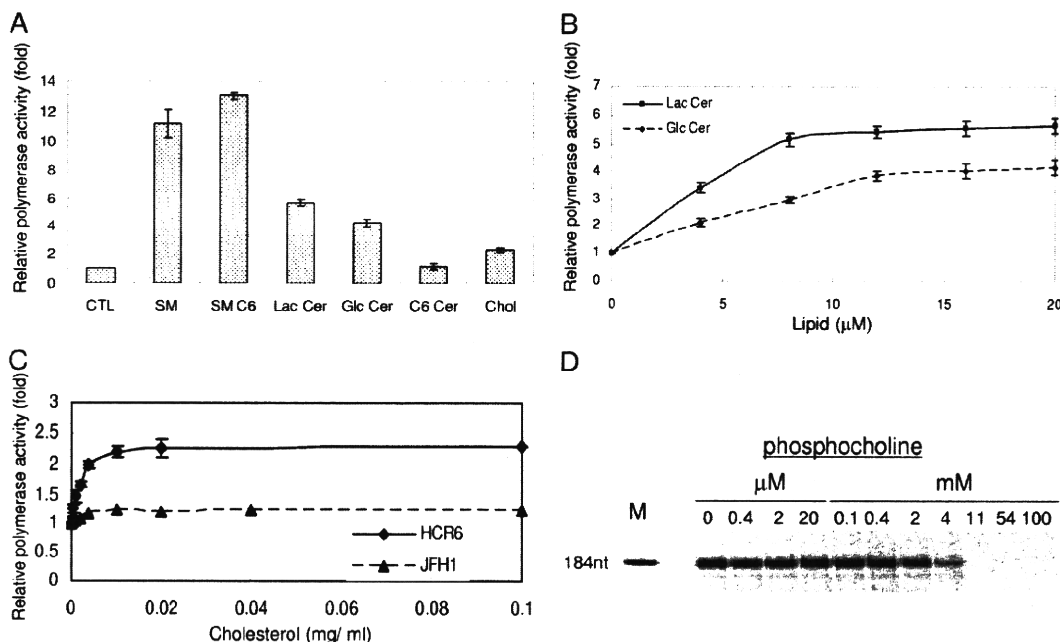

FIG. 3. HCV RNA polymerase activation effect of lipids. (A) Lipid activation of HCR6 (1b) RdRp wt. HCV HCR6 (1b) RdRp wt (100 nM) was incubated with or without (control [CTL]) 0.01 mg/ml egg yolk sphingomyelin (SM), 2 μM hexanoyl sphingomyelin (SM C6), 8 μM C_8-lactosyl(β) ceramide (Lac Cer), 12 μM C_8-β-D-glucosyl ceramide (Glc Cer), 12 μM C_6-ceramide (C6 Cer), or 0.02 mg/ml cholesterol (chol). (B) Activation kinetics of C_8-lactosyl(β) ceramide (Lac Cer) and C_8-β-D-glucosyl ceramide (Glc Cer) on HCR6 (1) RdRp. (C) Activation kinetics of cholesterol on HCR6 (1b) and JFH1 (12a) RdRps. (D) The effect of phosphocholine on HCR6 (1b) RdRp. The mean ± standard deviation of the activation ratio was calculated from three independent experiments.

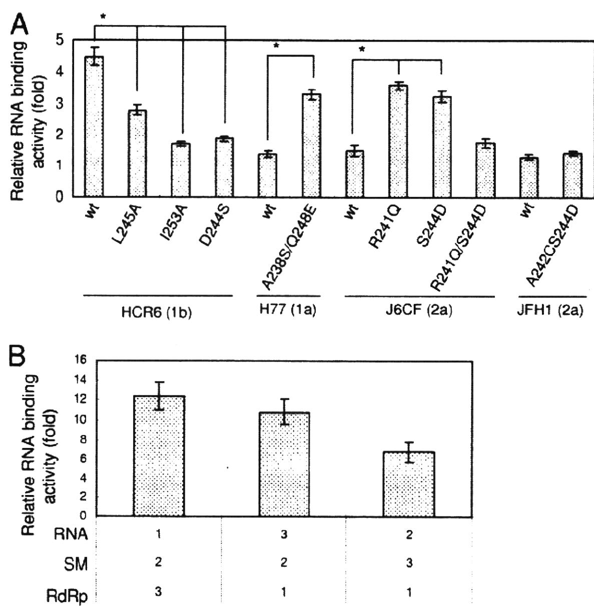

FIG. 4. Sphingomyelin activation of the RNA binding activity of HCV RNA polymerase. (A) Sphingomyelin activation of RNA filter binding of HCV RdRps (RdRp names are indicated below the graph). RdRps and ^{32}P-labeled RNA template (SL12-1S) were incubated with or without egg yolk sphingomyelin (SM), before filtration. (B) Effect of the order of sphingomyelin treatment. Numbers below the graph indicate the order in which the reagents were added. The graph represents the ratio to RNA binding without sphingomyelin. The mean ± standard deviation of the activation ratio was calculated from three independent experiments. *, $P < 0.01$.

function mutants H77 (1a) NS5B(A238S/Q248E) and JFH1 (2a) NS5B(A242C/S244D) were compared with 5 and 50 nM myriocin treatment for 72 h (Fig. 5).

First, HCV replicon activity was compared as the relative luciferase activity (Fig. 5A). Both JFH1 (2a) wt and NS5B(A242C/S244D) replicons showed similar and strong replicon activity ($133 \times 10^3 \pm 12 \times 10^3$ and $138 \times 10^3 \pm 8.5 \times 10^3$, respectively). JFH1 (2a) wt replicon was resistant to myriocin treatment, as reported by Aizaki et al. using other SPT inhibitors (3). The JFH1 (2a) NS5B(A242C/S244D) replicon became sensitive to myriocin but still showed higher replicon activity than NN (1b) or H77 (1a) replicons even at 50 nM myriocin.

To analyze the effect of mutations precisely, the replicon activity relative to each wt strain was compared (Fig. 5B). The JFH1 (2a) wt replicon with 50 nM myriocin showed the same luciferase activity as the wt without myriocin (102% ± 9.6%). JFH1 (2a) NS5B(A242C/S244D) replicon activity was the same as that of the wt without myriocin (103% ± 12%); with 5 nM myriocin it was 84.1% ± 6.6% of the wt level, but with 50 nM myriocin it was 70.3% ± 5.3% of the wt level, which was significantly lower ($P < 0.01$). NN (1b) wt replicon activity was 45.3% ± 6.6% with 5 nM myriocin and 21.7% ± 2.9% with 50 nM myriocin relative to the wt level without myriocin. NN (1b) NS5B(D244S) replicon activity was 72.2% ± 12% without myriocin ($P < 0.05$), 44.0% ± 7.4% with 5 nM myriocin, and 38.1% ± 4.2% with 50 nM myriocin relative to wt level without myriocin, which was significantly higher ($P < 0.01$). Thus, NN (1b) NS5B(D244S) showed lower replicon activity than the wt

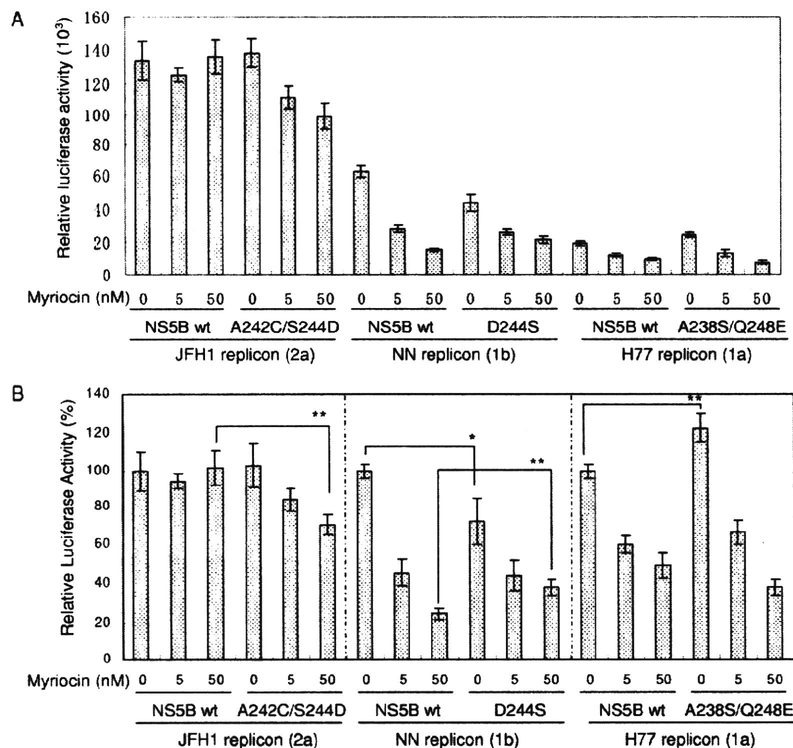

FIG. 5. Myriocin inhibition of HCV replicon activity. Huh7.5.1 cells were incubated with myriocin after transfection with the HCV replicons indicated below the graphs. Means ± standard deviations of the relative luciferase activity at 72 h after myriocin treatment compared to activity at 4 h after transfection (A) and to that of each wt without myriocin (B) were calculated from three independent measurements. *, $P < 0.05$; ** $P < 0.01$.

and was less sensitive to myriocin than the wt. H77 (1a) wt replicon activity was 59.9% ± 4.2% with 5 nM myriocin and 49.2% ± 6.4% with 50 nM myriocin relative to the wt level without myriocin. H77 (1a) NS5B(A238S/Q248E) replicon activity was 123% ± 7.1% without myriocin ($P < 0.01$), 66.1% ± 6.3% with 5 nM myriocin, and 38.0% ± 4.1% with 50 nM myriocin relative to wt level without myriocin. Both H77 (1a) wt and NS5B(A238S/Q248E) replicons were sensitive to myriocin, and the replicon activity of NS5B(A238S/Q248E) was higher than that of the wt.

JFH1 (2a) RdRp(A242C/S244D) localized in the DRM fractions. Myriocin sensitivity of JFH1 (2a) NS5B(A242C/S244D) replicon indicates the importance of 244D in JFH1 NS5B for sphingomyelin binding. To further confirm the role of 244D for recruitment of HCV RdRp to the detergent-resistant membrane (DRM), where the HCV replication complex exists, we compared the distribution of NS5A and NS5B of JFH1 (2a) wt and NS5B(A242C/S244D) in their replicon cells by sucrose density gradient centrifugation of the DRM (Fig. 6). NS5A proteins of both JFH1 (2a) wt and NS5B(A242C/S244D) replicons localized in the DRM fraction where caveolin-2 was present (11, 27), but most of NS5B wt localized in the Triton-soluble fractions. NS5B of JFH1 (2a) NS5B(A242C/S244D) replicon was shifted to the DRM fraction from the soluble fraction. The shift of NS5B(A242C/S244D) localization into the DRM demonstrated that SBD was the DRM localization domain of NS5B and that residue 244D was important for this localization.

DISCUSSION

Hepatitis C virus is an envelope virus, and the lipid components of the virion play important roles in HCV infectivity and virion assembly (3, 15, 20, 24). HCV replication complexes localize in lipid raft structures/DRMs in the membrane frac-

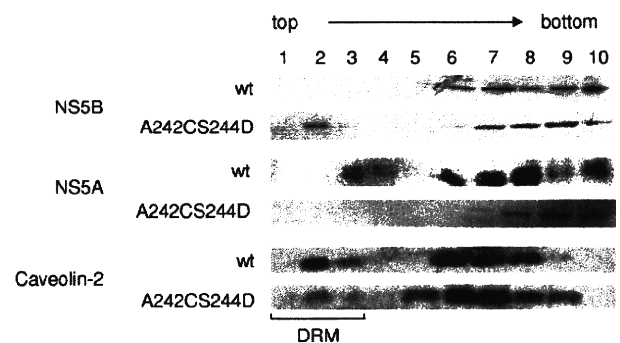

FIG. 6. Membrane floating assay of JFH1 wt and NS5B(A242C/S244D) replicon cells. The PNS fractions of HCV JFH1 (2a) wt and NS5B(A242C/S244D) replicon cells were treated with 1% Triton X-100 in TNE buffer for 30 min at 4°C and subjected to 10 to 40% sucrose gradient centrifugation in TNE buffer. Each fraction was subjected to 10% SDS-PAGE, followed by Western blotting with anti-NS5A, -NS5B, and -caveolin-2 antibodies. Fractions are numbered as indicated at the top of the panel. The DRM fractions (fractions 1 to 3) are indicated.

A

```
             231                                260
1b Con1      NDIRVEESIYQCCDLAPEARQAIRSLTERL
             CHHHHHHEEEEEEEHHHHHHHHHHHHHHHH
   HCR6      SDIRVEESIYQCCDLAPEARQAIKSLTERL
             HHHHHHHEEEEEEEHHHHHHHHHHHHHHHH
   NN        NDIRVEESIYQCCDLAPEAKLAIKSLTERL
             CHHHHHHEEEEEEEHHHHHHHHHHHHHHHH
1a H77       SDIRTEEAIYQCCDLDPQARVAIKSLTERL
             HHHHHHHCCCCCCCCCCCHHHHHHHHHHHH
   RMT       SDIRTEEAIYQCCDLDPQARVAIKSLTERL
             HHHHHHHCCCCCCCCCCCHHHHHHHHHHHH
2a JFH1      RDIRTEESIYQACSIPEEARTAIHSLTERL
             HHHCCCCCEEEEEEEEHHHHHHHHHHHHHH
   J6CF      RDIRTEESIYRACSLPEEAHTAIHSLTERL
             HHHCCCCCEEEEEHHHHHHHHHHHHHHHHH
3a           QDIRVEEEIYQCCNLEPEARKVISSLTERL
             HHHHHHHEEEEEEEEHHHHHHHHHHHHHHH
4a           KDIRVEEEVYQCCDLEPEARKVITALTDRL
             HHHHHHHEEEEEEEEHHHHHHEEEEEEEEE
5a           HDIMTEESIYQSCDLQPEARVAIRSLTQRL
             HHHHHHCEEEEEEEECCCEEEEEEEEEEEE
6a           RDIRTENDIYQSCQLDPVARRVVSSLTERL
             HHHCCCCCEEEEEEEEEEEEEEEECCCCCC
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B Con1 JFH1

231R H1 260L H2 241Q 244D 247P 242A 244S 246P

C Con1

FIG. 7. Sphingomyelin binding domain (SBD) of HCV RNA polymerase. (A) The SBDs (231N to 260L) of HCV RdRps are aligned together with their secondary structure predicted by the Chou-Fasman program (10). The predicted secondary structure is indicated below the sequence as follows: H, α-helix; E, β-sheet; and C, coil. The α-helix structures of HCV Con1 (1b) RdRp and JFH1 (2a) RdRp are boxed in red. Residues 241Q and 244D are indicated in red and green, respectively. The 238A and 248E of the H77 and RMT (1a) RdRps are indicated in purple. GenBank accession numbers of HCV genotypes 3a, 4a, 5a, and 6a are GU814263 (12), GU814265 (12), Y13184 (8), and Y12083 (1), respectively. (B) Comparison of the SBDs of HCV Con1 (1b) (yellow) and JFH1 (2a) RdRps (magenta). The starting and ending amino acids of H1 and H2 are indicated. The sphingomyelin binding site, 241Q, is indicated in red, and 244D of Con1 (1b) and 244S of JFH1 (2a) RdRp are indicated in green. (C) Surface model of HCV Con1 (1b) RdRp. SBD is indicated in yellow, and 241Q and 244D are indicated in red and green, respectively. The structures of the Con1 and JFH1 RdRps were constructed by PyMOL, version 1.1.1 (http://www.pymol.org/). PDB numbers of Con1 (1b) RdRp and JFH1 (2a) RdRp are 3FQL (14) and 3I5K (31), respectively.

tions of subgenomic replicon cells (30). Lipid rafts are composed mainly of sphingomyelin, cholesterol, and glycosphingolipids. Most reports regarding the relationship between lipids and HCV have examined virion assembly, infectivity, and the localization of HCV, but their biochemical interactions have not been reported. Our findings clearly demonstrate that sphingomyelin plays an important role not only in HCV replication complex formation and its localization but also in HCV RdRp activity.

The helix-turn-helix structure of the SBD (residues 230 to 263), which is located between RNA polymerase motifs A and B, has been proposed as the sphingomyelin binding domain of HCV RdRp (29). We compared the SBD of Con1 (1b) (Protein Data Bank [PDB] 3FQL) (14) and JFH1 (2a) (PDB 3I5K) (31) and the secondary structure of the amino acids (201 to 290) in the SBD predicted by the Chou-Fasman program (10) (Fig. 7; see also Fig. S5 in the supplemental material) because the helix structures of the SBD of Con1 (helix 1 [H1], 231N to 241Q; helix 2 [H2], 247A to 260L) and JFH1 (H1, 231R to 242A; H2, 246P to 260L) RdRp fit with those predicted by the Chou-Fasman program. The structures contributing to sphingomyelin binding and activation are H1 and H2 and the junction (turn) between the two helix structures that are similar to the human immunodeficiency virus (HIV) gp120 V3 domain, prion protein (PrP), and β-amyloid peptide (13, 22). Although Con1 (1b) RdRp has a shorter helix structure than JFH1 (2a) RdRp (Fig. 6B), the structures of their SBDs are very similar (Fig. 7; see also Fig. S5). When the helix-turn-helix structure of the SBD was destroyed (HCR6 genotype 1b RdRp mutants L245A and I253A), the RdRp lost sphingomyelin binding activity and lost its activation (Fig. 2).

In order to study the structure-function relationship of the SBD and sphingomyelin, we compared the SBD of genotype 1a, 1b and 2a RdRps and particularly focused on residue 244D in the turn and residues 241Q and 238S/248E in the helix domains. The polar amino acid 241Q and the negatively charged 244D of Con1 (1b) RdRp located on the surface of the RdRp molecule bind and interact with the positively charged choline residue of sphingomyelin (Fig. 7C; see also Fig. S5 in the supplemental material). The positively charged 241R repels the choline residue of sphingomyelin, and as a result, J6CF (a) RdRp wt did not bind to sphingomyelin. J6CF (2a) RdRp(R241Q) showed almost the same sphingomyelin binding activity as HCR6 (1b) RdRp wt. This ionic interaction between SBD and sphingomyelin agrees with the activation of lipids with different sphingosine structures and fatty acid chains (Fig. 3A). JFH1 (2a) RdRp does not interact well with sphingomyelin because it does not have the negatively charged

amino acids at the tip of its turn structure. Once its 244S was changed to D, more sphingomyelin bound to JFH1 (2a) RdRp and activated the RdRp (Fig. 2A and C). The reason for the low activation of J6CF (2a) RdRp(R241Q/S244D) is not clear. Sometimes mutations affect the entire conformation of the molecule. In conclusion, from the comparison of sphingomyelin binding and activation of HCR6 (1b), J6CF (2a), and JFH1 (2a) RdRp SBD mutants, 241Q is the essential amino acid for sphingomyelin binding in the SBD. Amino acid 244D enhanced both binding and RdRp activation.

The *in vitro* sphingomyelin binding and RdRp activation experiments indicate that sphingomyelin binding and its RdRp activation are different biochemical reactions because we found controversial activation rates for sphingomyelin binding and RdRp activation among J6CF (2a) RdRp mutants (Fig. 2). The relationship between sphingomyelin binding and the activation of polymerase activity was studied by comparing genotype 1b and 1a RdRps, both of which bind to sphingomyelin (Fig. 2). However, 1a RdRp is not activated by sphingomyelin because both of the helix structures of 1a RdRp are probably terminated at 238A and 248Q, making its helix structures shorter than those of 1b RdRp (Fig. 6A). The length of the helix structure may be essential for sphingomyelin activation because RdRp changes its structure to bind to template RNA when sphingomyelin binds to SBD (Fig. 4).

HCV RdRp changes its conformations at the early stages of transcription initiation, including the template RNA binding step (6, 9). Sphingomyelin binding is likely to change the conformation of 1b RdRp to recruit template RNA and initiate transcription efficiently. Comparison of the activation ratio of RNA binding and polymerase activity of 1b RdRp, J6CF (2a) RdRp wt and R241Q and S244D mutants, and JFH1 (2a) RdRp wt and mutant A242C/S244D suggests that steps other than RNA binding are also likely to be activated by sphingomyelin.

From a kinetic analysis of sphingomyelin activation (Fig. 1C and D), 20 sphingomyelin molecules are estimated to interact with the SBD of RdRp and activate it because sphingomyelin activation plateaued at 20 sphingomyelin molecules per HCV RdRp molecule. It is not clear whether 20 sphingomyelin molecules form a micelle or a layer structure. However, the structure of sphingomyelin is important for the activation of HCV RdRp because phosphocholine did not activate the RdRp (Fig. 3D).

To confirm these biochemical findings in HCV replication, we tested the effect of SBD mutations in HCV replicon systems with the SPT inhibitor myriocin (Fig. 5) (4, 33) because NA255 was not available. The loss-of-function mutant, HCV NN (1b) NS5B(D244S), showed lower replicon activity than NN (1b) wt and more resistance to 50 nM myriocin, which did not affect the viability of cells (4, 33), than the wt. The gain-of-function mutant, H77 (1a) NS5B(A238S/Q248E), showed higher replicon activity than H77 wt and retained myriocin sensitivity because it had the sphingomyelin binding sites 241Q and 244D. At 50 nM myriocin, another gain-of-function mutant, JFH1 (2a) NS5B(A242C/S244D), was inhibited although its activity was the same as that of JFH1 (2a) wt without myriocin because the JFH1 wt replicon had high replicon activity without myriocin (Fig. 5A). The JFH1 replicon activity may be maximal in the system; therefore, the JFH1 (2a) NS5B(A242C/S244D) replicon did not show higher activity than JFH1 (2a) wt without myriocin while H77 (1a) NS5B(A238S/Q248E) showed higher replicon activity than H77 wt.

The binding and RdRp activation activity of the amino acid 244 mutants by sphingomyelin did not differ greatly from the wt *in vitro*. However, the myriocin sensitivity of JFH1 (2a) NS5B(S244D) was demonstrated clearly. That of H77 (1a) NS5B(A238S/Q248E) indicated that sphingomyelin binding was the target of myriocin inhibition, not the sphingomyelin activation of RdRp. These data confirm the importance of 241Q, 244D, and the helix structure in SBD for HCV replication in the cells.

Sphingomyelin is the major component of the lipid raft structure/DRM where the HCV genome replicates. To confirm that the SBD is the membrane binding site of HCV RdRp, we analyzed the localization of NS5B of JFH1 (2a) wt and NS5B(A242C/S244D) replicons by membrane floating assay (Fig. 6). JFH1 (2a) NS5B wt did not localize in the DRM. However, the localization of NS5B of the JFH1 (2a) NS5B(A242C/S244D) replicon shifted to the DRM from the soluble fractions. Previously, HCV NS5B was believed to localize in the DRM by its C-terminal hydrophobic sequences (21). However, our data demonstrate that the SBD is the membrane localization domain of HCV NS5B, which agrees with the myriocin sensitivity of JFH1 (2a) NS5B(A242C/S244D) replicons (Fig. 5) and the release of HCV 1b NS5B from the DRM by another SPT inhibitor, NA255 (29).

This is the first report of RNA polymerase activation by lipids. Twenty sphingomyelin molecules interact with SBD, particularly with residues 241Q and 244D of HCV (1b) RdRp, and change the conformation of the RdRp in order to recruit RNA templates. At the same time, HCV RdRp molecules may be aligned on the sphingomyelin layer formed via interactions between the hydrocarbon chains of sphingosine and fatty acids via placement of their SBD into the layer (Fig. 7C). Consistent with previous research (3, 23, 37), our findings explain why the inhibitors of the sphingolipid biosynthetic pathway influence subgenomic replicons derived from HCV genotypes 1a and 1b but not those derived from JFH1 (2a) (Fig. 5). Most HCV isolates have 241Q in NS5B, and some of them also have 244D (Fig. 7A). These sphingomyelin interactions are new targets for the treatment of HCV.

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REFERENCES

1. **Adams, N. J., R. W. Chamberlain, L. A. Taylor, F. Davidson, C. K. Lin, R. M. Elliott, and P. Simmonds.** 1997. Complete coding sequence of hepatitis C virus genotype 6a. Biochem. Biophys. Res. Commun. **234:**393–396.
2. **Aizaki, H., K. J. Lee, V. M. Sung, H. Ishiko, and M. M. Lai.** 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology **324:**450–461.
3. **Aizaki, H., K. Morikawa, M. Fukasawa, H. Hara, Y. Inoue, H. Tani, K. Saito, M. Nishijima, K. Hanada, Y. Matsuura, M. M. Lai, T. Miyamura, T. Wakita, and T. Suzuki.** 2008. Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J. Virol. **82:**5715–5724.
4. **Amemiya, F., S. Maekawa, Y. Itakura, A. Kanayama, A. Matsui, S. Takano, T. Yamaguchi, J. Itakura, T. Kitamura, T. Inoue, M. Sakamoto, K. Yamau-**

chi, S. Okada, A. Yamashita, N. Sakamoto, M. Itoh, and N. Enomoto. 2008. Targeting lipid metabolism in the treatment of hepatitis C virus infection. J. Infect. Dis. 197:361–370.
5. Binder, M., D. Quinkert, O. Bochkarova, R. Klein, N. Kezmic, R. Bartenschlager, and V. Lohmann. 2007. Identification of determinants involved in initiation of hepatitis C virus RNA synthesis by using intergenotypic replicase chimeras. J. Virol. 81:5270–5283.
6. Biswal, B. K., M. M. Cherney, M. Wang, L. Chan, C. G. Yannopoulos, D. Bilimoria, O. Nicolas, J. Bedard, and M. N. James. 2005. Crystal structures of the RNA-dependent RNA polymerase genotype 2a of hepatitis C virus reveal two conformations and suggest mechanisms of inhibition by non-nucleoside inhibitors. J. Biol. Chem. 280:18202–18210.
7. Blight, K. J., J. A. McKeating, J. Marcotrigiano, and C. M. Rice. 2003. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J. Virol. 77:3181–3190.
8. Chamberlain, R. W., N. J. Adams, L. A. Taylor, P. Simmonds, and R. M. Elliott. 1997. The complete coding sequence of hepatitis C virus genotype 5a, the predominant genotype in South Africa. Biochem. Biophys. Res. Commun. 236:44–49.
9. Chinnaswamy, S., I. Yarbrough, S. Palaninathan, C. T. Kumar, V. Vijayaraghavan, B. Demeler, S. M. Lemon, J. C. Sacchettini, and C. C. Kao. 2008. A locking mechanism regulates RNA synthesis and host protein interaction by the hepatitis C virus polymerase. J. Biol. Chem. 283:20535–20546.
10. Chou, P. Y., and G. D. Fasman. 1974. Prediction of protein conformation. Biochemistry 13:222–245.
11. Fujimoto, T., H. Kogo, K. Ishiguro, K. Tauchi, and R. Nomura. 2001. Caveolin-2 is targeted to lipid droplets, a new "membrane domain" in the cell. J. Cell Biol. 152:1079–1085.
12. Gottwein, J. M., T. K. Scheel, B. Callendret, Y. P. Li, H. B. Eccleston, R. E. Engle, S. Govindarajan, W. Satterfield, R. H. Purcell, C. M. Walker, and J. Bukh. Novel infectious cDNA clones of hepatitis C virus genotype 3a (strain S52) and 4a (strain ED43): genetic analyses and *in vivo* pathogenesis studies. J. Virol. 84:5277–5293.
13. Hammache, D., G. Pieroni, N. Yahi, O. Delezay, N. Koch, H. Lafont, C. Tamalet, and J. Fantini. 1998. Specific interaction of HIV-1 and HIV-2 surface envelope glycoproteins with monolayers of galactosylceramide and ganglioside GM3. J. Biol. Chem. 273:7967–7971.
14. Hang, J. Q., Y. Yang, S. F. Harris, V. Leveque, H. J. Whittington, S. Rajyaguru, G. Ao-Ieong, M. F. McCown, A. Wong, A. M. Giannetti, S. Le Pogam, F. Talamas, N. Cammack, I. Najera, and K. Klumpp. 2009. Slow binding inhibition and mechanism of resistance of non-nucleoside polymerase inhibitors of hepatitis C virus. J. Biol. Chem. 284:15517–15529.
15. Huang, H., F. Sun, D. M. Owen, W. Li, Y. Chen, M. Gale, Jr., and J. Ye. 2007. Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. Proc. Natl. Acad. Sci. U. S. A. 104:5848–5853.
16. Ishii, N., K. Watashi, T. Hishiki, K. Goto, D. Inoue, M. Hijikata, T. Wakita, N. Kato, and K. Shimotohno. 2006. Diverse effects of cyclosporine on hepatitis C virus strain replication. J. Virol. 80:4510–4520.
17. Kashiwagi, T., K. Hara, M. Kohara, K. Kohara, J. Iwahashi, N. Hamada, H. Yoshino, and T. Toyoda. 2002. Kinetic analysis of C-terminally truncated RNA-dependent RNA polymerase of hepatitis C virus. Biochem. Biophys. Res. Commun. 290:1188–1194.
18. Kato, T., T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, and T. Wakita. 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 125:1808–1817.
19. Kiyosawa, K., T. Sodeyama, E. Tanaka, Y. Gibo, K. Yoshizawa, Y. Nakano, S. Furuta, Y. Akahane, K. Nishioka, R. H. Purcell, et al. 1990. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 12:671–675.
20. Lambot, M., S. Fretier, A. Op De Beeck, B. Quatannens, S. Lestavel, V. Clavey, and J. Dubuisson. 2002. Reconstitution of hepatitis C virus envelope glycoproteins into liposomes as a surrogate model to study virus attachment. J. Biol. Chem. 277:20625–20630.
21. Lemon, S., C. Walker, M. Alter, and M. Yi. 2007. Hepatitis C virus, p. 1253–1304. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
22. Mahfoud, R., N. Garmy, M. Maresca, N. Yahi, A. Puigserver, and J. Fantini. 2002. Identification of a common sphingolipid-binding domain in Alzheimer, prion, and HIV-1 proteins. J. Biol. Chem. 277:11292–11296.
23. Miyake, Y., Y. Kozutsumi, S. Nakamura, T. Fujita, and T. Kawasaki. 1995. Serine palmitoyltransferase is the primary target of a sphingosine-like immunosuppressant, ISP-1/myriocin. Biochem. Biophys. Res. Commun. 211:396–403.
24. Miyanari, Y., K. Atsuzawa, N. Usuda, K. Watashi, T. Hishiki, M. Zayas, R. Bartenschlager, T. Wakita, M. Hijikata, and K. Shimotohno. 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. 9:1089–1097.
25. Murayama, A., T. Date, K. Morikawa, D. Akazawa, M. Miyamoto, M. Kaga, K. Ishii, T. Suzuki, T. Kato, M. Mizokami, and T. Wakita. 2007. The NS3 helicase and NS5B-to-3′X regions are important for efficient hepatitis C virus strain JFH-1 replication in Huh7 cells. J. Virol. 81:8030–8040.
26. Murayama, A., L. Weng, T. Date, D. Akazawa, X. Tian, T. Suzuki, T. Kato, Y. Tanaka, M. Mizokami, T. Wakita, and T. Toyoda. 2010. RNA polymerase activity and specific RNA structure are required for efficient HCV replication in cultured cells. PLoS Pathog. 6:e1000885.
27. Ostermeyer, A. G., J. M. Paci, Y. Zeng, D. M. Lublin, S. Munro, and D. A. Brown. 2001. Accumulation of caveolin in the endoplasmic reticulum redirects the protein to lipid storage droplets. J. Cell Biol. 152:1071–1078.
28. Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, et al. 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. U. S. A. 87:6547–6549.
29. Sakamoto, H., K. Okamoto, M. Aoki, H. Kato, A. Katsume, A. Ohta, T. Tsukuda, N. Shimma, Y. Aoki, M. Arisawa, M. Kohara, and M. Sudoh. 2005. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat. Chem. Biol. 1:333–337.
30. Shi, S. T., K. J. Lee, H. Aizaki, S. B. Hwang, and M. M. Lai. 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J. Virol. 77:4160–4168.
31. Simister, P., M. Schmitt, M. Geitmann, O. Wicht, U. H. Danielson, R. Klein, S. Bressanelli, and V. Lohmann. 2009. Structural and functional analysis of hepatitis C virus strain JFH1 polymerase. J. Virol. 83:11926–11939.
32. Tsukiyama-Kohara, K., S. Tone, I. Maruyama, K. Inoue, A. Katsume, H. Nuriya, H. Ohmori, J. Ohkawa, K. Taira, Y. Hoshikawa, F. Shibasaki, M. Reth, Y. Minatogawa, and M. Kohara. 2004. Activation of the CKI-CDK-Rb-E2F pathway in full genome hepatitis C virus-expressing cells. J. Biol. Chem. 279:14531–14541.
33. Umehara, T., M. Sudoh, F. Yasui, C. Matsuda, Y. Hayashi, K. Chayama, and M. Kohara. 2006. Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model. Biochem. Biophys. Res. Commun. 346:67–73.
34. Wasley, A., and M. J. Alter. 2000. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin. Liver Dis. 20:1–16.
35. Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno. 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol. Cell 19:111–122.
36. Weng, L., J. Du, J. Zhou, J. Ding, T. Wakita, M. Kohara, and T. Toyoda. 2009. Modification of hepatitis C virus 1b RNA polymerase to make a highly active JFH1-type polymerase by mutation of the thumb domain. Arch. Virol. 154:765–773.
37. Yasuda, S., H. Kitagawa, M. Ueno, H. Ishitani, M. Fukasawa, M. Nishijima, S. Kobayashi, and K. Hanada. 2001. A novel inhibitor of ceramide trafficking from the endoplasmic reticulum to the site of sphingomyelin synthesis. J. Biol. Chem. 276:43994–44002.
38. Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari. 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. U. S. A. 102:9294–9299.

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Infectivity of Hepatitis C Virus Is Influenced by Association with Apolipoprotein E Isoforms[▽][†]

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Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. HCV in circulating blood associates with lipoproteins such as very low density lipoprotein (VLDL) and low-density lipoprotein (LDL). Although these associations suggest that lipoproteins are important for HCV infectivity, the roles of lipoproteins in HCV production and infectivity are not fully understood. To clarify the roles of lipoprotein in the HCV life cycle, we analyzed the effect of apolipoprotein E (ApoE), a component of lipoprotein, on virus production and infectivity. The production of infectious HCV was significantly reduced by the knockdown of ApoE. When an ApoE mutant that fails to be secreted into the culture medium was used, the amount of infectious HCV in the culture medium was dramatically reduced; the infectious HCV accumulated inside these cells, suggesting that infectious HCV must associate with ApoE prior to virus release. We performed rescue experiments in which ApoE isoforms were ectopically expressed in cells depleted of endogenous ApoE. The ectopic expression of the ApoE2 isoform, which has low affinity for the LDL receptor (LDLR), resulted in poor recovery of infectious HCV, whereas the expression of other isoforms, ApoE3 and ApoE4, rescued the production of infectious virus, raising it to an almost normal level. Furthermore, we found that the infectivity of HCV required both the LDLR and scavenger receptor class B, member I (SR-BI), ligands for ApoE. These findings indicate that ApoE is an essential apolipoprotein for HCV infectivity.

Hepatitis C virus (HCV) infection is a major global health problem. More than 170 million people worldwide are infected with HCV. HCV causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (18). A member of the family *Flaviviridae*, HCV has a positive-sense, single-stranded RNA genome that is packaged into an enveloped viral particle. The genome encodes a large precursor polyprotein, which is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope protein 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (12, 13). Core associates with the lipid droplet (LD). The role of this association remained elusive until robust HCV replication systems became available (32). We previously showed that the LD is an important organelle for HCV production (23). In hepatocytes, the LD is physiologically important as a lipid source for the production of lipoproteins such as very low density lipoprotein (VLDL) (11). VLDL is synthesized in the liver as a triglyceride/cholesterol ester-rich particle (diameter, 30 to 100 nm) surrounded by apolipoproteins such as apolipoprotein B100 (abbreviated as ApoB throughout), ApoC's, and ApoE. VLDL is released into blood vessels to be delivered as a lipid source to peripheral cells, and it is also readsorbed by liver cells after processing (5).

HCV particles circulating in the blood of HCV carriers associate with lipoproteins, such as low-density lipoprotein (LDL), VLDL, and chylomicrons; thus, these are termed lipoviro particles (LVPs) (1, 26). Purified LVPs from circulating blood contain triglyceride, ApoB, ApoB48, ApoCII, ApoCIII, ApoE, and virus components such as HCV RNA and core (8), indicating that the LVP has dual viral and lipoprotein characteristics. The HCVcc strain, which contains a chimeric HCV-2a genome with a structural region from HCV-J6 and nonstructural/noncoding regions from an infectious JFH1 virus, can establish long-term infection in chimpanzees. Viruses recovered from the chimpanzee contain infectious virus particles with a slightly low density, suggesting that an *in vivo* association with low-density factors influences infectivity (19). However, the role of a lipoprotein-like component of LVPs in virus replication is not clear. Moreover, the mechanism by which LVPs are generated during HCV production is unknown.

When HCV-producing cells are treated with an inhibitor of microsomal triglyceride transfer protein (MTP) or with ApoB-specific small interfering RNA (siRNA), the production of HCV particles is suppressed (10, 14, 25). Therefore, lipoprotein biosynthesis appears to play an important role in the production of infectious HCV and its egress from infected cells. ApoB, ApoC1, and ApoE associate with infectious virus

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TABLE 1. Primers used for the construction of expression plasmids[a]

Plasmid name	Primer sequence (5′ to 3′)	Template DNA	Restriction enzyme	Vector
pCAG-ApoE2	TGCCTGGCAGTGTACCAGGCCGGGGCCCGC	pCAG-ApoE3	EcoRI	pCAG
pCAG-ApoE2-HA	CTTCTGCAGGTCATCGGCATCGCGGAGGAG		XhoI	pCAG-HA
pCAG-ApoE3	ATGAAGGTTCTGTGGGCTGCG	Human liver cDNA	EcoRI	pCAG
pCAG-ApoE3-HA	GTGATTGTCGCTGGGCACAGG		XhoI	pCAG-HA
pCAG-ApoE4	CGCGGCCGCCTGGTGCAGTACCGCGGCGAG	pCAG-ApoE3	EcoRI	pCAG
pCAG-ApoE4-HA	CACGTCCTCCATGTCCGCGCCCAGCCGGGC		XhoI	pCAG-HA
pCAG-ApoE3-KDEL	TAACAATTCACTCCTCAGGTGCAGGCTGCC	pCAG-ApoE3	EcoRI	pCAG
	CAGTTCATCTTTGTGATTGTCGCTGGGCAC		XhoI	

[a] The sets of primers used to amplify the target genes, the template DNAs used in the PCRs, the restriction sites, and the destination plasmids into which the amplified DNA fragments were inserted are shown.

particles in the HCVcc infection/replication system (4, 6, 15, 22, 27). Furthermore, ApoE depletion suppresses the production of infectious HCV (4, 6, 15, 27). These reports strongly suggest the importance of lipoprotein function to the HCV life cycle. However, the precise roles of lipoproteins and apolipoproteins in virus production and infectivity are not fully understood.

We analyzed the production of HCV from cells in which apolipoprotein production was knocked down with siRNA. We found that ApoE is required for the infectivity of HCV, a finding consistent with other reports (4, 6, 15). ApoE is a polymorphic protein with three major isoforms: ApoE2, ApoE3, and ApoE4. The three isoforms differ by amino acid substitutions at one or two sites (residues 130 and 176) on the 317-amino-acid chain of the ApoE molecule. The polymorphism of ApoE influences its multiple functions due to isoform-dependent differences in receptor-binding activity and lipoprotein association preference. For example, ApoE2 has drastically lower LDL receptor (LDLR) binding activity than ApoE3 and ApoE4 (7). In the present study, we investigated the role of ApoE isoforms in virus production and infectivity.

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MATERIALS AND METHODS

Cell culture and viruses. The human hepatoma cell line HuH7.5 was grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml nonessential amino acids (Invitrogen), and 100 μg/ml of both penicillin and streptomycin sulfate (Nacalai Tesque, Kyoto, Japan). Infectious HCV in cell culture (HCVcc) was produced by transfection of HuH7.5 cells with *in vitro*-transcribed RNA derived from JFH1 or TNS2J1.

Generation of ApoE knockdown cells. Plasmids expressing short hairpin RNA (shRNA) targeting ApoE (5′-GCAGACACTGTCTGAGCAGGT-3′, 5′-CCGCCTCAAGAGCTGGTTCGA-3′, and 5′-GAAGGAGTTGAAGGCCTACAA-3′) or a control shRNA (5′-CAACAAGATGAAGAGCACCAA-3′) (Mission shRNA; Sigma) were transfected into HuH7.5 cells using TransIT-LT1 (Mirus). Cells were selected with puromycin (1.5 μg/ml; Invitrogen). Individual clones were screened by immunoblotting with an anti-ApoE antibody.

Plasmid construction and ectopic expression of ApoE in ApoE knockdown cells. Human ApoE3 cDNA was amplified from human liver cDNA and cloned into the pCAG and pCAG-HA vectors. A hemagglutinin (HA) tag was fused with the C terminus of ApoE. ApoE mutants were constructed by using a mutagenesis kit according to the manufacturer's instructions (Toyobo, Osaka, Japan). The plasmids, primer sequences, and PCR templates, and the restriction enzyme sites used to construct the plasmids, are listed in Table 1. The stable ApoE knockdown cells (sh-#3) were transfected with ApoE expression plasmids using TransIT-LT1 (Mirus) to obtain HuH7.5 cells expressing ApoE isoforms.

siRNA transfection. siRNA transfection was performed using Silentfect (Bio-Rad) according to the manufacturer's protocol. Duplex nucleotides of siRNA specific to mRNA for ApoE (5′-AGACAGAGCCGGAGCCCGA-3′), the LDLR (5′-GGACAGAUAUCAUCAACGA-3′), or scavenger receptor class B, member I (SR-BI) (5′-GCAGCAGGUCCUUAAGAAC-3′), and a control siRNA, si-control, were purchased from Sigma.

Antibodies and reagents. Rat anti-HA (3F10; Roche Applied Science), mouse anti-Flag (M2; Sigma), mouse anti-actin (AC-40; Sigma), goat anti-α1-antitrypsin (K15600G; Biodesign International), mouse anti-ApoE (13F45; Autogen Bioclear), goat anti-ApoE (AB947; Chemicon International), sheep anti-ApoB (K90086C; Biodesign International), mouse anti-core (CP11; Institute of Immunology), goat anti-LDLR (AF2148; R&D Systems), rabbit anti-SR-BI (EP1556Y; Abcam), mouse anti-CD81 (JS-81; BD Biosciences), and normal goat IgG (sc-2028; Santa Cruz Biotechnology) antibodies were purchased commercially. Rat anti-claudin 1 (anti-CLDN1) antibodies have been described previously (16). Rabbit polyclonal antibodies specific for NS5A were raised against a bacterially expressed glutathione *S*-transferase (GST)-NS5A (amino acids [aa] 1 to 406) fusion protein. Horseradish peroxidase-linked donkey antibodies to goat IgG (Santa Cruz Biotechnology) and donkey antibodies to sheep IgG (Jackson ImmunoResearch) were used. Horseradish peroxidase-linked goat antibodies to rat IgG, sheep antibodies to mouse IgG, and donkey antibodies to rabbit IgG were purchased from Amersham Biosciences. Human recombinant ApoE3 (A2331) was purchased from Sigma.

Focus-forming unit assay. The infectivity titer of HCV was determined on HuH7.5 cells by endpoint dilution and immunostaining of infected cells. Each sample was serially diluted 5-fold in DMEM, and 100 μl was used to inoculate 6×10^3 naïve HuH7.5 cells in a 96-well plate. Infection was examined 48 h postinoculation by immunofluorescence using a rabbit polyclonal anti-NS5A antibody and an Alexa 488-conjugated anti-rabbit IgG antibody (Invitrogen). Infectious foci were counted, and the titer was calculated and expressed in focus-forming units (FFU) per milliliter. The imaging analysis was conducted with an Axiovert 200 microscope (Carl Zeiss).

Intracellular and extracellular infectivity experiments. HCV-infected cells were washed twice with phosphate-buffered saline (PBS), collected by centrifugation, and then suspended in distilled water. Cells were sheared by 10 strokes with a 27-gauge needle (Terumo, Tokyo, Japan) before incubation at room temperature for 15 min. The lysate supernatant was collected after centrifugation, filtered through a 0.45-μm-pore-size filter (Iwaki, Tokyo, Japan), and concentrated with an Amicon Ultra-15 centrifugal filter (Millipore). The solvent was changed to DMEM, and the resulting solution was used as the intracellular HCV source. The cell culture medium was collected and filtered through a 0.45-μm-pore-size filter. The filtrate was concentrated with an Amicon Ultra-15 centrifugal filter and was then used as the extracellular virus source.

Quantification of HCV core protein. HCV core protein in the culture medium was quantified by using the Ortho HCV antigen enzyme-linked immunosorbent assay (ELISA) kit (Ortho-Clinical Diagnostics) according to the manufacturer's protocol.

Isopycnic gradient centrifugation of HCV particles. The 20-times-concentrated HCVcc was layered on top of 14 to 54% iodixanol gradients prepared in PBS. Gradients were centrifuged in an RPS40T rotor (Hitachi, Tokyo, Japan) at 36,000 rpm for 16 h at 4°C. Ten fractions (700 μl each) were collected from the top of the tube. The buoyant density of each fraction was calculated from the refractive index data, measured with an Abbe refractometer (Atago, Tokyo, Japan).

Real-time RT-PCR. RNA was extracted from 10-times-concentrated HCVcc for real-time reverse transcription-PCR (RT-PCR). Quantitative real-time RT-PCR analysis of the 5′ untranslated region of the HCV genome was performed as described previously (30). The forward and reverse primers were 5′-CCCTC CCGGGAGAGCCATAGTG-3′ and 5′-GTCTCGCGGGGGCACGCCCAAA T-3′, respectively. The TaqMan probe was 5′-6-carboxyfluorescein (FAM)-TCT GCGGAACCGGTGAGTACAC-BHQ1-3′.

Statistical analysis. Data are expressed as means and standard deviations (SD). Statistical analyses were performed using Student's *t* test, and a *P* value of <0.05 was considered statistically significant.

RESULTS

The production of infectious HCVcc from ApoE-depleted cells is suppressed. To clarify the roles of ApoE in HCV production, we infected ApoE knockdown cells with HCVcc and measured the amount of infectious HCV released into the culture medium. siRNA targeting ApoE or randomized control siRNA was introduced into HuH7.5 cells, and then the cells were infected with JFH1 4 h after transfection. The culture medium was inoculated into naïve HuH7.5 cells for infectivity analysis. The effect of ApoE knockdown was verified by Western blot analysis. ApoE siRNA treatment efficiently reduced the levels of ApoE in HuH7.5 cells, whereas the levels of actin, α1-antitrypsin, and ApoB remained unchanged (see Fig. S1A in the supplemental material). HCV genome replication, as determined by the amounts of virus proteins (core and NS5A) in cell lysates, was not affected by ApoE knockdown (see Fig. S1A). To determine if ApoE affects the secretion of HCV into culture medium, the amount of core in the medium was measured by a core-specific ELISA. We observed that there is no gross difference in the ratio of HCV core and HCV RNA between culture media harvested at different time points after virus infection, indicating that measurement of the level of core is relevant for representing HCV. The knockdown of endogenous ApoE reduced the level of core to 53% of that in control siRNA-treated cells (see Fig. S1B in the supplemental material). Next, we assessed the infectivity of extracellular virus particles. The infectivity of HCV in the culture medium of ApoE knockdown cells was strongly suppressed compared to that of HCV from control siRNA-treated cells (see Fig. S1B), a finding consistent with previous reports (4, 6, 15). Downregulated virus release and reduced production of infectious virus were also observed when the infectious chimeric HCV genome, TNS2J1 (30), which contains the HCV-1b-derived structural region and the JFH1-derived nonstructural region, was examined (see Fig. S1B, right). These results indicate that ApoE is a cellular factor essential for the production of infectious HCV.

To further clarify the role of ApoE in the HCV life cycle, we established HuH7.5 cells in which ApoE was stably knocked down with ApoE-specific short hairpin RNA and a control cell line that expressed normal control shRNA (NC). In the resultant two ApoE knockdown cell clones (sh-#3 and sh-#12), ApoE was barely detected in the cell lysate and medium (see Fig. S1C, top, in the supplemental material). The amounts of actin, α1-antitrypsin, and ApoB proteins were not affected compared to normal control shRNA-expressing cells (sh-NC) (see Fig. S1C in the supplemental material).

We examined whether or not the downregulation of ApoE influences the infection efficiency of HCV using these cell lines. Cells were infected with HCVcc, and infectivity was analyzed by a focus-forming unit assay 48 h after infection (see Fig. S1D in the supplemental material). We found no substantial difference in HCV infectivity in these cells. Therefore, endogenous ApoE is not required for HCV entry or for the establishment of infection. However, the production of infectious HCV from sh-ApoE cells was remarkably reduced, although the level of HCV core was only about 50% reduced (see Fig. S1E in the supplemental material). This result is consistent with the results obtained for cells in which ApoE was transiently knocked down (see Fig. S1B).

To exclude the possibility that the suppressed production of infectious HCV was caused by an off-target effect of shRNA, we examined whether or not HCV production was rescued by the ectopic expression of ApoE in the sh-ApoE cell lines. We inoculated infectious HCVcc into sh-ApoE cells that were either left untransfected or transfected with the ApoE expression plasmid. Forty-eight hours later, the culture medium and cell lysates were harvested in order to measure infectivity and the production of HCV proteins, respectively. Ectopic expression of ApoE or ApoE-HA increased ApoE protein levels in the cells, and ApoE was secreted into the medium. There were no changes in the levels of NS5A, actin, ApoB, and α1-antitrypsin in these cells (see Fig. S2A in the supplemental material). Interestingly, the ectopic expression of ApoE or ApoE-HA did not restore the secretion of HCV core to the level in control HCV-infected HuH7.5 cells (see Fig. S2B). However, HCV infectivity was restored to a level similar to that for sh-NC (see Fig. S2C). These results indicate that ApoE shRNA directly affects ApoE gene expression and that ApoE itself is an essential host factor for HCV infectivity.

ApoE interacts with infectious HCV particles released into the culture medium. To clarify the mechanisms underlying the role of ApoE in HCV infectivity, we examined the interaction between ApoE and HCV particles by performing coimmunoprecipitation experiments. The culture medium from cells bearing infectious JFH1 replicons was incubated either with an anti-ApoE antibody or with normal goat IgG. Immunocomplexes were recovered with protein G-Sepharose. RNA was extracted from the complex and was analyzed by quantitative RT-PCR. HCV RNA was detected mainly in the complex precipitated with the anti-ApoE antibody; only a little HCV RNA was detected with normal goat IgG (see Fig. S3A in the supplemental material). There is no substantial difference between the levels of HCV precipitated by different sources of anti-ApoE antibodies (data not shown). The fraction not precipitated by the anti-ApoE antibody had little infectivity, while the supernatant of the reaction mixture with normal goat IgG had significantly high infectivity (see Fig. S3B). These results provide evidence of a direct interaction between ApoE and HCV that is important for infectivity.

We conducted an iodixanol density gradient assay to find out if there was any qualitative difference between HCV produced from HuH7.5 cells and HCV produced from ApoE knockdown HuH7.5 cells (Fig. 1A). We could not see any difference in their buoyant densities to explain the difference in infectivity. This indicates that association with ApoE does not cause a drastic physical change in HCV.

Release of infectious HCV into the culture medium depends on the secretion of ApoE. Our results and those of other groups clearly indicate the importance of the association of HCV with

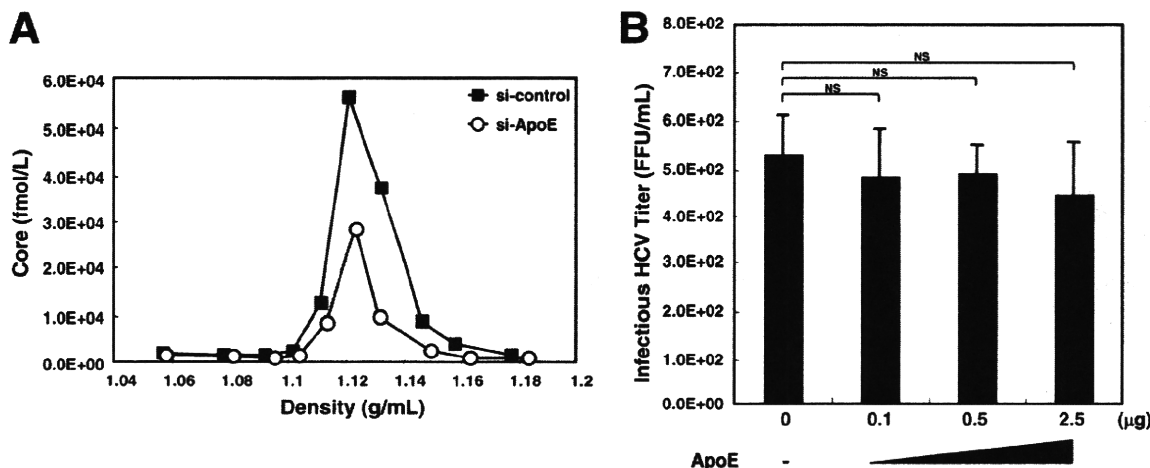

FIG. 1. Nature of HCV produced from ApoE knockdown HuH7.5 cells. (A) Buoyant density analysis. HuH7.5 cells were seeded onto 60-mm-diameter dishes. Cells were transfected with siRNA (si-control or si-ApoE). Four hours after transfection, cells were infected with HCVcc. Forty-eight hours after inoculation, the concentrated culture medium was fractionated using 14 to 54% iodixanol density gradient centrifugation at 36,000 rpm for 16 h at 4°C. The buoyant density profile is represented by the amount of core protein (in femtomoles per liter) in each fraction. Data from a representative of three experiments are shown. (B) Analysis of the infectivity of HCVcc produced from ApoE knockdown HuH7.5 cells after incubation with recombinant ApoE. HCVcc from cells in which ApoE expression was silenced was incubated alone or with different doses of human recombinant ApoE3 at 37°C for 2 h. Then the reaction mixtures were inoculated into naïve HuH7.5 cells. Forty-eight hours after infection, titers of infectious HCV were quantified by a focus-forming unit assay. The average values for three independent experiments are shown; error bars, standard deviations of the means. *P* values were determined by comparison (by Student's *t* test) with HCVcc that was not treated with recombinant ApoE3. NS, not significant ($P > 0.05$).

ApoE for infectivity (4, 6, 15, 27). However, it is uncertain when ApoE associates with HCV during the processes of morphogenesis and the secretion of infectious HCV particles. ApoE by itself can be released from ApoE-producing cells; thus, it is possible that HCV associates with ApoE after being secreted into the culture medium. However, this possibility is less likely, because the infectivity of HCV secreted from cells lacking ApoE expression was not rescued by incubation with different doses of recombinant ApoE (Fig. 1B).

To study the association of ApoE with HCV during the process of infectious virus production, we analyzed the production and infectivity of HCV produced by cells expressing mutant ApoE that is not secreted, due to the addition of Lys-Asp-Glu-Leu (KDEL), an endoplasmic reticulum (ER) retention signal, to its C terminus (Fig. 2A). sh-#3 cells were transfected with an ApoE3 or ApoE3-KDEL expression plasmid followed by HCV infection. Western blot analysis revealed that ApoE3 and ApoE3-KDEL were produced abundantly in transfected cells (Fig. 2B). As expected, ApoE3-KDEL accumulated inside cells, whereas ApoE3 localized both inside and outside cells (Fig. 2B). The expression of actin, NS5A, ApoB, and α1-antitrypsin in the transfected cells was unchanged (Fig. 2B). Additionally, the levels of extracellular and intracellular HCV core protein in the two types of transfected cells were not significantly different (Fig. 2C).

Next, we analyzed HCV infectivity in the extracellular and intracellular fractions of these cells. Infectious HCV was recovered from cells ectopically expressing ApoE3 (Fig. 2D, left). However, cells producing ApoE3-KDEL released very few infectious virus particles into the culture medium; instead, the infectious virus accumulated in the intracellular fraction (Fig. 2D, right). These results suggest that infectious virus particles constituted with ApoE are produced inside the cells and that the release of these infectious particles depends on the secretion of ApoE.

The ApoE isoform affects the infectivity of HCV. ApoE is a multifunctional protein that plays central roles in lipid metabolism and neurobiology. It has three major isoforms (ApoE2, ApoE3, and ApoE4) that have different effects on lipid and neuronal homeostasis. These isoforms differ by amino acid substitutions at one or two sites (residues 130 and 176). ApoE3 is the most common isoform, and there have been no reports of diseases associated with ApoE3. On the other hand, ApoE2 is the major risk factor for type III hyperlipoproteinemia, and ApoE4 is the major risk factor for Alzheimer's disease (20). ApoE2 has lower affinity for the LDLR than ApoE3 and ApoE4. Since lipoprotein receptors, including the LDLR and scavenger receptor class B, member I (SR-BI), are suspected of acting as receptors for HCV infection (3, 24, 27, 34), we hypothesized that the ApoE isoform may affect HCV infectivity. To clarify this hypothesis, we analyzed the production of infectious HCV from cells expressing different isoforms of ApoE.

Plasmids expressing ApoE isoforms were transfected into HuH7.5 cells in which endogenous ApoE3 had been knocked down (sh-#3). Although the level of ectopically expressed ApoE was higher than that of endogenous ApoE, the levels of ectopically expressed ApoE and the levels of ApoE secreted into the culture medium were not different for cell groups expressing different ApoE isoforms (Fig. 3A). The replication of the HCV genome, as determined by the amounts of NS5A and core, was unaffected by the expression of different isoforms of ApoE (Fig. 3A). The amount of extracellular core in ApoE isoform-expressing cells was one-half of that in control

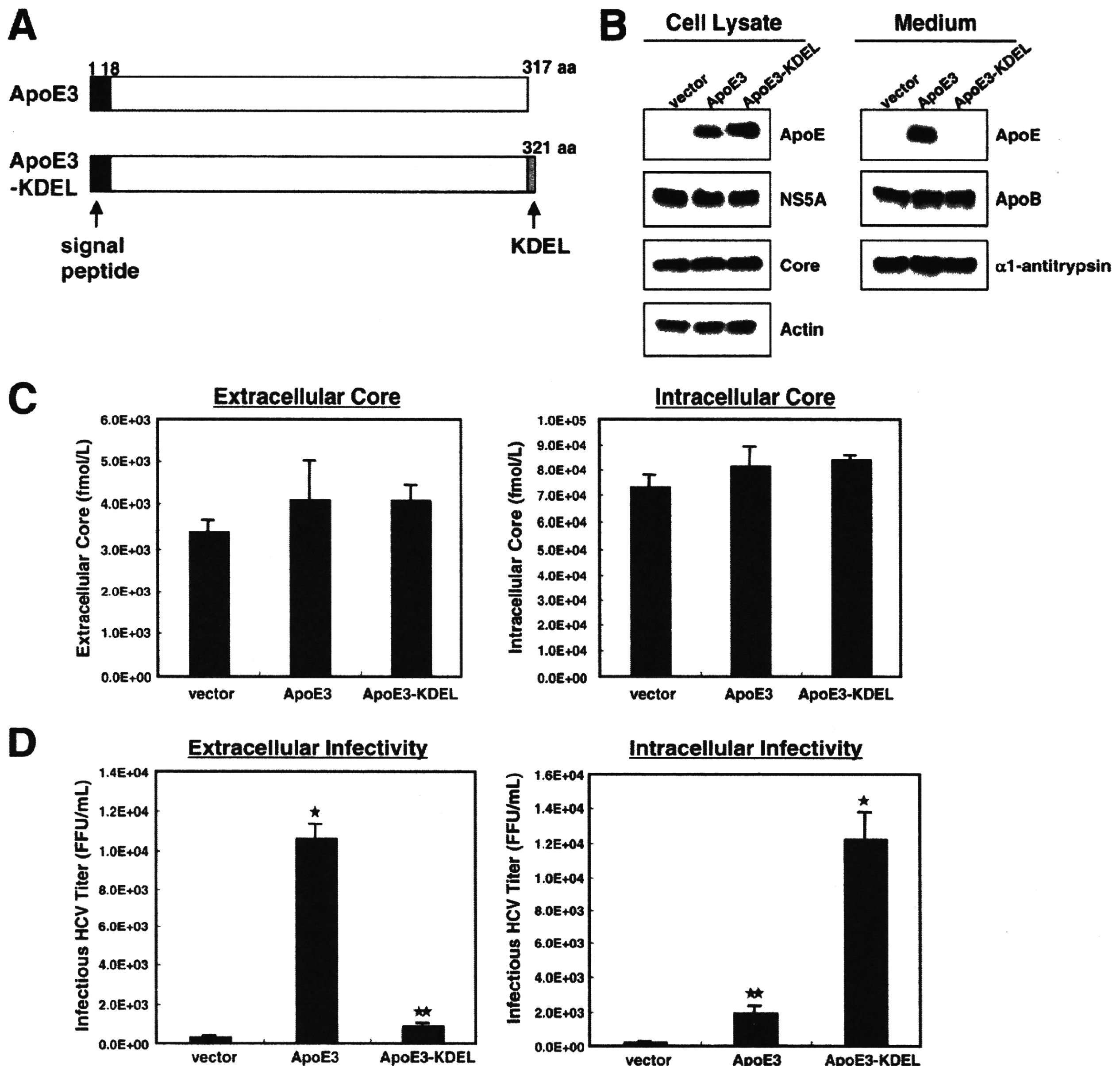

FIG. 2. The release of infectious HCV into the culture medium depends on the secretion of ApoE. (A) Schematic representation of ApoE3 and the ApoE3-KDEL mutant. (B) Verification of the expression of ectopic ApoE3 and ApoE3-KDEL. The sh-#3 cells were seeded onto 100-mm-diameter dishes. Cells were transfected with either pCAG (vector), pCAG-ApoE3 (ApoE3), or pCAG-ApoE3-KDEL (ApoE3-KDEL). Four hours after transfection, cells were inoculated with JFH1. Forty-eight hours after inoculation, cell lysates and supernatants were analyzed for the production of ApoE and its mutant by Western blotting with anti-ApoE antibodies. The expression of actin, α1-antitrypsin, ApoB, core, and NS5A was also examined. (C) The amounts of core in the culture supernatant and intracellular fractions were determined by a core-specific ELISA. The cells and transfections were the same as for panel B. (D) Analysis of HCV infectivity. The culture medium or cell lysate was inoculated into naïve HuH7.5 cells. The titers of infectious HCV were quantified by focus-forming unit assays. The average values for three independent experiments are shown; error bars, standard deviations of the means. The cells and transfections were the same as for panel B. *P* values were determined by comparison (by Student's *t* test) with cells expressing the control vector. *, $P < 0.0005$; **, $P < 0.005$.

cells and was not significantly different among cell groups expressing different ApoE isoforms (Fig. 3B, vector versus ApoE2 to ApoE4). We also noticed that the amounts of HCV RNA in extracellular fractions from cells expressing different ApoE isoforms were not significantly different (data not shown). To examine the production of infectious virus, the culture media of these cells were inoculated into naïve HuH7.5 cells, and infectivity was assayed 48 h after infection. Interestingly, ApoE2-expressing cells released substantially less infectious HCV into the culture medium than did ApoE3- or

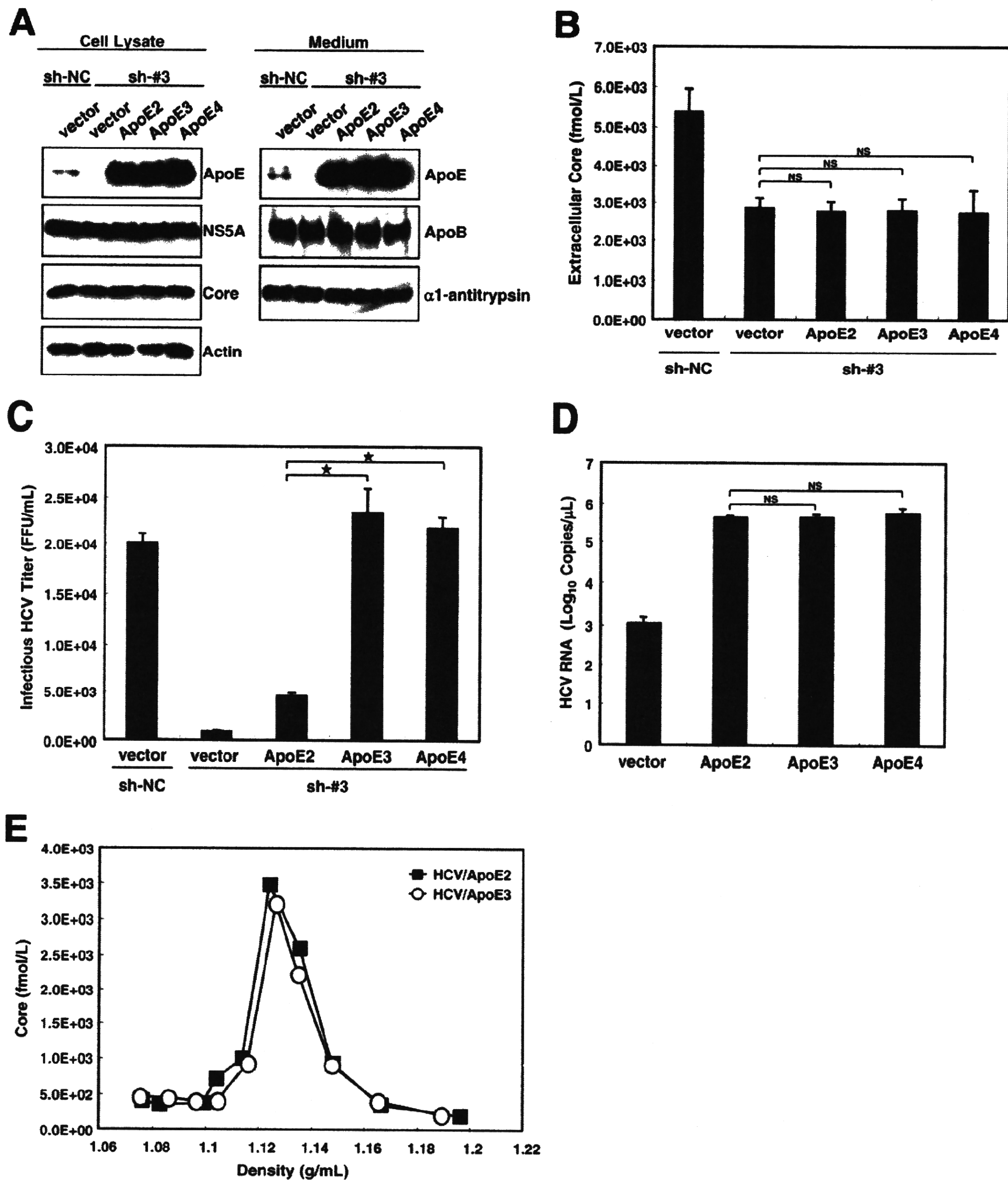

FIG. 3. ApoE isoforms affect HCV infectivity. (A) Verification of expression of ectopically introduced ApoE and the effect of ApoE isoforms on HCV genome replication. sh-NC cells and sh-#3 cells were seeded onto 60-mm-diameter dishes. Cells were transfected with either pCAG (vector), pCAG-ApoE2 (ApoE2), pCAG-ApoE3 (ApoE3), or pCAG-ApoE4 (ApoE4). Four hours after transfection, cells were inoculated with JFH1. Forty-eight hours after inoculation, the expression of ApoE, ApoB, NS5A, core, actin, and α1-antitrypsin in cell lysates and/or supernatants was analyzed by Western blotting using relevant antibodies. (B) The release of HCV core into the culture supernatant by cells expressing different ApoE isoforms was measured by a core-specific ELISA. The cells and transfections in panels B to D were the same as those in panel A. *P* values were determined by comparison (by Student's *t* test) with HCVcc from sh-#3 cells expressing the control vector. NS, not significant ($P > 0.05$). (C) Amount of infectious HCV that egressed from HCV-infected cells. Culture media of the indicated cells were inoculated into naïve HuH7.5 cells. Forty-eight hours after infection, naïve HuH7.5 cells were infected with the supernatant. Forty-eight hours after infection, the titers of infectious HCV were quantified by a focus-forming unit assay. *P* values were determined by comparison (by Student's *t* test) with HCVcc from sh-#3 cells expressing ApoE2. *, $P < 0.0005$. (D) Association of HCV with ApoE isoforms. HCV released into the culture medium from cells bearing each ApoE isoform was incubated with an anti-HA antibody. RNA was extracted from the immunoprecipitant and subjected to quantification by quantitative RT-PCR. *P* values were determined by comparison (by Student's *t* test) with HCVcc from sh-#3 cells expressing ApoE2. NS, not significant ($P > 0.05$). (E) Density gradient analysis of HCVcc containing ApoE2 or ApoE3. Concentrated HCVcc from cells expressing ApoE2 or ApoE3 was fractionated using 14 to 54% iodixanol density gradient centrifugation at 36,000 rpm for 16 h at 4°C. The buoyant density profile is represented by measuring the amount of core protein (in femtomoles per liter). HCV/ApoE3, HCV bearing ApoE3; HCV/ApoE2, HCV bearing ApoE2. Data from a representative of three experiments are shown.

ApoE4-expressing cells (Fig. 3C). We examined the possibility that the affinity of ApoE for HCV differs by isoform. HA-tagged ApoE isoform expression plasmids were transfected into sh-#3 cells, which were then infected with HCV. The culture media from the infected cells were incubated with an anti-HA antibody, and the immunocomplexes were recovered. RNA was extracted from the complexes and analyzed by quantitative RT-PCR. The amounts of HCV RNA in the immune complexes obtained from the culture media of cells expressing different ApoE isoforms were almost the same (Fig. 3D). Furthermore, we examined whether a difference between the densities of ApoE2-containing HCV and ApoE3-containing HCV can be correlated with the difference in infectivity (Fig. 3E). There is no significant density shift between these two viruses to explain the difference in infectivity, suggesting the importance of factors other than particle density for HCV infectivity. These results suggest that the ability of HCV-associated ApoE isoforms to bind to the LDLR seems to be responsible for isoform-based differences in the infectivity of HCV.

HCV requires the LDLR and SR-BI expression for full infectivity. Previously, the LDLR and SR-BI were shown to be involved in HCV infection, possibly through HCV-associating ApoE that functions as a ligand to those molecules. However, there is controversy as to which receptor, together with other receptors, such as CLDN1 and CD81, is involved in more importantly in HCV entry (3, 16, 24, 27, 34). To investigate this point, we conducted an infectivity analysis using ApoE2- and ApoE3-complemented HCV with HuH7.5 cells that were depleted of the LDLR and/or SR-BI by treatment with specific siRNAs. Western blot analysis showed that the levels of the LDLR and SR-BI were substantially reduced by treatment with their specific siRNAs (Fig. 4A). These cells were infected with HCV produced by HuH7.5 cells expressing ectopic ApoE3 (HCV bearing ApoE3 [HCV/ApoE3]) or ApoE2 (HCV bearing ApoE2 [HCV/ApoE2]), and infectivity was analyzed 48 h after infection (Fig. 4B). We observed significant reductions in HCV/ApoE3 infection of cells in which the LDLR or SR-BI was silenced, as expected on the basis of reports from other groups (3, 24, 27, 34). Assuming that LDLR and SR-B1 function independently in the step of HCV entry, a further reduction in infectivity would be expected for cells in which both the LDLR and SR-BI are silenced. However, this was not the case. The reduction in infectivity was almost the same as those for cells in which either the LDLR or SR-BI was silenced (Fig. 4B). The levels of HCV/ApoE2 entry into cells with both or either LDLR or SR-BI knocked down were also nearly the same (Fig. 4B).

Next, we conducted infectivity assays of HCV/ApoE2 and HCV/ApoE3 after treating them with antibodies against various candidate molecules for the HCV receptor (Fig. 4C). Anti-CLDN1 and anti-CD81 strongly inhibited HCV infection, as shown in Fig. 4C. Anti-LDLR and anti-SR-BI antibodies showed only moderate inhibition of HCV/ApoE3 infection (Fig. 4C, compare with inhibition by anti-CLDN1 and anti CD81 antibodies). Taken together, our results suggest the importance of both the LDLR and SR-BI for HCV infection, possibly through the function of HCV-associating ApoE.

DISCUSSION

Accumulating evidence suggests that HCV is complexed with lipoproteins and that it exhibits both viral and lipoprotein characteristics, leading to the recognition of HCV as an LVP. However, it is not known how the nature of lipoproteins associated with HCV is involved in the cycle of virus proliferation. HCV was found to be secreted in a manner that parallels the formation of VLDL by experiments that used an MTP inhibitor or ApoB knockdown (10, 14). In these studies, the suppression of ApoB significantly impaired the production of both VLDL and HCV. In contrast, another study reported a lesser contribution of ApoB to HCV production (15). On the other hand, ApoE knockdown severely interfered with the production of infectious virus (see below).

We showed here that ApoE is required for HCVcc infectivity for HuH7.5 cells, which is consistent with reports showing that the inhibition of ApoE production leads to reduced HCVcc infectivity (4, 6, 15, 27). Depletion of ApoE resulted in a significant reduction in the infectivity not only of JFH1 but also of TNS2J1, the chimeric HCVcc composed of the structural region of HCV-1b and a nonstructural region derived from JFH1, although the replication efficiencies of the genomes were unchanged (see Fig. S1B in the supplemental material). Thus, the requirement of ApoE for infectious HCV production may be unrelated to the HCV genotype.

ApoE seems to have an additional role in regulating virus assembly/release besides its role in virus entry. Chang et al. report a severe reduction in HCV particle assembly/release following ApoE knockdown (6). We analyzed the amount of HCV by measuring the level of core as well as virus RNA in the culture medium from ApoE-silenced HuH7.5 cells. Under this condition, the production and secretion of ApoE were severely suppressed (see Fig. S1A in the supplemental material). However, we observed only a 50% reduction of both core and HCV RNA levels in the culture medium (see Fig. S1B for core; data for HCV RNA not shown), in strong contrast to the data of Chang et al. Since ApoE knockdown does not affect replicon activity, we established several HuH7.5 clones that stably silenced the production of ApoE. Using some of those clones, sh-#3 and sh-#12, we observed the same result: only a ~50% reduction of HCV particle release upon HCV infection (see Fig. S1E). At present we cannot explain the difference between our results and those of Chang et al. However, it could be due to a difference in RNA transfection reagents that may affect cell variability or in HuH7.5 cells that might have been genetically modified during a prolonged period of cultivation after distribution from the original supplier.

ApoE associates with NS5A (4, 9, 15). Since NS5A is suggested to be involved in virus particle assembly (2, 21, 31), it is possible that ApoE participates, at least in part, in virus particle assembly by interacting with NS5A, as suggested by others (4, 15). However, the facts that the release of virus particles into the culture medium from cells in which ApoE is silenced is not completely suppressed and that HCV retained inside cells expressing the ApoE3-KDEL mutant and not released into culture medium indicate that ApoE is not an essential factor for assembly and release.

As for the effect of ApoE on HCV infectivity, we observed a dramatic reduction following ApoE knockdown (see Fig. S1B

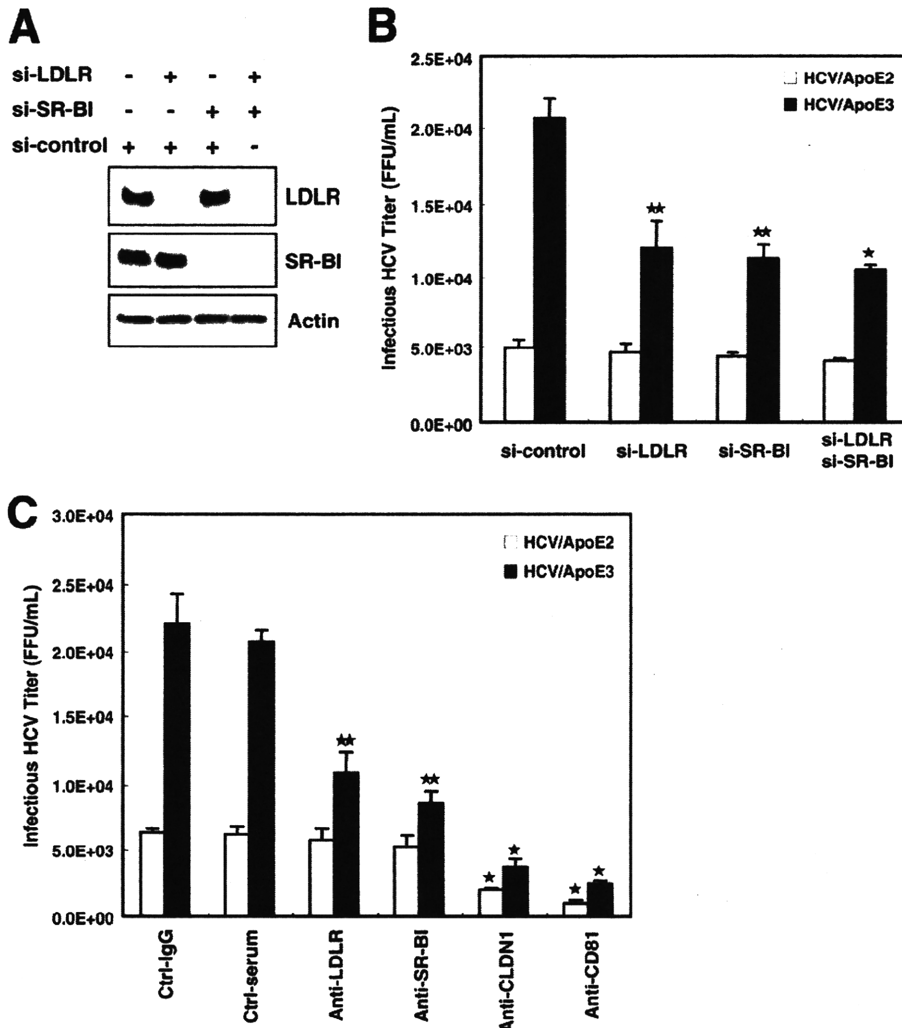

FIG. 4. Suppression of LDLR and SR-BI results in reduced HCV infection. (A) Knockdown of the LDLR and SR-BI by siRNA. HuH7.5 cells were seeded onto 24-well plates and transfected with siRNA (si-control, si-LDLR, and/or si-SR-BI). Twenty-four hours after transfection, cell lysates were analyzed for the expression of the LDLR, SR-BI, and actin by Western blotting. (B) Analysis of HCV infectivity for HuH7.5 cells in which either the LDLR, SR-BI, or both were silenced. Cells were transfected with siRNA (si-control, si-LDLR, and/or si-SR-BI). Twenty-four hours after transfection, HCVcc produced from cells bearing ApoE2 (open bars) or ApoE3 (filled bars) was serially diluted and inoculated. Forty-eight hours after infection, infected cells were counted by fluorescence microscopy after staining with an anti-NS5A antibody. HCV/ApoE3, HCV bearing ApoE3; HCV/ApoE2, HCV bearing ApoE2. *P* values were determined by comparison (by Student's *t* test) with si-control-treated cells. *, $P < 0.0005$; **, $P < 0.005$. (C) HuH7.5 cells were preincubated with control goat IgG (Ctrl-IgG), control rat preimmune serum (Ctrl-serum), or a goat anti-LDLR, rat anti-SR-BI, rat anti-claudin 1 (anti-CLDN1), or mouse anti-CD81 antibody for 1 h at 37°C before infection with serially diluted HCVcc from HuH7.5 cells expressing ApoE2 (open bars) or ApoE3 (filled bars). Forty-eight hours after infection, infected cells were stained with anti-NS5A antibodies and counted by fluorescence microscopy. The average values for three independent experiments are shown; error bars, standard deviations of the means. *P* values were determined by comparison (by Student's *t* test) against cells treated with preimmune serum. *, $P < 0.0005$; **, $P < 0.005$.

and S1E in the supplemental material). Thus, we think that ApoE affects HCV infectivity severely but affects virus assembly and/or release only slightly. We tried to find a difference between HCV derived from HuH7.5 cells and HCV from ApoE-depleted HuH7.5 cells by density gradient centrifugation, because a correlation between low HCV particle density and infectivity has been known. However, we could not see any difference in buoyant density to explain the difference in infectivity (Fig. 1A). We think that association with ApoE does not drastically change the physical nature of HCV. It will be important, however, to look for differences in the physical and biochemical nature of HCV in detail, including lipid contents, the status of glycoproteins such as E1 and E2, and so on, in the future.

An association between ApoE and secreted HCV was observed (see Fig. S3A in the supplemental material) (6, 15, 27). To obtain insight into the function of ApoE in the virus life cycle, and particularly in the step of infectious virus secretion,

we analyzed the infectious virus released into the culture medium from cells expressing the ApoE mutant ApoE3-KDEL, which is not secreted, because it contains the ER retention signal peptide KDEL. We confirmed that ApoE3-KDEL was not secreted into the culture medium (Fig. 2B). Indirect immunofluorescence showed that the majority of ApoE3-KDEL was retained on the ER, whereas ApoE3 was localized on the ER and the Golgi apparatus (data not shown). The amount of virus released into the culture medium from cells expressing ApoE3-KDEL was almost the same as that from cells expressing ApoE3 (Fig. 2C). However, the production of infectious virus in the culture medium was severely suppressed, since the infectious HCV accumulated in the cell lysate (Fig. 2D). The accelerated level of intracellular accumulation of infectious virus, which was higher than that in cells expressing ApoE3, suggests that the secretion of "infectious" virus depends on ApoE production and secretion. The amounts of HCV released into the culture medium from cells expressing ApoE3-KDEL and ApoE3 were almost the same as that released by cells lacking endogenous ApoE expression (Fig. 2C and D), indicating that ApoE affects the assembly and release of noninfectious virus into the culture medium only slightly but mainly affects the release of infectious virus. Our result also suggests that the association of ApoE and HCV occurs prior to the secretion of the virus from cells, which is required for the virus to gain infectivity. Incubation of recombinant ApoE with HCV produced from ApoE knockdown cells did not show a significant increase in HCV infectivity (Fig. 1B). Moreover, no interaction of ApoE with HCV *in vitro* was observed (data not shown); this observation confirms the establishment of the association before virus secretion.

The physical structure of HCV as an LVP is uncertain. It is not known if the association of ApoE with infectious virus depends on a coassociation with or integration of lipoprotein. We observed the importance of a substrate of lipoprotein lipase (LPL), which associates with HCV, for infectivity (29). LPL hydrolyzes triglycerides in VLDL and converts them to intermediate-density lipoproteins (IDL). When HCV produced from HuH7.5 cells was treated with LPL followed by hepatic lipase, the density of the virus was shifted higher than the density prior to treatment, and infectivity was simultaneously lost. Importantly, the amount of ApoE associated with HCV was reduced (29). This observation suggests that the interaction of ApoE with HCV depends on the presence of a virus-associated triglycerol ester, most likely a lipid component of lipoprotein. This observation also suggests the importance of an association of lipoprotein with HCV in order to maintain the function of ApoE for HCV infection. However, more study is needed to clarify how ApoE interacts with HCV to increase infectivity.

The ApoE gene is polymorphic, with three common alleles, *apoE2*, *apoE3*, and *apoE4*, which produce 3 isoforms of ApoE. Because the ApoE isoforms have different affinities for the LDLR (ApoE2 has low affinity, while ApoE3 and ApoE4 have high affinity), we analyzed the effect of ApoE isoforms on HCV infectivity. HCV/ApoE3 and HCV/ApoE4 showed almost the same infectivity as the control virus produced from HCV-infected HuH7.5 cells expressing endogenous ApoE3 (Fig. 3C). However, the infectivity of HCV/ApoE2 was about one-fifth that of HCV/ApoE3, even though the level of virus particles was almost the same as that of HCV/ApoE3 (Fig. 3B and C). Assuming that the LDLR plays a role as a receptor of HCV (24, 27, 33), this observation is in agreement with the fact that the binding affinity of ApoE for the LDLR is well correlated with the difference in HCV infectivity by ApoE isoforms.

HCV/ApoE2 showed reduced infectivity for LDLR-silenced HuH7.5 cells (Fig. 4B). Since the level of infectivity was almost the same as that for control cells, entry through SR-BI on HuH7.5 cells was not utilized by HCV/ApoE2. This is also suggested by analysis of the infectivity of HCV/ApoE2 for doubly silenced HuH7.5 cells (Fig. 4B). Because the possibility remains that ApoE2 interacts weakly with both the LDLR and SR-BI, although the binding regions for the LDLR and SR-BI on the ApoE molecule do not overlap (17), we analyzed the infectivity of HCV/ApoE3 for cells in which either the LDLR, SR-BI, or both were silenced (Fig. 4B). As expected on the basis of reports from other groups, suppression of infectivity for LDLR- or SR-BI-silenced HuH7.5 cells was observed. However, to our surprise, infectivity was not further reduced for doubly silenced cells (Fig. 4B). This result suggests that both the LDLR and SR-BI are required for virus entry. The absence of either of these proteins would result in a reduction in infectivity. It is important to determine whether these receptor molecules function independently or cooperatively for HCV entry.

When infectivity was analyzed by treating HCV with an anti-SR-BI or anti-LDLR antibody, the infectivity of HCV remained at a level higher than that of HCV treated with an anti-CLDN1 or anti-CD81 antibody (Fig. 4C). Further, a significant level of HCV/ApoE3 infectivity for LDLR- and SR-BI-silenced HuH7.5 cells was observed (Fig. 4B). These data suggest the presence of another receptor molecule(s) that is relevant to the LDLR and SR-BI regarding the ability to interact with ApoE.

The functional importance of ApoE for HCV infectivity is not limited to the HCVcc used in the present study. An epidemiological study of Caucasians with persistent chronic hepatitis indicates a notable absence of the ApoE2/ApoE2 genotype in HCV antibody-positive individuals (28), which is in agreement with the lower infectivity of ApoE2-bearing HCVcc. The authors did not describe any role for ApoE2 on HCV infectivity. However, our results strongly suggest that HCV produced from ApoE2-bearing individuals is eliminated quickly because it is less infectious. It will be important to conduct a virological study of HCV obtained from individuals carrying different isoforms of ApoE in the future.

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REFERENCES

1. **André, P., F. Komurian-Pradel, S. Deforges, M. Perret, J. L. Berland, M. Sodoyer, S. Pol, C. Brechot, G. Paranhos-Baccala, and V. Lotteau.** 2002. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J. Virol. **76:**6919–6928.
2. **Appel, N., M. Zayas, S. Miller, J. Krijnse-Locker, T. Schaller, P. Friebe, S. Kallis, U. Engel, and R. Bartenschlager.** 2008. Essential role of domain III

of nonstructural protein 5A for hepatitis C virus infectious particle assembly. PLoS Pathog. 4:e1000035.
3. **Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F. L. Cosset.** 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J. Biol. Chem. **278:**41624–41630.
4. **Benga, W. J., S. E. Krieger, M. Dimitrova, M. B. Zeisel, M. Parnot, J. Lupberger, E. Hildt, G. Luo, J. McLauchlan, T. F. Baumert, and C. Schuster.** 2010. Apolipoprotein E interacts with hepatitis C virus nonstructural protein 5A and determines assembly of infectious particles. Hepatology **51:**43–53.
5. **Blasiole, D. A., R. A. Davis, and A. D. Attie.** 2007. The physiological and molecular regulation of lipoprotein assembly and secretion. Mol. Biosyst. **3:**608–619.
6. **Chang, K. S., J. Jiang, Z. Cai, and G. Luo.** 2007. Human apolipoprotein E is required for infectivity and production of hepatitis C virus in cell culture. J. Virol. **81:**13783–13793.
7. **Davignon, J., R. E. Gregg, and C. F. Sing.** 1988. Apolipoprotein E polymorphism and atherosclerosis. Arteriosclerosis **8:**1–21.
8. **Diaz, O., F. Delers, M. Maynard, S. Demignot, F. Zoulim, J. Chambaz, C. Trepo, V. Lotteau, and P. André.** 2006. Preferential association of hepatitis C virus with apolipoprotein B48-containing lipoproteins. J. Gen. Virol. **87:**2983–2991.
9. **Evans, M. J., C. M. Rice, and S. P. Goff.** 2004. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. Proc. Natl. Acad. Sci. U. S. A. **101:**13038–13043.
10. **Gastaminza, P., G. Cheng, S. Wieland, J. Zhong, W. Liao, and F. V. Chisari.** 2008. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. J. Virol. **82:**2120–2129.
11. **Gibbons, G. F., K. Islam, and R. J. Pease.** 2000. Mobilisation of triacylglycerol stores. Biochim. Biophys. Acta **1483:**37–57.
12. **Grakoui, A., C. Wychowski, C. Lin, S. M. Feinstone, and C. M. Rice.** 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. J. Virol. **67:**1385–1395.
13. **Hijikata, M., H. Mizushima, T. Akagi, S. Mori, N. Kakiuchi, N. Kato, T. Tanaka, K. Kimura, and K. Shimotohno.** 1993. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J. Virol. **67:**4665–4675.
14. **Huang, H., F. Sun, D. M. Owen, W. Li, Y. Chen, M. Gale, Jr., and J. Ye.** 2007. Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. Proc. Natl. Acad. Sci. U. S. A. **104:**5848–5853.
15. **Jiang, J., and G. Luo.** 2009. Apolipoprotein E but not B is required for the formation of infectious hepatitis C virus particles. J. Virol. **83:**12680–12691.
16. **Krieger, S. E., M. B. Zeisel, C. Davis, C. Thumann, H. J. Harris, E. K. Schnober, C. Mee, E. Soulier, C. Royer, M. Lambotin, F. Grunert, V. L. Dao Thi, M. Dreux, F. L. Cosset, J. A. McKeating, C. Schuster, and T. F. Baumert.** 2010. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-claudin-1 associations. Hepatology **51:**1144–1157.
17. **Li, X., H. Y. Kan, S. Lavrentiadou, M. Krieger, and V. Zannis.** 2002. Reconstituted discoidal ApoE-phospholipid particles are ligands for the scavenger receptor BI. The amino-terminal 1–165 domain of ApoE suffices for receptor binding. J. Biol. Chem. **277:**21149–21157.
18. **Liang, T. J., L. J. Jeffers, K. R. Reddy, M. De Medina, I. T. Parker, H. Cheinquer, V. Idrovo, A. Rabassa, and E. R. Schiff.** 1993. Viral pathogenesis of hepatocellular carcinoma in the United States. Hepatology **18:**1326–1333.
19. **Lindenbach, B. D., P. Meuleman, A. Ploss, T. Vanwolleghem, A. J. Syder, J. A. McKeating, R. E. Lanford, S. M. Feinstone, M. E. Major, G. Leroux-Roels, and C. M. Rice.** 2006. Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. Proc. Natl. Acad. Sci. U. S. A. **103:**3805–3809.
20. **Mahley, R. W., and S. C. Rall, Jr.** 2000. Apolipoprotein E: far more than a lipid transport protein. Annu. Rev. Genomics Hum. Genet. **1:**507–537.
21. **Masaki, T., R. Suzuki, K. Murakami, H. Aizaki, K. Ishii, A. Murayama, T. Date, Y. Matsuura, T. Miyamura, T. Wakita, and T. Suzuki.** 2008. Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles. J. Virol. **82:**7964–7976.
22. **Meunier, J. C., R. S. Russell, R. E. Engle, K. N. Faulk, R. H. Purcell, and S. U. Emerson.** 2008. Apolipoprotein C1 association with hepatitis C virus. J. Virol. **82:**9647–9656.
23. **Miyanari, Y., K. Atsuzawa, N. Usuda, K. Watashi, T. Hishiki, M. Zayas, R. Bartenschlager, T. Wakita, M. Hijikata, and K. Shimotohno.** 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. **9:**1089–1097.
24. **Molina, S., V. Castet, C. Fournier-Wirth, L. Pichard-Garcia, R. Avner, D. Harats, J. Roitelman, R. Barbaras, P. Graber, P. Ghersa, M. Smolarsky, A. Funaro, F. Malavasi, D. Larrey, J. Coste, J. M. Fabre, A. Sa-Cunha, and P. Maurel.** 2007. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. J. Hepatol. **46:**411–419.
25. **Nahmias, Y., J. Goldwasser, M. Casali, D. van Poll, T. Wakita, R. T. Chung, and M. L. Yarmush.** 2008. Apolipoprotein B-dependent hepatitis C virus secretion is inhibited by the grapefruit flavonoid naringenin. Hepatology **47:**1437–1445.
26. **Nielsen, S. U., M. F. Bassendine, A. D. Burt, C. Martin, W. Pumeechockchai, and G. L. Toms.** 2006. Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. J. Virol. **80:**2418–2428.
27. **Owen, D. M., H. Huang, J. Ye, and M. Gale, Jr.** 2009. Apolipoprotein E on hepatitis C virion facilitates infection through interaction with low-density lipoprotein receptor. Virology **394:**99–108.
28. **Price, D. A., M. F. Bassendine, S. M. Norris, C. Golding, G. L. Toms, M. L. Schmid, C. M. Morris, A. D. Burt, and P. T. Donaldson.** 2006. Apolipoprotein ε3 allele is associated with persistent hepatitis C virus infection. Gut **55:**715–718.
29. **Shimizu, Y., T. Hishiki, K. Sugiyama, K. Ogawa, K. Funami, A. Kato, Y. Ohsaki, T. Fujimoto, H. Takaku, and K. Shimotohno.** 3 September 2010. Lipoprotein lipase and hepatic triglyceride lipase reduce the infectivity of hepatitis C virus (HCV) through their catalytic activities on HCV-associated lipoproteins. Virology. doi:10.1016/j.virol.2010.08.011.
30. **Sugiyama, K., K. Suzuki, T. Nakazawa, K. Funami, T. Hishiki, K. Ogawa, S. Saito, K. W. Shimotohno, T. Suzuki, Y. Shimizu, R. Tobita, M. Hijikata, H. Takaku, and K. Shimotohno.** 2009. Genetic analysis of hepatitis C virus with defective genome and its infectivity in vitro. J. Virol. **83:**6922–6928.
31. **Tellinghuisen, T. L., K. L. Foss, and J. Treadaway.** 2008. Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. PLoS Pathog. **4:**e1000032.
32. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11:**791–796.
33. **Wünschmann, S., J. D. Medh, D. Klinzmann, W. N. Schmidt, and J. T. Stapleton.** 2000. Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and the low-density lipoprotein receptor. J. Virol. **74:**10055–10062.
34. **Zeisel, M. B., G. Koutsoudakis, E. K. Schnober, A. Haberstroh, H. E. Blum, F. L. Cosset, T. Wakita, D. Jaeck, M. Doffoel, C. Royer, E. Soulier, E. Schvoerer, C. Schuster, F. Stoll-Keller, R. Bartenschlager, T. Pietschmann, H. Barth, and T. F. Baumert.** 2007. Scavenger receptor class B type I is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81. Hepatology **46:**1722–1731.

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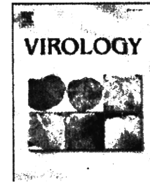

Cell culture and in vivo analyses of cytopathic hepatitis C virus mutants☆,☆☆

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ABSTRACT

HCV-JFH1 yields subclones that develop cytopathic plaques (Sekine-Osajima Y, et al., Virology 2008; 371:71). Here, we investigated viral amino acid substitutions in cytopathic mutant HCV-JFH1 clones and their characteristics in vitro and in vivo. The mutant viruses with individual C2441S, P2938S or R2985P signature substitutions, and with all three substitutions, showed significantly higher intracellular replication efficiencies and greater cytopathic effects than the parental JFH1 in vitro. The mutant HCV-inoculated mice showed significantly higher serum HCV RNA and higher level of expression of ER stress-related proteins in early period of infection. At 8 weeks post inoculation, these signature mutations had reverted to the wild type sequences. HCV-induced cytopathogenicity is associated with the level of intracellular viral replication and is determined by certain amino acid substitutions in HCV-NS5A and NS5B regions. The cytopathic HCV clones exhibit high replication competence in vivo but may be eliminated during the early stages of infection.
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Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality (Alter, 1997). Antiviral therapeutic options against HCV have been limited to type I interferons and ribavirin and have yielded unsatisfactory responses (Fried et al., 2002). Given this situation, a precise understanding of the molecular mechanisms of interferon resistance has been a high priority of research in academia and industry.

Molecular analyses of the HCV life cycle, virus–host interactions, and mechanisms of liver cell damage by the virus are not understood completely, mainly because of the lack of cell culture systems. These problems have been overcome to some extent by the development of the HCV subgenomic replicon (Lohmann et al., 1999) and HCV cell culture systems (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The HCV-JFH1 strain, which is a genotype 2a clone derived from a Japanese fulminant hepatitis patient and can replicate efficiently in Huh7 cells (Kato, 2001; Kato et al., 2003), has contributed to the establishment of the HCV cell culture system. Furthermore, the Huh7-derived cell lines, Huh-7.5 and Huh-7.5.1 cells, allow production of higher viral titers and have a greater permissivity for HCV (Koutsoudakis et al., 2007; Lindenbach et al., 2005; Zhong et al., 2005). The HCV-JFH1 cell culture system now allows us to study the complete HCV life cycle: virus-cell entry, translation, protein processing, RNA replication, virion assembly and virus release.

HCV belongs to the family *Flaviviridae*. One of the characteristics of the *Flaviviridae* is that they cause cytopathic effects (CPE). The viruses have positive strand RNA genomes of ~10 kilo-bases that encode polyproteins of ~3000 amino acids. These proteins are processed post-translationally by cellular and viral proteases into at least 10 mature proteins (Sakamoto and Watanabe, 2009). The viral non-structural proteins accumulate in the ER and direct genomic replication and viral protein synthesis (Bartenschlager and Lohmann, 2000; Jordan et al., 2002; Mottola et al., 2002). It has been recently

Abbreviations: HCV, hepatitis C virus; CPE, cytopathic effect; ER, endoplasmic reticulum; RdRp, RNA dependent RNA polymerase.

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reported that HCV-JFH1 transfected Huh-7.5.1 cells die when all of the cells are infected and intracellular HCV RNA reaches maximum levels (Zhong et al., 2006). These findings suggest HCV-induced cytopathogenicity. However, the mechanisms have not been well documented.

In a previous study, we investigated the cellular effects of HCV infection and replication using the HCV-JFH1 cell culture system and we reported that HCV-JFH1 transfected and infected cells show substantial CPE that are characterized by massive apoptotic cell death with expression of several ER stress-induced proteins. Taking advantage of the CPE, we developed a plaque assay for HCV in cell culture and isolated subclones of HCV that showed enhanced replication and cytopathogenicity (Sekine-Osajima et al., 2008). We have demonstrated that these viral characters were determined by mutations at certain positions in the structural and nonstructural regions of the HCV genome, especially the NS5A and NS5B regions.

In this study, we investigated the mechanisms and viral nucleotide sequences involved in HCV-induced cytopathic effects using HCV-JFH1 cell culture and a newly developed cytopathic plaque-forming assay. We demonstrated that introduction of NS5A and NS5B mutations into the JFH1 clone resulted in a higher replication efficiency, although introduction of these mutations into the JFH1 subgenomic replicon has no effect on viral replication. These mutations do not affect virion entry or release of viral particles but regulate virus replication, and high levels of virus replication result in cytopathogenicity.

Results

Development of cytopathic plaques by HCV infection of Huh-7.5.1 cells

A plaque assay was performed to investigate the morphological CPE following HCV-JFH1 infection (see Materials and methods). Culture supernatants from JFH1-transfected cells were diluted serially and inoculated onto uninfected Huh-7.5.1 cells. The cells were subsequently cultured in medium containing agarose. On 9 days after the inoculation, viable cells were stained and plaques were visualized (Fig. 1A). HCV-inoculated cell cultures developed plaques as unstained areas, accompanied by rounded cells in the periphery (Fig. 1B). The formation of cytopathic plaques was not observed in a parental Huh7 cell line (data not shown). Those results were consistent with our previous study (Sekine-Osajima et al., 2008).

Introduction of mutations in the NS5A and NS5B regions of the JFH1 clone augmented its cytopathic effects

Among the amino acid substitutions that developed in the plaque-derived HCV-JFH1 strains, 6 of the 9 amino acid changes appeared redundantly among 5 independently isolated plaques, and clustered in the C terminal part of the NS5A and NS5B regions. To investigate the phenotype of each amino acid substitution, we constructed mutant JFH1

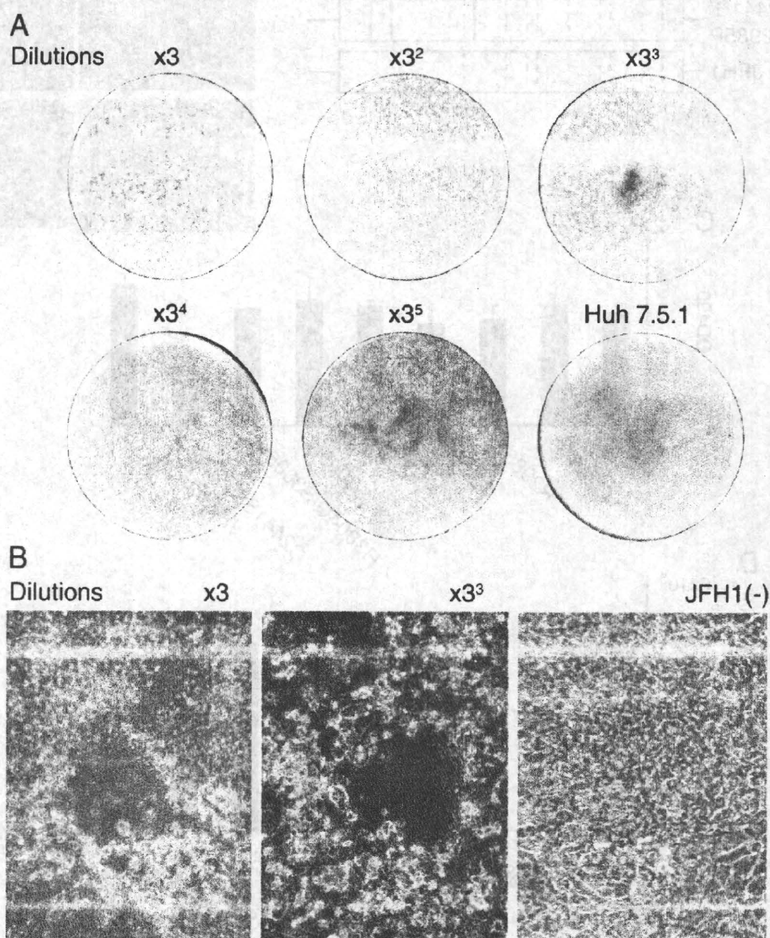

Fig. 1. The cytopathic effects of HCV-JFH1 *in vitro*. A. Plaque assay. Huh-7.5.1 cells were seeded in collagen-coated 60mm-diameter plates at density of 4×10^5 cells per plates and were incubated at 37 °C under 5.0% CO_2 (as described above). After overnight incubation, HCV-infected culture supernatants were serially diluted in a final volume of 2 ml per plates and transferred onto the cell monolayers. After ~5 h of incubation, the inocula were removed and the infected cells were overlaid with 8 ml of culture medium containing 0.8% methyl-cellulose and incubated under normal conditions. After 7 days culture, formation of cytopathic plaque was visualized by staining with 0.08% crystal violet solution. B. The cytopathic plaques were observed by phase-contrast microscopy at day 7 after HCV-JFH1 infection.

clones in which we introduced separately one amino acid substitution in NS5A and five substitutions in NS5B (Fig. 2A) and transfected the mutant HCV RNAs into Huh-7.5.1 cells. To compare the electroporation efficiencies of viral RNAs, Huh-7.5.1 cells were harvested 8 h after transfection and the levels of intracellular core antigen were measured. There was no difference in the efficiencies of electroporation (Fig. 2D). The substitutions G2964D, H3004Q, and S3005N did not lead to cytopathic effects but three mutant subclones (C2441S, P2938S and R2985P) produced much more cell death compared to the wild type JFH1 (Fig. 2B). To assess the quantitative cytopathic effect seen in host cells for each of the mutants, we also performed MTS assay at 6 days post transfection. It showed that Huh-7.5.1 cells transfected with the triple mutants (C2441S, R2938S, or R2985P) induced apparently much more cytopathic effect compared to the parental JFH1 and other mutant clones, although the three mutant clones encoding the substitutions C2441S, P2938S, or R2985P did not show significant difference but

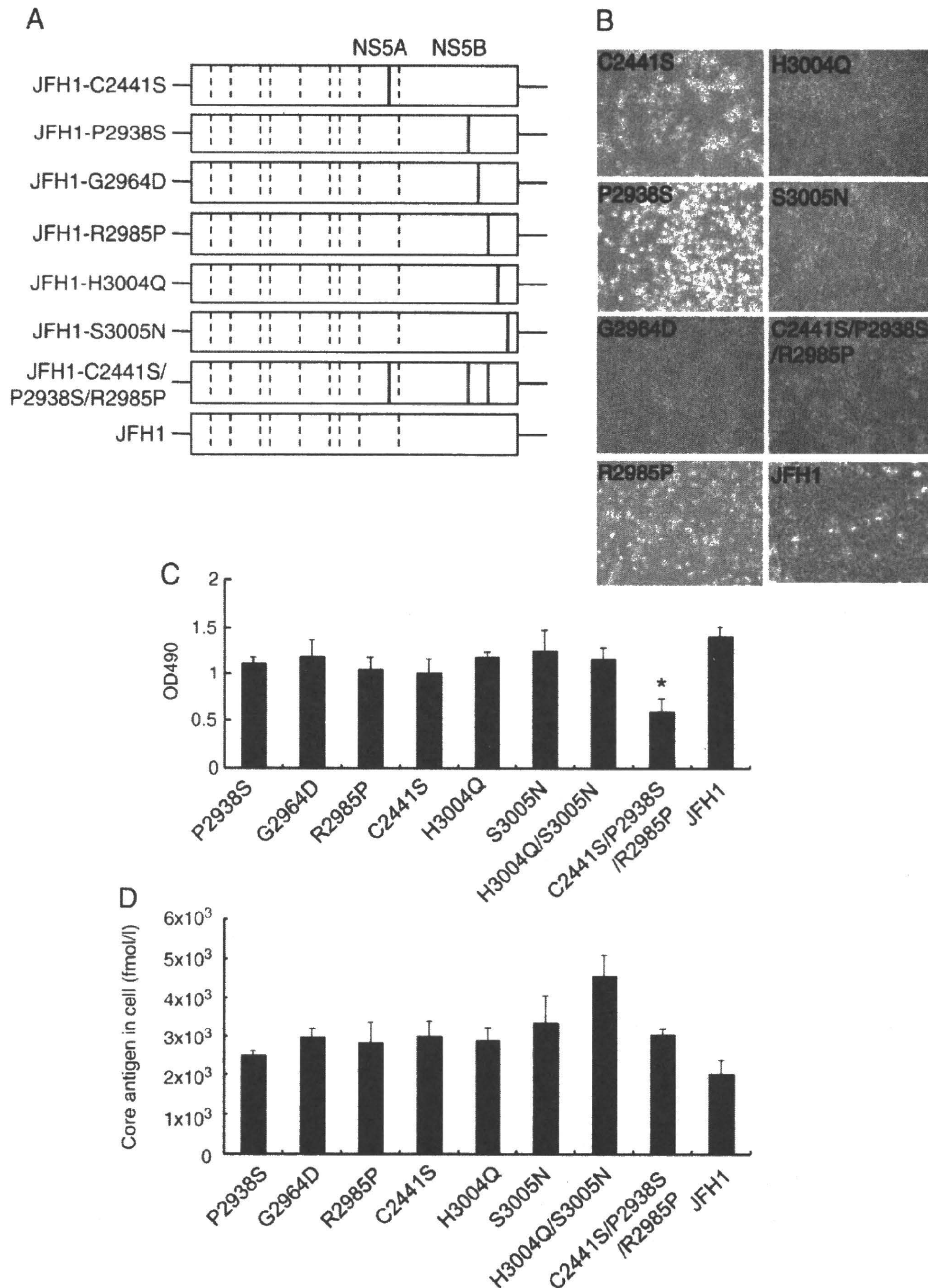

Fig. 2. Introduction of mutations into the NS5A and NS5B regions of JFH1. A. The mutations identified in the cytopathic plaque were introduced individually into the parental JFH1. Each JFH1 mutant was transfected into Huh-7.5.1 cells by electroporation. B. Huh-7.5.1 cells transfected with JFH1-mutants were observed by phase-contrast microscopy at day10 after transfection. C. MTS assay was performed to assess the quantitative cytopathic effect seen in Huh-7.5.1 cells for each of the mutants 6 days post transfection. Asterisks indicate p-values of less than 0.05 as compared with JFH1. D. Huh-7.5.1 cells were harvested at 8 h after transfection and the levels of intracellular core antigen were measured.

showed tendency to introduce more cytopathic effect than the parental JFH1 and the mutant clones encoding the substitutions G2964D, H3004Q, and S3005N (Fig. 2C).

Introduction of NS5A and NS5B mutations into the JFH1 clone led to a greater replication efficiency

To compare the expression levels of each mutant subclone, each HCV RNA was transfected and core antigen was detected subsequently in the culture medium. Similar to Fig. 2B, HCV clones with individual substitutions G2964D, H3004Q and S3005N produced significantly less core antigen or did not replicate at all. In contrast, the C2441S, P2938S and R2985P mutants produced significantly more core antigen than the wild type JFH1. In addition, an HCV clone with all 3 adaptive substitutions (C2441S, P2938S and R2985P) produced more core antigen than any other clone (Fig. 3A).

Next, we harvested the infected cells at 5 days after electroporation and performed western blotting. As shown in Fig. 3B, the three clones encoding the substitutions C2441S, P2938S, or R2985P, and the clone with all three mutations, expressed far more core protein than the parental JFH1, although the clones encoding the substitutions G2964D, H3004Q and S3005N did not express core protein. We also transferred culture media from the mutant clones onto uninfected Huh-7.5.1 cells and performed western blotting and the cells infected with the same mutant subclones as Fig. 3B expressed more core protein (Fig. 3C).

Introduction of NS5A and NS5B mutations into the JFH1 subgenomic replicon

To investigate the primary phase of replication of JFH1 mutants, we constructed JFH1 subgenomic replicons by introducing individually the six mutations in NS5A and NS5B. We transfected each replicon RNA into Huh7 cells and compared their replication levels according to the luciferase activities. Consistently with the mutant viruses, the subgenomic replicon encoding the changes C2441S, P2938S or R2985P, which produced higher amounts of core antigen, did replicate at higher levels than the other subgenomic replicons with single mutation, G2964D, H3004Q and S3005N. However, none of these mutants replicated at higher than the parental JFH1 subgenomic replicon. Furthermore, replicon with triple mutations of C2241S, P2938S and R2985P did not replicate (Fig. 4).

Introduction of NS5A and NS5B mutations into the JFH1 clone had no effect on the production of infectious virions

We sought to investigate the effects of the NS5A and NS5B mutations on virus replication and virion secretion independent of re-infection and spread of the viruses produced. Therefore, we used the S29-subclone of Huh7 cells, which cannot be infected by HCV because of a defect in CD81 expression but does support viral genomic replication and releases infectious HCV particles after transfection (Russell et al., 2008). The Huh7-S29 cells enabled us to evaluate a single cycle of infection and production of virions. Those cell lines did not show apparent cytopathic effects after transfection with HCV RNAs (data not shown). To analyze HCV particle production from cells transfected with the viral genomic RNAs transcribed in vitro, we harvested culture media and cells at 72 h post transfection and measured the core antigen levels in culture media and intracellular HCV RNA by real-time RT-PCR. The C2441S, P2938S, and R2985P mutants produced significantly greater amounts of core antigen in the culture medium than the wild type JFH1. The HCV clone carrying all three mutations produced the greatest amount of core antigen (Fig. 5A, top). Consistent with the core antigen levels in the culture media, intracellular HCV RNA levels were also higher in the cells transfected with the mutated genomes encoding separately C2441S,

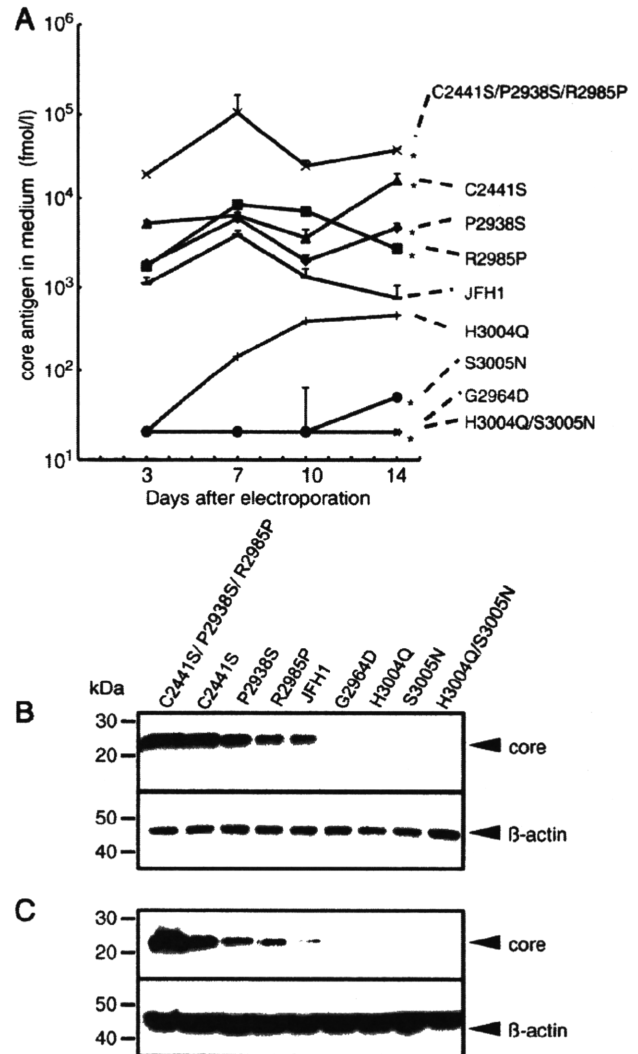

Fig. 3. Replication competences of HCV subclones with NS5A and NS5B mutations. A. Levels of core antigen in the culture medium. The culture media from transfected cells were collected on the days indicated and the levels of core antigen were measured. Asterisks indicate p-values of less than 0.05 as compared with JFH1. B. Huh-7.5.1 cells transfected with JFH1 mutants were harvested at 5 days after transfection and western blotting was performed. C. The culture media from Huh-7.5.1 cells transfected with JFH1 mutants were transferred onto uninfected Huh-7.5.1 cells. The cells were harvested at 3 days after infection. Western blotting was performed using anti-core and anti-beta-actin. kDa: kilo dalton.

P2938S, and R2985P, and that with all three mutations (Fig. 5A, middle), indicating that these mutations affected virus replication. Fig. 5A bottom shows the efficiency of infectious viral particle release from each transfectant, this being expressed as the core antigen level in the culture medium adjusted by dividing by the levels of intracellular HCV RNA. There was no difference in the efficiency of release of virions by the wild type JFH1 and the genomes carrying the C2441S, P2938S or R2985P changes. These results indicated that these three mutations in NS5A and NS5B did not affect virion entry or viral particle release but did regulate virus replication, and a high level of viral replication induces cytopathogenicity. Similarly, as shown in Fig. 3B, the three clones with C2441S, P2938S or R2985P, or all three mutations expressed much higher levels of core protein than the parental JFH1, while clones with G2964D, H3004Q or S3005N mutations did not express detectable amounts of core protein (Fig. 5B).

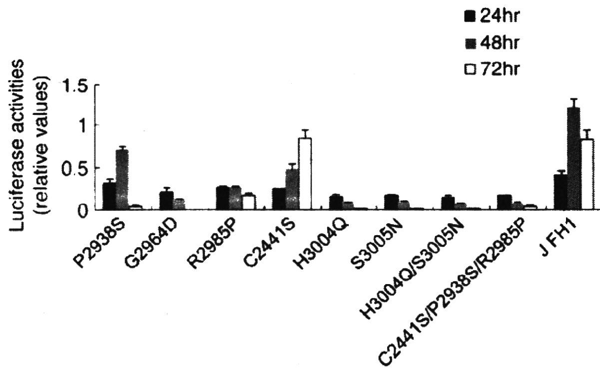

Fig. 4. Luciferase assay of the cytopathic JFH1-subgenomic replicon. Mutations were introduced into 2a-Feo subgenomic replicon and transcribed RNA for each replicon was transfected into Huh7 cells by electroporation. The cells were harvested at 24 h, 48 h and 72 h after electroporation and were used for Luciferase assay. Values are relative values to those of 8 h.

Mutations of NS5A and NS5B are associated with replication competence at earlier stages in vivo

We next used human hepatocyte chimeric mice to investigate the infectivity of the triple mutant of NS5A and NS5B. We confirmed the mouse liver chimerism greater than 70% by immunohistochemical analysis (data not shown). Culture media of the parental JFH1 and the mutant subclone with three mutations (C2441S, P2938S, and R2985P), were collected following transfection of Huh-7.5.1 cells, concentrated, and inoculated intravenously into human hepatocyte chimeric mice. We confirmed that the three mutations in NS5A and NS5B were conserved in the virus genome sequence of cell culture supernatants that were used for inoculation (data not shown). Two mice were inoculated with JFH1 and three were inoculated with the mutant virus. HCV RNA and human albumin in the sera of the mice were detected sequentially.

We repeated the same exam twice and confirmed consistency of the results. In the early phase post inoculation, the concentration of HCV RNA in serum was significantly higher in mice inoculated with the culture medium from the mutant subclone (Fig. 6A), suggesting that the mutations in NS5A and NS5B (C2441S, P2938S, and R2985P) are associated with virus replication in vivo. However, there was no difference in the level of HCV RNA in later period. The disparity of viral production at early time point could be influenced by the disparate numbers of infectious virus between the 2 initial inoculums. However, the sharp elevation of serum HCV RNA at day 5 after dropping at day 3 indicates that the mutants (C2441S, P2938S plus R2985P) are more replication competent at early stages in vivo. Serum levels of human albumin remained constant throughout the observed periods and showed no significant differences between wild and mutant-infected mice (Fig. 6B).

We also investigated expression of ER stress-related proteins, the glucose regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP), in liver of chimeric mice infected with JFH1 or the mutant in the early phase post inoculation. Human hepatocyte chimeric mice were inoculated in the same way as described above, and we verified that the level of virus titer in serum of each mouse was same as presented in Fig. 6A (data not shown). We sacrificed one each mouse that was infected with wild type or mutant JFH1 at 5 day of infection and investigated hepatic expression of GRP78 and CHOP. Liver histology showed no sign of inflammation or cytopathic cell death. However, as shown in Fig. 7, the expression level of both GRP78 and CHOP was higher in mice inoculated with the mutant viruses than the parental JFH1. There was no apparent difference in percents of hepatic chimerism between each mouse. These finding suggested that ER stress-related proteins were upregulated in the liver of HCV-infected mouse and that these responses were more strongly induced in the liver of mutant-infected mouse.

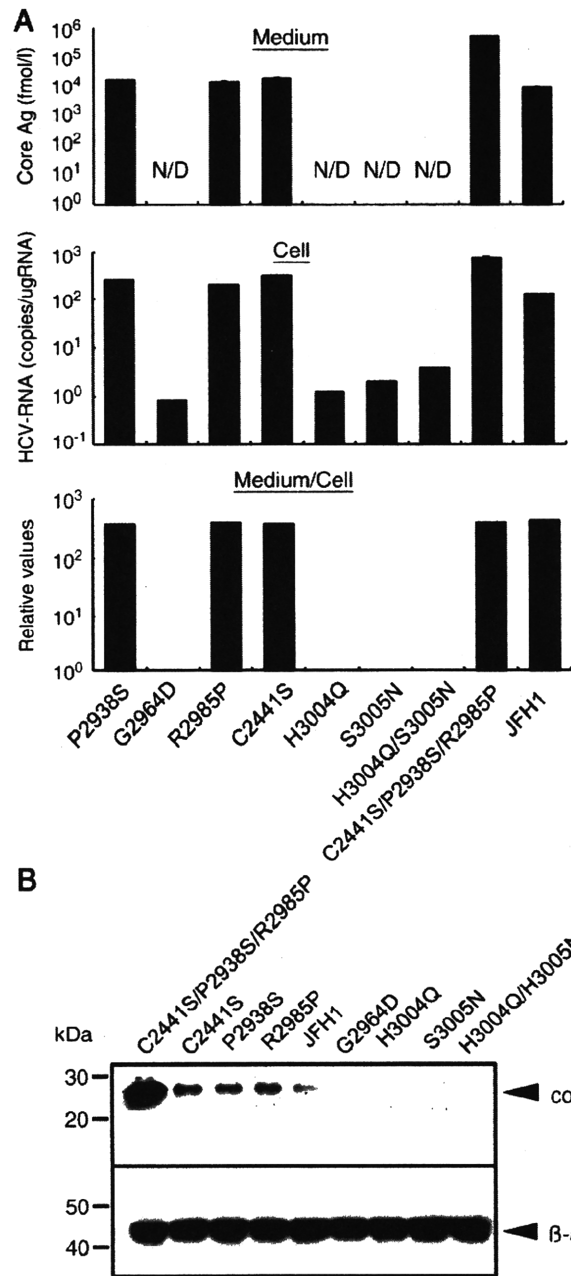

Fig. 5. Analysis of viral replication and production of viral particles using a single-cycle assay. A. Levels of core antigen in the culture media 3 days after transfection of JFH1 mutants into CD81-deficient Huh7-S29 cells (top). Levels of intracellular HCV RNA were quantified by real-time RT-PCR 3 days after transfection of JFH1 mutants into Huh7-S29 cells (middle). To determine the efficiency of infectious viral particle release from Huh7-S29 cells transfected with JFH1 mutants, the levels of core antigen in the culture media were adjusted by dividing by the levels of intracellular HCV RNA (bottom). Core Ag: Core antigen, N/D: not detectable. B. Huh7-S29 cells were harvested at 3 days after transfection of JFH1 mutants and western blotting was performed using anti-core and anti-beta-actin. kDa: kilo dalton.

Highly adapted cytopathic mutations reverted to wild type in vivo

Finally, we analyzed the serum viral sequence at the specified time points. On days 1 and 5, the HCV genomic sequences of the mice