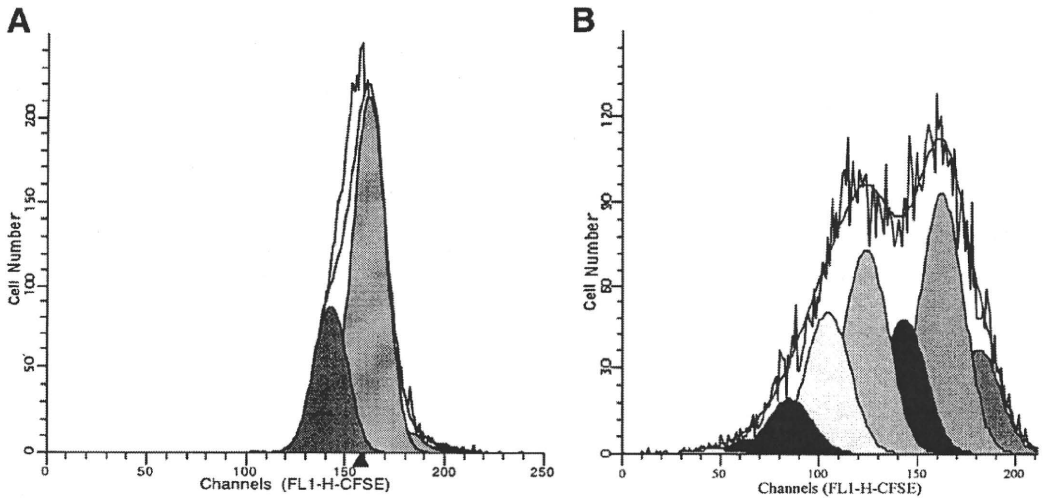


Fig. 2. Primary human hepatocytes (45-day-old PPH cultures) were stained with CellTrace CFSE (Invitrogen). (A) As a control, primary hepatocytes were synchronized at G2/M with Nocodazole (1 μg/mL for 30 minutes) and chased in HDM medium for 24 hours. The synchronized cells undergo one cell generation. (B) Unsynchronized cells were similarly pulse-labeled with CSFE for 30 minutes followed by chase in fresh HDM medium for 6 days. Dilution of CFSE in successive generations of cells is shown (based on a computer enhanced program, ModFit LT, Verity Software House, Topsham, ME).

days (Fig. 2B). Replication of the primary hepatocytes in PPH cultures was also monitored by bromodeoxyuridine (BrdU) incorporation in a 30-minute pulse followed by a 3-day chase (Supporting Information Fig. S1, Supporting Information Materials).

Morphologic Features of PPH Cultures. Figure 3 (upper panels), shown as phase contrast images of a 180-day-old maintenance culture, suggests vertical accumulation of primary hepatocytes in the maintenance cultures. The spherical masses of primary hepatocytes show morphologic properties of hepatocytes in normal liver tissues (Fig. 3, lower panels). Histological (hematoxylin and eosin [H&E]-stained) sections through the spheroid mass indicates clusters of binucleated cells (lower panel, A,B). Immunohistochemical staining for factor VIII indicated sinusoidal endothelial cell lining (lower panel, C).

Biochemical Characteristics of PPH Cultures. To assess whether markers of normal liver cell differentiation are expressed in long-term PPH cultures (ranging in age up to 180 days), we performed RT-PCR assays utilizing primers specific for human transcripts and compared them with the respective rat primers (primer sequences are shown in Supporting Information Table S2). Among the markers of hepatic differentiation we examined expression of plasma proteins (albumin [Alb], alpha-fetoprotein [α-FP], epithelial markers cytokeratins [CK18], TGF-β [Supporting Information Fig. S2A], type-1 collagen, matrix metalloproteases [MMP] 2 and MMP13), IFN receptor (IFN-R1 and IFN-R2, Supporting Information Fig. S2C), and drug metabolism (cytochrome P450) (Supporting Information Fig. S2D).

Expression of these hepatic markers, along with the endothelial cell marker (Factor VIII, Fig. 3C), suggests an overall capacity of the primary hepatocytes to sustain normal liver function during their long-term cultures in HDM, arguing that the human primary hepatocytes maintained in *in vitro* cultures assumed morphologic features and functional characteristics of liver differentiation *in vivo*. The expression of hepatic markers in the primary hepatocytes was independent of measurable contributions from the feeder cell layer.

Ethanol-induced hepatocyte injury in great part is due to generation of reactive oxygen species (ROS) on exposure of hepatocytes to ethanol, as it is oxidized by enhanced cytochrome P450 2E1 (CYP2E1).[14,15] To determine whether the primary human hepatocytes propagated *in vitro* cultures will be suitable for alcohol toxicity studies, we evaluated CYP2E1 expression by western blotting. Supporting Information Fig. S2D shows an accumulation of CY2E1 in primary hepatocytes during a 3-week growth in HDM. For comparison we observed the accumulation of albumin in the same cells (Supporting Information Fig. S2D, lower panel).

We analyzed the expression CK18 in freshly plated primary hepatocytes and compared it with cells grown for 30 days in cocultures. As controls, we compared the expression of albumin and AFP in freshly plated and 30-day-old cocultures (Supporting Information Fig. S3). Low levels of CK18 synthesis were detectable

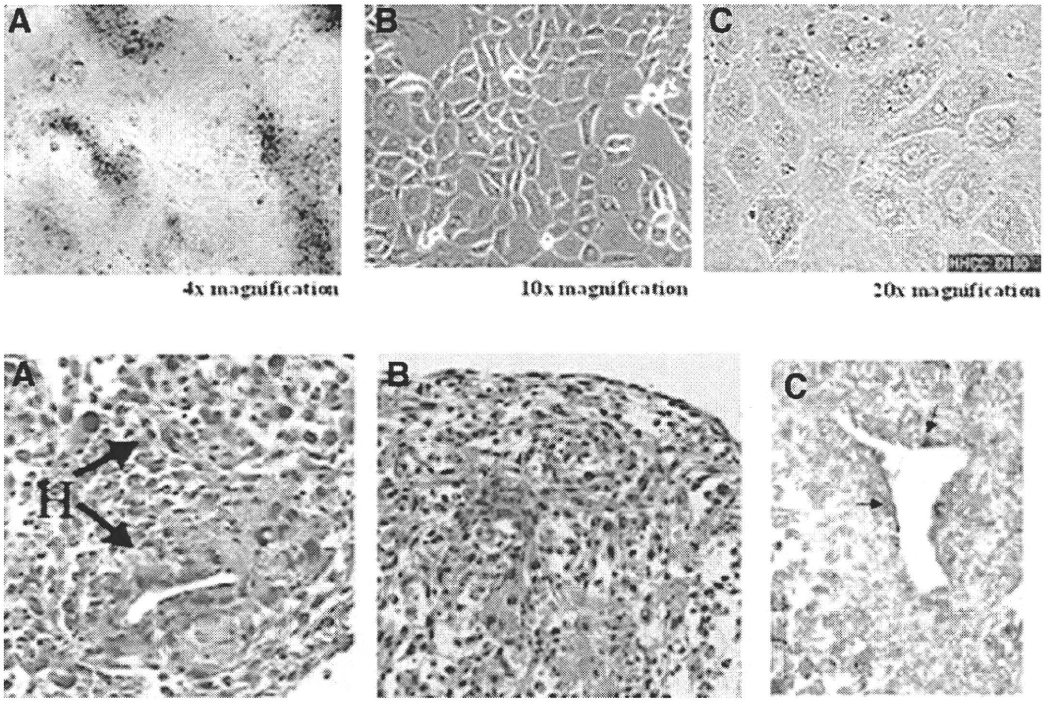


Fig. 3. Morphologic features of primary human hepatocyte (PPH) cultures. (A, upper panel) Maintenance culture (180-day-old from the time they were harvested from the coculture) are shown as phase-contrast images at different magnifications, suggesting three-dimensional growth of primary hepatocytes "spherical mass" (4× magnification). (B) 10× magnification focused on the upper cell layer. (C) 20× magnification of primary hepatocytes showing large nuclei and binucleated cells. Lower panel (A,B) H&E staining of 5-μm sections through the spherical mass of primary hepatocytes showing hepatocytes organized in clusters with defined nucleus and binucleated cells (arrows). (C) Immunohistochemical staining for Factor VIII indicates sinusoidal endothelial cell lining (arrow).

in freshly cocultured primary hepatocytes; however, we observed an induction of CK18 expression in 30-day cocultures. Overall, the results define phenotypic restoration of long-term cultures of primary hepatocytes consistent with normal hepatic functions.

In addition to comparing the synthesis of CYP 2E I and CK-18 in freshly plated primary hepatocytes and 30-day-old cultures, we analyzed the expression of hepatic functions in normal and HCV1a-infected cells from long-term cultures (Supporting Information Tables S3, S4). In contrast to the results reported by Richert et al.,[16] where hepatic functions are drastically reduced after reseeding, our PPH culture maintained hepatic functions through repeated reseeding in long-term culture.

Primary Hepatocyte Cultures Are Permissive to HCV-GFP Replication. To ascertain whether they support HCV replication, we introduced run-off transcripts of full-length HCV-GFP replicons[17] into PPH cultures. An increase in the GFP-positive foci is observed at 3-6 days posttransfection (Supporting Information Fig. S4). As control, cells transfected with the polymerase mutant virus showed background GFP fluorescence (Supporting Information Fig. S4).

Newly synthesized viral RNA was visualized by Br-UTP incorporation. Virus replication was carried out in the presence of 5 μg/mL actinomycin-D (to suppress Br-UTP incorporation into host cell transcripts); as a result, we observed background level of Br-UTP incorporation in uninfected cells (Fig. 4, control). Cells transfected with polymerase mutant showed no BrUTP incorporation (Fig. 4[I], pPol$^-$/5A), as compared with cells infected with wildtype HCV (Fig. 4[I], pI/5A). The Br-UTP-labeled viral RNA colocalized with GFP-positive foci in the cytoplasmic structures (Fig. 4[II]).

To validate the inhibition of viral RNA accumulation in cells transfected with the polymerase mutants, we compared viral RNA levels in primary hepatocytes 6 days after the introduction of either the wildtype or the polymerase mutant HCV genome. There was marked inhibition of viral RNA accumulation in cells transfected with polymerase mutant (Supporting Information Fig. S5). Overall, the results suggest that primary hepatocyte cultures are permissive for replication of HCV genome.

Replication of Full-Length HCV Genomic RNA in Primary Hepatocyte Cultures. We next asked whether

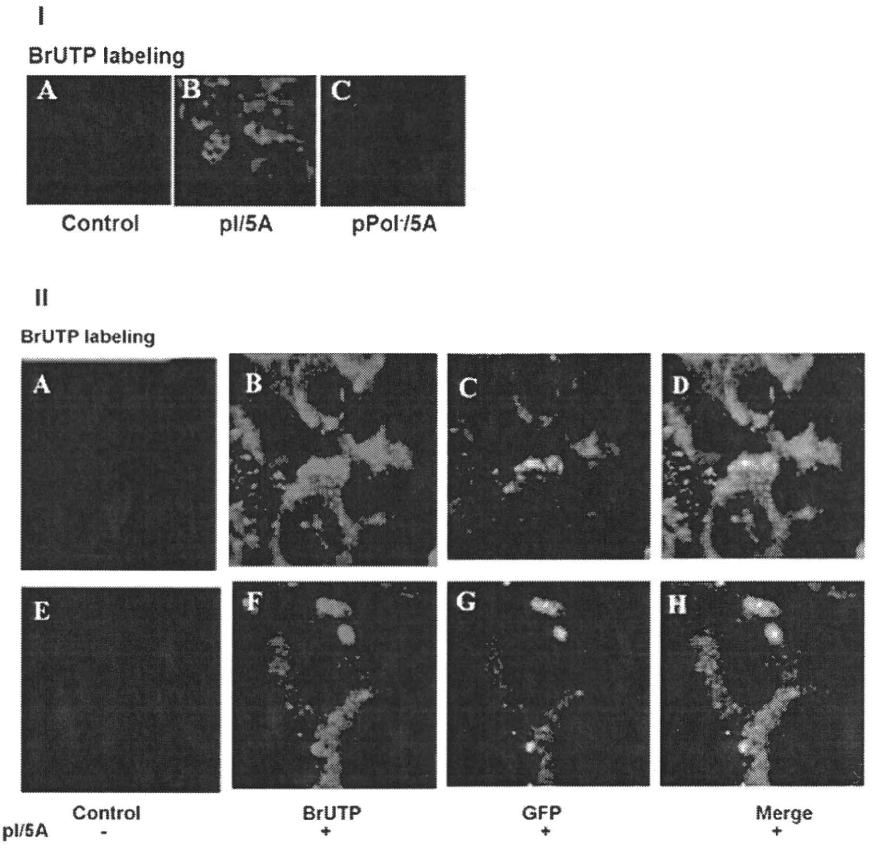


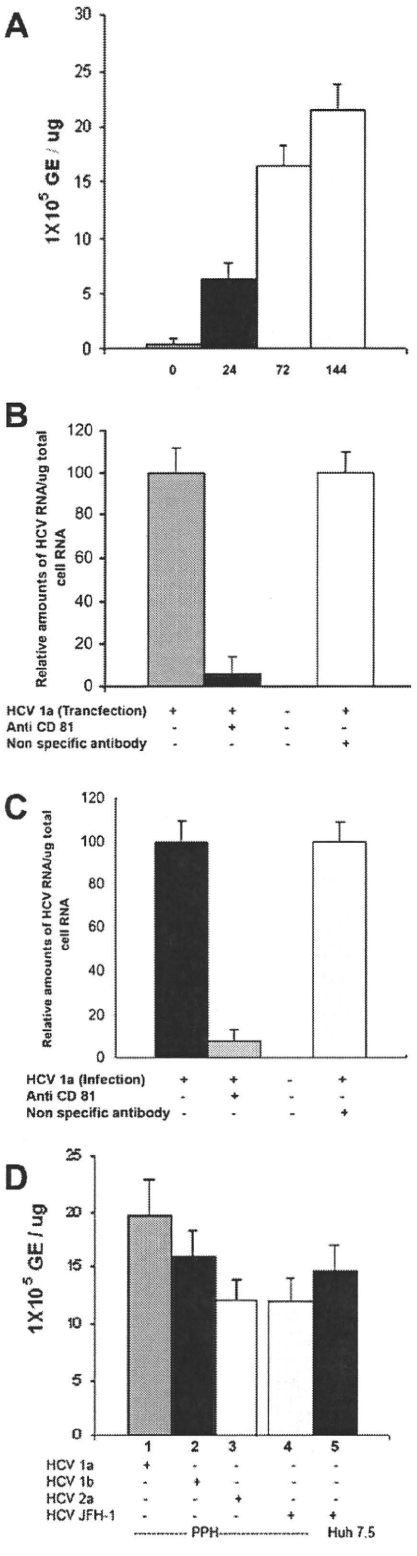
Fig. 4. (I, upper panel) Br-UTP labeled nascent HCV RNA detected (by immunostaining with anti-Br-UTP antibody): (A) Uninfected host cell control: primary hepatocyte cultures were incubated with 5 μM actinomycin D for 30 minutes prior to 45 minutes Br-UTP (25 μM) incubation at 37°C, followed by two 15-minute chases with fresh medium supplemented with 5% FBS. (B) Wildtype HCV transcript (3 days posttransfection) was detected (by immunostaining with anti-Br-UTP antibody) in either the "wildtype" HCV transfected cells (pI/5A) or (C) The polymerase mutant (pPol$^-$/5A) HCV RNA transfected cells. (II, lower panel) The confocal image of a group of cells showing: (A,E) uninfected control cells; (B,F) Viral RNA (red) in cultures transfected with pI/5A-GFP (wildtype) HCV replicon; (C,G) nascent viral protein (green); (D,H) the viral RNA (red) and protein (green) are merged.

the primary hepatocytes support replication of genomic clones of HCV by transfecting the PPH cultures with full-length HCV genotype 1a RNA (Fig. 5A). Equal amounts of HCV1a genomic RNAs were introduced into PPH cultures using the FuGENE (Roche) transfection method. Transfection efficiency was monitored by cotransfection with a control GFP expression plasmid. We noted the accumulation of viral RNA in HCV transfected primary hepatocytes over a 6-day period. The input viral RNA was detected in cells immediately following transfection (shown as "Day 0" in Fig. 5A). The results indicate an accumulation HCV RNA in the primary hepatocytes over 6 days posttransfection. Control cells transfected with RNA polymerase mutants (Supporting Information Fig. S5) showed no viral RNA accumulation during a similar 6-day period.

CD81 is an essential HCV entry factor expressed on most cells.[18] Next, we determined whether its binding to viral envelope glycoproteins, an essential step in HCV infection, is required for the propagation of HCV in PPH cultures. Pretreatment of cells with monoclonal antibody against CD81 prior to transfection with HCV1a genomic RNA inhibited HCV RNA accumulation (Fig. 5B).

In the FuGENE[6] transfection protocol, HCV genomic RNA is "artificially" introduced into the PPH cultures. To determine whether CD81 entry factor is important in reinfection of naïve hepatocytes we preblocked the cells with CD81 antibody prior to infection with virus (recovered from the culture medium of cells transfected with HCV1a genomic RNA). Untreated and CD81 entry receptor blocked cells were infected with equal amounts (GE) of virus recovered from the spent medium. HCV infection and propagation in primary hepatocytes appears to be dependent on the availability of CD81 entry receptor (Fig. 5C).

Infection of Naive Cells with Virus from Filtered Culture Media. Next we asked whether HCV infection could be transferred to naïve cells by inoculating primary hepatocytes with equal amounts (genomic RNA equivalents) of virus recovered from filtered culture media of cells 6 days posttransfection with full-length HCV genotypes 1a, 1b, and 2a. For comparison, we replicated equal amounts HCV JFH-1 full-length genomic RNAs introduced either into the primary hepatocytes or the Huh-7.5 hepatoma cells (Fig. 5D, columns 4 and 5, respectively).



Viral RNA replication in cells infected with three different HCV strains was compared by transfecting PPH cultures with equal amounts of full-length genomic RNAs of HCV genotypes 1a (pCV-H77c), 1b (pCV-J4L6S), and 2a (pJ6CF). Replication of HCV RNA was assessed by nested RT-PCR reactions of total cell RNA. Transfer of HCV infection to naïve hepatocytes was carried out with equal amounts of virus recovered from the culture media and was quantitated by qRT-PCR (TaqMan, Applied Biosystems), using WHO standards for HCV virus (Acrometrix). Comparable GEs of the three strains of HCV (recovered from 0.25-μm filtered culture media) were used to inoculate primary hepatocyte cultures (same passage as PPH cultures). Replication of viral RNA in cells infected with the three HCV genotypes (shown in Fig. 5D, columns 1-3) was determined by qRT-PCR 6 days postinfection. Replication of HCV1a appeared consistently higher as compared to HCV genotypes 1b or 2a. For comparison, we transfected genomic RNA from the JFH-1 strain into either the primary hepatocyte (PPH) cultures or the Huh-7.5 hepatoma cells and utilized the virus particles recovered from the culture media to inoculate either naïve primary hepatocytes (Fig. 5D, column 4), or the Huh-7.5 hepatoma cells (Fig. 5D, column 5). The accumulation of viral RNA (6 days postinfection) suggests that the replication of JFH-1 strain in primary hepatocytes is equally

Fig. 5. (A) Kinetics of HCV genotype 1a RNA replication: primary hepatocyte cultures were transfected with full-length run-off transcripts of HCV genotype 1a using FuGene 6. Total cell RNA was prepared from infected cells at indicated times. Results represent input viral RNA (0 hours), immediately after transfection. Viral RNA accumulation at 0, 24, 72, and 144 hours after the introduction of full-length genomic RNA was analyzed as indicated. The results are represented as GEs/μg total cell RNA. Estimates of the HCV RNA were normalized with WHO HCV standard. The data shown represent three independent assays. (B) Inhibition of HCV RNA replication by anti-CD-81 monoclonal antibody. (B) Transfection: primary hepatocyte cultures were either preincubated with CD-81 monoclonal antibody (10 μg/mL, Santa Cruz Biotechnology) for 30 minutes prior to transfection or with nonspecific antibody as negative control followed by transfection with full-length genomic RNA of HCV genotype 1a. (C) Infection: primary hepatocytes were preincubated with CD-81 antibody or a nonspecific control as in (B), followed by infection with HCV1a virus (recovered from filtered media of HCV1a-infected cells). Viral RNA was analyzed as in (A). Viral RNA levels are shown from 6 days postinfection. Data represent average of three experiments. $P < 0.05$. (D) Infection of primary hepatocytes with virus recovered from filtered culture media. Primary hepatocyte cultures were incubated with an equal amount of virus recovered from the culture media of cells transfected with full-length genomic clones of HCV genotype 1a (pCV-H77c) (lane 1), 1b (pCV-J4L6S) (lane 2), and 2a (pJ6CF) (lane 3). Viral RNA replication was determined by RT-PCR 6 days postinfection (as in A). The replication of JFH-1 genomic RNA in primary hepatocytes or Huh 7.5 cells (lanes 4 and 5, respectively) is shown for comparison. The error bars represent the average of three experiments.

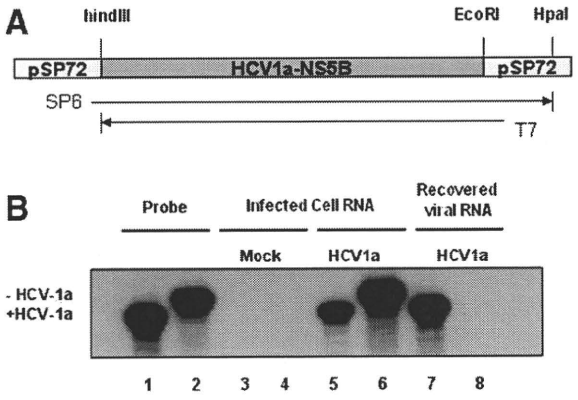


Fig. 6. RNase-protection assay for ± sense viral RNAs. (A) The RPA probes were prepared by cloning a 344 bp Hind III/Eco R1 fragment of NS5B region from pH77c into a pSP72 vector. (B) Lanes 1 and 2 show the positive and the negative probes, respectively. The RPA products using either positive and negative probes are shown, from total cell RNA of mock-infected (lanes 3 and 4), HCV1a-infected cells (lanes 5 and 6), or the RNA from virus particles recovered from the culture medium of HCV1a-infected cells (lanes 7 and 8).

efficient (Fig. 5D, column 4) as compared to its replication in Huh-7.5 hepatoma cells (Fig. 5D, column 5). Overall, the results argue that infectious virus particles are recovered from culture media of primary hepatocytes transfected with full-length genomic clones of HCV genotypes 1a, 1b, and 2a.

Formation of the negative sense RNA replicative intermediate is an important indicator of virus replication in infected cells. We monitored the relative ratios of positive and negative sense viral RNAs in cells infected with HCV1a and, as a control, in the virus particles recovered from the culture medium. The viral RNA concentration was monitored by RPA using run-off probes of metabolically labeled transcripts from the NS5B region. Figure 6A outlines the probes used for detecting the positive and negative sense viral RNAs. Comparison of the positive sense and the negative sense viral RNAs 6 days postinfection showed higher levels of the replicative intermediate (negative sense RNA) in HCV1a-infected primary hepatocytes (Fig. 6B). Estimates of the copy number, based on titration of the probes, suggested that the negative sense RNA in HCV1a-infected hepatocytes was 2.5 times more abundant than the positive sense viral RNA (Fig. 6B, lanes 5 and 6). As controls, the RPA assay showed only the positive sense genomic RNA in mature virus particles, recovered from the culture media (Fig. 6B, lanes 7 and 8); no viral RNA was detected in the mock-transfected cells (Fig. 6B, lanes 3 and 4).

Replication of HCV in Primary Hepatocytes Is Sensitive to IFN. Patients with HCV genotype 2a infection respond to IFN therapy better than HCV1a infections. As shown in Fig. 7, HCV genotype 1a appears relatively resistant to IFN treatment as compared with the genotype 2a-infected primary hepatocytes cells (HCV 2a replication is undetectable at 400 IU of IFN-β).

Replication of Viral RNA and the Release of Infectious Virus in the Culture Medium. We next asked whether the HCV particles released in the culture medium could passage infectivity to naïve hepatocytes. HCV genomic RNA units were estimated by qRT-PCR in the virus fraction recovered from the culture medium of infected primary hepatocytes. In the experiments shown in (Fig. 8A) we compared the viral RNA of HCV1a-infected primary hepatocytes (PPH) with that of Huh-7.5 hepatoma cells infected with HCV1a (in each case, infection was initiated with an equal amount of virus recovered from HCV1a-infected primary hepatocytes). We estimated the efficiency of virus released in the culture media of either the infected primary hepatocytes or Huh-7.5 hepatoma cells, based on qRT-PCR analysis of HCV genomic RNA. The results (Fig. 8B) represent virus released in the culture media for up to 20 days postinfection. As shown, efficient virus recovery was sustained from infected primary hepatocytes. By contrast, the virus released from Huh-7.5 hepatoma cells infected with HCV1a rapidly declines.

Interestingly, although full-length HCV1a genomic RNA when introduced directly into Huh-7.5 hepatoma cells (by transfection) failed to replicate (Fig. 8A, lane 5), Huh-7.5 hepatoma cells infected with the HCV1a virus (recovered from infected primary hepatocytes)

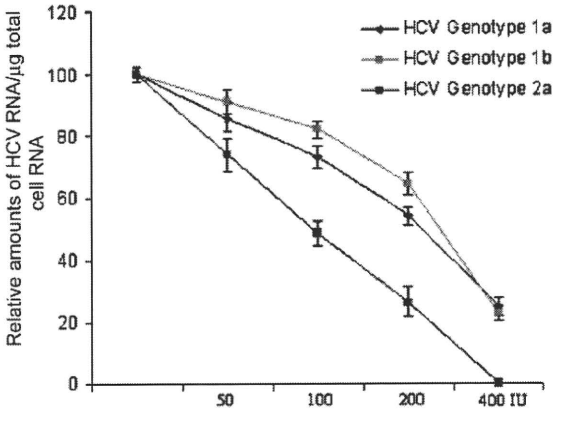


Fig. 7. Sensitivity to IFN-α treatment of the HCV-infected primary hepatocytes. Primary hepatocytes infected with HCV1a, 1b, or 2a were either untreated or treated with increasing concentration (IU) of IFN-α, and the inhibition of virus RNA replication was evaluated at 6 days postinfection. The level of inhibition is presented as a fraction of the untreated controls.

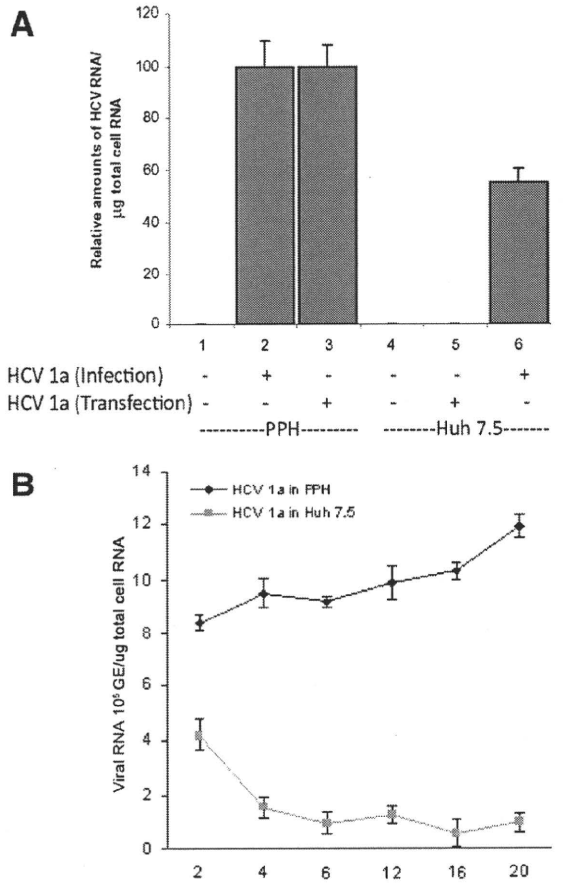


Fig. 8. (A) HCV RNA accumulation in infected PPH and Huh 7.5 cells: quantitative RT-PCR analysis of viral RNA from total cell RNA of PPH and Huh 7.5 cells 20 days postinfection is presented as relative amounts of HCV RNA/μg total cell RNA, lane 1, RNA from uninfected control PPH cells; lane 2, RNA from PPH infected with HCV1a; lane 3, RNA from cells transfected with run-off transcripts of HCV1a in PPH; lane 4, uninfected Huh 7.5 cells; lane 5, RNA from Huh-7.5 cells transfected with HCV1a genomic RNA; lane 6, RNA from Huh-7.5 cells infected with HCV1a virus recovered from infected primary hepatocytes. (B) Release of infectious virus particles: primary human hepatocytes were infected with equal amounts of HCV1a virus and the virus released in the culture media was collected at the indicated days postinfection. Quantitative RT-PCR analysis of released viral RNA was validated with OptiQuant (Applied Biosystem, AcroMetrix) using WHO standard HCV RNA. The efficiency of virus released from HCV1a-infected primary hepatocytes is maintained over 20 days postinfection (by contrast, the virus released from Huh-7.5 hepatoma cells declines rapidly).

initiates virus replication, albeit at a lower efficiency (Fig. 8A, compare lanes 2 and 6). However, as noted above (Fig. 8B), the release of mature virus from Huh-7.5 hepatoma cells infected with HCV1a is restricted.

Discussion

Long-term cultures of primary hepatocytes are desirable *in vitro* as a model for studying effects of hepatotoxins on liver function and host response to hepatopathogens. In this study we examined the expression of normal human liver cell markers to determine whether primary human hepatocytes sustain hepatic functions through long-term cultures and whether the primary hepatocyte cultures can support productive HCV replication, a major hepatopathogen. Essential hepatic functions sustained in long-term cultures include synthesis of plasma proteins, drug metabolism, and signal transduction.

In the present study we established a culture system of primary human hepatocytes (derived from liver biopsy tissues of donors ranging in age from 13 months to 58 years; Supporting Information Table S1), and validated their physiological relevance as an *in vitro* cell culture model for productive replication of an important hepatic pathogen. The primary hepatocyte cultures described here are able to sustain replication through multiple passages as well, as they are able to retain liver-specific functions, making it possible to use the *in vitro* culture system for repeat applications in studies with hepatopathogens and hepatotoxicity.

We validated the physiological relevance of primary hepatocyte cultures by studying productive replication of infectious clones of the three prevalent HCV genotypes. Virus recovered from the culture media of HCV-infected primary cells was able to transfer infection to naïve hepatocytes. Recovery of infectious virus from Huh-7.5 is limited as compared with the infected primary hepatocytes. Supporting Information Fig. S8 provides a schematic presentation of replication-competent virus produced from primary cells. Although the reasons for restriction of native HCV replication in hepatoma cell line Huh-7.5 is yet unclear, our studies point to an important role of primary human hepatocytes as appropriate host cells for productive infection with a wider repertoire of native HCV genotypes and examination of host response to hepatopathogens.

We observed robust replication of infectious virus particles from HCV1a, 1b, and 2a-infected primary hepatocytes. The efficiency of HCV replication in its natural host, human hepatocytes, reflects the innate replicative properties of the HCV genotypes (Supporting Information Table S3). In natural infections, HCV genotype 1a is known to cause the most severe liver disease; as well, infection with HCV genotype 1a/b is less responsive to IFN therapy. We observed that the replication of HCV1a/1b is less sensitive to inhibition by IFN, suggesting that HCV infection in primary hepatocytes imitates natural HCV infections. The infectivity of recovered HCV1a virus, however, declines over a month following the infection. It is likely that

the effect of HCV replication on cholesterol biosynthesis might interfere with sustained virus production, packaging, and release of stable virus particles.[19,20]

The results described here provide a physiologically relevant *in vitro* system for efficient replication of full-length infectious strains of HCV genotypes 1a, 1b, and 2a. An *in vitro* system for the replication of infectious HCV clones in primary hepatocytes should facilitate analysis of host factors that modulate virus replication and will be useful in *in vitro* models for molecular analysis of virus-host interactions and studies with hepatotoxins.

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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.

(Part of this study was presented at the 16th International Symposium on Hepatitis C Virus and Related Viruses, Nice, France, 3 to 7 October 2009.)

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′-CCGC CTCAAGAGCTGGTTCGA-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

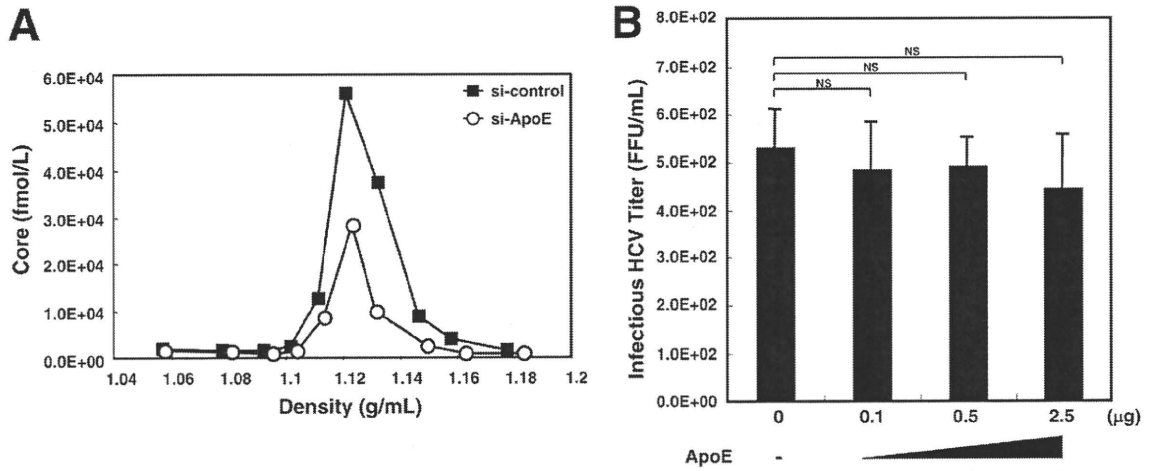

FIG. 1. Nature of HCV produced from ApoE knockdown HuH7.5 cells. (A) Buoyant density analysis. HuH7.5 cells were seeded onto 60-mm-diameter dishes. Cells were transfected with siRNA (si-control or si-ApoE). Four hours after transfection, cells were infected with HCVcc. Forty-eight hours after inoculation, 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

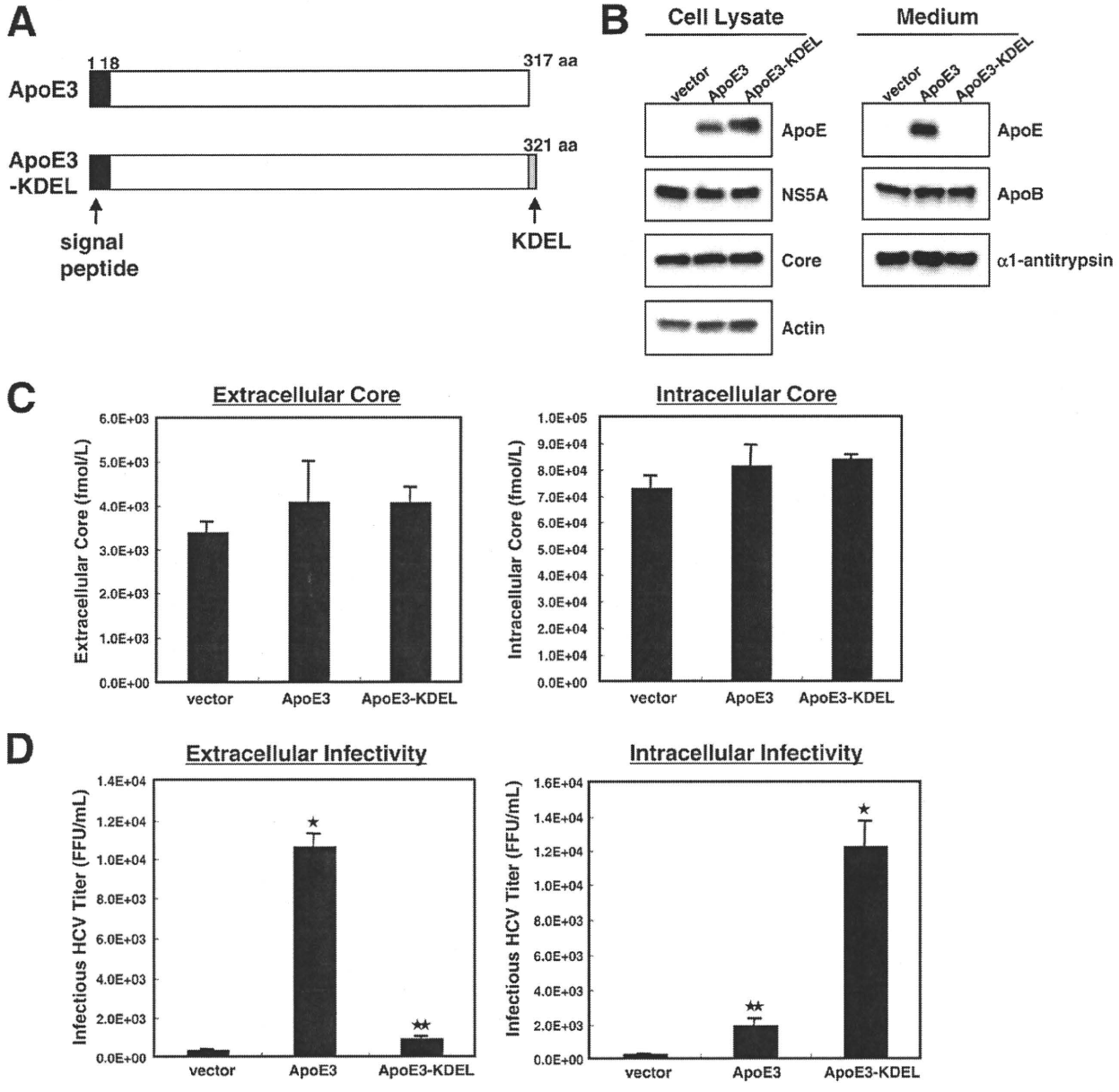

FIG. 2. The release of infectious HCV into the culture medium depends on the secretion of ApoE. (A) Schematic representation of ApoE3 and the ApoE3-KDEL mutant. (B) Verification of the expression of ectopic ApoE3 and ApoE3-KDEL. The sh-#3 cells were seeded onto 100-mm-diameter dishes. Cells were transfected 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. Interest ingly, ApoE2-expressing cells released substantially less infectious HCV into the culture medium than did ApoE3- or

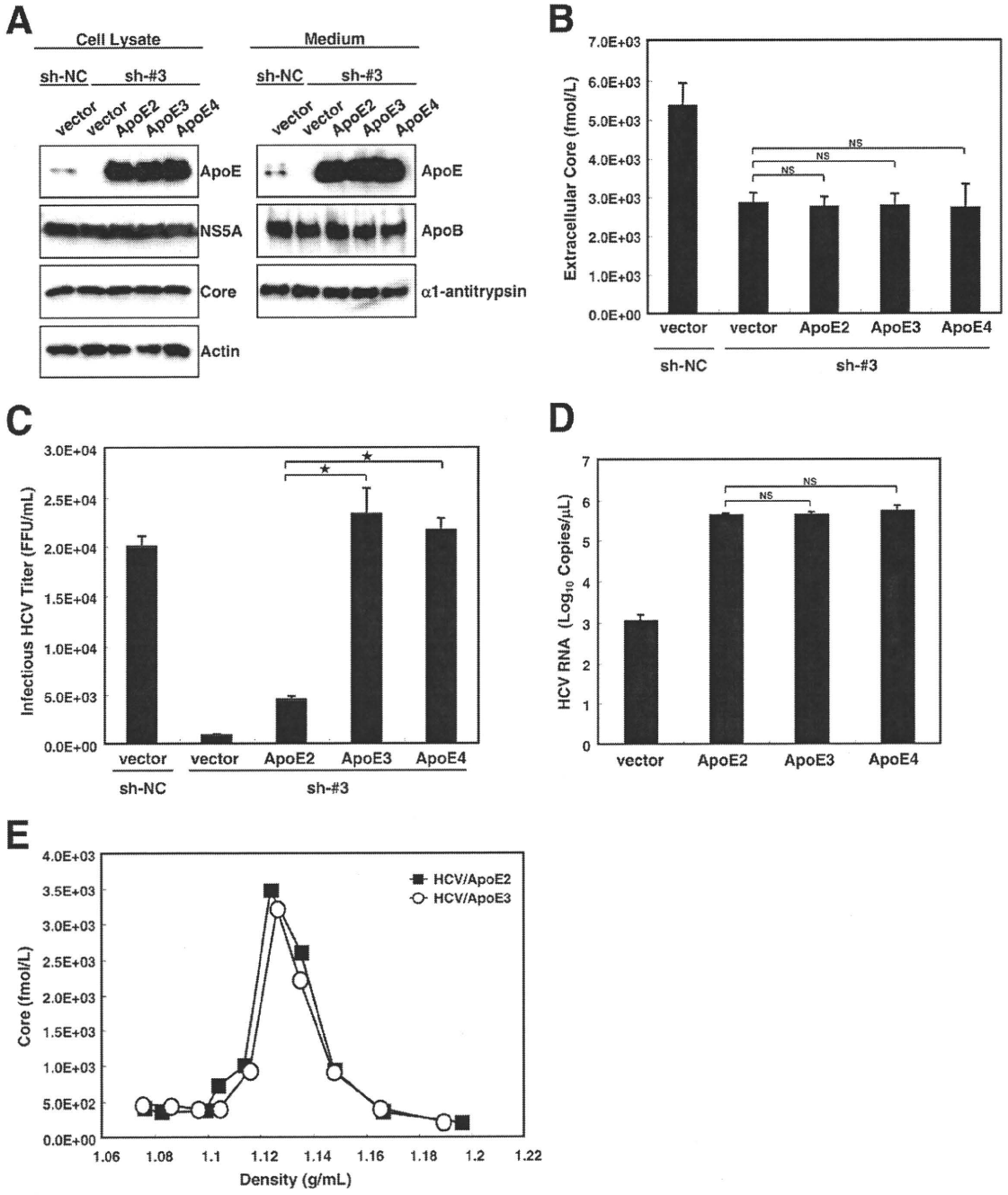

FIG. 3. ApoE isoforms affect HCV infectivity. (A) Verification of expression of ectopically introduced ApoE and the effect of ApoE isoforms on HCV genome replication. sh-NC cells and sh-#3 cells were seeded onto 60-mm-diameter dishes. Cells were transfected with either pCAG (vector), pCAG-ApoE2 (ApoE2), 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

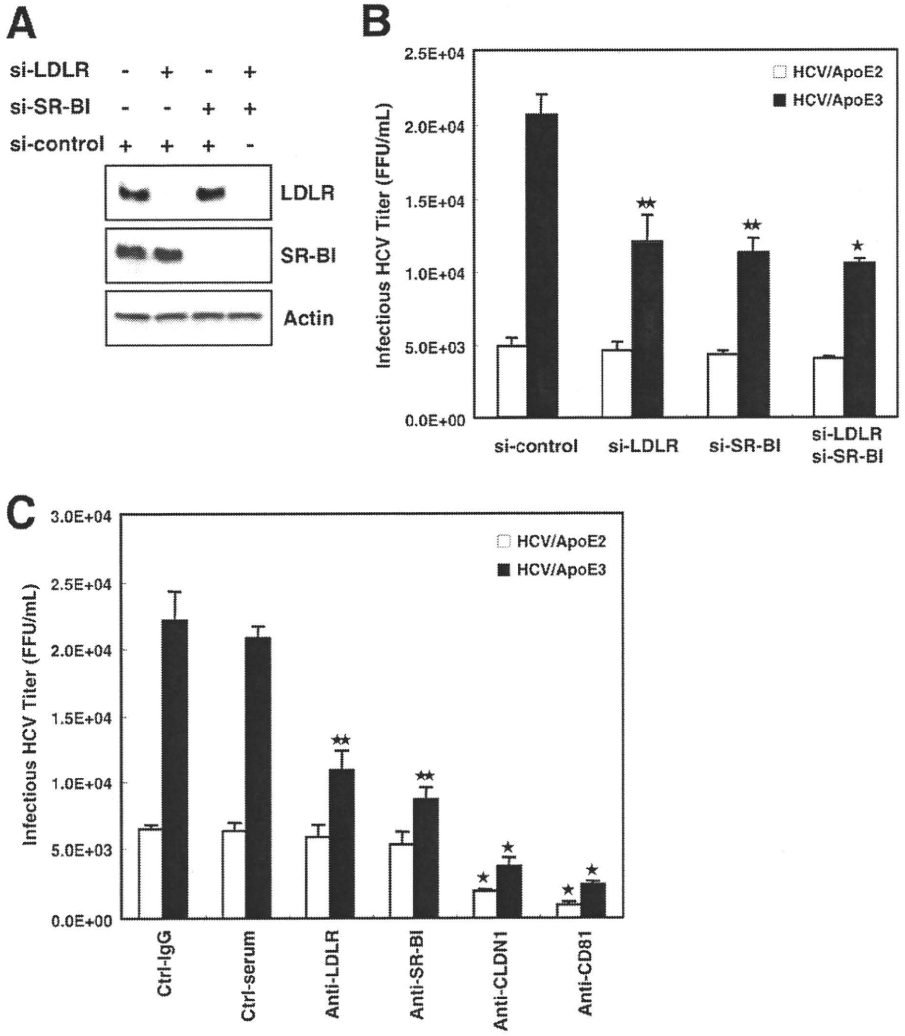

FIG. 4. Suppression of LDLR and SR-BI results in reduced HCV infection. (A) Knockdown of the LDLR and SR-BI by siRNA. HuH7.5 cells were seeded onto 24-well plates and transfected with siRNA (si-control, si-LDLR, and/or si-SR-BI). Twenty-four hours after transfection, cell lysates were analyzed for the 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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A Disulfide-Bonded Dimer of the Core Protein of Hepatitis C Virus Is Important for Virus-Like Particle Production▽†

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Hepatitis C virus (HCV) core protein forms the nucleocapsid of the HCV particle. Although many functions of core protein have been reported, how the HCV particle is assembled is not well understood. Here we show that the nucleocapsid-like particle of HCV is composed of a disulfide-bonded core protein complex (dbc-complex). We also found that the disulfide-bonded dimer of the core protein (dbd-core) is formed at the endoplasmic reticulum (ER), where the core protein is initially produced and processed. Mutational analysis revealed that the cysteine residue at amino acid position 128 (Cys128) of the core protein, a highly conserved residue among almost all reported isolates, is responsible for dbd-core formation and virus-like particle production but has no effect on the replication of the HCV RNA genome or the several known functions of the core protein, including RNA binding ability and localization to the lipid droplet. The Cys128 mutant core protein showed a dominant negative effect in terms of HCV-like particle production. These results suggest that this disulfide bond is critical for the HCV virion. We also obtained the results that the dbc-complex in the nucleocapsid-like structure was sensitive to proteinase K but not trypsin digestion, suggesting that the capsid is built up of a tightly packed structure of the core protein, with its amino (N)-terminal arginine-rich region being concealed inside.

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, affecting approximately 200 million people worldwide (13, 29, 44). Current treatment strategies, including interferon coupled with ribavirin, are not effective for all patients infected with HCV. An error-prone replication strategy allows HCV to undergo rapid mutational evolution in response to immune pressure and thus evade adaptive immune responses (10). New approaches to HCV therapy include the development of specifically targeted antiviral therapies for hepatitis C (STAT-Cs) which target such HCV proteins as the nonstructural 3/4A (NS3/4A), serine protease, and RNA-dependent RNA polymerase NS5B proteins (3). Despite the potent antiviral activities of some of these approaches, many resistant HCV strains have been reported after treatment with existing STAT-Cs (23, 48, 51). Therefore, identification of new targets that are common to all HCV strains and that are associated with low mutation rates is an area of active research.

HCV has a 9.6-kb, plus-strand RNA genome composed of a 5′ untranslated region (UTR), an open reading frame that encodes a single polyprotein of about 3,000 amino acids, and a 3′ UTR. The polyprotein is processed by host and viral proteases to produce three structural proteins (the core, envelope 1 [E1], and E2 proteins) and seven nonstructural proteins (the p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B proteins) (14, 16, 17, 22, 49). The HCV core protein is produced cotranslationally via carboxyl (C)-terminal cleavage to generate an immature core protein, 191 amino acids in length, on the endoplasmic reticulum (ER) (16). This protein consists of three predicted domains: the N-terminal hydrophilic domain (D1), the C-terminal hydrophobic domain (D2), and the tail domain (33), which serves as a signal peptide for the E1 protein. D1 includes a number of positively charged amino acids responsible for viral RNA binding (amino acids 1 to 75) (43) and the region involved in multimerization of the core protein via homotypic interactions (amino acids 36 to 91 and 82 to 102) (32, 40) (see Fig. S1 in the supplemental material). Hydrophobic D2 includes the region responsible for core protein association with lipid droplets (LDs; amino acids 125 to 144) (7, 18, 37), which accumulate in response to core protein production (1, 6).

Many functions of the core protein have been reported (13, 38, 50), yet because infectious HCV particles cannot be appropriately produced in currently available experimental systems, HCV particle assembly has not been elucidated to date. A cell culture system that reproduces the complete life cycle of HCV *in vitro* was developed by Wakita et al. using a cloned HCV genome (JFH1) (53). Using this system, the assembly of infectious HCV particles was found to occur near LDs and ER-derived LD-associated membranes (36, 47). Neither the structures nor the functions of the virus proteins involved in virus particle assembly are known, however. To elucidate this point, we have analyzed the biochemical characteristics of the proteins within the fraction containing the HCV particle and found a disulfide-bonded core protein complex (dbc-complex). We revealed that the disulfide-bonded dimer of core protein (dbd-core) was formed by a single cysteine residue at amino

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acid position 128 on the ER. The roles of the disulfide bond of the core protein in virus-like particle formation are discussed in this paper.

MATERIALS AND METHODS

Cell culture. Cells of the HuH-7 and HuH-7.5 human hepatoma cell lines were grown in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum, 100 U/ml nonessential amino acids (Invitrogen, Carlsbad, CA), and 100 μg/ml each penicillin and streptomycin sulfate (Invitrogen).

Antibodies. The antibodies used for immunoblotting and indirect immunofluorescence analysis were specific for core protein (antibody 32-1), FLAG M2 (Sigma-Aldrich, St. Louis, MO), c-myc (Sigma-Aldrich), NS5A protein (CL1), adipocyte differentiation-related protein (ADRP; StressGen, Victoria, British, Columbia, Canada), calnexin (Calnexin-NT; StressGen), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Temecula, CA). Antibodies specific for core protein (antibody 32-1) were a gift from M. Kohara (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Rabbit polyclonal anti-NS5A protein CL1 antibodies have been described previously (36).

Plasmid construction. All plasmids were generated by inserting PCR-amplified fragments into expression plasmids. The plasmids, primer sequences, templates for the PCRs, and restriction enzyme sites used to construct the plasmids are listed in Table S1 in the supplemental material. Plasmids pJFH1^{E2FL} (encoding the full-length HCV genome with the FLAG epitope in the E2 hypervariable region), pJFH1^{AAA99} (encoding a NS5A mutant of JFH1^{E2FL}, resulting in noninfectious HCV particles), pJFH1$^{PP/AA}$ (encoding a core protein mutant of JFH1^{E2FL}, which allows replication in cells but prevents HCV particle production), and pcDNA3-core^{WT} (an expression plasmid encoding the full-length core protein of JFH1) have been described previously (36). Plasmid pJ6/JFH1, which contains the full-length HCV genome encoding structural proteins from the J6 strain and nonstructural proteins from the JFH1 strain, was kindly provided by Charles M. Rice (The Rockefeller University, New York, NY).

***In vitro* transcription.** RNA for transfection was synthesized as described previously (36). In brief, plasmids carrying the HCV RNA sequence were linearized with XbaI and used as templates for *in vitro* transcription with MEGAscript T7 (Ambion, Austin, TX).

Transfection. Ten micrograms of JFH1^{E2FL}, JFH1^{C128A}, JFH1^{C184A}, JFH1$^{C128/184A}$, JFH1$^{PP/AA}$, or JFH^{AAA99} and J6/JFH1 or J6/JFH1^{AAA99} RNAs were transfected into HuH-7 and HuH-7.5 cells (1.0×10^7 cells) by electroporation (260 V, 0.95 μF) using a Gene Pulser II system (Bio-Rad, Hercules, CA). Core protein expression plasmids were transfected into HuH-7 cells using Lipofectamine LTX (Invitrogen), according to the manufacturer's protocol.

HCV particle precipitation. Culture medium from HCV RNA-transfected cells were concentrated using Amicon Ultra-15 centrifugal filters with Ultracell-100 membranes (Millipore, Billerica, MA) and mixed with sucrose solution in phosphate-buffered saline (PBS) to a final sucrose concentration of 2%. This mixture was ultracentrifuged (100,000 × *g*, 4°C for 2 h), and the HCV particles were obtained as a pellet. The pellet was then suspended in culture medium for infection experiments or PBS for immunoblot analysis.

Indirect immunofluorescence analysis. Indirect immunofluorescence analyses of HCV infection and the cellular localization of the HCV proteins were performed as described previously (36).

Protease protection assay. Concentrated culture medium from JFH1^{E2FL} RNA-transfected HuH-7 cells was fractionated using 20 to 50% sucrose density gradients, and the HCV RNA titer was measured in quantitative reverse transcription-PCRs (RT-PCRs) as described below. Fractions with high HCV RNA titers were collected, and JFH1^{E2FL} particles were obtained as a pellet after ultracentrifugation (100,000 × *g*, 4°C for 2 h). The pellet was suspended in PBS and treated with 10 μg/ml trypsin or 5 μg/ml proteinase K in the presence or absence of 1% Nonidet P-40 (NP-40) at 37°C for 15 min, unless otherwise indicated. The reaction was quenched by the addition of protease inhibitor cocktail (Nacalai Tesque), followed by SDS-PAGE under nonreducing conditions and immunoblotting specific for core protein.

Immunoblot analysis. Samples were subjected to SDS-PAGE in sample buffer (62.5 mM Tris-HCl [pH 7.8], 1% SDS, 10% glycerol) with or without 5% β-mercaptoethanol (β-ME) or 50 mM dithiothreitol (DTT) for reducing and nonreducing conditions, respectively. *N*-Ethylmaleimide (NEM; Nacalai Tesque) was added to the sample buffer to a final concentration of 5 mM for the indicated samples. Proteins were transferred to a polyvinylidene difluoride membrane and blocked in blocking buffer for 1 h at room temperature with gentle agitation. After incubation with primary antibodies overnight at 4°C, the membrane was washed three times for 5 min in washing buffer at room temperature with gentle agitation. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. After three washes in washing buffer, proteins were detected using Western Lightning reagent (PerkinElmer, Waltham, MA) or ECL Advance (GE Healthcare, Buckinghamshire, England) and Kodak MXJB Plus medical X-ray film (Kodak, Rochester, NY) or an LAS-4000 system (Fujifilm, Tokyo, Japan).

Preparation of LDs. LDs were prepared as described previously (36).

Preparation of MMFs. Microsomal membrane fractions (MMFs) were collected as described previously (15) with some modifications. In brief, cells were collected in homogenization buffer (20 mM Tris-HCl [pH 7.8], 250 mM sucrose, and 0.1% ethanol supplemented with protease inhibitor cocktail) and homogenized on ice using 40 strokes of a Dounce homogenizer. The samples were then centrifuged at 1,000 × *g* for 10 min at 4°C. The supernatant was collected in a new tube and centrifuged again at 16,000 × *g* for 20 min at 4°C. The supernatant was further centrifuged at 100,000 × *g* for 1 h at 4°C. The MMF precipitate was homogenized in lysis buffer (1% NP-40, 0.1% SDS, 20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, and 10% glycerol supplemented with protease inhibitor cocktail) using a Dounce homogenizer.

qRT-PCR analysis. Quantitative RT-PCR (qRT-PCR) analysis for determination of the HCV RNA titer was performed as described previously (36).

ELISA specific for core protein. The core protein in culture medium was quantified using an enzyme-linked immunosorbent assay (ELISA; HCV antigen ELISA; Ortho-Clinical Diagnostics, Raritan, NJ), according to the manufacturer's protocol.

RNA-protein binding precipitation assay. Core^{WT} or core^{C128A} was translated *in vitro* from pcDNA3-core^{WT} and pcDNA3-core^{C128A}, respectively, using a TNT-coupled rabbit reticulocyte lysate system (Promega, Madison, WI), according to the manufacturer's protocol. These proteins were incubated with poly(U) agarose (Sigma) in binding buffer (50 mM HEPES (pH 7.4)–100 mM NaCl–0.1% NP-40–20 U RNase inhibitor) at 4°C for 2 h with or without RNase A. After five washes, the resin-bound core proteins were immunoblotted.

RESULTS

The HCV particle contains core protein complex formed by a disulfide bond. To analyze the core protein of the HCV particle, we first subjected the concentrated culture medium of HuH-7 cells transfected with *in vitro*-transcribed JFH1^{E2FL} RNA to ultracentrifugation. After the resulting pellet was resuspended in culture medium, we confirmed the presence of infectious HCV particles on the basis of the infectivity of HuH-7.5 cells (Fig. 1a). The infectious JFH1^{E2FL} particle-containing pellet was separated by SDS-PAGE under nonreducing conditions, and immunoblot analysis showed the presence of a core antibody-reactive protein that was approximately twice the size of the core protein (38 kDa), in addition to the expected 19-kDa core protein (Fig. 1b, lane 1). Because treatment with DTT eliminated the larger core protein antibody-reactive band while the levels of the core protein monomer increased (Fig. 1b, lanes 2 to 6), the larger protein likely represented a dbc-complex. This complex was also found in J6/JFH1-derived particles (see Fig. S2 in the supplemental material), indicating that the complex was not specific for JFH1^{E2FL}.

To determine whether the dbc-complex is a component of the HCV particle, a protease protection assay was performed using RNase-resistant HCV particles fractionated on the basis of their buoyant densities. Concentrated culture medium from HuH-7 cells transfected with *in vitro*-transcribed JFH1^{E2FL} RNA was fractionated using a 20 to 50% sucrose density gradient; and JFH1^{E2FL} particles, which were presumed to contain both infectious and noninfectious particles, were collected from fractions with high HCV RNA titers using ultracentrifugation (Fig. 2a, fractions 8 to 13). The core protein from the collected fractions was analyzed by immunoblotting after SDS-

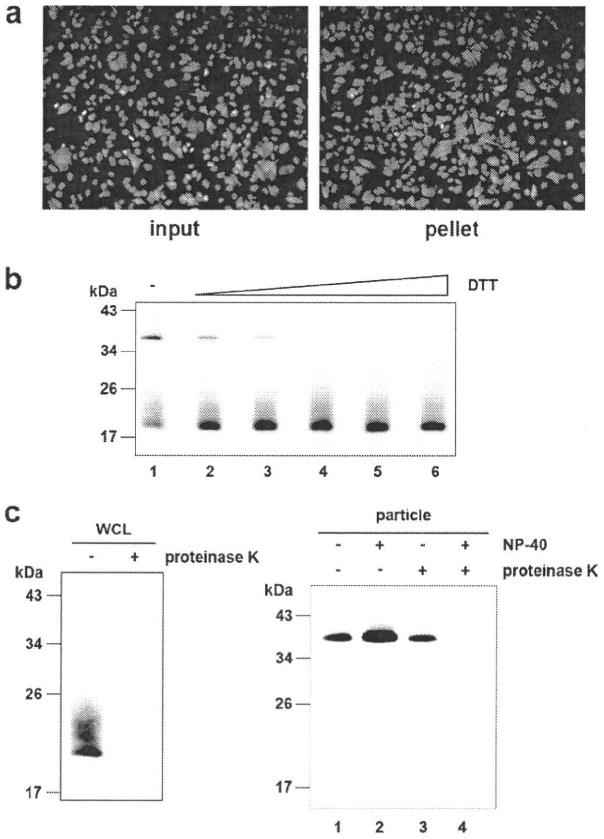

FIG. 1. The HCV-like particle consists of a core complex formed by a disulfide bond. (a) The infectivity of the pellet fraction collected from concentrated culture medium from JFH1^{E2FL} RNA-transfected HuH-7 cells was analyzed as described in Materials and Methods. Input indicates the same volume of concentrated culture medium used to pellet the virus-like particles. (b) Immunoblot analysis of the core protein in pellets containing JFH1^{E2FL} virus particles treated with various levels of DTT (lanes 1, 2, 3, 4, 5, and 6, 0, 1.56, 3.13, 6.25, 12.5, and 25 mM DTT, respectively). (c) Immunoblot analysis of the core protein in JFH1^{E2FL} particles collected from sucrose density gradient fractions with high HCV RNA titers (particle) (Fig. 2a, fractions 8 to 13) and treated with 5 μg/ml proteinase K at 37°C for 15 min in the presence or absence of 1% NP-40 (right panel). As a positive control, WCL prepared from JFH1^{E2FL} RNA-transfected HuH-7 cells in lysis buffer was treated with 5 μg/ml proteinase K at 37°C for 15 min (left panel). Data are representative of three independent experiments.

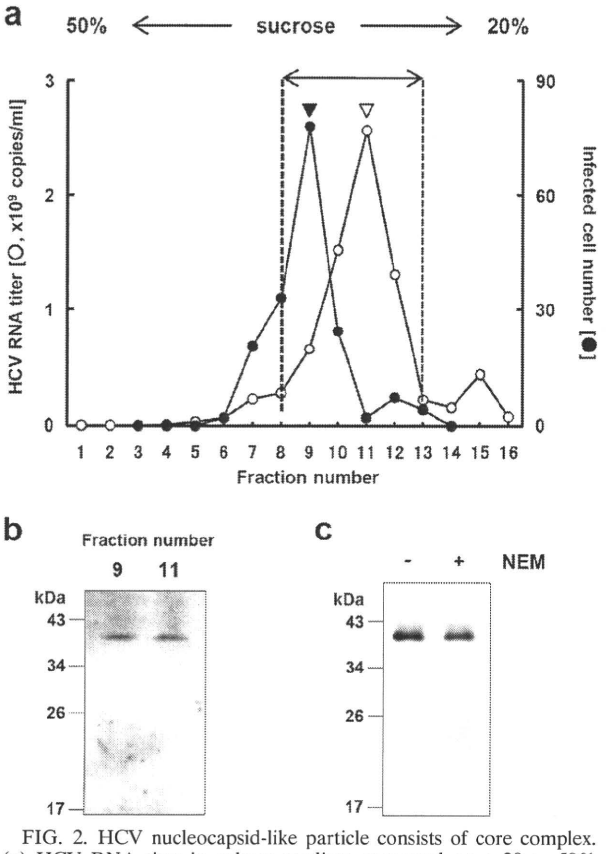

FIG. 2. HCV nucleocapsid-like particle consists of core complex. (a) HCV RNA titer in culture medium separated on a 20 to 50% sucrose density gradient. Concentrated culture medium from JFH1^{E2FL} RNA-transfected HuH-7 cells were treated with RNase and separated on a 20 to 50% sucrose density gradient. Fractions 1 to 16 were obtained from the bottom to the top of the tube, respectively. The HCV RNA titer and infectivity of each fraction were analyzed by real-time qRT-PCR (for fractions 1 to 16) and counting the number of cells infected with HCV-like particle detected by immunofluorescence (for fractions 3 to 14), respectively, as described in Materials and Methods. In brief, each fraction was diluted with 1× PBS and HCV-like particles were collected by ultracentrifugation, and then the pellets were suspended in culture medium and used for infection. (b) HCV-like particle collected from the infectious HCV peak (from panel a, filled arrowhead) and the HCV RNA peak (from panel a, open arrowhead) were collected by ultracentrifugation, subjected to nonreducing SDS-PAGE, and detected by immunoblotting against the core protein. (c) HCV-like particles collected from fractions 8 to 13 (a) were subjected to nonreducing SDS-PAGE in the presence (lane +) or absence (lane −) of 5 mM NEM and analyzed by immunoblotting against the core protein. Data are representative of two (a, infectivity of fractions) or three independent experiments.

PAGE under nonreducing conditions and showed only the dbc-complex (Fig. 1c, right panel).

To examine whether the complex contributes to the infectivity of the particles, we analyzed the dbc-complex in the fractions containing infectious and noninfectious HCV particles (fractions 9 and 11 of Fig. 2a, filled and open arrowheads, respectively). Both the infectious and noninfectious HCV particle-containing fractions contained the dbc-complex (Fig. 2b). To confirm this further, a pellet containing particles of mutant JFH1^{AAA99}—a mutant of JFH1^{E2FL} that primarily produces noninfectious particles (36)—was analyzed in a similar manner. These dbc-complexes were found in pelleted particles of both JFH1^{AAA99} and J6/JFH1^{AAA99}, which was a mutant J6/JFH1 with a similar substitution to JFH1^{AAA99} (see Fig. S2 in the supplemental material). These results indicated that the dbc-complex was present in both the infectious and noninfectious HCV-like particles.

The core protein monomer observed in the pellet samples (Fig. 1b) may be from the secreted core protein or the debris of apoptotic cells, because the core protein is known to be secreted from cells expressing this protein under particular conditions (42) and strain JFH1 is known to cause apoptosis (45). The dbc-complex-specific signals in the HCV particles seem to be increased in the NP-40-treated samples for some