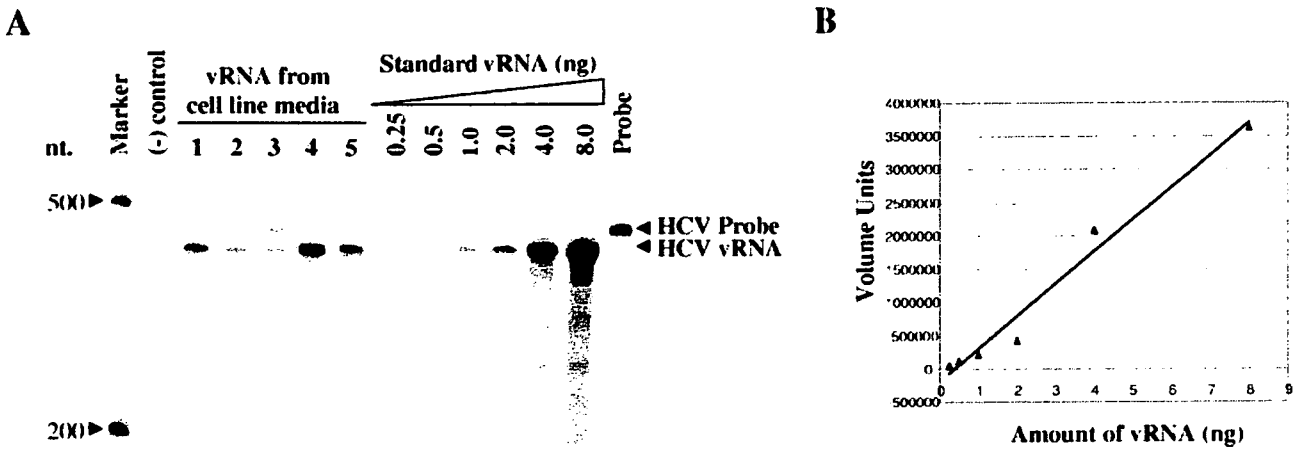

FIG. 5. (A) Determination of the HCV vRNA by RPA. HCV virions in the culture medium (20 to 25 ml) were collected by centrifugation through a 20% sucrose cushion. The vRNA was then extracted with Trizol reagent and dissolved in 50 μl of RNase-free water. A total of 20 μl of vRNA was used to hybridize with 10^5 cpm of the [α-^{32}P]UTP-labeled negative-strand 3′ UTR RNA probe. Various amounts (0.25 to 8 ng as indicated at top) of the in vitro T7 transcripts of the JFH1 HCV vRNA were used as a standard. The RNA products were analyzed in a 6% polyacrylamide–7.7M urea gel, autoradiographed, and quantified with a phosphorimager. Sizes of the RNA molecular markers are indicated at the left and the RNA probe and products are highlighted by arrows at the right. The vRNAs from the culture medium of different cell lines are numbered on the top. The culture medium of naïve Huh7 cells was used as a negative control. (B) Standard curve. The RNA products protected by HCV vRNA transcribed by T7 RNA polymerase in vitro (standard RNA) were quantified with a phosphorimager. The RNA product intensity (*y* axis, volume units) was plotted against the amounts of HCV vRNA (*x* axis).

sequence (Fig. 1). The DNA-based HCV RNA replication system was initially examined by transfection of a subgenomic HCV RNA-expressing DNA into Huh7 cells. The HCV RNA produced by the cellular Pol II polymerase transcription was expressed and replicated in Huh7 cells, as determined by cell colony formation experiments (G. Luo, unpublished results) (8, 25). Additionally, sequence analysis revealed that the cDNA-derived HCV RNA in the cell contained the precise 5′ and 3′ ends of the HCV RNA genome (data not shown).

To construct stable cell lines that produce infectious HCV, the pcDNA6/TR-Tight/JHF1-FL/AR DNA was transfected into Huh7 cells. Cell colonies were selected in the presence of blasticidin (Fig. 1). Initially, stable cell lines were screened for the expression of the HCV NS3 protein by Western blotting using an NS3-specific monoclonal antibody. Among approximately 100 cell lines screened, 9 were found to express high levels of HCV proteins (Fig. 2 and data not shown). Some of the stable cell lines were further characterized (Fig. 2, 3, and 4). High levels of the HCV NS3 and E2 proteins were detected to various extents by Western blotting, depending on the cell line (Fig. 2). When visualized by IFA, nearly all cells expressed high levels of NS3 protein (Fig. 3). The NS3 protein was detected only in cells stained with anti-NS3 monoclonal antibody but not with normal mouse IgG1 (Fig. 3). Additionally, high levels of both positive- and negative-strand HCV RNAs were detected by RPA in the stable cell lines (Fig. 4). The levels of both positive- and negative-strand RNAs are similar among these cell lines. The ratio between the positive- and negative-strand RNAs varies from 25 to 60 (Fig. 4), which differs from a ratio of 5 to 10 found in the HCV replicon-harboring Huh7 cells (23) or in the HCV-infected Huh7.5 cells (see Fig. 9D), suggesting that the positive-strand HCV RNA produced by the Pol II polymerase transcription likely accounted for the higher ratio between the positive- to negative-strand RNA. Collectively, these results demonstrate that the HCV RNA genome produced from the chromosomally integrated cDNA by cellular Pol II polymerase transcription was expressed and replicated efficiently in the cell.

Determination of HCV virions released from the stable cell lines. The question arose whether the stable cell lines containing a chromosomally integrated cDNA of the JFH1 HCV RNA actually secreted virions into the culture medium. The culture medium of each stable cell line was collected and clarified by centrifugation. The HCV virions were then pelleted down by ultracentrifugation through a 20% sucrose cushion. The virus pellet was initially assayed for the HCV E2 protein by Western blotting and then by RPA for HCV vRNA. The Western blotting experiment detected the HCV E2 protein in the pellet (data not shown). The vRNA extracted from the virus pellet was quantified by RPA using an [α-^{32}P]UTP-labeled negative-strand HCV 3′ UTR RNA as a probe (Fig. 5). At the same time, the purified T7 transcripts of the JFH1 HCV RNA genome were used in parallel as a standard for RNA quantification. The standard curve shown in Fig. 5B indicates that RNA products nicely correlate with the amounts of RNA used in the RPA. The HCV vRNA extracted from the culture medium was detected to high levels for all stable cell lines tested (Fig. 5A and Table 1). When quantified with phosphorimager analysis and compared to the standard curve (Fig. 5B), it is estimated that 1 ml of the culture medium contains approximately 2.4×10^7 to 7.5×10^7 copies of HCV vRNA, varying between different cell lines (Fig. 5A and Table 1). These findings suggest that the stable cell lines robustly produced and secreted HCV virions into the culture medium.

Infectivity of HCV virions produced by stable cell lines. To determine the infectivity of HCV virions secreted by the stable cell lines, the naïve Huh7.5 cells were infected with the HCV-containing culture medium. The expression of viral proteins in

TABLE 1. vRNA copies and infectious titers of HCV virions in the culture medium of stable cell lines

Stable cell lines	vRNA copy numbers per ml	Infectious titer per ml
1	4.5×10^7	8×10^3
2	3.0×10^7	9×10^3
3	2.4×10^7	2×10^4
4	7.5×10^7	9×10^4
5	5.4×10^7	4×10^4

the HCV-infected Huh7.5 cells was determined by Western blot analysis, while the positive-strand RNA was detected by RPA. As shown in Fig. 6, the HCV NS3 protein was detected by Western blotting in the HCV-infected Huh7.5 cells (Fig. 6A). Consistent with the levels of the NS3 protein, the positive-strand HCV RNA was also detected by RPA (Fig. 6B). To further determine the infectious titer of HCV, the culture medium was serially diluted by 10-fold and used to infect Huh7.5 cells. The infectious titers were determined by the number of cell foci stained for NS3 by IFA at the lowest dilution point in multiplication with the dilution factor (n-fold) (44). Results are summarized in Table 1. Surprisingly, the infectious titer of HCV was lower than the vRNA copy number detected by RPA (Table 1) by approximately 1,000-fold, suggesting that a large number of HCV RNAs present in the culture are not in an infectious form. These results are consistent with those obtained from transient transfection of HCV RNA (22, 37, 44). To examine the properties of infectious versus noninfectious HCV virions, we performed a continuous 20 to 60% sucrose density gradient sedimentation analysis. HCV virions in each fraction were analyzed by the detection of vRNA and HCV infectivity. The vRNA was mainly detected in the top fractions (fractions 2 to 8), with the most abundance detected in fractions 5 and 6 (Fig. 7A). Similar to findings reported by others (22), the buoyant density of virion-containing fractions varies from 1.06 to 1.16 g/ml (Fig. 7A). By contrast, fractions 3 and 4, with a buoyant density of 1.11 and 1.12, respectively, contain most of the infectious HCV virions, as determined by NS3 expression (Fig. 7B), and positive-strand HCV RNA (Fig. 7C) in Huh7.5 cells infected with HCV of each fraction. Interestingly, fractions 5 and 6, which contain most of the vRNA, were much less infectious (fraction 5) or noninfectious (fraction 6) (Fig. 7). However, the properties of infectious and noninfectious HCV virions remain to be determined.

In an effort to examine the HCV infectious cycle, we performed a time course study of the HCV infection (Fig. 8). The HCV NS3 protein was detected at 36 h p.i. and was increasingly expressed thereafter (Fig. 8A), while the virus egresses into the culture medium as early as 24 h p.i., as shown for the presence of the HCV vRNA determined by RT-PCR (Fig. 8B). The discrepancy in timing of HCV protein and vRNA detection was most likely due to different sensitivity between Western blotting and RT-PCR analyses. Nevertheless, these findings suggest that the HCV RNA was expressed and that it replicated efficiently upon infection. Taken together, our results clearly demonstrate that the stable cell lines produced and secreted infectious HCV into the culture medium.

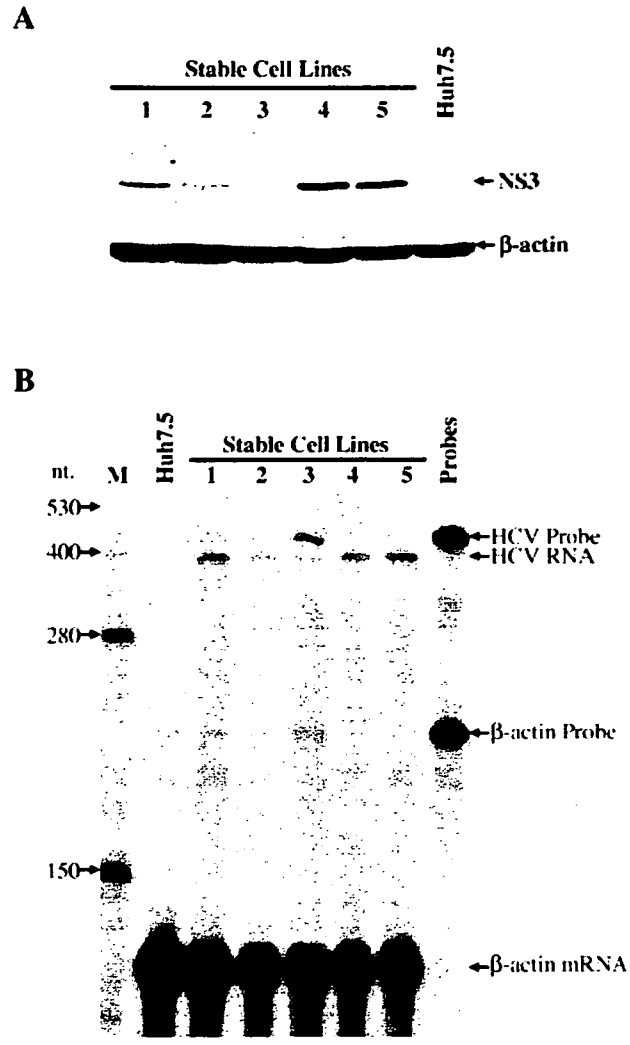

FIG. 6. Determination of the infectivity of the HCV virions secreted by the stable cell lines. The naïve Huh7.5 cells in a six-well plate were incubated with 1 ml of culture medium of each cell line. At 3 h p.i., the HCV-containing medium was replaced with 2 ml of fresh DMEM, and the cells were incubated for 3 days. One set of cells was used for Western blot analysis of the NS3 protein, and the other set was used for isolation of total cellular RNA. (A) Western blotting of the NS3 protein. (B) Determination of positive-strand HCV RNA by RPA. RPA was carried out as described in the legend of Fig. 4. The RNA sizes are indicated at the left. The culture media of the stable cell lines used for infection are numbered at the top.

Inhibition of the HCV infectivity by monoclonal antibodies specific to the HCV envelope glycoproteins E1 and E2 and the HCV putative receptor CD81. To further prove the infectivity of HCV virions produced by the stable cell lines, we performed infectivity neutralization and inhibition experiments using monoclonal antibodies specifically against the HCV E1 and E2 proteins and CD81. Results are shown in Fig. 9. The levels of the HCV NS3 protein were unaffected by increasing concentrations of purified normal mouse IgG1 (Fig. 9A). By contrast, the levels of the NS3 protein were proportionally reduced by treatment with increasing concentrations of the anti-E1/E2

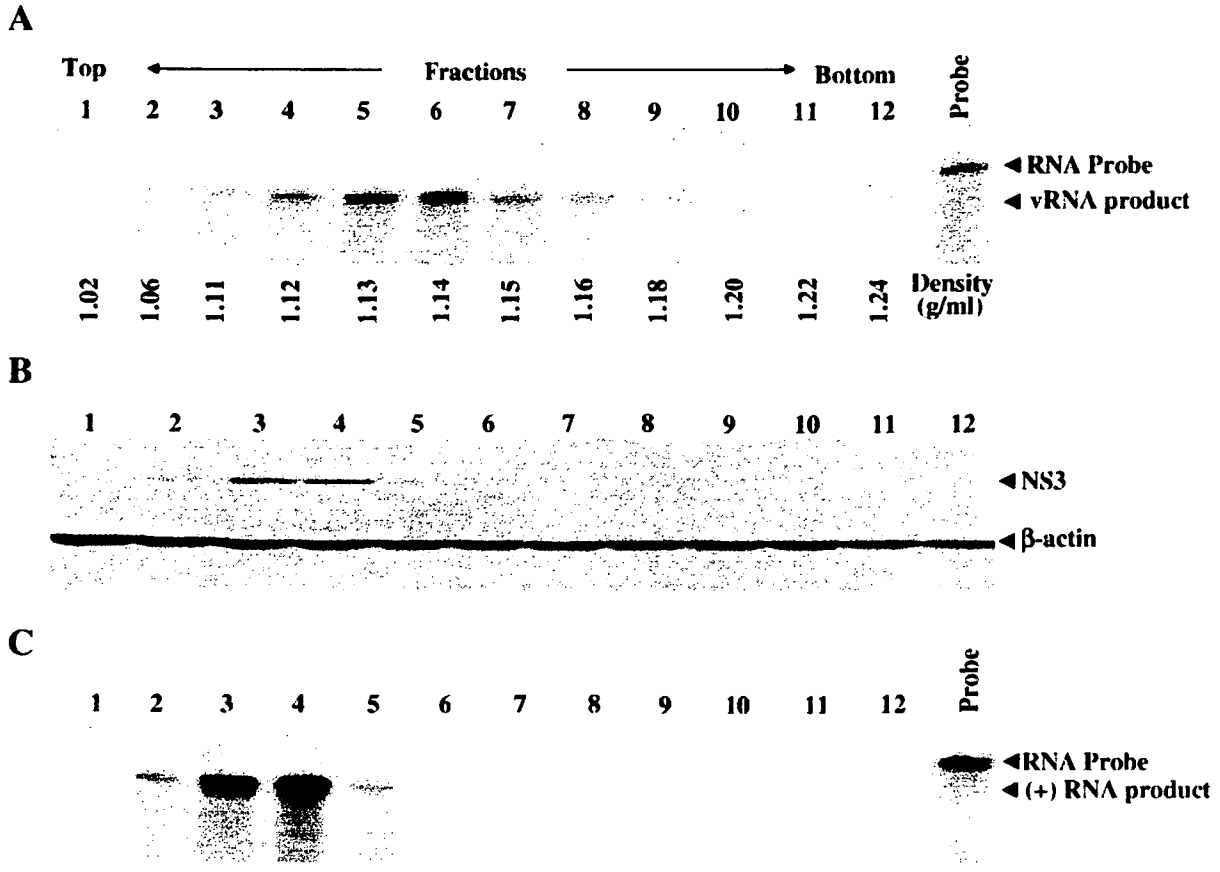

FIG. 7. (A) Sucrose gradient sedimentation analysis of HCV virions in the culture medium. HCV virions in the culture medium were concentrated by centrifugation through 20% sucrose cushion and then fractionated through 20 to 60% continuous sucrose gradient. Fractions (1 ml each) were collected from the top of the sucrose gradient. The HCV vRNA in each fraction (numbered on the top) was extracted with Trizol reagent and then determined by RPA using the [^{32}P]UTP-labeled negative-strand 3′ UTR RNA probe, as described in the legend of Fig. 4. The buoyant density (g/ml) of each fraction is indicated at the bottom. (B) Determination of HCV infectivity in different fractions by Western blotting analysis of NS3 in the infected cells. A total of 100 μl of each fraction was used to infect Huh7.5 cells in one of the six-well plates. Cells were lysed at 3 days p.i., and the NS3 protein was detected by Western blotting. (C) Determination of positive-strand HCV RNA in cells infected with different fractions by RPA. Huh7.5 cells in a six-well culture plate were infected with 100 μl of each fraction. Total RNA was extracted with Trizol reagent at 3 days p.i. The positive-strand HCV RNA was determined by RPA, as described in the legend of Fig. 4. Fraction numbers are indicated at the top.

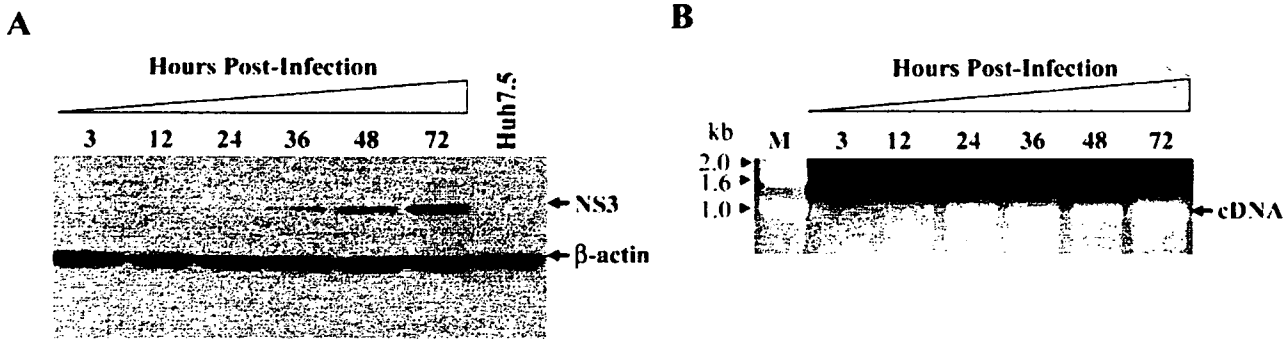

FIG. 8. Time course study of the HCV infection. Huh7.5 cells in a six-well plate were infected with 1 ml of medium of cell line 4. At 3 h p.i., the virus was removed, and cells were washed with PBS and then incubated with 2 ml of DMEM containing 10% FBS. The HCV-infected cells and culture medium were harvested at different time points (indicated at the top of each panel). (A) Detection of the NS3 protein by Western blotting. (B) Detection of the HCV vRNA in the culture medium by RT-PCR. The vRNA was extracted with Trizol reagent and was detected by using a Titan one-step RT-PCR system (Roche). The DNA products were analyzed in a 1% agarose gel and photographed.

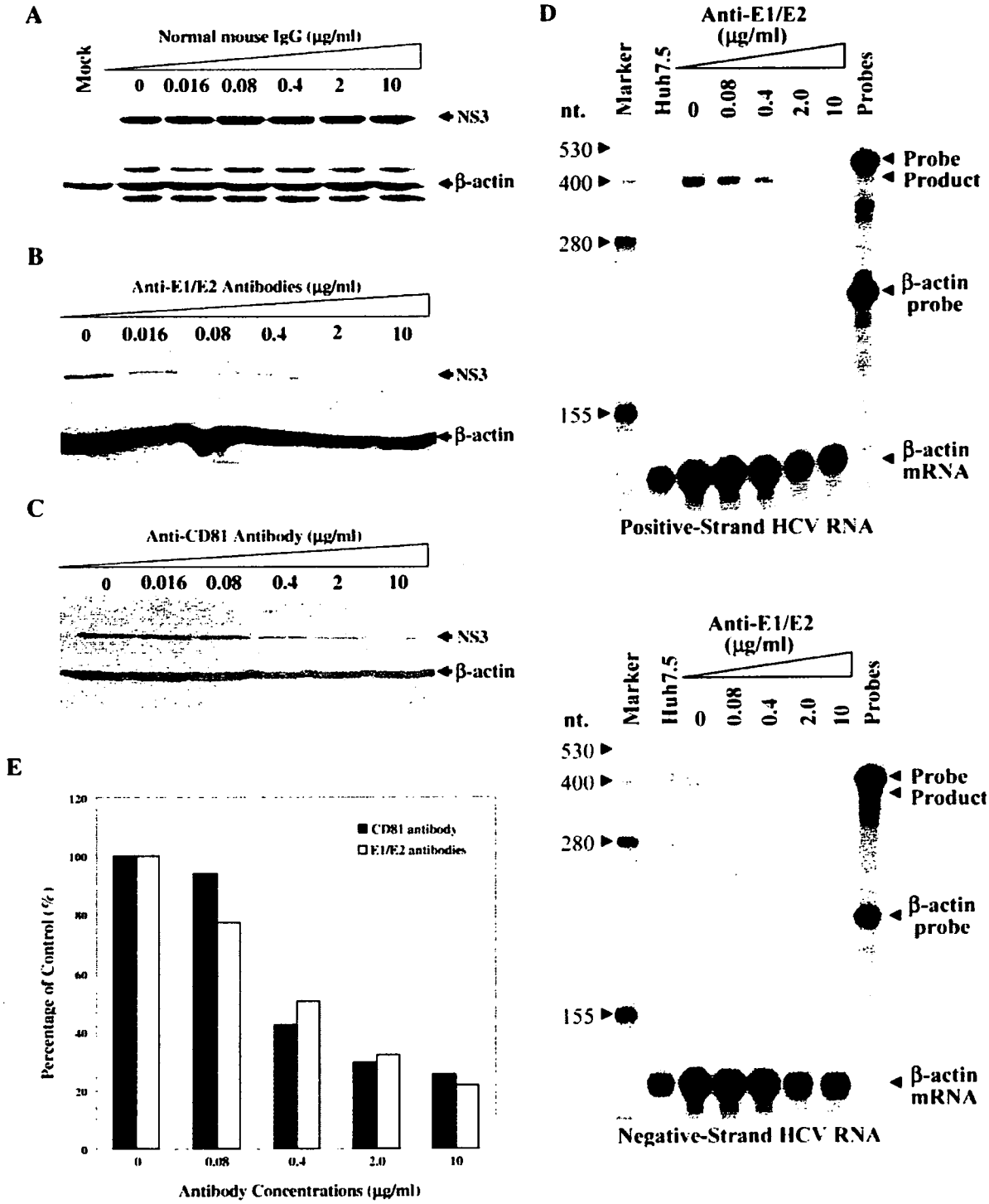

FIG. 9. (A) Effect of normal mouse IgG1 on HCV infection. Huh7.5 cells in a six-well plate were infected with 1 ml of culture medium in the presence of increasing concentrations (μg/ml) of normal mouse IgG1. At 3 h p.i., the HCV-containing medium was removed, and the cells were washed with PBS and incubated with DMEM containing 10% FBS for 3 days. The levels of the NS3 protein were determined by Western blotting. (B) Neutralization of HCV infectivity by anti-E1 and E2 antibodies. The HCV infection is the same as described for panel A except for the use of anti-E1 and anti-E2 antibodies (a mixture of two antibodies in equal amounts). The levels of the NS3 protein were determined by Western blotting. (C) Inhibition of HCV infectivity by CD81 monoclonal antibody. HCV infection was carried out in the presence of increasing concentrations of the CD81 monoclonal antibody; otherwise, the experiment was performed as described for panel A. (D) Determination of the positive- and negative-strand RNAs in the HCV-infected cells treated with anti-E1/E2 antibodies during infection. Half of the RNA sample was used for the determination of the positive-strand RNA, while the other half was used for detection of the negative-strand RNA, as indicated at the bottom. RPA was performed as described in the legend of Fig. 3. The concentrations of anti-E1/E2 antibodies are shown on the top. (E) Dose-dependent neutralization and suppression of the HCV infectivity by monoclonal antibodies against HCV E1/E2 proteins and CD81. The levels of positive-strand HCV RNA were quantified by phosphorimager analysis. The percentage of the RNA level relative to control cells without antibody treatment (100%) was calculated. The percentage of control is plotted against the antibody concentrations.

antibodies (Fig. 9B) or anti-CD81 antibody (Fig. 9C) during HCV infection. Likewise, the levels of both positive- and negative-strand HCV RNAs were reduced by anti-E1/E2 antibodies in a dose-dependent manner (Fig. 9D and E). At 10 μg/ml concentration, both anti-E1/E2 antibodies and CD81 antibody neutralized or inhibited the HCV infectivity by approximately 80% (Fig. 9E). These results demonstrate that the infectivity of HCV virions secreted by the stable cell lines was potently neutralized or suppressed by anti-E1/E2 and CD81 monoclonal antibodies.

Inhibition of HCV replication by IFN-α. We next determined the inhibitory activity of IFN-α against HCV replication. At 3 h p.i., the HCV-infected Huh7.5 cells were incubated with increasing concentrations of IFN-α. At 3 days p.i., cells were harvested, and the level of the NS3 protein was determined by Western blotting. As shown in Fig. 10, IFN-α efficiently inhibited HCV replication in the HCV-infected Huh7.5 cells with a similar efficacy to the inhibition of a subgenomic HCV replicon replication (34).

DISCUSSION

We have established a robust cell culture system that continuously produces and secretes infectious HCV into the culture medium by stable human hepatoma cell lines. The stable Huh7 cell lines were isolated following DNA transfection and selection with blasticidin. The transcriptional expression of the HCV RNA was under the control of a minimal CMV promoter. Upon transcription by the cellular polymerase II and self-cleavage by the HDV ribozyme at the 3′ end, the resulting HCV RNA was expressed and replicated efficiently in the stable cell lines (Fig. 2, 3, and 4). High levels of HCV proteins in the stable cell lines were detected by Western blotting using anti-NS3 and anti-E2 monoclonal antibodies (Fig. 2). Additionally, the IFA experiments revealed that nearly all cells of the stable cell lines expressed HCV proteins, as shown by NS3 staining in nearly every cell (Fig. 3). Furthermore, high levels of both positive- and negative-strand HCV RNAs were detected by RPA (Fig. 4). More importantly, the stable cell lines robustly secret HCV virions into the culture medium. The HCV virions were determined by the detection of the viral envelope glycoprotein E2 and vRNA extracted from the virus pellet (Fig. 5 and data not shown). Based on the amount of HCV vRNA determined by RPA, it is estimated that the titer of HCV virions was detected to nearly 10^8 copies of vRNA per milliliter of culture medium, although this value varied slightly between different cell lines (Fig. 5 and Table 1). However, the infectious titer of HCV virions in the culture medium was significantly lower than its vRNA copy numbers (Table 1) (see below). This finding indicates that many HCV virions are not infectious.

Substantial evidence derived from our studies demonstrates that infectious HCV virions were produced and secreted by the stable cell lines. Both the HCV NS3 protein and the positive-strand HCV RNA were detected by Western blotting and RPA, respectively, in the HCV-infected naïve Huh7.5 cells (Fig. 6 and 7). Upon HCV infection, the HCV proteins could be detected at 36 h p.i. by Western blotting, and the virus released into the culture medium was detected as early as 24 h p.i. by RT-PCR (Fig. 8). The delayed detection of the NS3 protein in the HCV-infected cells was likely due to the differences in assay sensitivity between Western blotting and RT-PCR, as virus must replicate in order to produce progeny virions. Furthermore, the infectivity of HCV virions was potently neutralized by monoclonal antibodies against the HCV envelope glycoproteins E1 and E2 (Fig. 9), consistent with recent findings that the viral entry of recombinant pseudotype viruses was specifically inhibited by anti-E1 and anti-E2 antibodies (2, 3, 21). It is thought that the heterodimeric E1/E2 complex mediates virus entry to target cells by binding to the cell surface receptor(s) like CD81 (27, 28, 43). The infectivity of HCV virions was also inhibited by a CD81 monoclonal antibody in a dose-dependent manner (Fig. 9C and E). It is believed that CD81 serves as an HCV receptor or coreceptor for virus attachment during infection (28). Anti-CD81 antibody was shown to potently block HCV infection and the viral entry of a recombinant human immunodeficiency virus pseudotyped with the HCV E1 and E2 proteins (22, 27). Similar to the HCV replicon replication, the HCV replication in the cell was also suppressed by the treatment with IFN-α (14). Taken together, our findings unambiguously demonstrate the infectivity of the HCV virions produced and secreted by the stable cell lines. Recently, several other independent groups also demonstrated the infectivity of HCV virions produced in Huh7.5 cells that were transiently transfected with in vitro T7 transcripts of JFH1 HCV cDNAs (22, 37, 44).

It is puzzling that the infectious titer of HCV virions produced and secreted by the stable cell lines was about 1,000-fold lower than the vRNA copies (Table 1). This finding is not unique to HCV virions produced by our stable cell lines since it was independently observed for HCV virions generated by transient transfection of HCV RNA into Huh7.5 cells, as described by others (22, 37, 44). Findings derived from sucrose density gradient sedimentation experiments demonstrate that a lower infectious titer of HCV virions than its vRNA copies was due to the presence of a large quantity of noninfectious virions with a buoyant density of above 1.13 g/ml (Fig. 7). This finding is similar to the one reported by Lindenbach et al. (22). However, it is not clear whether the noninfectious virions are defective-interfering particles or the result of a lack of components required for infectivity. Future studies are warranted to further determine the structural properties of HCV virions important for initiating a productive infection.

The HCV replicon systems developed in the past several years have made enormous contributions to the understanding of the mechanisms of HCV replication and anti-HCV drug discovery (1, 4, 23). However, it has not been amenable to perform genetic analysis and manipulation of the infectious HCV. Our recent studies demonstrate that a cDNA-derived HCV genotype 1b RNA generated in the cell was able to produce high levels of HCV virions (15). In this study, we further demonstrate that the HCV of a genotype 2a secreted by stable cell lines is infectious and efficiently replicates in the naïve Huh7.5 cells. The establishment of a stable robust culture system for HCV production and propagation opens up many new avenues to determine each aspect of the entire HCV life cycle. These include the molecular mechanisms of viral entry, assembly, and egression. The infectious virus produced by stable cell lines can be used to determine the cell tropism of infection and replication, including determination of cell re-

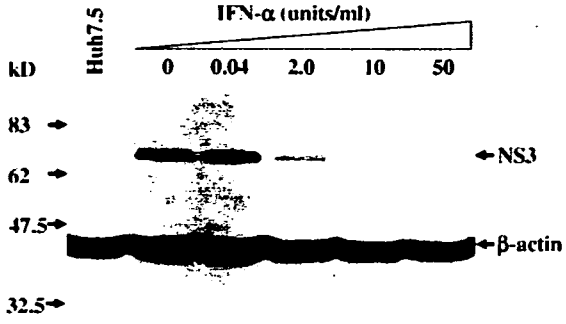

FIG. 10. Inhibition of HCV RNA replication by IFN-α. At 3 h p.i., cells were incubated with DMEM containing increasing concentrations of IFN-α for 3 days. The levels of the NS3 protein were determined by Western blotting. The concentrations of IFN-α are shown at the top.

ceptors and/or coreceptors. Additionally, the stable HCV culture system provides a unique source for robust production of infectious virus that can be used for anti-HCV drug discovery. The HCV infection system is superior to the HCV replicon system as to the identification of antiviral inhibitors in many ways. It covers any target of the HCV life cycle such as the viral entry, viral RNA translation, polyprotein processing and maturation, RNA replication, virus assembly and budding. In fact, the efficient inhibition of the HCV infection by anti-E1/E2 antibodies (Fig. 9) and IFN-α (Fig. 10) provides a proof-of-concept that such a system will be valuable for evaluation of different antiviral strategies to ultimately control HCV infection.

It was previously reported that HCV infection caused significant apoptosis (34). We have not determined whether the replication of HCV in the stable cell lines or in the infected naïve Huh7.5 cells results in any cell death, lytic infection, or cytopathic effects. Grossly, there are no significant changes in cell morphology either in the stable cell lines or in the HCV-infected naïve Huh7.5 cells. It should be noted that the stable cell lines initially grew at a slower rate than the parent cells although they are viable even after numerous (>30) passages. These observations suggest that HCV production and infection did not cause significant lytic and/or cytopathic effects. However, we do not know whether continuous passages of these stable cell lines affect HCV production. It appeared that cells grew at a much slower rate in early passages compared to later passages, suggesting that an adaptation might occur in the cell. It will be interesting to determine the effects of HCV replication on normal cell growth pathways by future investigations. Additionally, it is noteworthy that HCV appears to replicate more efficiently in Huh7.5 cells than in Huh7 cells upon infection (Z. Cai et al., unpublished data). This observation is consistent with a recent finding that the RIG-I (retinoic acid-inducible gene I) with a single amino acid mutation found in Huh7.5 cells caused a defect in the intracellular interferon response to HCV RNA replication, resulting in higher levels of HCV RNA replication (12, 33). It remains to be determined whether HCV infection was able to activate the intracellular antiviral response via a pathway independent of the RIG-I-mediated interferon response, which is specifically induced by double-strand viral RNA.

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Robi ititis C virus infection *in vitro*

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Contributed by Francis V. Chisari, May 1, 2005

The absence of a robust cell culture model of hepatitis C virus (HCV) infection has severely limited analysis of the HCV life cycle and the development of effective antivirals and vaccines. Here we report the establishment of a simple yet robust HCV cell culture infection system based on the HCV JFH-1 molecular clone and Huh-7-derived cell lines that allows the production of virus that can be efficiently propagated in tissue culture. This system provides a powerful tool for the analysis of host–virus interactions that should facilitate the discovery of antiviral drugs and vaccines for this important human pathogen.

CD81 | Huh-7 | viral entry | viral spread | interferon

Hepatitis C virus (HCV) is a noncytopathic positive-stranded RNA virus that causes acute and chronic hepatitis and hepatocellular carcinoma (1). The hepatocyte is the primary target cell, although various lymphoid populations, especially B cells and dendritic cells, may also be infected at lower levels (2–4). A striking feature of HCV infection is its tendency toward chronicity, with at least 70% of acute infections progressing to persistence (1), which is often associated with significant liver disease, including chronic active hepatitis, cirrhosis, and hepatocellular carcinoma (5). Thus, with >170 million people currently infected (5), HCV represents a growing public health burden.

The HCV life cycle and host–virus interactions that determine the outcome of infection have been difficult to study, because cell culture and small animal models of HCV infection are not available. Thus, HCV infection studies to date have involved infected patients (6–8) and chimpanzees (9–12). The recent development of HCV replicon systems has also permitted the study of HCV translation and RNA replication in human hepatoma-derived Huh-7 cells *in vitro* (13, 14), revealing some of the host–virus interactions that regulate these processes (15–19). Nonetheless, these replicons do not replicate efficiently without adaptive mutations (20, 21), nor do they produce infectious virions. Thus, the relevance of replicons to HCV infection is unclear, and they do not permit analysis of the complete viral life cycle.

Wakita and colleagues (22, 23), however, have developed an HCV genotype 2a replicon (JFH-1) that replicates efficiently in Huh-7 cells, other human hepatocyte-derived cells (e.g., HepG2 and IMY-N9) (24), and nonhepatic cells (e.g., HeLa and HEK293) (25) without adaptive mutations. This group also recently reported that Huh-7 cells transfected with *in vitro* transcribed JFH-1 genomic RNA can secrete infectious viral particles.‖ Unfortunately, the infection efficiency observed was low, and infectious particles could not be propagated in naïve Huh-7 cells (26, ‖).

In contrast, we now report the establishment of a robust highly efficient *in vitro* infection system based on Huh-7-derived cell lines and the JFH-1 consensus clone. This system yields viral titers of 10^4-10^5 infectious units per ml of culture supernatant; infection spreads throughout the culture within a few days after inoculation at low multiplicities of infection (moi), and the virus can be serially passaged without loss in infectivity.

Materials and Methods

HCV Constructs and Transcription. The HCV consensus clone used was derived from a Japanese patient with fulminant hepatitis and has been designated JFH-1 (23). Wakita *et al.* (22) cloned this HCV cDNA behind a T7 promoter to create the plasmid pJFH-1, as well as a replication-defective NS5B negative control construct pJFH-1/GND (22). To generate genomic JFH-1 and JFH-1/GND RNA, the pJFH-1 and pJFH-1/GND plasmids were linearized at the 3′ end of the HCV cDNA by XbaI digestion. The linearized DNA was then purified and used as a template for *in vitro* transcription (MEGAscript; Ambion, Austin, TX).

Cell Culture. The hepatic (Huh-7 and Huh-7.5.1) and nonhepatic HEK293 (27) and HeLa (28) cells were maintained in complete DMEM supplemented with 10% FCS/10 mM Hepes/100 units/ml penicillin/100 mg/ml streptomycin/2 mM L-glutamine (Invitrogen) at 5% CO_2. The human promyeloblastic HL-60 and monoblastoid U-937 cell lines were obtained from American Type Culture Collection and cultivated as recommended. The human hepatocarcinoma cell line HepG2 (American Type Culture Collection) (29) and Epstein–Barr virus-transformed B cells were maintained in RPMI medium 1640 with the same supplements described above (Invitrogen).

Huh-7.5.1 cells were derived from the Huh-7.5 GFP-HCV replicon cell line I/5A-GFP-6 (30), kindly provided by Charles Rice (Rockefeller University, New York). The I/5A-GFP-6 replicon cells were cultured 3 weeks in the presence of 100 units/ml human IFN-γ to eradicate the I/5A-GFP-6 replicon. Clearance of the HCV replicon bearing the neomycin resistance gene was confirmed by G418 sensitivity and HCV-specific reverse transcription real-time quantitative PCR (RT-QPCR) analysis.

HCV RNA Transfection. *In vitro* transcribed genomic JFH-1 RNA was delivered to cells by electroporation or liposome-mediated transfection. Electroporation was performed as described by Krieger *et al.* (31). Briefly, trypsinized cells were washed twice with and then resuspended in serum-free Opti-MEM (Invitrogen) at 1×10^7 cells per ml. Ten micrograms of JFH-1 RNA was mixed with 0.4 ml of the cells in a 4-mm cuvette, and a Bio-Rad Gene Pulser system was used to deliver a single pulse at 0.27 kV, 100 ohms, and 960 μF and the cells were plated in a T162 Costar flask (Corning). Liposome-mediated transfection was performed with Lipofectamin 2000 (Invitrogen) at an RNA/lipofectamin ratio of 1:2 by using 5 μg of JFH-1 RNA in cell suspensions containing 10^4 cells. Cells were then plated in DMEM with 20% FCS for overnight incubation. In both cases,

Abbreviations: HCV, hepatitis C virus; moi, multiplicity of infection; RT-QPCR, reverse transcription real-time quantitative PCR; ffu/ml, focus-forming units per milliliter; p.i., postinfection.

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transfected cells were transferred to complete DMEM and cultured for the indicated period. Cells were passaged every 3–5 days; the presence of HCV in these cells and corresponding supernatants were determined at the indicated time points.

RNA Analysis. Total cellular RNA was isolated by the guanidine thiocyanate method by using standard protocols (32). RT-QPCR analysis (for primer sequences, see Fig. 7, which is published as supporting information on the PNAS web site) was performed as described (19, 33), and HCV and GAPDH transcript levels were determined relative to a standard curve comprised of serial dilutions of plasmid containing the HCV JFH-1 cDNA or human GAPDH gene.

Indirect Immunofluorescence. Intracellular staining was performed as described (33). Polyclonal anti-NS5A rabbit antibody MS5 [a gift from Michael Houghton (Chiron)] was used at a dilution of 1:1,000 followed by incubation with a 1:1,000 dilution of Alexa555-conjugated goat anti-rabbit IgG (Molecular Probes) for 1 h at room temperature. Cell nuclei were stained by Hoechst dye.

Titration of Infectious HCV. Cell supernatants were serially diluted 10-fold in complete DMEM and used to infect 10^4 naïve Huh-7.5.1 cells per well in 96-well plates (Corning). The inoculum was incubated with cells for 1 h at 37°C and then supplemented with fresh complete DMEM. The level of HCV infection was determined 3 days postinfection by immunofluorescence staining for HCV NS5A. The viral titer is expressed as focus-forming units per milliliter of supernatant (ffu/ml), determined by the average number of NS5A-positive foci detected at the highest dilutions.

Amplification of HCV Viral Stocks. To generate viral stocks, infectious supernatants were diluted in complete DMEM and used to inoculate naïve 10–15% confluent Huh-7.5.1 cells at an moi of 0.01 in a T75 flask (Corning). Infected cells were trypsinized and replated before confluence at day 4–5 postinfection (p.i.). Supernatant from infected cells was then harvested 8–9 days p.i. and aliquoted for storage at −80°C. The titer of viral stock was determined as described above.

Concentration and Purification of HCV. Sucrose density-gradient ultracentrifugation analysis was performed as described (34). Pooled supernatant from two mock or two HCV-infected T162 cm^2 Costar flasks (Corning) were centrifuged at 4,000 rpm for 5 min to remove cellular debris and then pelleted through a 20% sucrose cushion at 28,000 rpm for 4 h by using a SW28 rotor in an L8–80M ultracentrifuge (Beckman). The pellet was resuspended in 1 ml of TNE buffer (50 mM Tris·HCl, pH 8/100 mM NaCl/1 mM EDTA) containing protease inhibitors (Roche Applied Science, Indianapolis), loaded onto a 20–60% sucrose gradient (12.5-ml total volume), and centrifuged at 120,000 × *g* for 16 h at 4°C in a SW41Ti rotor (Beckman). Fractions of 1.3 ml were collected from the top of the gradient. The fractions were analyzed by RT-QPCR to detect HCV RNA. To determine the infectivity titer of each fraction, fractions were titrated on Huh7.5.1 cells as described above.

Blocking Infection with CD81- and E2-Specific Antibodies. Recombinant human monoclonal (IgG1) anti-E2 antibody was derived from a cDNA expression library (prepared from mononuclear cells of a HCV patient) that was screened against recombinant HCV genotype 1a E2 protein (GenBank accession no. M62321) by phage display. The antibody was serially diluted and preincubated with 15,000 ffu of JFH-1 virus in a volume of 250 μl for 1 h at 37°C. The virus antibody mixture was then used to infect 45,000 Huh-7.5.1 cells in a 24-well plate (Corning) for 3 h at 37°C. Mouse monoclonal anti-human CD81 antibody 5A6 (35) at 1 mg/ml (a gift from Shoshana Levy, Stanford University, Stanford, CA) was serially diluted (1:2,000, 1:200, and 1:20) and preincubated in a volume of 50 μl with 10^4 Huh-7.5.1 cells seeded in a 96-well plate for 1 h at 37°C. Cells were subsequently inoculated with infectious JFH-1 supernatant at an moi of 0.3 for 3 h at 37°C. The efficiency of the infection in the presence of antibodies was monitored 3 days p.i. by RT-QPCR and immunofluorescence.

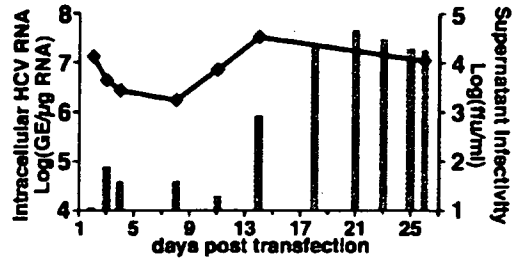

Fig. 1. Production of infectious HCV after transfection of genomic JFH-1 RNA. Ten micrograms of *in vitro* transcribed JFH-1 RNA was electroporated into 4 × 10^6 Huh-7.5.1 cells. Transfected cells and supernatant were harvested at the indicated time points posttransfection. Intracellular HCV RNA was analyzed by RT-QPCR and displayed as genome equivalents (GE)/μg total RNA (line). Supernatant infectivity titers were determined in naïve Huh-7.5.1 cells and are expressed as ffu/ml (bars).

Results

Production of Infectious HCV Particles in HCV RNA-Transfected Hepatoma Cells. Blight *et al.* (36) have established an Huh-7-derived cell line, termed Huh-7.5, that is highly permissive for replication of HCV replicons, including the I/5A-GFP-6 replicon (30) that expresses an NS5A-GFP fusion protein. For the current study, we cured I/5A-GFP-6 replicon cells with IFN-γ (see *Materials and Methods*) establishing an HCV-negative Huh-7.5-derived cell line, which we refer to as Huh-7.5.1. In a first set of experiments, 10 μg of *in vitro* transcribed genomic JFH-1 RNA was delivered into Huh-7.5.1 cells by electroporation. Transfected cells were then passaged when necessary (usually every 3–4 days) to maintain subconfluent cultures throughout the experiment. At the indicated times, total RNA was isolated from the transfected Huh-7.5.1 cells, and the level of HCV RNA was determined by HCV-specific RT-QPCR. Two days posttransfection, 1.3 × 10^7 copies of HCV RNA per μg of cellular RNA were detected (Fig. 1), probably reflecting a combination of input RNA and RNA produced by intracellular HCV replication. HCV RNA levels subsequently decreased, reaching a minimum level of 1.6 × 10^6 copies per μg of cellular RNA at day 8 posttransfection (Fig. 1). Importantly, however, intracellular HCV RNA levels began to increase thereafter, reaching maximal levels of >10^7 copies per μg of total RNA by day 14 posttransfection, and these levels were maintained until the experiment was terminated on day 26 (Fig. 1). These results suggested that HCV was actively replicating in transfected Huh-7.5.1 cells. This notion is supported by a rapid disappearance of a replication-incompetent JFH-1 RNA genome after transfection (Fig. 7).

Interestingly, immunofluorescence staining for NS5A indicated that the percentage of NS5A-positive cells in the transfected cell cultures increased from 2% on day 5 (Fig. 2*A*) to almost 100% on day 24 (Fig. 2*B*). These results were consistent with the amplification of HCV RNA and further suggested either that HCV-transfected cells had acquired a selective growth advantage or that HCV was spreading to untransfected cells within the culture.

To determine whether the JFH-1-transfected Huh-7.5.1 cells were releasing infectious virus, we inoculated naïve Huh-7.5.1

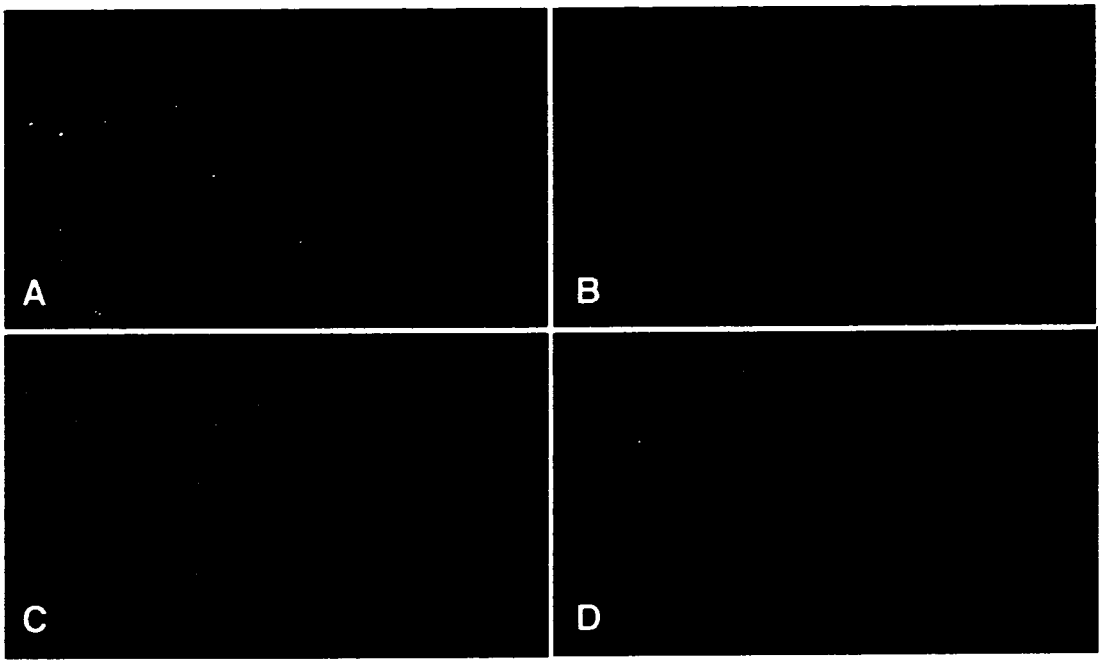

Fig. 2. Detection of infected cells by NS5A immunofluorescence. (*Upper*) Immunofluorescent detection of NS5A in transfected cells: (*A*) day 5 and (*B*) day 24 posttransfection. (*Lower*) Infectivity titration of transfected cell supernatant on naïve Huh-7.5.1 cells; (*C*) undiluted supernatant; (*D*) 10-fold diluted supernatant. NS5A staining in red; nuclei stained with Hoechst (blue) (×50).

cells with supernatants collected at different time points during the transfection experiment. Not only did immunofluorescence staining 3 days postinoculation reveal NS5A positive cells in the culture (Fig. 2*C*) but also, when the supernatants were serially diluted, the infection resulted in discrete foci of NS5A-positive cells (Fig. 2*D*), which allowed us to determine the ffu/ml in the supernatants collected at different times posttransfection. This type of supernatant titration was performed for the transfection experiment described in Fig. 1 and is indicated by vertical bars (Fig. 1). Infectious virus was detected in the culture medium 3 days after transfection (80 ffu/ml) and then increased, reaching a maximum of 4.6×10^4 ffu/ml by day 21 posttransfection, concomitant with the amplification of intracellular JFH-1 RNA.

Taken together, these results strongly suggest that Huh-7.5.1 cells transfected with genomic JFH-1 RNA were able not only to support HCV replication but also to produce infectious HCV particles. Notably, similar results were obtained when JFH-1 RNA was delivered to Huh-7.5.1 cells by an alternative transfection method (i.e., liposomes; Fig. 8, which is published as supporting information on the PNAS web site).

Propagation of HCV Virus Generated by Transfection. Next, we determined whether cells infected with JFH-1-transfected cell supernatant produced progeny virus that could be serially passaged to naïve Huh-7.5.1 cells. To do so, we infected naive Huh-7.5.1 cells at low multiplicity (moi = 0.01) with infectious supernatants collected from two independent transfection experiments and monitored infectious virus production by titrating the infected cell supernatants at the indicated time points. On day 1 after inoculation, no infectious particles were detectable in the supernatant of cells infected with either transfection cell inoculate (Fig. 3*A*). However, infectious particles exponentially accumulated in the supernatant thereafter, reaching a maximal titer of at least 10^4 ffu/ml on day 7 after both infections (Fig. 3*A*). Thus, within 7 days p.i., HCV produced in two independent transfection experiments was amplified in naïve Huh-7.5.1 cells >100-fold with very similar kinetics. Of note, we also performed additional experiments in which we monitored the intracellular levels of HCV RNA and proteins. This analysis confirmed that the appearance of infectious virus in the cell culture supernatant directly correlated with the amplification and subsequent translation of the input HCV RNA (Fig. 9, which is published as supporting information on the PNAS web site).

To determine whether the progeny virus produced by infection could be further passaged, we infected naive Huh-7.5.1 cells with the virus collected from one of the experiments shown in Fig. 3*A* (lipofection). As shown in Fig. 3*B*, this secondary infection progressed with kinetics similar to the primary infection (Fig. 3*A*), again reaching maximal levels on day 7. This was reflected by increasing numbers of NS5A-positive cells over the time course of the infection, with almost all of the cells being positive for NS5A at day 7 (Fig. 3*C*). These results indicate that the JFH-1 virus generated by transfection can be passaged in Huh-7.5.1 cells without a detectable loss in infectivity, and that it infects a high proportion of the cells in a relatively short period.

HCV Infection Is Inhibited by Anti-E2 Antibodies. Previous studies using HCV surface glycoprotein (E1/E2) pseudotyped viruses (37, 38) have suggested that E1 and/or E2 mediate the interaction with cellular receptors that are required for viral adsorption. To verify whether such an interaction is required for HCV infection *in vitro*, we performed neutralization experiments using anti-E2 antibodies in which the JFH-1 virus was preincubated with serial dilutions of a recombinant human monoclonal antibody specific for HCV E2 or an isotype-negative control antibody for 3 h at 37°C before infection.

Huh-7.5.1 cells infected with JFH-1 virus (moi = 0.3) in the presence of 100 μg/ml of anti-E2 antibody were found to have 5-fold lower intracellular HCV RNA levels compared with cells infected in the presence of the same amount of an isotype control antibody (Fig. 4*A*), and this was reflected by a reduction in NS5A-positive cells as determined by immunofluorescence

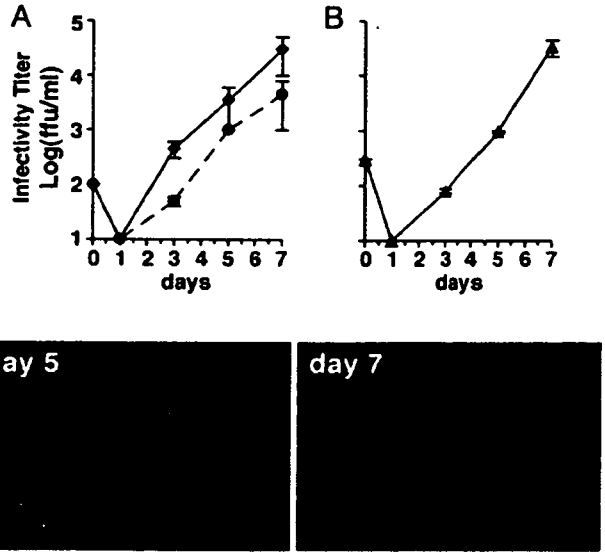

Fig. 3. HCV infection kinetics and passage in tissue culture cells. Naïve Huh 7.5.1 cells were inoculated with culture supernatants at an moi of 0.01. Supernatants from the inoculated cells were collected at the indicated times p.i. and evaluated for infectivity (ffu/ml). Data represent the average of two or more experiments with error bars. (*A*) Huh-7.5.1 cells inoculated with supernatant harvested at day 19 after transfection of Huh-7.5.1 cells with JFH-1 genomic RNA by electroporation (dashed line) or day 24 after lipofection (solid line). (*B*) Huh-7.5.1 cells inoculated with supernatant collected at day 5 from the infection shown as a solid line in *A*; (*C*) Increasing NS5A immunostaining in Huh-7.5.1 cells between days 5 and 7 p.i. in the experiment shown in *B*.

(data not shown). Titration of the anti-E2 antibody indicated that 10 μg/ml of antibody was required for a 50% reduction in intracellular HCV RNA 3 days p.i. (Fig. 4*A*). These results are therefore consistent with the conclusion that *in vitro* HCV infection in this system is at least in part mediated by the viral envelope E2 protein.

HCV Infection Is Inhibited by Anti-CD81 Antibodies. Previous studies using pseudotyped viruses that express HCV E1/E2 have also suggested that the interaction between HCV E2 and CD81 is crucial for viral entry (39). To determine whether CD81 is required in this HCV infection system, anti-CD81 antibody-pretreated naïve Huh-7.5.1 were infected with JFH-1 virus at an moi of 0.3 and analyzed 3 days p.i. Intracellular HCV RNA levels

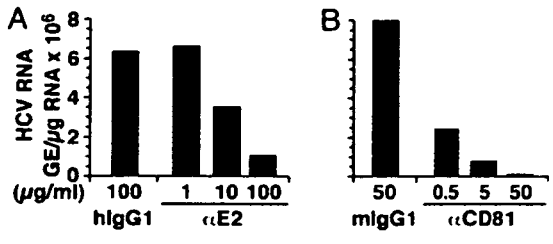

Fig. 4. Inhibition of HCV infection by anti-E2 and anti-CD81 antibodies. (*A*) JFH-1 virus was preincubated with the indicated concentrations of anti-E2 antibody or irrelevant human IgG1 antibody for 1 h at 37°C before inoculating Huh-7.5.1 cells. Total cellular RNA was analyzed by RT-QPCR at day 3 p.i. (*B*) Huh-7.5.1 cells were preincubated with the indicated concentrations of anti-human CD81 or control mouse IgG1 antibody for 1 h at 37°C before inoculation with JFH-1 virus at an moi of 0.3. Total cellular RNA was analyzed by RT-QPCR at day 3 p.i.

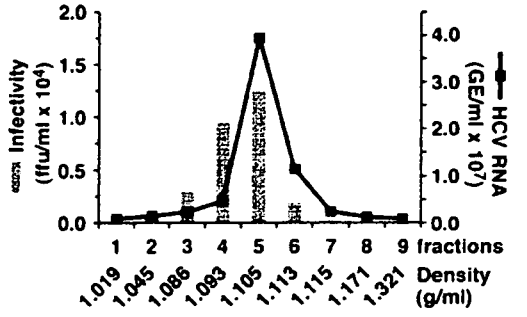

Fig. 5. Sucrose gradient sedimentation of infectious HCV. Supernatant from infected Huh-7.5.1 cells was fractionated as described in *Materials and Methods*. Fractions (1–9) were collected from the top of the gradient and analyzed by RT-QPCR for HCV RNA (line). The infectivity of each fraction was determined (bars) by titration. Fraction densities are expressed as g/ml.

were reduced in a dose-dependent manner with a 50-fold reduction at 50 μg/ml anti-CD81 antibody compared with the control antibody-treated cells (Fig. 4*B*).

Biophysical Properties of Infectious HCV JFH-1 Particles. To examine the density of the secreted infectious HCV virions, supernatants collected from uninfected and HCV-infected Huh7.5.1 cells were subjected to sucrose gradient centrifugation. Gradient fractions were collected after centrifugation and analyzed for the presence of HCV RNA and infectivity (Fig. 5). Maximal infectivity titers (1.25×10^4 ffu/ml) were present in fraction 5 and coincided with the peak of HCV RNA. The ≈1.105 g/ml apparent density of the peak infectivity fraction is consistent with that previously reported for HCV virions isolated from patient sera (40, 41), indicating that the density of the recombinant JFH-1 virus is similar to that of human isolates.

***In Vitro* Tropism of JFH-1 HCV.** To determine whether infection with the JFH-1 virus was restricted to Huh-7.5.1 cells, we attempted to infect a panel of hepatic (Huh-7 and HepG2) and nonhepatic cell lines (HeLa, HEK293, HL-60, U-937, and EBV-transformed B cells). Besides the Huh-7.5.1 cells, only the Huh-7 cells were permissive for HCV infection, as determined by immunofluorescent staining for the viral NS5A protein at day 3 p.i. (data not shown).

To determine whether there are quantitative differences in infection efficiency between the Huh-7.5.1 and Huh-7 cells, we infected both cell lines in parallel. As shown in Fig. 6, infectious particle release into the supernatant of infected Huh-7 cells

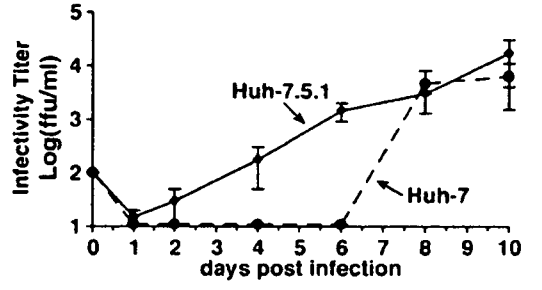

Fig. 6. Kinetics of JFH-1 HCV infection in Huh-7.5.1 and Huh-7 cells. A virus stock generated in Huh-7.5.1 was diluted to infect Huh-7.5.1 and Huh-7 cells at an moi of 0.01. Culture supernatant was collected at the indicated times and titrated. Infectious titers in Huh-7.5.1 (solid lines) and Huh-7 cells (dashed lines) are expressed as ffu/ml. Average values of two independent infection experiments are shown.

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appears tc ompared with the particle production by H vertheless, Huh-7 cells produced similar am particles by days 8 and 10. Similar delayed ki ... amplification of intracellular HCV RNA was also observed in the Huh-7 cells (Fig. 10, which is published as supporting information on the PNAS web site). Taken together, these results demonstrate that Huh-7 cells can produce similar amounts of progeny virus as Huh-7.5.1 cells but with delayed kinetics.

Discussion

The absence of a robust cell culture model of HCV infection has limited the analysis of the HCV life cycle and the development of effective antivirals and vaccines. Despite intensive efforts by many groups and the finding that *in vitro* transcribed HCV RNA is infectious when transfected into the liver of chimpanzees (42, 43), production of infectious HCV *in vitro* has remained problematic. Although a cell line capable of producing HCV-like virions was recently reported, the infectivity of those particles was not demonstrated (34). Furthermore, until recently, the replication efficiency of HCV replicons has depended on adaptive mutations (reviewed in ref. 44) that are attenuating in chimpanzees (45).

Interestingly, Kato *et al.* (22) produced an HCV genotype 2a replicon (JFH-1) that replicates efficiently in Huh-7 cells without adaptive mutations. Importantly, Wakita *et al.* (26, ‖) have also reported that JFH-1 RNA generated from the genomic JFH-1 viral clone can produce virus when transfected into Huh-7 cells. The limitation of this discovery has been the low efficiency of infection achieved and the resulting inability to perform controlled infection experiments.

Here, we report the establishment of a simple yet robust cell culture HCV infection system based on the reverse genetics system designed by Wakita *et al.* (26, ‖), which allows the rescue of infectious virus from the JFH-1 consensus cDNA clone. Our results demonstrate that transfection of JFH-1 RNA into the Huh-7-derived cells allows for the recovery of a viable JFH virus that can then be serially passaged and used for infection-based experimentation. Impressively, infection with serial dilutions of the virus resulted in the formation of infected cell foci that allowed us to quantitatively titrate the HCV being produced. Although the molecular basis of this viral spread pattern requires additional investigation, one can speculate that after infection at low multiplicity, HCV may spread by multiple mechanisms such as by attaching preferentially to adjacent cells after secretion and/or by spreading via cell-to-cell transmission.

The ability to monitor the complete HCV infection process *in vitro* has already provided some insight into the HCV life cycle. Specifically, the disappearance of input virus from the supernatant within 24 h p.i. suggests that particles were able to enter the cells within this time frame. As infectious viral titers rose from these undetected levels to 10^4-10^5 ffu/ml, the number of NS5A-positive cells also increased, suggesting that the virus was spreading to new cells (Fig. 3*C*). Importantly, when passaged to naïve Huh-7.5.1 cells, the virus produced by both transfected and infected cells exhibited the same infection kinetics with an HCV doubling time of ≈22 h. This doubling time is longer than the 6–8 h previously reported in infected patients (46) and chimpanzees (47); however, technical and biological factors may be responsible for this discrepancy. For example, earlier estimates were based on the number of HCV genome equivalents detected in the serum of infected individuals, not the infectivity titer as in the current study. The different doubling times may also reflect genotypic differences between the infecting viruses in the various studies and/or biological differences between Huh-7.5.1 cells and primary hepatocytes in the context of the liver.

The tropism of the JFH-1 virus thus far appears to be limited to Huh-7-derived cell lines. Even HepG2, HeLa, and HEK293 cells, which have been shown to support replication of the subgenomic JFH-1 replicon (22, 24, 25), failed to become infected. This suggests that the block to HCV infection in these cells may exist before RNA replication and translation. Importantly, that an antibody directed against the viral surface glycoprotein E2 reduced the infectivity of the JFH-1 virus suggests the process of viral adsorption and entry can be studied in this system. Consistent with this claim, HCV infection of Huh-7-derived cells was inhibited by an antibody against CD81 (Fig. 4), an extensively characterized putative HCV receptor (48).

Our initial efforts focused on the use of Huh7.5.1 cells, a cell line derived from Huh7.5 cells that has been shown to be highly permissive for HCV genotype 1 replicon replication (36). Importantly, however, non-HCV-adapted Huh-7 cells were also found to be susceptible to infection with the JFH-1 virus (Fig. 6). Virus amplification in Huh-7 cells did tend to display slower kinetics, but the Huh-7 cells eventually produced viral titers comparable to those attained in Huh-7.5.1 cells. The specific characteristics of the Huh-7.5.1 cells might be crucial in the future, because we expect mutants with reduced viability to be produced during reverse genetics studies investigating HCV biology.

Although the different kinetics of virus production in these two Huh-7-based cell lines is still under continued investigation, at this point, the differences observed are compatible with the idea that HCV infection may induce an innate antiviral response in the Huh-7 cells that is compromised in the Huh-7.5.1 cells. Specifically, it has been demonstrated that Huh-7.5 cells contain an inactivating mutation in RIG-I (49), a key component of the cellular double-stranded RNA-sensing machinery (50). Hence, our results suggest that HCV infection may induce a double-stranded RNA antiviral defense pathway in the Huh-7 cells, which transiently delays viral replication and/or spread. That HCV eventually overcomes the limitations present in Huh-7 cells and reaches titers similar to those produced by Huh-7.5.1 cells further suggests that expression of one or more viral encoded functions (e.g., NS3 or NS5A) may block or negate the intracellular antiviral defense(s). HCV infection, however, remained sensitive to the effects of exogenously added IFN, because both IFN-α and -γ prevented JFH-1 virus infection of Huh-7.5.1 cells (Fig. 11, which is published as supporting information on the PNAS web site). Interestingly, these *in vitro* observations appear to parallel those seen clinically (5, 46, 51), in which IFN therapy is able to reduce viral titers in some patients regardless of the mechanisms the virus has evolved that allow it to persist in the presence of the IFN it induces. Ongoing comparison of HCV infection in these different Huh-7-based cell lines should provide a unique opportunity to dissect and understand these critical virus–host interactions.

Conclusion

We have established a robust cell culture model of HCV infection in which infectious HCV can be produced and serially passaged to naïve cells. The availability of this system creates the opportunity to address aspects of the virus life cycle, including entry, trafficking, viral assembly, and egress, that have not been previously approachable. Likewise, many aspects of the host–virus relationship can now be explored, particularly the proviral factors that are required for viral production and the antiviral factors that control HCV infection. In addition to the deeper understanding of basic HCV infection biology that should be forthcoming from this system, it creates new investigative opportunities that could greatly accelerate the discovery of antiviral drugs and contribute to the development of an HCV vaccine.

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TECHNICAL REPORTS

Production of infectious hepatitis C virus in tissue culture from a cloned viral genome

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Hepatitis C virus (HCV) infection causes chronic liver diseases and is a global public health problem. Detailed analyses of HCV have been hampered by the lack of viral culture systems. Subgenomic replicons of the JFH1 genotype 2a strain cloned from an individual with fulminant hepatitis replicate efficiently in cell culture. Here we show that the JFH1 genome replicates efficiently and supports secretion of viral particles after transfection into a human hepatoma cell line (Huh7). Particles have a density of about 1.15–1.17 g/ml and a spherical morphology with an average diameter of about 55 nm. Secreted virus is infectious for Huh7 cells and infectivity can be neutralized by CD81-specific antibodies and by immunoglobulins from chronically infected individuals. The cell culture–generated HCV is infectious for chimpanzee. This system provides a powerful tool for studying the viral life cycle and developing antiviral strategies.

HCV is a major cause of chronic liver diseases[1,2]. Development of selectable drugs and efficient vaccines has been hampered by poor virus growth in cell culture[3]. Although subgenomic replicons replicate efficiently in cultured cells[4], for unknown reasons infectious viral particles are not produced[5,6]. Subgenomic replicons of the HCV genotype 2a JFH1 strain cloned from an individual with fulminant hepatitis[7] replicate efficiently in Huh7 cells and do not require cell culture–adaptive mutations[8–10]. In this study, we show that transfection of *in vitro*–transcribed full-length JFH1 RNA into Huh7 cells results in secretion of viral particles that are infectious for cultured cells and a chimpanzee.

RESULTS

Virus production from cells transfected with full-length JFH1 RNA

We transfected *in vitro*–transcribed RNAs corresponding to the full-length JFH1 genome (**Fig. 1a**) and a replication-incompetent mutant (JFH1/GND) into Huh7 cells. Total RNAs from cells harvested at different time points were analyzed by northern hybridization (**Fig. 1b**). Up to 12 h after transfection, we detected only degraded input RNA. But genome-length RNA was found in JFH1-transfected cells after 24 h, and remained detectable up to 72 h. The same was true for expression of viral proteins (**Fig. 1c** and **Supplementary Fig. 1** online). Immunofluorescence analysis of JFH1-transfected cells showed that 70–80% of the cells were positive for core and nonstructural (NS) 3 proteins at 72 h after transfection (**Fig. 1d**), indicating that this genome replicates to high levels in transfected Huh7 cells.

As a surrogate for virus production, we quantified secretion of core protein into the culture medium of cells transfected with JFH1 mutants JFH/GND and JFH1/ΔE1-E2 or full-length genomes of different origin (J6CF[11] or JCH1 (ref. 7)). Core protein was secreted efficiently from JFH1 RNA but much less efficiently from JFH1/ΔE1-E2 RNA–transfected cells (**Fig. 1e**), despite comparable core protein levels. No core protein was secreted from the other RNA-transfected cells, consistent with their lack of replication (data not shown). These results suggest that core secretion depends on HCV RNA replication and that envelope glycoproteins are required, although the RNA segment deleted in JFH1/ΔE1-E2 may contain signal(s) required for core secretion.

To determine whether JFH1 RNA–transfected cells can sustain continuous HCV replication, we serially passaged cells. Cells transfected with JFH1/ΔE1-E2 RNA or a subgenomic replicon (SGR-JFH1)[8] lacking the core to NS2 region served as controls. We determined HCV RNA titer using real-time detection reverse transcription (RTD)-PCR[12]. In JFH1-transfected cells, viral RNA and core protein titers in the medium increased rapidly at 5 d after transfection, and remained high for the next 7 d, followed by a slow decrease (**Fig. 2a**). In contrast, RNA levels in supernatant of control cells gradually decreased with increasing passage. At day 30, background levels were reached, which were 4.5% for JFH1/ΔE1-E2– and 0.7% for SGR-JFH1 RNA–transfected cells as compared to JFH1 (**Fig. 2a**). During the first two passages, transfected cells had similar levels of intracellular HCV RNA, but they declined much more rapidly in the controls (**Fig. 2a**). We also determined levels of core protein and obtained similar results (**Supplementary Fig. 2**

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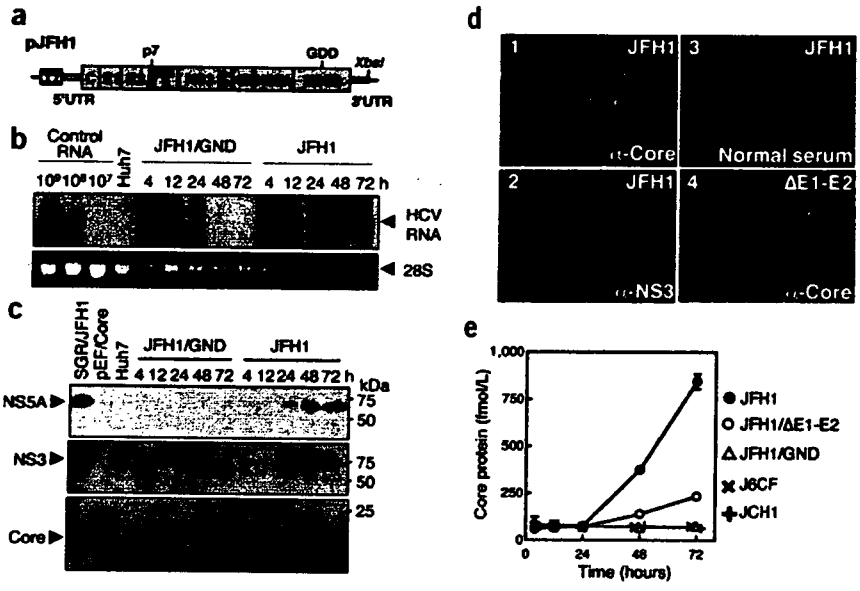

Figure 1 Transient replication of JFH1 RNA in transfected Huh7 cells. (**a**) Organization of the full-length HCV construct pJFH1. Open reading frames (thick boxes) are flanked by the 5′- and 3′-UTRs (thin boxes). T7, T7 RNA polymerase promoter; GDD, active-site motif of NS5B polymerase; *Xba*I, restriction site. (**b**) Northern blot analysis of total RNA prepared from cells transfected with full-length JFH1 and JFH1/GND RNA. Control RNA, given numbers of synthetic HCV RNA; Huh7, RNA isolated from naive cells. Arrowheads indicate full-length HCV RNA and 28S ribosomal RNA (28S). Upper panel, northern blot; lower panel, ethidium bromide staining. (**c**) Western blot analysis of transfected cells for HCV proteins NS5A, NS3 and core. Lysates of SGR/JFH1-RNA[8] or pEF/Core[29] DNA–transfected Huh7 cells and naive Huh7 cells served as positive and negative controls, respectively. (**d**) Immunofluorescence assay of cells fixed 72 h after transfection with JFH1 (1–3) or JFH1/ΔE1-E2 RNA (4). Magnification, ×200. (**e**) HCV core protein secretion into culture medium after HCV transfection of Huh7 cells.

online). Immunofluorescence assay showed that 3 d after transfection, JFH1 RNA and JFH1/ΔE1-E2 RNA transfection gave similar numbers of HCV-positive cells (**Fig. 1d**). After 13 d, 50–60% of JFH1 RNA–transfected cells were still positive for core protein, but only a few with the mutant (**Fig. 2b**). These results indicate a limited spread of infection. Alternatively, JFH1 RNA–replicating cells may have a longer half-life than cells containing JFH1/ΔE1-E2 or SGR-JFH1 RNAs.

Biophysical properties of HCV particles

To characterize secreted viral particles, we analyzed supernatant of JFH1-transfected cells using a sucrose density gradient. Core protein and nuclease-resistant HCV RNA sedimented to the same density of 1.17 g/ml (**Supplementary Fig. 3** online). To show co-sedimentation of all structural proteins, we generated a JFH1-E2HA construct containing a hemagglutinin tag replacing part of the E2 hypervariable region (HVR). HCV lacking the HVR is infectious in chimpanzee[13], and JFH1-E2HA virus remained infectious for Huh7 cells. Similar to the wild type, viral particles of this mutant had a density of ~1.15 g/ml (**Fig. 3a**). Moreover, we detected core, E1 and E2 proteins in the same peak fraction of the sucrose density gradient (**Fig. 3b**), indicating the production of complete viral particles.

Detection of HCV particles by electron microscopy

Cell culture–derived JFH1 particles were visualized by immunoelectron microscopy, using an E2-specific antibody (CBH5)[14]. We detected gold-labeled spherical structures with an electron-dense inner core using a concentrated virus preparation (**Fig. 3c–e**), whereas we found only unstructured aggregates with the mock-transfected control (**Fig. 3f**). The inner ring has a slightly angular morphology and a diameter of 30–35 nm, consistent with nucleocapsids[15]. The overall diameter of the structures (50–65 nm) is compatible with the predicted size of HCV[15–18].

Infectivity of HCV particles

We inoculated naive Huh7 cells with supernatant harvested from JFH1 RNA– or JFH1/ΔE1-E2 RNA–transfected cells, and 48 h later we double-stained cells for core and NS5A (**Fig. 4a**). Only cells inoculated with JFH1 medium were positive for these proteins (10–20 cells/cover slip, **Fig. 4a**), with the number increasing to about 390 core protein–positive cells/cover slip using 1/30 concentrated medium (~0.5% of inoculated cells; **Fig. 4a**). To exclude the possibility that residual *in vitro* transcripts were captured by inoculated cells, we prepared supernatant from cells treated with the same amount of JFH1 RNA but without the electroporation. Upon inoculation of naive Huh7 cells, we observed no core protein–positive cells (**Fig. 4a**), as was the case with supernatant from JFH1/ΔE1-E2 RNA–transfected cells. Furthermore, ultraviolet irradiation of the inoculum substantially reduced the number of positive cells. Finally, we found no productive infection with HepG2, IMY-N9 and HeLa cells (**Fig. 4a**), consistent with their lower permissiveness for HCV RNA replication[9,10].

To compare infectivity of culture supernatant of JFH1 RNA– and JFH1/ΔE1-E2 RNA–transfected cells, we inoculated Huh7 cells with concentrates containing equivalent RNA copy numbers (1.17×10^8 copies/ml) and determined amounts of cell-associated RNA at 0, 12, 24 and 48 h after inoculation. We detected similar amounts of RNA in cells after an adsorption period of 3 h and found similar decreases up to 12 h (**Fig. 4b**). But 12 h later, RNA titers increased only in JFH1-inoculated cells, suggesting that productive infection depends on HCV envelope glycoproteins (**Fig. 4b**). This conclusion was supported by results obtained with single E-gene deletions (**Supplementary Fig. 4** online). JFH1-E2HA particles were also infectious for Huh7 cells, but the RNA titer in infected cells was approximately 10 times lower compared to JFH1 virus infection (data not shown). Finally, HCV can be passaged by infection but virus titers decrease upon serial passages (data not shown).

Neutralization of HCV infection by CD81-specific antibody

CD81 was shown to be involved in HCV entry using HCV pseudoparticles[19,20]. To determine whether authentic particles follow the same entry route and to confirm specificity of uptake, we incubated Huh7 cells with JFH1 or JFH1/ΔE1-E2 inocula in the presence of CD81-specific or nonspecific antibodies. We scored infection 48 h later, using NS3-specific immunofluorescence (**Supplementary Fig. 5** online) or RTD-PCR (**Fig. 4c**). CD81-specific antibodies reduced both the number of infected cells and the amount of HCV RNA associated with the cells by about 90% as compared to control antibody, confirming specificity of the infection and the important role of CD81 in HCV entry.

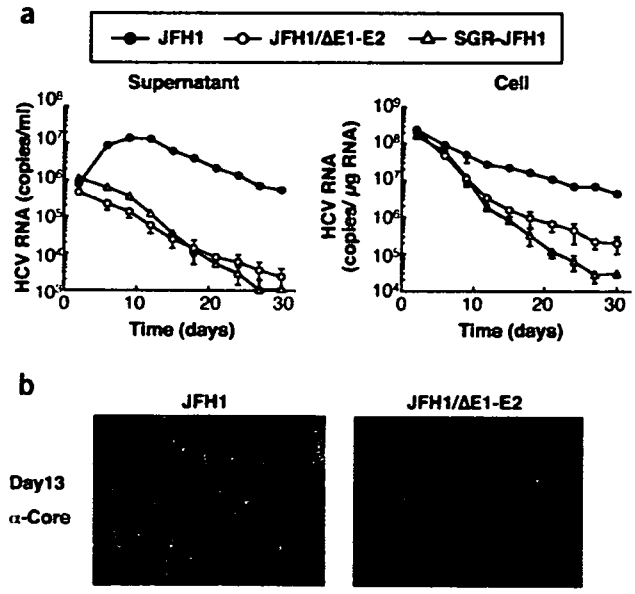

Figure 2 Time course of HCV RNA replication and core protein expression in transfected cells. (a) Levels of HCV RNA in transfected cells (right) and corresponding culture supernatants (left). Huh7 cells were transfected with given HCV RNAs and passaged up to 30 d. Viral RNA was determined 2, 5, 8, 12, 15, 18, 21, 24, 27 and 30 d after transfection. (b) Immunofluorescence assay of HCV core protein in passaged cells transfected with JFH1 and JFH1/ΔE1-E2 RNA. Transfected cells were harvested 1 d after the fourth passage (day 13 after transfection). Magnification, ×100.

Neutralization of infection by patient sera

To facilitate quantification of infection, we generated a bicistronic JFH1 luciferase reporter construct (**Fig. 4d** and **Supplementary Fig. 6** online). Infectious titers attainable by JFH1 and Luc-JFH1 genomes are similar, indicating that added sequences do not markedly affect production of infectious particles (data not shown). Taking advantage of this system, we performed neutralization experiments using sera of individuals infected with HCV genotype 2 or 1. Culture supernatants containing Luc-JFH1 virus particles were mixed with serum dilutions and infection was determined by luciferase assay (we considered a reduction to at least 50% significant). At a dilution of 1:20, all HCV sera showed neutralizing activity comparable to 0.08 μg/ml CD81-specific antibody (**Fig. 4d**). Neutralization was dose dependent and highest with sera 3 and 4. None of the serum samples inhibited entry of HIV-based pseudoparticles bearing murine leukemia virus–derived envelope proteins into Huh7 (data not shown). Finally, antibodies purified from patient 3 but not from control serum B inhibited infection with similar efficiency as the original serum, whereas immunoglobulin depletion prevented neutralization (**Fig. 4d**). These data show that antibodies in sera from infected individuals are capable of neutralizing JFH1 viruses.

Infectivity of cell culture–derived HCV particles *in vivo*

To show the infectivity of cell culture–grown JFH1 virus *in vivo*, we intravenously inoculated medium from JFH1 RNA transfected cells into a chimpanzee. The inoculum contained 7.65×10^6 RNA copies/ml and had a core protein concentration of 4,630 fmol/L. To ensure that the HCV RNA remaining in the medium after transfection did not cause infection, we collected medium from a mock-transfected culture, in which HCV RNA was added to the cells without transfection. This sample contained 3.47×10^4 RNA copies/ml and had undetectable levels of core protein. Chimpanzee X0215 was inoculated with 1 ml of undiluted control medium and showed no signs of infection for 6 weeks (**Fig. 5**). Thereafter, we inoculated the chimpanzee with 1 ml of a 10^4 dilution of supernatant from JFH1 RNA–transfected cells. After 6 weeks of monitoring with no signs of infection, we re-inoculated the chimpanzee with 1 ml of a 10^3 dilution. HCV RNA became detectable in the serum at week 2, persisted until week 5, and thereafter became undetectable. Viremia was low with highest HCV RNA titer of 2.04×10^3 copies/ml at week 4. Infection was cleared without HCV-specific seroconversion, elevation of alanine aminotransferase or histological evidence of liver injury

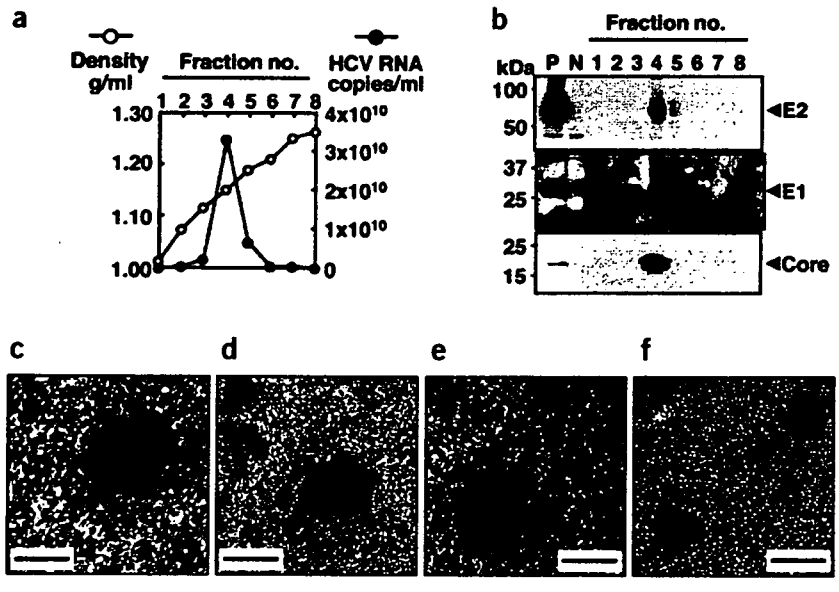

Figure 3 Density gradient and electron microscope analysis of recombinant HCV particles. (a,b) Co-sedimentation of viral RNA and structural proteins. (a) Concentrated culture medium collected from JFH1/E2HA RNA–transfected cells was fractionated using a 10–60% sucrose density gradient. HCV RNA titer in each fraction was determined. (b) Density gradient fractions were further concentrated and analyzed by western blotting for core, E1 or E2-hemagglutinin. P, cell lysate prepared from JFH1/E2HA RNA–transfected Huh7 cells; N, cell lysate from untransfected Huh7 cells. Arrowheads indicate positions of HCV proteins. (c–f) Electron micrograph of spherical structures shown by immunogold labeling. Grids were incubated with a concentrated JFH1 virus stock and then with the E2 monoclonal antibody CBH5 (ref. 14). Bound antibodies were detected with Protein A coupled to gold particles 10 nm in diameter. (c–e) Three representative examples showing the same structure. (f) Control grid coated with concentrated cell-free supernatant derived from mock-transfected cells. In rare cases, we observed gold particles attached to unstructured protein aggregates. Scale bar, 50 nm.

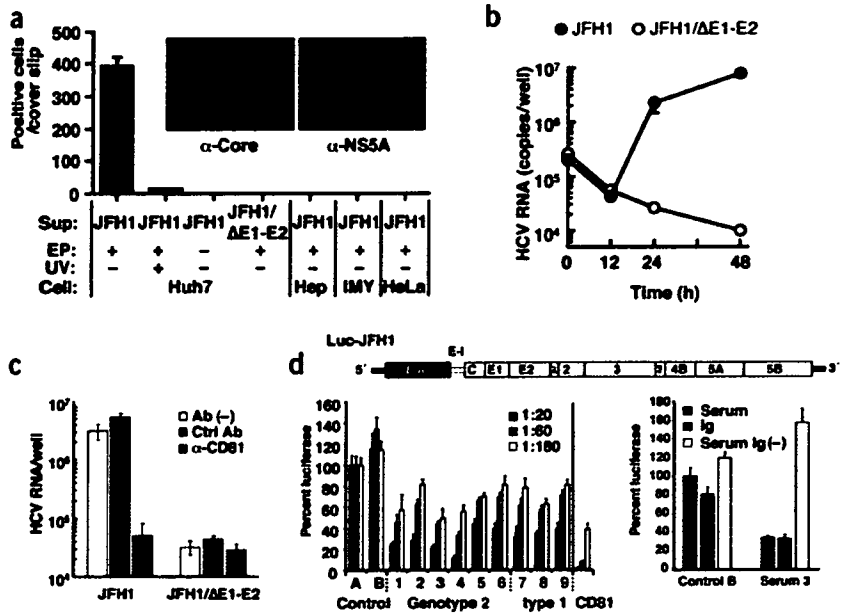

Figure 4 Infectivity of viral particles and neutralization of infection. (**a**, insert) Immunofluorescence analysis of cells infected with viral particles for core (left) and NS5A (right). (**a**) Cell lines specified on the bottom were also inoculated with a 1/30 concentrated supernatant from full-length JFH1 RNA– or JFH1/ΔE1-E2 RNA–transfected cells (Sup). In some experiments, culture supernatant of nontransfected cells was used (EP–), or culture supernatant was irradiated with ultraviolet light before inoculation of cells (UV+). (**b**) Comparison of infectivity of culture supernatant from JFH1 RNA– and JFH1/ΔE1-E2 RNA–transfected cells. (**c**) Inhibition of infection by CD81-specific antibody. We used 1/20 concentrated supernatants from cells transfected with given genomes for infection of Huh7 cells in the presence of a CD81-specific (α-CD81, black bars) or a control antibody (Ctrl Ab, gray bars), or in the absence of antibody (Ab(–), white bars). Inoculated cells were analyzed by RTD-PCR. (**d**) Production of infectious HCV particles carrying the firefly luciferase reporter gene and neutralization of infection by sera from infected individuals. Upper panel, schematic representation of Luc-JFH1 construct with luciferase (Luc) reporter gene (**Supplementary Fig. 6** online). E-I, EMCV-IRES. Bottom left panel, neutralization of Luc-JFH1 virus by sera from infected individuals. Luc-JFH1 viral particles were mixed with given dilutions of sera from healthy donor (Control), or sera from individuals infected with given genotypes (lanes 1–9). Results of CD81-specific antibody neutralization are shown in the right; black bar, 2 μg/ml; gray bar, 0.4 μg/ml; white bar, 0.08 μg/ml. Luciferase activity is expressed relative to the values obtained with control serum A. Bottom right panel, neutralization by immunoglobulins purified from infected individuals' sera. Luc-JFH1 virus particles were mixed with control serum B or patient serum 3 (serum, black bars), 2 mg protein of the same sera depleted of immunoglobulins (Serum Ig(–), open bars), or 130 μg of corresponding purified immunoglobulins (Ig, gray bars). Infectivity is expressed relative to control serum B.

(**Fig. 5**). We sequenced parts of the 5′-untranslated region (UTR; nucleotides 128–331), the E2 HVR (nucleotides 1,438–1,828) and NS5B (nucleotides 9,049–9,382) of the circulating viral RNA at week 4 after the last inoculation. The sequences were identical to the JFH1 strain. These results show the *in vivo* infectivity of JFH1 virus produced in culture.

DISCUSSION

This study shows that recombinant HCV particles are produced and secreted from JFH1 RNA–transfected cells, and secreted viruses are infectious for both Huh7 cells and a chimpanzee. Biochemical analyses show that cell culture–grown virus particles have a density of 1.15–1.17 g/ml and are spherical in size, with an average outer diameter of about 55 nm. Infectivity can be neutralized by CD81-specific antibodies, supporting the importance of CD81 in HCV entry[19,20]. Neutralization and cross-neutralization were achieved with immunoglobulins from infected individuals' sera. Cross-neutralization was also found with chimeric viruses composed of the core to NS2 region of the Con1 isolate (genotype 1) and the JFH1 replicase (T.P., G. Koutsoudakis, S. Kallis, T.K., T.W & R.B., unpublished data), indicating conserved epitopes involved in HCV entry. The possibility to create chimeric infectious virus broadens the scope of this system and allows (cross-)neutralization to be addressed in more detail.

It is unclear why the spread of infection in Huh7 cells is limited. Activation of innate immunity in transfected or infected cells is one possibility. Alternatively, JFH1 RNA–containing cells may have a growth disadvantage and therefore be displaced during passage. Moreover, formation of assembly-competent envelope protein complexes and virus release may be a rate-limiting step, reducing virus production to a level insufficient to sustain infection.

Earlier attempts to infect Huh7 cells with sera from infected individuals were not successful. But recombinant viral particles have a homogeneous density, whereas HCV in human sera shows much lower and heterogeneous densities, suggesting an association with cellular com-

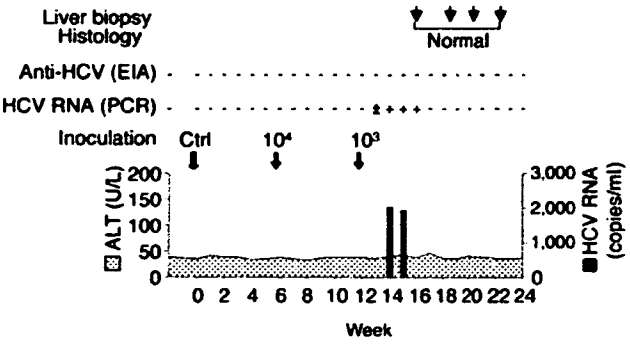

Figure 5 *In vivo* infectivity of JFH1 virus produced in tissue culture. Chimpanzee X0215 was first inoculated with 1 ml of the undiluted culture medium from mock-transfected Huh7 cells (Ctrl). We re-inoculated the chimpanzee 6 weeks later with 1 ml of a 10^4 dilution of culture medium from full-length JFH1 RNA-transfected cells (10^4). After 6 more weeks, we repeated inoculation with 1 ml of a 10^3 dilution (10^3). The course of infection is shown with arrows indicating the three inoculations. HCV RNA (copies/ml) and ALT (units/L) levels are plotted; HCV-specific, HCV RNA and liver biopsy results are shown above the graph.

ponents, especially lipoproteins[21], that may interfere with infection. Further studies are required to analyze the mechanism underlying the different infectivities of recombinant and serum-derived virus particles, especially using the serum from JFH1 virus–infected chimpanzee.

Culture-grown JFH1 viral particles are infectious in chimpanzee. The ratio of RNA titer versus infectious titer of the culture medium is about 1,000, which is lower than reported for infectious human and chimpanzee serum (10–100)[22,23]. Possible explanations are more defective viruses produced by the JFH1 strain or overestimation of viral RNA because of the input RNA used for transfection. Notably, human sera with lower infectivity have a density of about 1.17 g/ml, whereas those with higher infectivity sediment primarily at a lower density[24].

The transient course of infection of the JFH1 strain differs from most infectious clones[22,25,26], usually causing higher viremia and occasionally HCV-specific seroconversion or hepatocellular injury. This may result from the inoculum (culture grown virus versus inoculation of genomic *in vitro* transcripts) or the rather old age of the chimpanzee (20–30 years). Alternatively, the JFH1 strain, although replicating efficiently *in vitro*[8–10], may be less infectious *in vivo*[27]. Nevertheless, this is the first report describing the production of HCV in cell culture, which can infect both cells and primates.

METHODS

For details of Methods, please see **Supplementary Methods** *online.*

Plasmid construction. Based on the consensus sequence of JFH1 (ref. 7), we assembled plasmid pJFH1 containing the full-length JFH1 cDNA downstream of the T7 RNA promoter. We generated the following mutants of pJFH1: pJFH1/GND carrying a mutation in the NS5B GDD motif, which abolishes RNA polymerase activity[4,5]; pJFH1/ΔE1-E2 with a deletion of 351 amino acids (amino acids 217–567); pJFH1/E2HA with the hemagglutinin tag (YPYDVPDYA) replacing part of the E2 HVR (amino acids 394–402). pJ6CF is the prototype genotype 2a clone[11]. We constructed plasmid pJCH1 analogous to pJFH1 (ref. 7). In plasmid pFK-Luc-JFH1, the 5′-UTR and part of the core region were fused to the firefly luciferase gene. The second cistron is expressed through the encephalomyocarditis virus internal ribosomal entry site and encodes the complete JFH1 polyprotein.

RNA transfection and analysis of transfected cells. *In vitro* synthesis of HCV RNA, electroporation and northern blot analysis were performed as described previously[4,8]. For detection of HCV proteins by western blot, we used NS5A (ref. 9), NS3 (ref. 8) and core-specific (clone 2H9) antibodies and peroxidase-labeled rabbit-specific goat immunoglobulin (Biosource) or mouse-specific sheep immunoglobulin (Amersham Pharmacia). E1- and E2-specific polyclonal antibodies were raised by immunization of rabbits with synthetic peptides. We used rat monoclonal hemagglutinin-specific antibody (Roche) and peroxidase-labeled rat-specific goat IgG to detect hemagglutinin-tagged E2 protein. Immunofluorescence was performed using the same primary antibodies.

Quantification of HCV core protein and RNA. We quantified HCV core protein in culture supernatant or cell lysate using a new immunoassay described previously[28]. Total RNA was isolated from cell lysates or culture media by Isogen (Nippon Gene). We determined RNA copy numbers of HCV by RTD-PCR as described[12].

Sucrose density gradient analysis. We cleared culture medium collected 6 d after transfection using low-speed centrifugation, and passed it through a 0.45-μm filter. We layered filtrate on a sucrose gradient (60% to 10%, wt/vol) and centrifuged it for 16 h. We harvested and analyzed fractions for HCV RNA titers using RTD-PCR.

Infection of cells with secreted HCV. We collected culture medium 72 h after transfection, cleared it using low-speed centrifugation and passed it through a 0.45-μm filter. Part of the filtrate was concentrated 1/30 using an Amicon Ultra-15 (cut off: 1×10^5 Da; Millipore). We seeded cells 24 h before infection at a density of 5×10^4 cells/well in a 12-well plate, or at 1×10^5 cells/well in a 6-well plate. We infected cells with 100 μl of inoculum for 3 h, washed them, added complete medium and cultured cells for 12, 24, 48, 72 and 96 h. We performed immunofluorescence 2 d after infection.

Electron microscopy. We concentrated supernatant harvested from JFH1-transfected Huh7 cells and mock-transfected cells using ultracentrifugation and adsorbed it onto carbon-coated grids. Grids were fixed with 3% paraformaldehyde, and blocked in a solution of 0.8% BSA, 1% coldwater fish skin gelatin (Sigma) and 20 mM glycine. We performed immunogold labeling with an E2-specific antibody (CBH5) and Protein A coupled to 10-nm gold particles. After extensive washing, we stained grids with 1.8% methylcellulose and 0.3% uranyl acetate.

Virus neutralization assays. Target cells were infected with culture supernatants supplemented with JS-81 (BD Biosciences) or Mab46D2 (Dengue type 2–specific antibody) at a final concentration of 10 μg/ml (unless otherwise stated). After inoculation, we supplemented cells with fresh medium. We lysed cells 72 h after infection for RTD-PCR or luciferase assays.

For neutralization with sera from infected individuals, we mixed virus-containing supernatants with dilutions of heat-inactivated serum. After incubation for 1 h, we added mixtures to target cells and measured infection. Immunoglobulins contained in human sera were purified by using a HiTrap Affinity Protein G column (Amersham Pharmacia).

***In vivo* infection.** The chimpanzee experiment was conducted at Southwest Foundation for Biomedical Research (SFBR), San Antonio, Texas, in an American Association of Laboratory Animal Care–accredited animal facility under animal protocol approved by SFBR Institutional Animal Care and Use Committee. We collected culture medium from Huh7 cells transfected with the JFH1 genome and cleared them by centrifugation before inoculation into chimpanzee X0215. Serum samples were tested for alanine aminotransferase levels, HCV-specific antibodies (EIA2.0, Abbott Laboratories) and HCV RNA that was quantified by the Roche Amplicor Cobas Monitor II (Roche). We collected liver biopsies for histological analysis.

Accession numbers. The Genbank accession numbers for the consensus sequence of JFH1 is AB047639 and for JCH1 it is AB047640.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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CORRIGENDUM: Old fat, make way for new fat

Geoff Gibbons
Nat. Med. 11, 722–723 (2005)

The sentence beginning at the bottom of page 722 should read: "Abnormal cholesterol metabolism is also a characteristic of PPARα deficiency and is associated with a downregulation of the cholesterogenic enzyme HMG-CoA reductase[9], the molecular target of the statin family of hypocholesterolemic drugs."

CORRIGENDUM: Production of infectious hepatitis C virus in tissue culture from a cloned viral genome

Takaji Wakita, Thomas Pietschmann, Takanobu Kato, Tomoko Date, Michiko Miyamoto, Zijiang Zhao, Krishna Murthy, Anja Habermann, Hans-Georg Kräusslich, Masashi Mizokami, Ralf Bartenschlager & T Jake Liang
Nat. Med. 11, 791–796 (2005)

The chimpanzee used in this study is referred to as X0215 in several parts of the text and supplementary information. Its correct number is X0205.