cesses involved in a productive, and thus authentic, infection cycle, and studies remain limited by the very low efficiency.[2] A breakthrough in HCV research was achieved in 2005 when it became possible to grow the virus in cell culture thanks to the cloning of JFH1, a genotype 2a-HCV isolate with exceptional efficiency at viral genome replication.[3] Highly permissive subclones of the human hepatocellular carcinoma–derived cell line Huh-7 have been shown to produce workable titers of cell-culture–derived virus (HCVcc) when replicating the full-length genome of JFH1, or chimeras consisting of the nonstructural genes (encoding the viral proteins sufficient for genome replication) of JFH1 fused to the structural genes (encoding the viral core protein and envelope glycoproteins E1 and E2) of other HCV isolates.[3,10–13] However, the transformed, rapidly dividing, and poorly differentiated Huh-7 cells differ significantly from normal human adult hepatocytes, raising doubts as to the in vivo relevance of this now widely used in vitro system.[2–4] Thus, it has remained a challenging priority to develop a cell-culture–based model supporting the complete, productive infection cycle of HCV in the context of normal, and thus physiologically relevant, human liver cells.

These considerations prompted us to investigate whether HCVcc could be used as a source of virus to achieve productive infection in PHH. With this strategy we have established a simple yet robust system supporting all steps of the HCV infection cycle in highly differentiated hepatocytes. Indeed, this system is shown here to support not only replication of the viral genome but also production of highly infectious progeny virus, which has unique properties compared with HCVcc, and which will be termed *primary-culture–derived HCV (HCVpc)*.

Materials and Methods

Isolation and Primary Culture of Human Adult Hepatocytes

Normal-appearing liver tissue was obtained from adult patients undergoing partial hepatectomy in Cochin Hospital for the therapy of metastases or benign tumors and seronegative for HCV, hepatitis B virus, and human immunodeficiency virus. Experimental procedures were performed in accordance with French laws and regulations. Immediately after surgical resection, the liver pieces were stored in Celsior solution (IMTIX-SangStat, Lyon, France), and dissociation of cells was performed no more than 3 hours later by a 2-step perfusion method essentially as described previously[14] with some modifications, as follows. Visible vessels were first perfused for 15 minutes with Liver Perfusion Medium (Invitrogen, Cergy Pontoise, France) at 37°C to eliminate blood cells. A second perfusion then was performed with collagenase- and dispase-containing Liver Digest Medium (Invitrogen) at 37°C, at a flow rate of 10 mL/catheter/min (Masterflex peristaltic pump; Fisher Scientific, Illkirch, France), until the tissue was fully digested (typically, 30 min). Liver fragments were shaken gently in Hepatocyte Wash Medium (Invitrogen) to free loose cells, which then were filtered through a 70-μm nylon mesh before centrifugation at 200 × g for 1 minute. The fibroblast- and Küppfer cell–containing supernatant was discarded, and hepatocytes were washed a second time before assessing viability by trypan blue dye exclusion. Cells were resuspended in complete hepatocyte medium consisting of Leibovitz's L-15 medium (Invitrogen) supplemented with 26 mmol/L $NaHCO_3$, 100 IU/L insulin (Novo Nordisk, Puteaux, France), and 10% heat-inactivated fetal calf serum (Biowest, Nuaillé, France), and seeded at a density of 1.2–1.6 × 10^5 viable cells/cm^2 onto 6- or 12-well plates that had been precoated with a solution (1 mg/mL in 0.1 mol/L acetic acid) of Bornstein and Traub type I collagen from calf skin (catalog #C8919; Sigma-Aldrich, St. Louis, MO) between 1 and 10 hours previously (plates were covered with the minimal volume of collagen solution, then left to dry at room temperature). The medium was replaced 16–20 hours later with fresh complete hepatocyte medium supplemented with 1 μmol/L hydrocortisone hemisuccinate (SERB, Paris, France), and cells were left in this medium until HCV inoculation. The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

HCV Inoculation

High-titer stocks of JFH1- or Con1/C3-HCVcc and HCVpc were prepared as described in the Supplementary Materials and Methods section. PHH were inoculated 3 days after seeding. Unless otherwise stated, the multiplicity of infection (MOI) was 0.1 or 0.04 focus-forming units (ffu) per cell for HCVcc and HCVpc, respectively. The medium was replaced with the inoculum diluted in the smallest volume of fresh complete hepatocyte medium sufficient for covering the cells. After a 6- to 16-hour incubation at 37°C, the inoculum was removed, and monolayers were washed 3 times with phosphate-buffered saline. The cultures then were maintained during 15 days in complete hepatocyte medium. Half of the medium volume was changed every third day.

For drug inhibition assay and for E1 and CD81 neutralization assays, the conditions of HCV inoculation and culture are described in the Supplementary Materials and Methods section.

Phenotypic Characterization of PHH

To characterize the differentiation status of PHH, the expression of hepatocyte-specific genes and the inducibility of cytochrome P450 (CYP)3A4 expression were analyzed by a quantitative reverse-transcription real-time polymerase chain reaction technique, as described in the Supplementary Materials and Methods section.

Quantification of HCV RNA and Core Antigen and Titration of Infectivity

Intracellular levels of positive- and negative-strand HCV RNA were quantified by a strand-specific reverse-

transcription real-time polymerase chain reaction technique described previously (threshold of detection, 25 copies/reaction).[7]

HCV-RNA levels in filtered culture supernatants or in gradient fractions were quantified by reverse-transcription real-time polymerase chain reaction using the m2000sp and m2000rt instruments and RealTime HCV kit (Abbott Diagnostics, Rungis, France) (lower limit of quantification, 12 IU/mL), in accordance with the manufacturer's instructions.

HCV core antigen levels in filtered culture supernatants were quantified by enzyme immunoassay using the Ortho HCV Ag enzyme-linked immunosorbent assay kit (Ortho-Clinical Diagnostics, Tokyo, Japan) (threshold of detection, 50 fmol/L).

Infectivity titers in filtered culture supernatants or in gradient fractions were determined by focus-formation assay (threshold of detection, 10 ffu/mL), as previously described.[15]

Western Blotting

Western blotting was performed as described previously.[16] Primary antibodies were monoclonal antibodies against HCV core protein (C7-50; Alexis Biochemicals, Lausen, Switzerland) or HCV nonstructural protein 3 (NS3) (1847; Virostat, Portland, MA).

Isopycnic Iodixanol Gradient Ultracentrifugation

The buoyant densities of viral particles were determined by isopycnic iodixanol gradient ultracentrifugation essentially as described by Lindenbach et al[10] with minor modifications, as follows. A 9-mL continuous iodixanol gradient was prepared with Gradient Former 485 (Bio-Rad, Marne La Coquette, France) by using 10% and 40% (wt/vol) OptiPrep (Axis-Shield, Oslo, Norway) solutions in gradient buffer (10 mmol/L HEPES [pH 7.55], 0.02% bovine serum albumin). To maintain iso-osmotic conditions throughout the gradient, a reverse gradient of sodium chloride was made by adding 120 and 50 mmol/L NaCl to the 10% and 40% OptiPrep solutions, respectively. This continuous iodixanol gradient was overlaid with a 1-mL layer of 8% (wt/vol) OptiPrep, which was covered with a 0.6-mL layer of 6% (wt/vol) OptiPrep in gradient buffer. The filtered culture supernatant to be tested (typically 1 mL) was loaded onto this preformed gradient, and isopycnic ultracentrifugation was performed in a Beckman SW41Ti rotor (Beckman Coulter, Villepinte, Prance) at 110,000 × g for 20 hours at 4°C before fractionation from the top of the tube into 5 fractions of 1.4 mL followed by 6 fractions of 0.8 mL. The density of each fraction was determined by measuring the refractive index of a 10-μL aliquot with an Abbe refractometer (Atago, Tokyo, Japan) at a constant temperature of 20°C. Each fraction was probed for HCV-RNA level and infectivity titer, as described previously.

Lipoprotein Analysis

To compare PHH and Huh-7.5.1 cells for the secretion of lipoproteins containing apolipoprotein B (ApoB), cells were cultured for 3 days in the presence or absence of oleic acid (see the Supplementary Materials and Methods section for the preparation of cell culture media containing oleic acid). Culture supernatants were collected and subjected to sequential ultracentrifugation in KBr to separate lipoproteins in the density range of the major classes of human plasma lipoproteins, that is, very-low-density lipoproteins (VLDL), low-density lipoproteins, and high-density lipoproteins, as previously described.[17] After dialysis against phosphate-buffered saline to remove KBr, fractions were analyzed by Western blotting with monoclonal antibody against human ApoB (1D1; Heart Institute, Ottawa, Canada).

Results

Maintenance of Differentiation in PHH Culture

Freshly isolated hepatocytes from HCV-seronegative adult subjects were seeded at high density onto collagen-coated plates for inoculation with JFH1-HCVcc, and maintained in primary culture for 2 weeks to monitor kinetics of HCV infection. We first evaluated whether PHH retained their phenotypic characteristics during culture under our conditions (Figure 1). Indeed, cultured PHH displayed typical morphology at all time points (Figure 1*A*). Cell viability was maintained over the entire period of investigation of 15 days, with less than 10% apoptotic or necrotic cells in the culture at any time point (not shown). The expression of hepatocyte-specific genes also was maintained, albeit at varying levels, throughout the culture period, including CCAAT/enhancer binding protein β, hepatocyte nuclear factor-4, hepatocyte nuclear factor-3β, and hepatocyte nuclear factor-1β (key regulators for the maintenance of hepatocyte phenotype), albumin (a marker for liver synthesis function), and CYP2E1 and CYP3A4 (markers for liver detoxification function)[4] (Figure 1*B*). Finally, CYP3A4 expression remained inducible by rifampicin, its prototypical inducer, with a more than 10-fold increase at all time points (not shown), as expected for differentiated hepatocytes.[4]

Cell viability and expression of hepatocyte-specific genes also were evaluated when cultures were left uninfected. Similar results were obtained irrespective of whether PHH were inoculated or not (data not shown), indicating that infection of these cells with HCVcc did not induce any obvious cytopathic effect.

Finally, we compared uninfected PHH and Huh-7.5.1 cells for expression of hepatocyte-specific genes. For all markers tested, expression levels were significantly higher in PHH at the end of the 2-week culture period than they were in Huh-7.5.1 cells, whether at

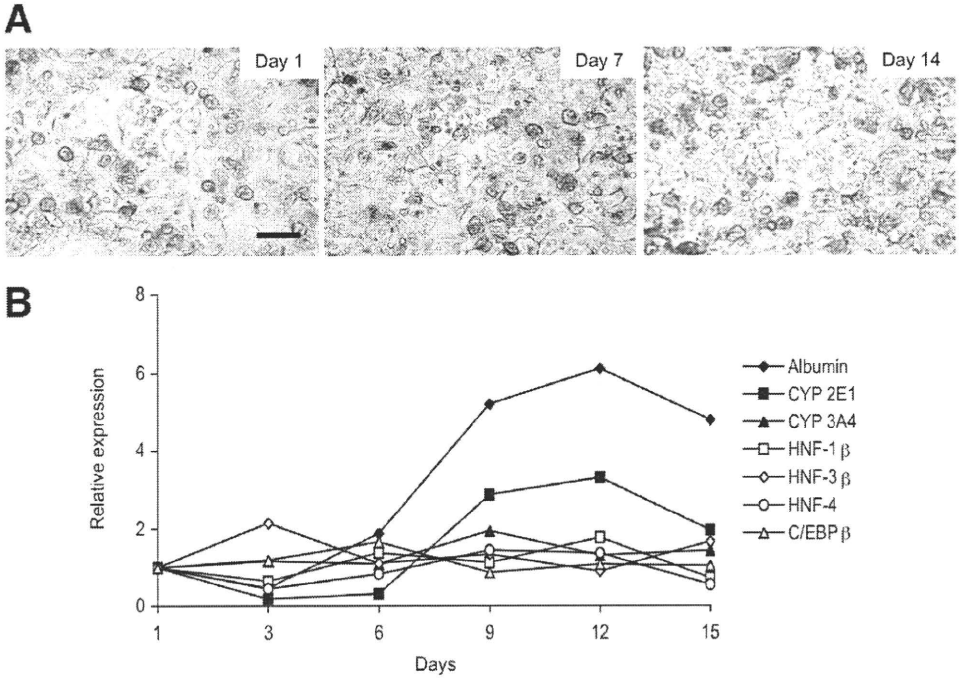

Figure 1. Phenotypic characteristics of PHH are maintained during culture. A representative experiment with PHH inoculated with HCVcc is shown. (*A*) Maintenance of hepatocyte morphology. Shown are phase-contrast micrographs of the culture at the indicated days (scale bar, 100 μm). Cell–cell contacts were established upon attachment of hepatocytes to the collagen-coated plates and maintained thereafter; the integrity of the monolayer was not compromised during culture. At all time points, cultured PHH appeared as mononucleated and binucleated cells displaying typical pavement-like morphology. (*B*) Maintenance of hepatocyte-specific gene expression. Results are shown as relative changes (*n*-fold) of transcript levels during culture for the indicated genes. Of note, although in most cultures albumin transcript levels showed only minor variations over time, they were found to increase during the second week in the culture presented here; such variations in cultured PHH have been reported by other investigators.[34] C/EBPβ, CCAAT/enhancer binding protein; HNF, hepatocyte nuclear factor.

confluence or in an exponential growth phase (Supplementary Figure 1). Thus, even at the end of the investigation period chosen for our study, PHH retained a higher degree of differentiation than did Huh-7.5.1 cells at any growth stage.

Replication and Expression of HCV Genome in PHH

To investigate whether viral replication occurred in PHH inoculated with JFH1-HCVcc, we used a technique allowing specific quantification of each strand of HCV RNA.[7] Negative-strand HCV RNA, a hallmark of HCV genome replication, could be detected as early as day 1 after inoculation, and the intracellular levels of both negative and positive strands increased during the culture period, indicating efficient replication (Figure 2*A*). Similar results were obtained upon inoculation of PHH with Con1/C3-HCVcc, a JFH1-derived chimeric virus whose structural proteins are encoded by the genotype 1b-HCV sequence Con1[13] (Figure 2*D*).

To further confirm that inoculation of PHH with HCVcc leads to authentic de novo replication, we tested the effect of telaprevir, a peptidomimetic inhibitor of the HCV serine protease.[18] Indeed, this specific antiviral drug reduced the accumulation of negative-strand HCV RNA in PHH in a dose-dependent manner (Figure 3*A*, negative strand). No cytotoxic effects were observed in PHH at the drug concentrations used, as assessed by both microscopic evaluation of the cultures (not shown) and lactate dehydrogenase leakage assay (Figure 3*B*). The concentration of telaprevir required for achieving 50% inhibition in our system was comparable with or slightly lower than the values reported with other in vitro systems.[18,19]

It also was important to verify that infection of PHH occurred via an authentic envelope-glycoprotein-mediated process. Indeed, an antibody to HCV E1 glycoprotein previously shown to neutralize infection of Huh-7 cells with HCVcc of various genotypes[13] also neutralized infection of PHH in a dose-dependent manner (Figure 4*A*, black bars). In addition, HCV infection of PHH was neutralized in a dose-dependent manner by a monoclonal antibody to the human tetraspanin CD81, an essential component of HCV receptor complex[3] (Figure 4*B*, black bars). We noted, however, that a 10-μg/mL concentration of this antibody only partly neutralized infection of PHH, whereas it almost completely abolished infection of Huh-7.5.1 cells (not shown), in agreement with previous reports.[11,20] Collectively, the results indicate that in PHH inoculated with HCVcc, infection occurred in an E1- and CD81-dependent way.

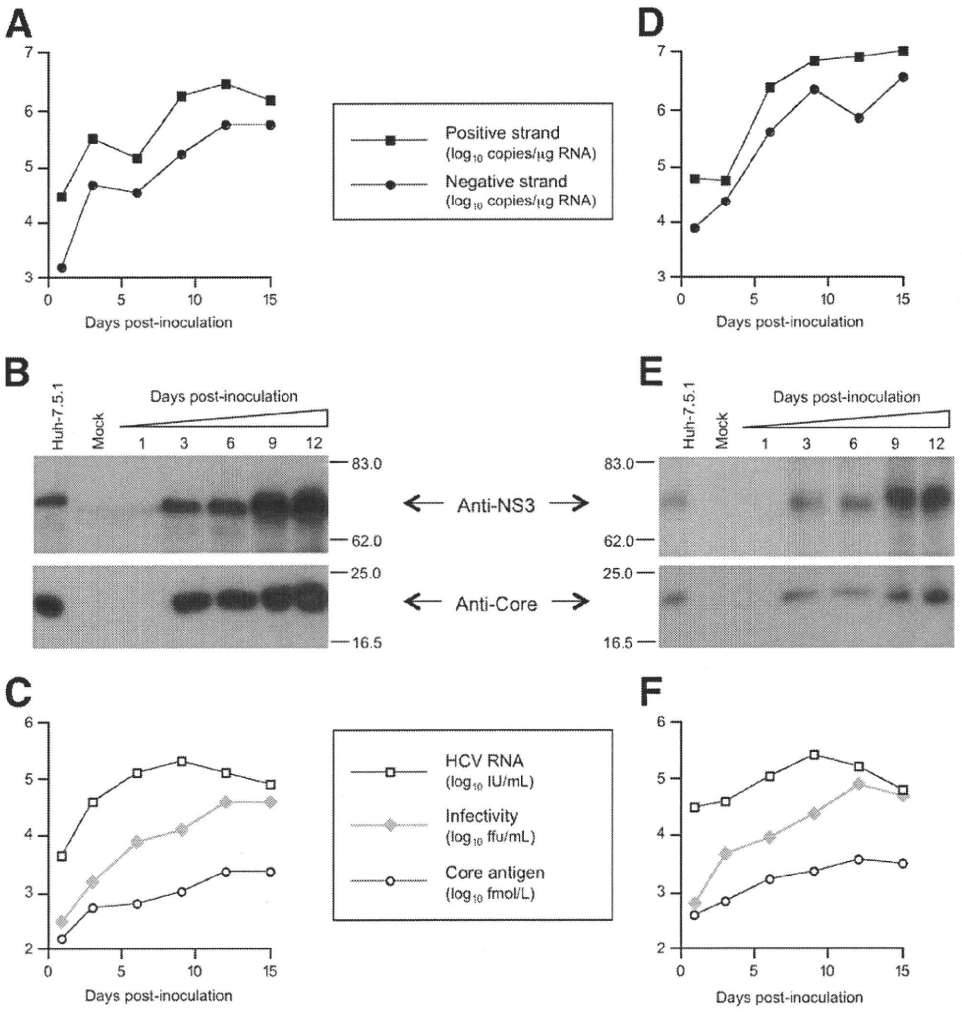

Figure 2. PHH support the complete productive HCV infection cycle. PHH were inoculated with (*A–C*) JFH1-HCVcc or (*D–F*) Con1/C3-HCVcc, and the parameters of HCV infection were monitored at the indicated days after inoculation. (*A* and *D*) Cells were lysed to evaluate intracellular levels of positive- and negative-strand HCV RNA. (*B* and *E*) Cells were lysed for Western blot analysis with monoclonal antibodies against HCV NS3 (anti-NS3) or core protein (anti-core). Lysates of HCV-replicating Huh-7.5.1 cells (Huh-7.5.1) and naive PHH (Mock) were run in parallel to serve as positive and negative controls, respectively. Positions of molecular mass markers are indicated (in kilodaltons). (*C* and *F*) Filtered culture supernatants were probed for HCV RNA, core antigen, and infectivity titer. None of these markers were detected in control PHH left uninfected.

The efficiency of HCV replication prompted us to investigate intracellular accumulation of viral proteins. HCV core and NS3 proteins could be detected by Western blot analysis as early as 3 days after inoculation, and at increasing levels thereafter (see Figure 2*B* and *E* for JFH1 and Con1/C3, respectively). Thus, HCV polyprotein was expressed and processed in PHH inoculated with HCVcc.

Production of Infectious HCV in PHH

To investigate whether progeny virus was produced from infected PHH, filtered culture supernatants were first examined for HCV core antigen with a commercial immunoassay, and for HCV RNA with a commercial standardized assay used for viral load quantification in clinical practice.[21] For both JFH1 (Figure 2*C*) and the intergenotypic chimeric virus Con1/C3 (Figure 2*F*), these 2 markers were detected at increasing levels, indicating production of viral particles. Most importantly, these particles were found to be infectious in a classic titration assay based on inoculation of naive Huh-7.5.1 cells followed by in situ immunofluorescence to detect foci of HCV-infected cells.[12,15] In typical experiments infectivity increased during culture, reaching peak titers of up to 10^4–10^5 ffu/mL by day 12 after inoculation (Figure 2*C* and *F*). Similar to replication of the viral genome, production of infectious particles was inhibited in a dose-dependent manner by antibodies to HCV E1 (Figure 4*A*, white bars) or to human CD81 (Figure 4*B*, white bars), or by HCV-specific antiviral drug (Figure 3*A*, infectivity), confirming the occurrence of de novo morphogenesis of HCVpc. We conclude that our system permits production of high titers of particles of different genotypes.

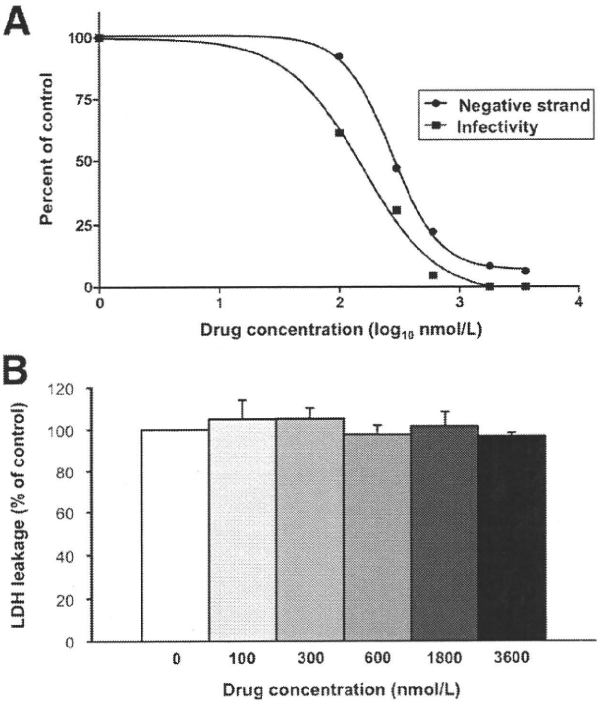

Figure 3. Dose-dependent inhibition of HCV replication in PHH by antiviral drug. PHH were inoculated with HCVcc, then cultured in the presence of the indicated concentrations of the HCV-specific drug telaprevir, or dimethylsufoxide as carrier control. (*A*) Antiviral effect of telaprevir in PHH. Intracellular levels of negative-strand HCV RNA (NEGATIVE STRAND) or infectivity titers in culture supernatants (INFECTIVITY) were expressed as percentages of values obtained upon culture in carrier control medium. Data are means of values obtained in 2–4 independent experiments. The 50% inhibitor concentrations of antiviral drug found for reducing the intracellular level of negative-strand HCV RNA and the culture supernatant infectivity titer were 269 nmol/L (95% confidence interval, 224–322 nmol/L) and 173 nmol/L (95% confidence interval, 124–241 nmol/L), respectively. (*B*) No significant cytotoxicity of telaprevir in PHH. Percentages of lactate dehydrogenase (LDH) leakage relative to carrier control are shown as the mean and standard error of the mean of 3 independent experiments performed in triplicate.

The efficiency of infectious virus production prompted us to compare the permissiveness of PHH and Huh-7.5.1 cells upon inoculation with the same input virus (see a representative experiment in Supplementary Figure 2). Upon inoculation at high MOI (10 ffu/cell), a condition ensuring that most cells of the culture can be infected at the time of virus inoculation, infectivity titers at day 1 were as high in PHH as in Huh-7.5.1 cells, suggesting that the 2 types of cells are similarly permissive to HCV infection. Nevertheless, only in Huh-7.5.1 cells did the titer increase from day 1 to day 3 after inoculation, probably because these cells are actively dividing, contrary to PHH. Upon inoculation at a lower MOI (0.1 ffu/cell), infectivity titers increased from day 1 to day 3 in both PHH and Huh-7.5.1 cells, suggesting propagation of progeny virus to cells that had not been infected at the time of inoculation with input virus.

Finally, we verified that HCVpc could be passaged in PHH cultures. Indeed, upon inoculation of PHH with either JFH1- or Con1/C3-HCVpc at a low MOI (0.04 ffu/cell), production of infectious virus was detected in filtered culture supernatants (Supplementary Figure 3*A* and *B*). Interestingly, infectivity titers achieved under these conditions reached up to 10^5–10^6 ffu/mL in the second week after inoculation. Thus, HCVpc could be propagated in PHH with high efficiency.

Distinctive Properties of HCV Grown in PHH

Infectivity in the blood of HCV-infected patients is mainly owing to particles of exceptionally low buoyant density, corresponding to virus in complex with ApoB-containing triglyceride-rich lipoproteins.[22–25] Moreover, virus recovered from animals infected with HCVcc showed higher specific infectivity (a reflection of the number of infectious particles normalized to the total number of physical viral particles) and lower buoyant density than input virus.[26] This has raised doubts as to whether HCVcc faithfully represents the properties of HCV in vivo, and prompted us to characterize HCVcc and HCVpc comparatively.

We found that specific infectivity was consistently higher for HCVpc than for the input virus HCVcc, regardless of both the source of hepatocytes and the HCVcc stock used (Table 1). To investigate whether this phenomenon was related to the nature of the cells used to grow the virus, we inoculated naive Huh-7.5.1 cells with HCVpc. Specific infectivity of progeny virus recovered after a 2-week period of re-culture in these cells (HCVrecc) was lower than that of HCVpc used as input virus, and again approximated that of HCVcc (Table 1). These results are consistent with the host cell playing a critical role in determining specific infectivity of produced virus.

To characterize possible biophysical differences between HCV grown in PHH and in Huh-7.5.1 cells, we subjected HCVcc, HCVpc, and HCVrecc to isopycnic ultracentrifugation through iodixanol gradients. Similar results were observed with JFH1 and Con1/C3 (see representative experiments in Figure 5*A* and *B*, respectively). Irrespective of whether HCV was grown in PHH or in Huh-7.5.1 cells, infectivity was broadly distributed across the fractions of lowest buoyant density, and was not detected above 1.15 g/mL. Although in the case of HCVcc the peak of viral RNA was found between 1.10 and 1.14 g/mL with little associated infectivity (Figure 5*A* and *B*, left panels), as previously reported,[10,27] in the case of HCVpc viral RNA peaked between 1.07 and 1.11 g/mL and coincided with the fractions of highest infectivity (Figure 5*A* and *B*, center panels). For HCVrecc, the peak of HCV RNA was shifted again to a higher buoyant density, with low associated infectivity (Figure 5*A* and *B*, right panels). As previously reported for HCVcc of various genotypes,[10,27] we found that the fractions of highest specific infectivity had low densities (≤1.10 g/mL) for HCV grown in Huh-7.5.1 cells, whether it was HCVcc or

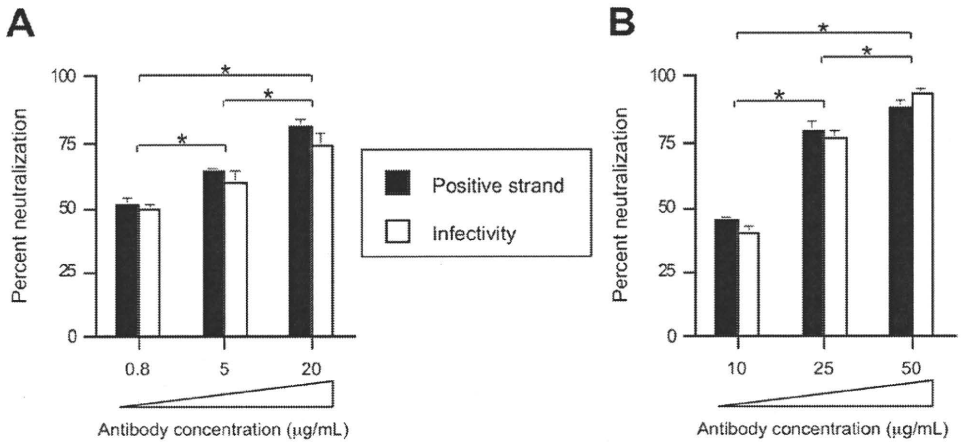

Figure 4. Dose-dependent neutralization of HCV infection of PHH by antibodies to HCV E1 and to human CD81. (*A*) HCVcc particles were pre-incubated with the indicated concentrations of mouse polyclonal antibody raised against E1, or else with polyclonal immunoglobulin G from a control mouse, and the virus/antibody mixtures then were used to inoculate PHH. (*B*) PHH were pre-incubated with the indicated concentrations of mouse monoclonal antibody against human CD81 JS-81 or isotype-matched control antibody (BD Biosciences, Le Pont de Claix, France) before inoculation with HCVcc. The neutralization percentages were calculated by comparing the intracellular levels of positive-strand HCV RNA (POSITIVE STRAND) or infectivity titers in culture supernatants (INFECTIVITY) upon incubation with specific antibodies, relative to the respective control antibodies. Data are mean and standard error of the mean of at least 3 independent experiments. Comparisons were performed using the Mann–Whitney rank-sum test (*$P < .05$).

HCVrecc (see Supplementary Figure 4*A* and *B* for JFH1 and Con1/C3, respectively). Specific infectivity also peaked at these densities in the case of HCVpc, but, compared with HCVcc or HCVrecc, was higher by 1–2 orders of magnitude. Collectively, the results indicate that compared with HCV grown in the Huh-7 established cell line, HCV grown in PHH has both lower average buoyant density of the viral-RNA–containing particles and higher ratio of infectious to noninfectious virus even among particles with low density.

Triglyceride-rich lipoproteins are synthesized in the liver in the form of VLDL. The lower average buoyant density of HCV grown in PHH vs Huh-7 cells prompted us to compare the ability of these cells to secrete VLDL. Upon sequential ultracentrifugation of culture supernatants, ApoB was found mainly in the VLDL fraction in the case of PHH, whereas it was found mainly in the density range of low-density lipoproteins in the case of Huh-7.5.1 cells (Figure 6). Importantly, even when these cells were cultured in the presence of oleic acid as a source of exogenous supply of fatty acids, ApoB only barely was detected in the density range of VLDL, indicating an intrinsic inability of the Huh-7 established cell line to secrete authentic VLDL. The PHH and Huh-7.5.1 cell profiles of secretion of ApoB-containing lipoproteins remained unchanged upon infection with HCVcc (data not shown). We conclude that highly differentiated PHH and transformed Huh-7 cells differ in their ability to secrete VLDL, providing a possible explanation for the distinct properties of HCV grown in these cells.

Table 1. HCV Grown in PHH Has Higher Specific Infectivity Than HCV Grown in Huh-7.5.1 Cells

Experiment		HCVcc			HCVpc[a]		HCVrecc[b]	
No.	Source of hepatocytes	Virus	Stock	Specific infectivity[c]	Specific infectivity[c]	Fold-increase in specific infectivity[d]	Specific infectivity[c]	Fold-reduction in specific infectivity[e]
1	Liver A	JFH1	J1	17	343	20	–	–
2	Liver B	JFH1	J2	10	634	63	21	30
3	Liver C	JFH1	J2	10	156	16	–	–
4	Liver D	JFH1	J3	8	254	32	–	–
5	Liver D	Con1/C3	C1	3	232	77	14	17
6	Liver E	Con1/C3	C1	3	118	39	–	–
7	Liver F	Con1/C3	C2	5	655	131	15	44

[a]Progeny virus recovered from PHH inoculated with HCVcc at the end of the 2-week culture period.
[b]Progeny virus recovered from Huh-7.5.1 cells inoculated with HCVpc at the end of the 2-week re-culture period.
[c]HCV-RNA levels and infectivity titers in filtered culture supernatants were measured with the Abbott viral load test (Abbott Laboratories, Abbott Park, IL) and focus-formation assay, respectively, and specific infectivity values were calculated as follows: 10^3 × infectivity titer (ffu)/HCV-RNA level (IU).
[d]Relative to HCVcc.
[e]Relative to HCVpc.

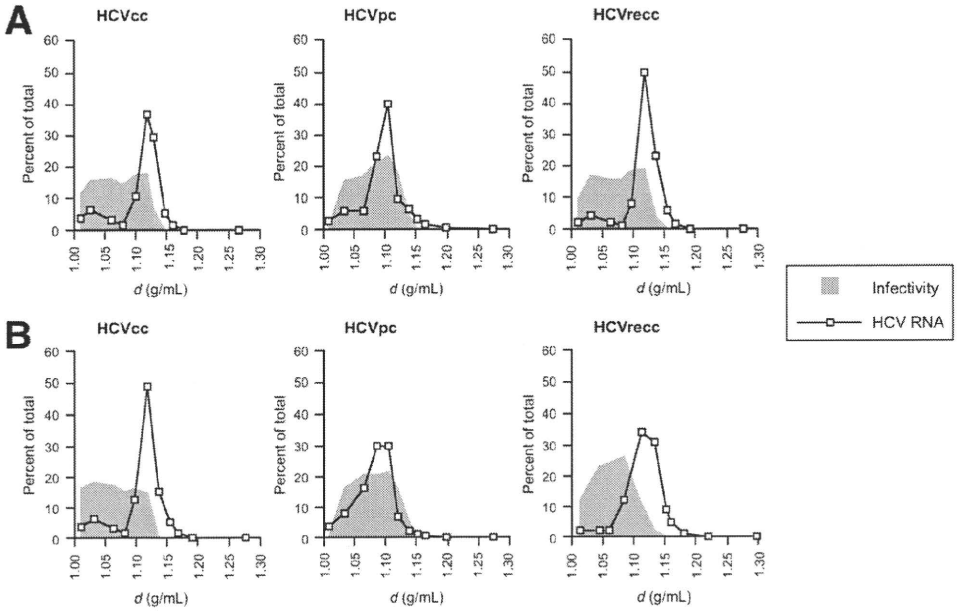

Figure 5. HCV grown in PHH has lower average buoyant density than HCV grown in Huh-7.5.1 cells. Representative experiments with (*A*) JFH1 or (*B*) Con1/C3 are shown. In each case, HCVcc, HCVpc, and HCVrecc were subjected to isopycnic centrifugation analysis through iodixanol gradients. HCV-RNA levels and infectivity titers expressed as percentages of the total in the gradient are plotted against the buoyant density (*d*).

Discussion

A robust system enabling HCV to be grown in cell culture has been made available only recently, using hepatocellular carcinoma–derived Huh-7 subclones.[3,10-13] In the course of efforts to develop presumably more physiological cell-culture–based models, workable titers of infectious HCV also were obtained in growth-arrested Huh-7 cells,[28] immortalized human hepatocytes,[29] and human fetal hepatocytes.[30] All these cells, however, diverge to various extents from the primary natural host cell of HCV, that is, nontransformed, nondividing, highly differentiated human adult hepatocytes, implying that any hijacking or perturbation of normal hepatocyte functions by HCV cannot be studied completely or accurately with these systems.[2,3] Here, we have developed a long-awaited simple yet robust system enabling similarly high infectivity titers to be achieved in PHH, which more closely mimic natural host cells of HCV (for critical experimental conditions, see the Supplementary Discussion section). Obvious limitations of choosing PHH as a model are the restricted availability of liver specimens of good quality to isolate them and the shortness of the window to study HCV infection. Even so, the system we have developed provides a valuable complement to existing systems for validating data on HCV/host cell interactions in a more relevant context and for studying the impact of HCV infection on hepatocellular physiology.

Unlike sera from HCV–infected patients,[5,6,8] HCVcc used as inoculum was found here to reproducibly allow production of

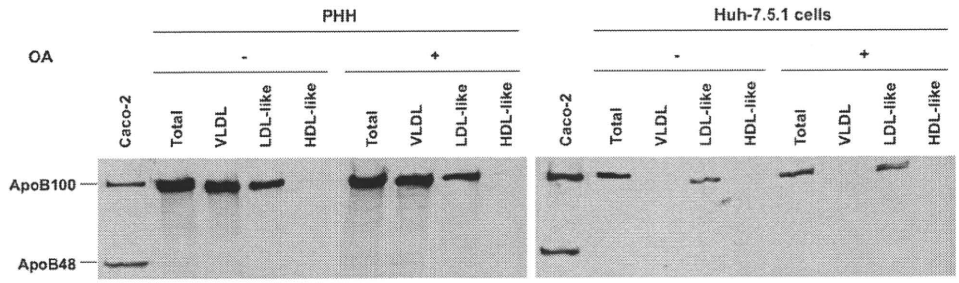

Figure 6. Comparison between PHH and Huh-7.5.1 cells: secretion of ApoB-containing lipoproteins. PHH and Huh-7.5.1 cells were cultured in the presence (+) or absence (−) of oleic acid (OA), and culture supernatants were subjected to sequential ultracentrifugation in KBr to separate lipoproteins with a density less than 1.006 g/mL (VLDL), 1.006 < density < 1.063 g/mL (low-density lipoprotein [LDL]-like), and 1.063 < density < 1.21 g/mL (high-density lipoprotein [HDL]-like). Unfractionated supernatants (TOTAL) and fractions were analyzed by Western blotting with monoclonal antibody against human ApoB. Culture supernatant from Caco-2 cells (CACO-2) was run in parallel to calibrate the blots because these cells secrete lipoproteins containing both isoforms of human ApoB, hepatic ApoB100, and intestinal ApoB48.[17] Note that with longer exposure of the blots, ApoB100 only barely was detected in the VLDL fraction of Huh-7.5.1 cell culture supernatant.

measurable titers of infectious progeny virus in PHH. An advantage of using HCVcc as source of virus is that the inoculum is well defined, thus avoiding confounding factors and allowing inoculation at a chosen MOI; by contrast, patient sera contain mixtures of HCV genomes of usually unknown sequences and other components likely to influence infectivity such as lipoproteins and neutralizing antibodies, which may help explain why their ability to allow replication in cultured hepatocytes is inconstant and unpredictable.[2,3,5,6,30] Nevertheless, for reasons that are not completely understood, production of HCVcc itself has so far depended on the nonstructural genes of JFH1, a single genotype 2a-clone with apparent peculiarities.[3] Thus, although JFH1-derived chimeric viruses bearing structural proteins of different genotypes can be grown in vitro,[3,13] as shown for Con1/C3 in our model, it would be desirable to extend cell-culture–based systems in both the Huh-7 cell line and PHH to include a representative range of HCV isolates covering all 7 genotypes.

It is remarkable that, compared with HCV particles produced to date in cell culture, HCVpc shows distinctive properties, that is, lower average buoyant density and higher specific infectivity, reminiscent of what is seen for virus recovered from the blood of animals infected with HCVcc.[26] HCV circulating in the blood of patients is heterogeneous, but the most infectious fraction is that of lowest buoyant density, corresponding to virus particles in complex with ApoB-containing triglyceride-rich lipoproteins.[22–25] Thus, the properties of HCVpc mimic those of infectious HCV produced in vivo. That these properties were lost after re-culture in Huh-7.5.1 cells designates a host cell factor as playing a major role in determining the characteristics of the virions that are produced. Studies in the Huh-7 cell-based system have highlighted a critical role of lipoprotein biogenesis in HCV morphogenesis, although the requirement for ApoB in this process remains controversial.[31–33] In fact, we have shown here that Huh-7 cells secrete ApoB-containing lipoproteins in the density range of low-density lipoproteins, indicating that these transformed cells do not fully reproduce the physiological pathways of assembly and maturation of authentic, triglyceride-rich VLDL. By contrast, PHH were found to retain (among other differentiation characteristics) the ability to secrete ApoB-containing lipoproteins at the expected density of VLDL. Hence, the lower buoyant density shared by HCVpc and animal-derived HCV[26] is probably at least partly because, in both cases, most of the viral RNA-containing particles have assembled with authentic VLDL in normal hepatocytes. The proper composition of HCV particles produced in normal hepatocytes may in turn facilitate virus entry, explaining the observed gain in specific infectivity; by contrast, Huh-7 cells appear to release a poor ratio of properly assembled, and hence infectious particles even among the particles with low density. Thus, our system of HCV production in PHH provides a most relevant in vitro model for studying the structure, morphogenesis, and infectivity of virus/lipoprotein complexes produced during infection in vivo.

Of the estimated 170 million individuals infected with HCV, many do not qualify for or tolerate the only approved treatment, and almost half of the patients treated do not achieve sustained virologic response, urging on the development of novel, more effective, and safer drugs.[1] Our data with telaprevir, the most advanced HCV-specific drug under development, provide a proof of concept that our system is applicable to testing candidate antiviral drugs in the context of physiological hepatocytes. Indeed, because this system supports productive HCV infection, it can be used to assess the antiviral activity of drugs targeting any step of the virus life cycle. In addition, because cultured PHH retain expression and inducibility of CYP3A4, which is involved in the metabolism of the majority of clinically used drugs, our system is suitable for predicting the drug metabolic profile and for testing pro-drugs.[4] Most importantly, the use of differentiated hepatocytes at least partially enables drug toxicity in human beings to be anticipated,[4] a major concern highlighted by the withdrawal of several otherwise promising HCV-specific drugs in clinical trials.[1] Finally, although the heterogeneity of PHH originating from different donors with various ethnic and genetic backgrounds might at first glance appear as a source of confounding factors, for therapeutic applications it may in fact prove instrumental to revealing possible interindividual variability in susceptibility to drugs.[4]

In conclusion, the ability of this newly developed cell-culture–based system to mimic as closely as possible the complete life cycle of HCV in its natural host cell makes it a model of choice for the study of HCV infection physiopathology and for the ultimate, most relevant validation of novel drugs in vitro.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.06.058.

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Received August 31, 2009. Accepted June 21, 2010.

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Acknowledgments

The authors are indebted to Brett D. Lindenbach for sharing with us his detailed protocol for isopycnic ultracentrifugation; the authors also thank Francis V. Chisari for the gift of Huh-7.5.1 cells; and the authors wish to thank Sylvie Lagaye for her contribution to the development of virologic tools, and Morgane Bomsel and Linda L. Pritchard for critical reading of the manuscript.

P.P. and A.C. contributed equally to this work, and A.R.R. and Y.C. contributed equally to this work.

Conflicts of interest

The authors disclose no conflicts.

Funding

Supported by a "Contrat d'initiation" from the Agence Nationale de Recherches sur le Sida et les hépatites virales (France) (P.P.), and by an "Avenir" grant from the INSERM (A.R.R.); A.C. was supported by a fellowship from the Agence Nationale de Recherches sur le Sida et les hépatites virales; V.P. and L.A. received fellowships from the Fondation Recherche Médicale (France) and the Société Francophone de Transplantation (France), respectively; and C.H. is a recipient of a fellowship from the Agence Nationale de Recherches sur le Sida et les hépatites virales.

Supplementary Materials and Methods

Phenotypic Characterization of PHH

Viability of cultured PHH was evaluated by flow cytometry analysis using annexin V and propidium iodide staining to detect apoptotic or necrotic cells, as previously described.[1]

To characterize the differentiation status of PHH, the expression of hepatocyte-specific genes was analyzed by quantitative reverse-transcription real-time polymerase chain reaction (see Supplementary Table 1 for primer sequences and reaction parameters). Briefly, total RNA was extracted from cells using RNeasy mini kit (Qiagen, Courtaboeuf, France), and reverse transcription was carried out using oligo$(dT)_{15}$ primer and Moloney murine leukemia virus reverse transcriptase (Promega, Charbonnières, France) as recommended by the manufacturer. Real-time polymerase chain reactions were performed using the LightCycler^R instrument and FastStart DNA Master SYBR Green I kit (Roche Applied Science, Grenoble, France), in accordance with the procedure recommended by the manufacturer. Expression of each specific transcript was normalized to that of both β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. The $2^{-\Delta\Delta Ct}$ method[2] was used to analyze the changes in transcript expression during culture.

To compare the differentiation status of PHH and Huh-7.5.1 cells, PHH were collected at the end of the 2-week culture period; Huh-7.5.1 cells were seeded at a density of 2.5×10^4 cells/cm^2, and then collected either 2 days (i.e., when the cells were in mid-log phase) or 4 days (i.e., at 1 day after the cells had reached confluence) later. The $2^{-\Delta\Delta Ct}$ method[2] was used to calculate the relative expression of hepatocyte-specific genes analyzed as described above.

To test for inducibility of CYP3A4 expression, cultured PHH at various time points were subjected or not to a 24-h incubation with 10 μmol/L rifampicin (Sanofi-Aventis, Paris, France), and the CYP3A4 transcript levels were quantified as described above. The $2^{-\Delta\Delta Ct}$ method[2] was used to calculate the relative induction of CYP3A4 transcript expression in treated cells compared to untreated cells at the same time point.

Preparation of Virus Stocks

Huh-7 or Huh-7.5.1 cells[3] (a kind gift from Francis V. Chisari, the Scripps Research Institute, La Jolla, CA) were grown in complete Huh-7 medium consisting of Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10 mmol/L HEPES (pH 7.3), non-essential amino acids (Invitrogen), 2 mmol/L L-glutamine (Invitrogen), and 10% heat-inactivated fetal calf serum (PAA Laboratories, Pasching, Austria), at 37°C in a humidified 5% CO_2 atmosphere. To prepare HCVcc stocks, cells were first electroporated with genomic HCV RNA transcribed in vitro from the plasmids pJFH1[4] or pFK-Con1/C3[5] used as templates, as previously described.[6] Transfected cells were grown in complete Huh-7 medium, and culture supernatants were harvested after several cell passages when infectivity titers reached at least 1×10^5 ffu/mL. For some HCVcc preparations, additional amplification of the virus was performed by passage of the culture supernatant:[3] naïve 20%-confluent Huh-7.5.1 cells were inoculated at a low MOI (0.01 ffu/cell), and grown until infectivity titers reached at least 1×10^5 ffu/mL. HCVcc-containing supernatants were passed through a 0.45-μm filter, then concentrated to ~1/10 of the original volume in an Amicon Ultra-15 100,000 molecular weight cutoff ultrafiltration device (Millipore, Billerica, MA), and viral stocks were aliquoted and stored at −80°C until use.

To prepare HCVpc stocks, culture supernatants of PHH harvested on day 12 after inoculation with HCVcc were filtered and concentrated as described above for the preparation of HCVcc stocks.

To derive HCVrecc, Huh-7.5.1 cells were inoculated with HCVpc, and then grown for 2 weeks in complete Huh-7 medium before harvesting the culture supernatant.

Drug Inhibition Assay

PHH were inoculated with HCVcc as described in Materials and Methods. After the inoculum was removed, monolayers were washed three times with phosphate-buffered saline, and incubated for 3 days in complete hepatocyte medium containing 0.5% dimethylsufoxide as carrier control, or else increasing concentrations of telaprevir (VX-950, Vertex Pharmaceuticals, Cambridge, MA), before determination of intracellular levels of negative strand HCV RNA and infectivity titers in culture supernatants. Data were expressed as percentages of values obtained upon culture in carrier control medium, and plotted against the antiviral drug concentration. Prism 5 (GraphPad, La Jolla, CA) was used to generate best-fit sigmoidal curves and determine the IC_{50} values with 95% confidence intervals.

To assess the potential cytotoxicity of increasing concentrations of telaprevir in PHH, the activity of lactate dehydrogenase released into culture supernatants was measured in triplicate using the CytoTox 96^R Non-Radioactive Cytotoxicity Assay (Promega), and the percentages of lactate dehydrogenase leakage relative to carrier control were calculated as previously described.[7]

Neutralization Assays

For E1 neutralization assays, HCVcc particles were pre-incubated for 1 h at 37°C with increasing concentrations of a previously described mouse polyclonal antibody raised against E1 or polyclonal IgG from a control mouse,[8] and the virus/antibody mixtures then were used to inoculate PHH as described in Materials and Methods. For CD81 neutralization assays, PHH were pre-incubated for 1 h at 37°C with increasing concentrations of mouse monoclonal antibody against human CD81 JS-81 or isotype-matched (mouse IgG1, κ) control antibody (BD Biosciences, Le Pont de Claix, France) in medium without

fetal calf serum, then washed three times with phosphate-buffered saline before inoculation with HCVcc. Intracellular levels of positive-strand HCV RNA and infectivity titers in culture supernatants were determined on day 3 and day 12 after inoculation, respectively. The percentages of neutralization with specific antibodies were calculated relative to the respective control antibodies. Statistical analysis was performed with the Mann-Whitney rank-sum test using SigmaStat software (Systat Software Inc, Chicago, IL).

Preparation of Cell Culture Media Containing Oleic Acid

To prepare cell culture media containing oleic (OA), a stock solution of 100 mmol/L OA (Sigma-Aldrich) was first prepared in chloroform-methanol 2:1 (v/v). OA (6 μL of stock solution for each mL of medium to prepare) was then dried in a sterile glass tube under a stream of nitrogen. OA in complex with bovine serum albumin was finally obtained by addition of either complete hepatocyte medium or complete Huh-7 medium to dried OA and incubation for 1 h at 37°C, with shaking. The resulting OA concentration and the molar ratio of fatty acid to albumin were 0.6 mmol/L and 1:1, respectively.

Discussion

In this work, PHH were isolated from normal-appearing tissue within surgical liver resection specimens from 48 consecutive patients. A differentiated monolayer could be established with 35 of these 48 preparations (73%). Upon inoculation with HCVcc of either genotype, production of infectious virus with titers $\geq 10^3$ ffu/mL was achieved in 29 of the 35 successful cultures (83%). Infectivity titers $\geq 10^4$ ffu/mL were reached in 14 of these 29 cases (48%). Thus, once a differentiated monolayer could be achieved, productive HCV infection was generally obtained. The patients used as liver donors had various clinical characteristics, but all of them were Caucasian, and the vast majority underwent partial hepatectomy for the therapy of metastatic colorectal cancer. Because of the failure of all preliminary experiments using liver samples from patients who either had excessive alcohol consumption or received chemotherapy within the 8 weeks preceding surgery, donors with these characteristics were excluded from further experiments. The culture of PHH from one donor with extensive hepatic steatosis could also not be maintained beyond 1 week. These observations support the recommendation that liver samples with extensive fibrosis or fatty changes should not be used as a source of PHH.[9] Other critical parameters to achieve maintenance of the differentiated state of hepatocytes include the following: (i) dissociation of liver cells was performed no more than 3 h after surgical resection; a delay >6 h was found to decrease cell viability dramatically, confirming that liver resection specimens should be perfused as soon as possible to obtain PHH of good quality;[9] (ii) plates were coated with collagen within 10 h before hepatocyte seeding, in accordance with the recommendation that the collagen matrix should be freshly prepared;[10] (iii) at the time of seeding, the cell suspension contained more than 75% viable hepatocytes as assessed by trypan blue dye exclusion; and (iv) hepatocytes were seeded at a density of at least 1.15×10^5 viable cells/cm^2, otherwise a monolayer was not achieved. In fact, most of these culture conditions, especially the requirement for a high percentage of viable hepatocytes and seeding at confluence, were used in previous attempts to achieve HCV infection in PHH, but the lower efficiency and reproducibility of HCV replication reported in those studies are most certainly attributable to the fact that sera from HCV-infected patients were used as sources of virus.[11,12] The newly available JFH1 virus and derived chimeras have provided a uniform source of infectious virus, but in the few studies reporting the use of JFH1-HCVcc as input virus in PHH, production of new progeny virus was not assessed.[9,13] A very recent study reported de novo production of infectious virus upon HCVcc inoculation of micropatterned co-cultures of PHH and supportive stroma, but the levels were extremely low, and passage onto naïve cultures was unsuccessful, most probably due to the microscale format used.[14] Nevertheless, it should be noted that replication of the HCV genome was more efficient upon inoculation with HCVcc compared to sera from HCV-infected patients,[13,14] justifying our strategy of using HCVcc as input virus (also compare intracellular levels of both positive- and negative-strand HCV RNA between our previous work with patient sera[15] and the present study). Here we have described simple conditions enabling productive infection to be obtained in a high percentage of cases with this strategy. Importantly, titers were high enough to allow further biophysical analysis of the progeny virus.

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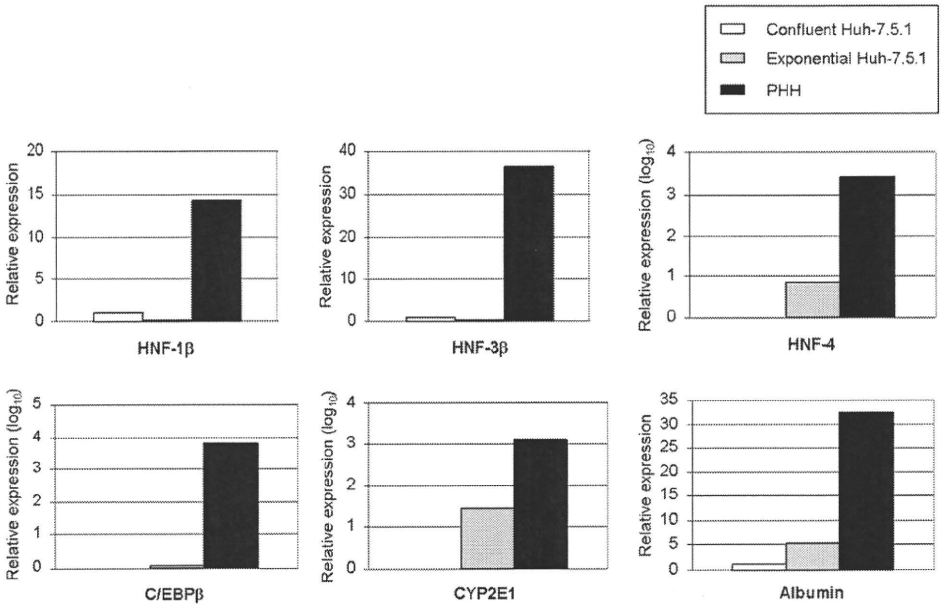
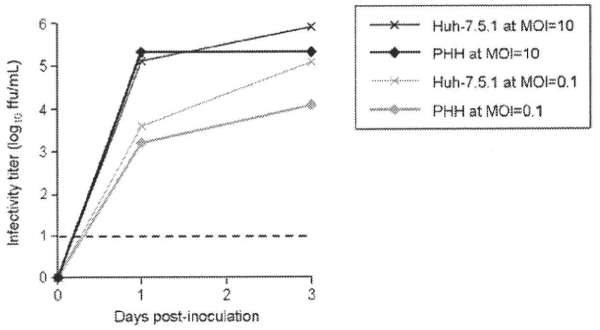
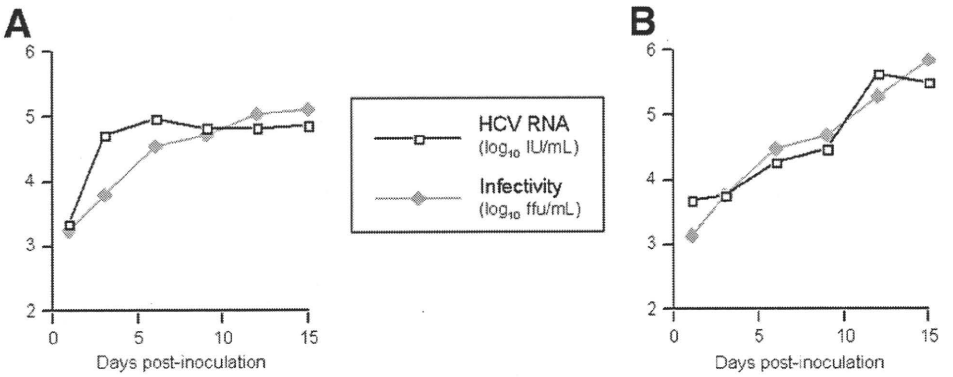


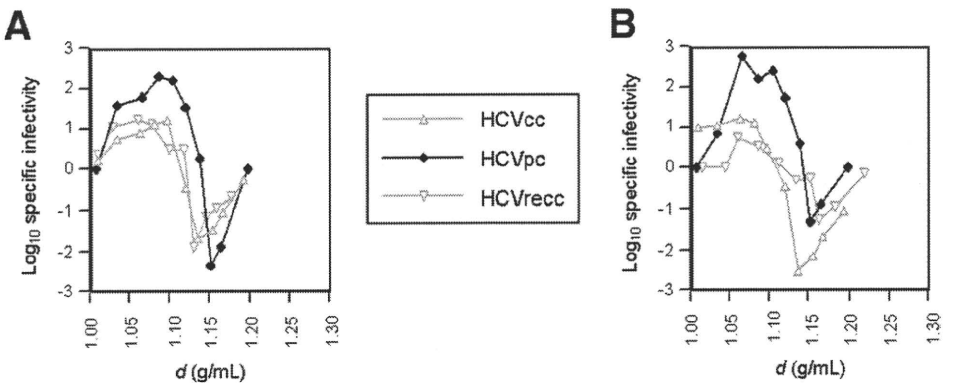
Supplementary Figure 1. Comparison between PHH and Huh-7.5.1 cells for expression of hepatocyte-specific genes. For the indicated genes, results are shown as relative expression (*n*-fold) of transcript levels in PHH at the end of the 2-week culture period (*PHH*) or Huh-7.5.1 cells in mid-log phase (*exponential Huh-7.5.1*) versus Huh-7.5.1 cells at confluence (*confluent Huh-7.5.1*). Of note, expression of CYP3A4 was undetected in Huh-7.5.1 cells irrespective of their growth stage.



Supplementary Figure 2. Comparison between PHH and Huh-7.5.1 cells for permissiveness to HCVcc infection. PHH or Huh-7.5.1 cells were inoculated with the same stock of HCVcc at a multiplicity of infection of 0.1 ffu/cell (*MOI=0.1*) or 10 ffu/cell (*MOI=10*). At the indicated days post-inoculation, filtered culture supernatants were probed for infectivity titer. The threshold of detection of the assay is indicated by a dotted line.



Supplementary Figure 3. Passage of HCVpc in PHH cultures. Naïve PHH were inoculated with JFH1-HCVpc (*A*) or Con1/C3-HCVpc (*B*). At the indicated days post-inoculation, filtered culture supernatants were probed for HCV RNA and infectivity titer.



Supplementary Figure 4. Correlation between specific infectivity and buoyant density. Representative experiments with JFH1-HCV (*A*) or Con1/C3-HCV (*B*) are shown. In each case, HCVcc, HCVpc, and HCVrecc were subjected to isopycnic iodixanol gradient ultracentrifugation. Each gradient fraction was then probed for HCV RNA level and infectivity titer with the Abbott viral load test and focus-formation assay, respectively. Specific infectivity, calculated as 10^3 × infectivity titer (ffu)/HCV RNA level (IU), is plotted against the buoyant density (*d*).

Supplementary Table 1. Analysis of Hepatocyte Gene Expression by qRT-PCR: Primer Sequences and Reaction Conditions

Gene	Sequence of forward and reverse primers (5' to 3')	Annealing temperature, °C	Elongation time, s
Albumin	F: TTAGGATCCCCCAGGAAGACATCCTTTGC R: CCTGAGCCAGAGATTTCC	55	45
CYP 2E1	F: AGCACAACTCTGAGATATGG R: ATAGTCACTGTACTTGAACT	60	20
CYP 3A4	F: CTCTCATCCCAGACTTGGCCA R: ACAGGCTGTTGACCATCATAAAAG	67	10
HNF-1β	F: GAAACAATGAGATCACTTCCTC R: CTTTGTGCAATTGCCATGACTC	66	10
HNF-3β	F: ATGGAAGGGCACGAGCC R: TACGTGTTCATGCCGTTCAT	65	3
HNF-4	F: CTGCTCGGAGCCACAAAGAGATCCATG R: ATCATCTGCCACGTGATGCTCTGCA	76	13
CCAAT/enhancer binding protein β	F: GCGCGAGCGCAACAACA R: TGCTTGAACAAGTTCCGCAG	69	6
β-actin	F: TGCTATCCAGGCTGTGCTA R: ATGGAGTTGAAGGTAGTTT	58	17
Glyceraldehyde-3-phosphate dehydrogenase	F: GCCAAGGTCATCCATGACAAC R: GCCTGCTTCACCACCTTCTTGA	73	11

NOTE. The polymerase chain reaction protocol consisted of one step of initial denaturation for 10 minutes at 95°C, followed by 40 cycles of denaturation (at 95°C for 5 s), annealing (at the indicated temperature for 5 s), and elongation (at 72°C for the indicated time). A melting curve analysis was performed immediately after amplification to verify the specificity of polymerase chain reaction products.
F, forward; HNF, hepatocyte nuclear factor; qRT-PCRT, quantitative reverse-transcription real-time polymerase chain reaction; R, reverse.

MAJOR ARTICLE

La Protein Required for Internal Ribosome Entry Site–Directed Translation Is a Potential Therapeutic Target for Hepatitis C Virus Replication

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Background. Translation of the hepatitis C virus (HCV) is mediated by an internal ribosome entry site (IRES). Here, we analyzed the functional relevance of La protein for replication of HCV using an infectious HCV clone, JFH-1.

Methods. A single-nucleotide mutation from A to U was introduced at the 338th nucleotide in the stem-loop domain IV structure of HCV IRES, which stabilized stem-loop IV and abolished translation and replication of JFH-1 almost completely.

Results. During JFH-1 replication, translation initiation factors required for HCV IRES activity, including La protein, polypyrimidine tract binding protein (PTB), PSMA7, and PCBP2, were significantly induced in Huh-7.5 cells. Interestingly, JFH-1 infection increased telomerase activity and induced the expression of human telomerase RNA (hTR) in Huh-7.5 cells. In 37 tissue specimens from patients with chronic hepatitis C, La protein significantly correlated with the representative essential telomerase components hTR, p23, and HSP90 ($P < .001$). Recombinant adenovirus that expressed short-hairpin RNA against La protein successfully suppressed the levels of La protein and core protein of JFH-1 to 30% of that in the control cells.

Conclusions. HCV infection might be strongly related to telomerase activity in the liver through La protein induction. Inhibition of La protein substantially repressed JFH-1 replication; therefore, La protein is a potential therapeutic target for HCV.

Hepatitis C virus (HCV) is a positive-strand, enveloped RNA virus that belongs to the genus *Hepacivirus* in the family *Flaviviridae.* A human liver infected with HCV develops chronic hepatitis, cirrhosis, and in some instances, hepatocellular carcinoma [1]. Although a combination of ribavirin and interferon has become a routine means of treating infected patients, the results are often unsatisfactory, especially in patients with a high viral load [2]. Identification of host factors that regulate HCV replication in infected patients could be helpful in the development of a novel antiviral treatment strategy. It has been reported that various host factors are associated with HCV infection; however, only a few proteins have been functionally shown with an infectious HCV clone to regulate HCV replication [3].

The translation of HCV is initiated by a highly structured RNA segment, the internal ribosome entry site (IRES), which occupies most of the 5′ nontranslated RNA [4]. Many canonical and noncanonical translation initiation factors, such as La protein [5], polypyrimidine tract binding protein (PTB) [6], and eukaryotic initiation factor 3 (eIF3), interact with HCV IRES and might regulate HCV translation. Previously, we reported that HCV IRES activity is highly dependent on these initiation factors, and it correlated with the expression of La protein [7, 8]. However, the functional relevance of these translation initiation factors on HCV

Received 17 September 2009; accepted 6 January 2010; electronically published 24 May 2010.

Potential conflicts of interest: none reported.

Financial support: This study is partially supported by the Grants-in-Aid for Scientific Research by Japan Society for the Promotion of Science (project 17591036).

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The Journal of Infectious Diseases 2010;202(1):75–85

0022-1899/2010/20201-0009$15.00
DOI: 10.1086/653081

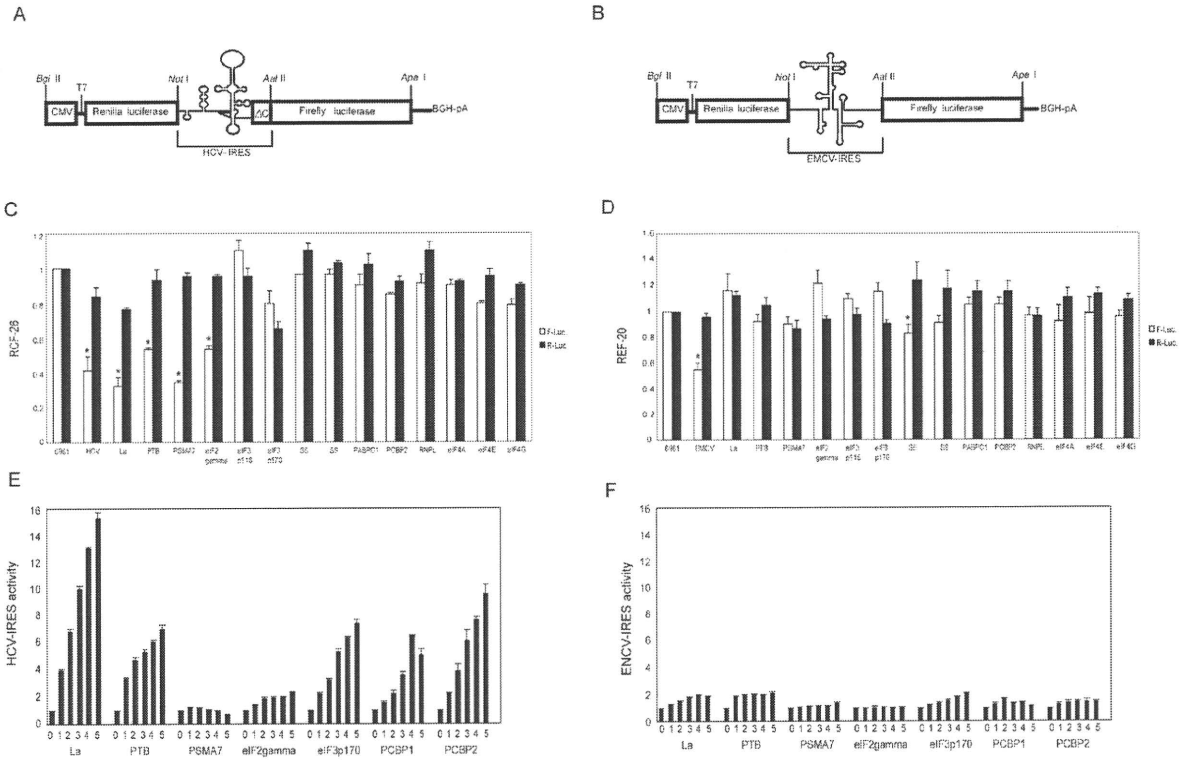

Figure 1. Organization of the transcriptional unit of plasmids pRL-HL (*A*) and pRL-EL (*B*). *C* and *D*, Suppression of 14 canonical and noncanonical initiation factors by specific antisense oligonucleotides. Changes in *Renilla* luciferase (RL) and firefly luciferase (FL) (hepatitis C virus–internal ribosome entry site [HCV-IRES]–directed translation) activities in RCF-26 (*C*). Changes in RL and FL (encephalomyocarditis virus [ENCV]–IRES–directed translation) activities in REF-20 (*D*). *$P < .05$. *E* and *F*, In vitro translation of pRL-HL and pRL-EL in rabbit reticulocyte lysate. The plasmids pRL-HL or pRL-EL (0.05 μg) and increasing amounts of expression vectors (0–0.125 μg) of La protein, polypyrimidine tract binding protein (PTB), eIF3p 170, eIF2γ, PSMA7, PCBP1, and PCBP2 were co-translated in rabbit reticulocyte lysate. The fold increases in relative HCV IRES activity (*E*) and ENCV-IRES activity (*F*) are shown. *$P < .05$. *Lane 0*, 0 μg; *lane 1*, 0.025 μg; *lane 2*, 0.05 μg; *lane 3*, 0.075 μg; *lane 4*, 0.1 μg; *lane 5*, 0.125 μg. *$P < .05$.

replication had not been fully evaluated. In this study, we found that the expression of La protein is induced by HCV infection, and this induced La protein–activated telomerase activity in a human hepatoma cell line. The results indicate La protein is a potential therapeutic target for HCV infection.

EXPERIMENTAL PROCEDURES

Expression vector plasmids. The FLAG tag fusion La protein expression vector (pCMV-La-FLAG) was created by polymerase chain reaction (PCR), using the La protein expression vector (pCMV-La) as the template [8]. The forward primer 5′-AAT GAA ATC AGA AGA AA-3′ contains an *Xba* I site, and the reverse primer 5′-TGA TCT AGA TTA CTT ATC GTC GTC ATC CTT GTA ATC CTG GTC TCC AGC ACC ATT TTC TGT TTT CTG TTG -3′ contains *Xba* I and FLAG sites.

Cell lines. Human hepatocellular carcinoma 7 (Huh-7) cells and Huh-7.5 cells (provided by Professor C. M. Rice, Rockefeller University) were maintained in Dulbecco modified Eagle medium (DMEM; Gibco BRL), which contained 10% fetal bovine serum and 1% penicillin/streptomycin. The RCF-26 was a stably transformed cell line from Huh-7 cells that constitutively expressed dicistronic RNA transcripts containing sequences encoding 2 reporter proteins—*Renilla* luciferase and firefly luciferase—separated by a functional HCV IRES of genotype 1b (Figure 1*A*) [7]. The REF-20 was a stably transformed cell line from Huh-7 cells that constitutively expressed dicistronic RNA transcripts in which HCV IRES was replaced with encephalomyocarditis virus (EMCV) IRES (Figure 1*B*).

Antisense oligodeoxynucleotide. The antisense phosphorothioate oligodeoxynucleotides (oligos) designed for HCV IRES, La protein, PTB, eIF3, eIF2γ, S9, poly(A)-binding protein cytoplasmic 1 (PABPC1), PCBP2, RNPL, and control randomized oligo 6961 were described elsewhere [8]. Antisense oligos for PSMA7, S5, eIF4A, eIF4E, eIF4G, and EMCV IRES were synthesized. The nucleotide sequences of the antisense oligos were 5′-CTC ATG CCG GCG GGC GGC CG-3′ for PSMA7,

5′-GTC ATC CTG AGA ACA CAG CC-3′ for S5, 5′-GAC ATG ATC CTT AGA AAC TA-3′ for eIF4A, 5′-GCC ATC TTA GAT CGA TCT GA-3′ for eIF4E, 5′- GAC ATG ATC TCC TCT GTG AT-3′ for eIF4G, and 5′-TCC ATA TTA TCA TCG TGT TT-3′ for EMCV IRES. The antisense oligos (1.0 μmol/L) were transfected into RCF-26 (Figure 1*C*) or REF-20 (Figure 1*D*). After 24 h of transfection, *Renilla* luciferase (cap-dependent translation) and firefly luciferase (HCV or EMCV-directed translation) activities were measured with the Dual-Luciferase Reporter Assay System (Promega).

In vitro translation of pRL-HL and pRL-EL in rabbit reticulocyte lysate. In vitro translation of pRL-HL and pRL-EL was carried out in transcription and translation–coupled rabbit reticulocyte lysate systems (Promega). In 25 μL of the transcription and translation reaction mixture, 0.05 μg of pRL-HL or pRL-EL was cotranslated with an increasing amount of plasmid DNA (up to 0.125 μg) of La, PTB, PSMA7, eIF2-γ, eIF3p170, PCBP1, and PCBP2, which were cloned using the T7 promoter. A 3-μL aliquot was then used to measure *Renilla* luciferase and firefly luciferase activities using the Dual-Luciferase Reporter Assay System (Promega).

Site-directed mutagenesis. The plasmid pJFH-1 was used as the template for introduction of the site-directed mutation at nucleotide 338 in the 5′ nontranslated RNA. The site-directed mutagenesis reaction was performed using the Pfu Turbo DNA polymerase PCR system (Stratagene), according to the manufacturer's instructions.

Transfection of JFH-1 and JFH-1 338U into Huh-7.5 cells. Ten micrograms of synthetic RNA transcribed from pJFH-1 or pJFH-1 338U was used for electroporation. Cells were then pulsed at 260 V and 950 μF using the Gene Pulser II apparatus (Bio-Rad Laboratories).

Infection of Huh-7.5 cells with JFH-1. Seventy-two hours after transfection, the culture medium was collected, cleared by low-speed centrifugation at 2000 revolutions per minute at 760g for 10 min, and passed through a Millipore filter (pore size, 0.45 μm; Millipore Corporation). Part of the filtered culture medium was diluted 50-fold or 10-fold with DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin. Diluted culture medium (1 mL) was used for injection of cells into a well of a 6-well plate or a well containing cover slips and incubated for 4 h. At 3 days after infection, inoculated cells grown on cover slips were fixed and stained using anti-core antibody, as described below. The amounts of HCV RNA, La-RNA, and human telomerase RNA (hTR)-RNA in inoculated cells were determined by quantitative real-time detection (RTD)-PCR.

Western blot analysis and immunofluorescence staining. The expression levels of La protein and PTB in cells were evaluated by Western blotting using mouse anti-La antibody (SW5) and rabbit anti-PTB antibody, as described elsewhere [9]. The expression of HCV core protein, PSMA7, eIF2γ, PCBP2, and FLAG-tagged La protein was evaluated with mouse anti-core antibody (Affinity BioReagents), mouse anti-PSMA7 antibody (Antibodies Direct), rabbit anti-eIF2γ antibody (Abcam), mouse anti-huRNP E2 (23-G) antibody (Santa Cruz Biotechnology), and mouse anti-FLAG antibody (Sigma), respectively. For immunofluorescence staining, anti–core monoclonal antibodies and Alexa Fluor 488 goat anti–mouse immunoglobulin G antibody (Invitrogen) were used.

Quantitative RTD-PCR. The primer pairs and probes for La protein, PTB, eIF3 p170, GAPDH, and HCV were obtained as described elsewhere [8]. The primer pairs and probes for PSMA7, eIF2γ, PCBP2, hTR, p23, Hps90, and β-actin were obtained from the TaqMan assay reagents library. One microgram of isolated RNA was reverse-transcribed to complementary DNA using SuperScript II RT (Invitrogen) according to the manufacturer's instructions, and the resulting complementary DNA was amplified with appropriate TaqMan assay reagents [10].

Telomerase activity assay. The plasmids pCMV-La-FLAG and pCR3.1 were transfected into Huh-7 cells using Fugene 6 transfection reagent (Roche Applied Science). Forty-eight hours after transfection, the amounts of hTR-RNA in the transfected cells were determined by RTD-PCR. The expression of the FLAG-tag fusion La protein was evaluated by Western blot analysis. Telomerase activity was measured with a PCR-based telomerase repeat amplification protocol (TRAP) assay, performed with the TRAPEZE kit (Invitrogen) according to the manufacturer's instructions. Each reaction product was amplified in the presence of a 36-base pair internal telomerase assay standard. The PCR products were fractionated by electrophoresis on a 10% polyacrylamide gel and then visualized by staining with SYBR Green (Molecular Probes).

Construction of recombinant adenovirus expressing short-hairpin RNA for La protein. The short-hairpin RNA expression plasmid (pSh-La), which expresses short-hairpin RNA for La protein (seq: 5′-CCG GCC AAG GCA GAA CTC ATG GAA ACT CGA GTT TCC ATG AGT TCT GCC TTG GTT TG-3′), was purchased from Sigma. The pSh-La was digested with the enzymes *Hind* III and *Bam*HI, and the excised fragment, including the short-hairpin RNA, was transferred to the adenoviral expression plasmid. The adenoviral expression plasmid and bovine growth hormone plasmid were cotransfected into 293A cells using the CellPhect Transfection kit (GE Healthcare) to produce crude adenoviral stocks. These stocks were purified using the Adeno-X Virus Purification kit (Clontech Laboratories) and stored at −80°C. The titers of the adenoviral stocks were adjusted to 4.0×10^9 PFU/mL.

Twelve hours after JFH-1 RNA transfection, the cells were washed 3 times with phosphate-buffered saline, and then Ad-shLa or Ad-Null was added at a multiplicity of infection of 10.

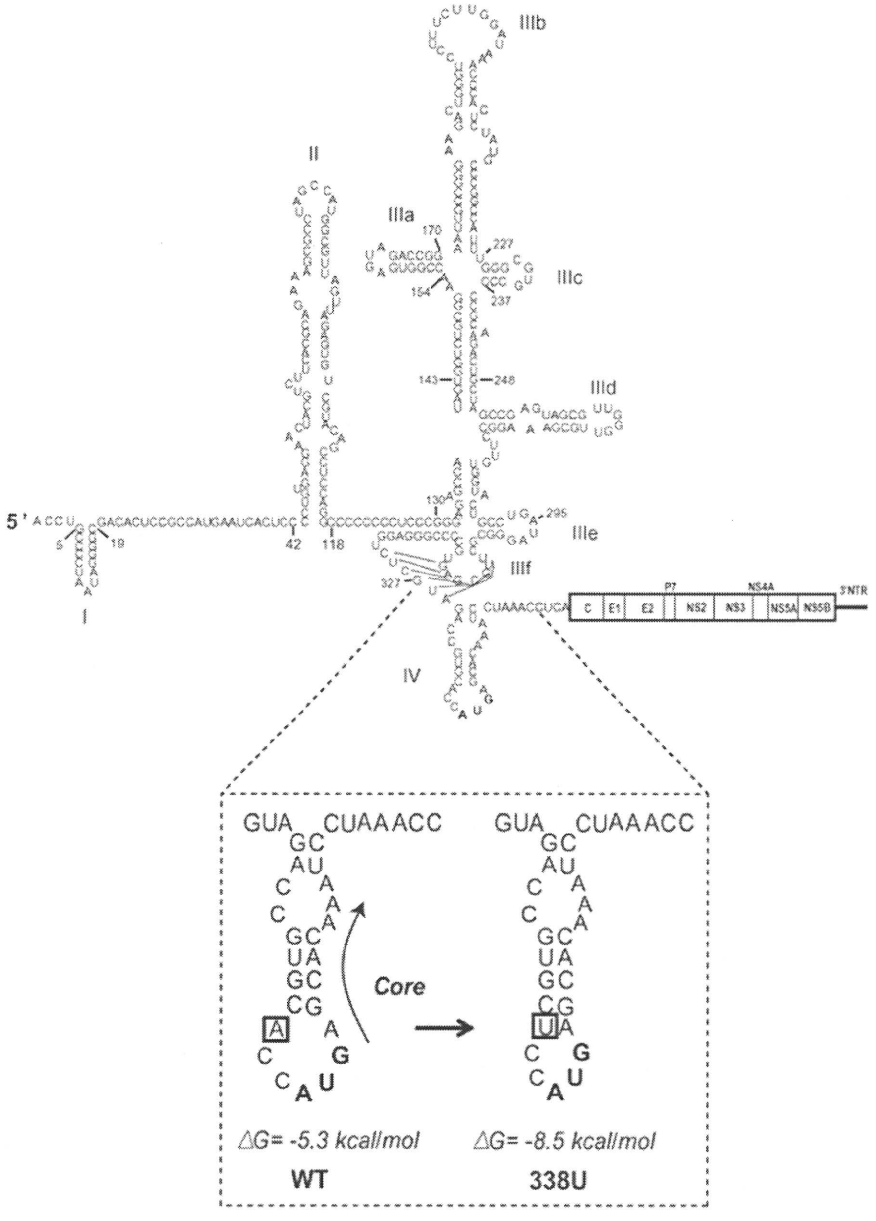

Figure 2. Organization of the full-length JFH-1 and the mutation at nucleotide 338 of stem loop IV.

One hour after injection, the cells were washed 3 times with phosphate-buffered saline, and complete culture medium was added.

Statistical analysis. Results were expressed as mean values ± standard deviation. Significance was tested by 1-way analysis of variance with Bonferroni methods, and differences were considered statistically significant at $P < .05$.

RESULTS

Dependence of HCV IRES activity on translation initiation factors. To confirm that HCV IRES activity was highly dependent on translation initiation factors, antisense oligonucleotides designed for 14 translation initiation factors were transfected into RCF-26 and REF-20 cells, and HCV or EMCV IRES

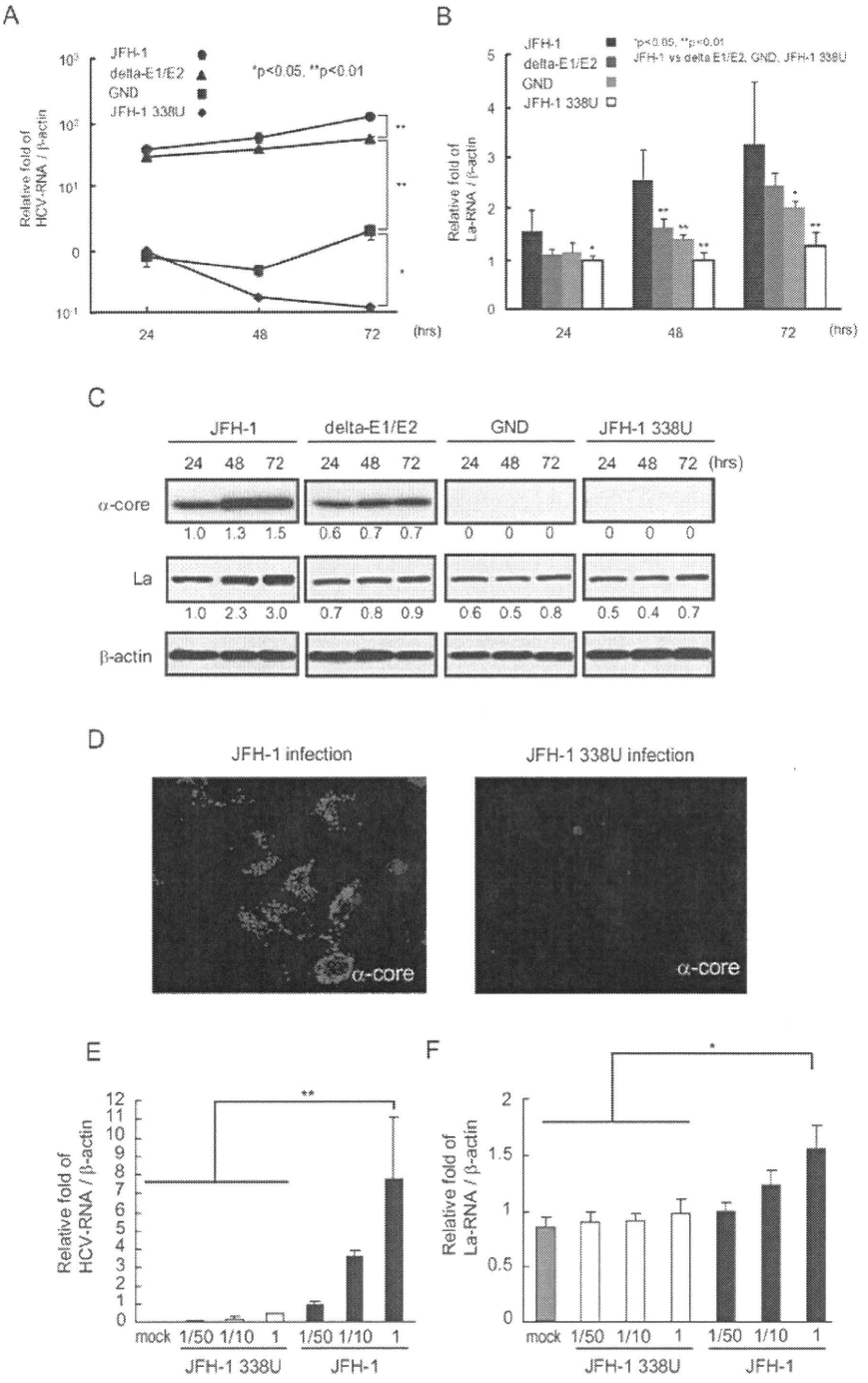

Figure 3. *A*, Hepatitis C virus (HCV) RNA replication determined by real-time detection–polymerase chain reaction (RTD-PCR) in JFH-1, JFH-1/delta E1-E2, JFH-1/GND, and JFH-1 338U transfected cells. *$P < .05$. **$P < .01$. *B*, La RNA expression determined by RTD-PCR in JFH-1, JFH-1/delta E1-E2, JFH-1/GND, and JFH-1 338U transfected cells. *$P < .05$. **$P < .01$. *C*, Western blots for detection of HCV core protein and La protein in JFH-1, JFH-1/delta E1-E2, JFH-1/GND and JFH-1 338U transfected cells. *D*, Immunofluorescence staining of core protein in Huh-7.5 cells infected with JFH-1 or JFH-1 338U. *E* and *F*, HCV RNA and La RNA determined by RTD-PCR in Huh-7.5 cells infected with serial dilution of JFH-1 or JFH-1 338U. *$P < .05$, **$P < .01$.