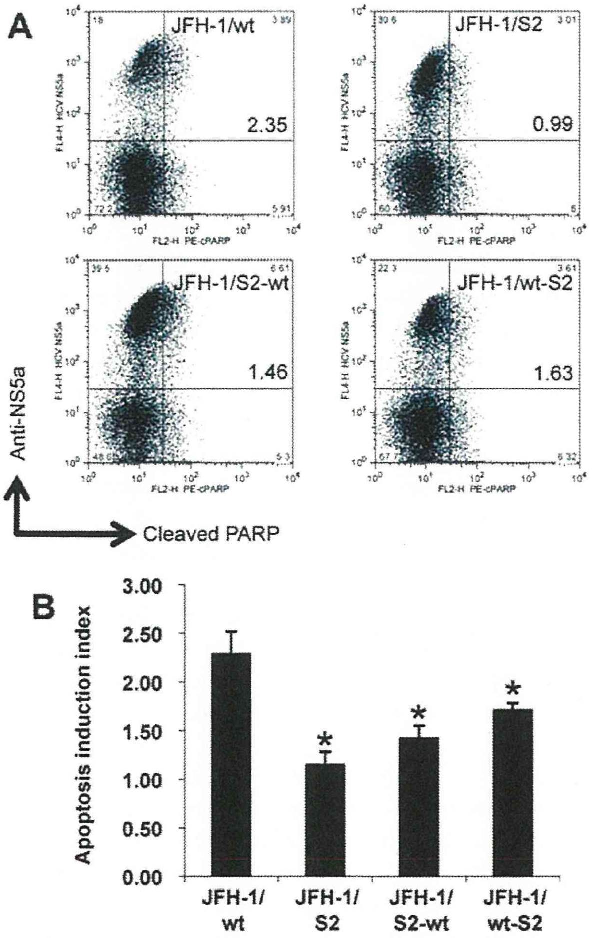

Fig. 5. Apoptosis induction in Huh-7.5.1 cells transfected with JFH-1/wt, JFH-1/S2, and their chimeric constructs. (A) Three million cells were transfected with 3 μg *in vitro*-transcribed full-genome RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2. Apoptosis was induced by exposing cells to 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 ± 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