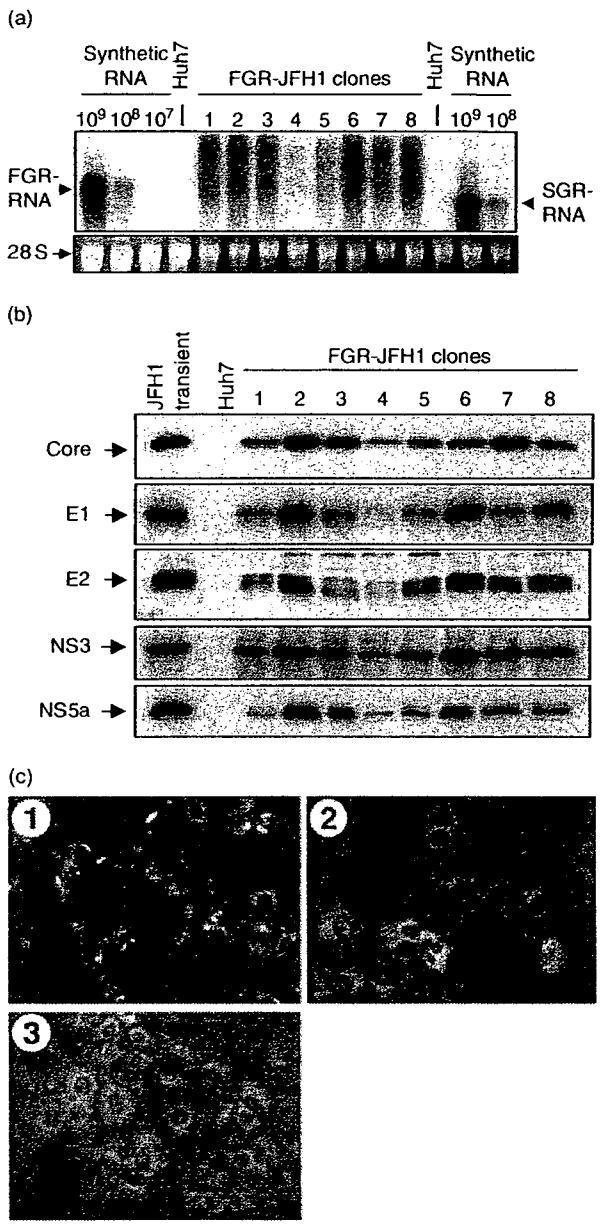

Figure 2 Analysis of isolated full-genome replicon cells. (a) Northern blot analysis of replicon RNA. Total RNA from eight isolated replicon cell clones (FGR-JFH1 clones 1–8) was analyzed by northern blotting with DNA probes of the *neo*^{r} – EMCV IRES and β-actin genes. We performed *in vitro* synthesis of 10^9, 10^8 and 10^7 copies of transcribed positive-strand full-genome replicon JFH-1 RNA (FGR-RNA) and 10^8 and 10^7 copies of positive-strand subgenomic JFH-1 replicon RNA (SGR-RNA). The synthesized RNA was loaded onto the gel as positive controls (left 3 lanes and right 2 lanes, respectively). Left and right arrowheads indicate target positions of full-genome and subgenomic replicon RNAs, respectively. Arrow indicates position of β-actin. 'Huh7' indicates cellular RNA of normal Huh7 cells, which was used as a negative control. (b) Western blot analysis. Cell lysates were prepared from clones of Huh7 cells transfected with FGR-JFH1 RNA (FGR-JFH1 clones 1–8). Huh7 cells transfected with full-length JFH-1 RNA were used as positive controls (JFH1 transient), and untransfected parental Huh7 cells (Huh7) were used as negative controls. Anti-core monoclonal antibodies and anti-E1, -E2, -NS3 and -NS5A polyclonal antibodies were used to detect HCV antigens. Target sizes of HCV proteins are indicated by arrows. (c) Subcellular localization of HCV antigens determined by immunofluorescence. Isolated FGR-JFH1 replicon cell clone 3 was cultured on cover slips. Cultured cells were fixed before being incubated with anti-Core (1, α-Core), anti-NS3 (2, α-NS3) and anti-NS5A (3, α-NS5A) antibodies. (Original magnification ×200).

(Fig. 5a). Core protein titers in culture supernatants gradually decreased in one series of the passages (Fig. 5a, Transfection 1). However, in another series (Fig. 5a, Transfection 2), core protein titer in culture supernatant increased beginning with the fourth inoculation. Colony formation of cells inoculated with culture supernatant of transfection 1 gradually decreased during the observation period. Colony formation of cells inoculated with culture supernatant of transfection 2 increased beginning with the fourth passage (Fig. 5b, P4/d118).

DISCUSSION

IN THE PRESENT study, we established a selectable, infectious full-length HCV replicon. Transcribed full-length replicon RNA was transfected into Huh7 cells. Cells transfected with the full-length replicon formed colonies at reduced efficiency, compared with cells transfected with the subgenomic replicon. However, expanded replicon cells supported efficient replicon RNA replication. Furthermore, although the replicon genome (approximately 11 kb) is longer than the wild-type genome of HCV (approximately 9.6 kb), culture supernatant from the replicon cells were infectious for naïve Huh7 cells. After long-term culture, replicon cells did not stop replicating replicon RNA, but they did stop secreting infectious viral particles. Viral adaptation might occur during repeated serial infection of the

Table 2 Mutations and titers of JFH-1 replicon

Clone	Nucleotide†	Amino acid‡	Region	Replicon RNA titer§ (copies/μg RNA)	Core protein titer¶ (fmol/L)	Colony formation efficiency†† (c.f.u./mL)
1	3893 A > C	707 Y > S	E2	2.71×10^7	64	0.3
	5610 T > A	1279 N > K	NS3			
	7236 G > A	Synonymous‡‡	NS4b			
	10161 C > A	Synonymous	NS5b			
2	None			5.19×10^7	826	63.3
3	None			4.47×10^7	3450	133.3
4	6599 A > C	1609 D > A	NS3	1.14×10^7	33	1.0
	8902 T > A	2377 S > T	NS5a			
5	9653 C > A	2627 A > E	NS5b	1.60×10^7	2904	89.3
6	None			7.09×10^7	363	15.3
7	394 C > A	Synonymous	Core§§	1.51×10^7	571	41.0
	5295 C > A	Synonymous	NS3			
	7189 T > C	1806 S > P	NS4b			
	8076 G > A	Synonymous	NS5a			
8	6483 A > G	Synonymous	NS3	1.15×10^7	387	11.3
	8972 G > A	2400 G > E	NS5a			
	9216 T > C	Synonymous	NS5b			

†Position of mutated nucleotide within replicon; ‡position of mutated amino acid within complete open reading frame of full-length JFH-1; §HCV RNA copy titer in replicon cell; ¶core protein concentration in culture supernatant of replicon cells; ††naïve Huh7 cells were inoculated with concentrated culture supernatants from replicon cells, and inoculated cells were cultured for 3 weeks in medium supplemented with G418 (0.3 mg/mL); ‡‡synonymous mutation does not change amino acid sequence; §§sequential region from 5′-untranslated region upstream of *neo*^{r} gene.

replicon virus. Importantly, selectable infection was established using HCV replicon containing full-length genotype 2a JFH-1 cDNA. This system may be useful for HCV research.

Several full-length HCV cDNAs have been cloned, and their infectivity has been confirmed *in vivo* using chimpanzee models.[15,16] However, it has been difficult to produce recombinant viral particles and test their infectivity using cell culture systems,[4,7] and this limits the ability to perform detailed analyses of the HCV life cycle and pathogenesis in cell culture. The JFH-1 strain was isolated from a patient with fulminant hepatitis, and it efficiently replicates in Huh7 cells and other hepatic and non-hepatic cell lines in subgenomic replicon form.[10,11,17] Full-length wild-type JFH-1 RNA and chimeric JFH-1 RNA can replicate in Huh7 cells and produce infectious virus.[12,18,19] Sequence analysis has revealed that the JFH-1 strain clusters with genotype 2a HCV isolates, and exhibits 89–90% homology with other genotype 2a strains at the nucleic acid level and 91–92% homology at the amino acid level.[9] The relationship between the high levels of replication and virus production of JFH-1 in cell culture is unclear. Chimeric virus, which contains structural region of J6CF strain and non-structural region from JFH-1, replicates as well as wild-type JFH-1 and produces infectious virus in cell culture.[19,20] However, wild type J6CF strain or another chimeric virus containing structural region of JFH-1 and non-structural region from J6CF did not replicate in tissue culture (unpublished data).[12] It is thus clear that non-structural proteins or genome are important for the efficient replication of JFH-1 strain.

In the present study, the full-genome JFH-1 replicon produced infectious virus particles. Full-genome replicon clones have previously been developed using genotype 1a and 1b HCV clones, but none of those replicons produced viral particles from replicon cells.[6,7,21,22] This inability to produce virus particles may be related to adaptive mutations in the replicon genome, because adaptive mutations increase replication of replicons in cultured cells. However, H77-S strain was recently reported to produce infectious virus particles into culture medium from the transfected cells, although this strain contains at least five adaptive mutations.[23] The full-length JFH-1 replicon does not require adaptive mutations to efficiently replicate in cultured cells.[10,11,17] In the present study, the full-genome replicon cells with amino acid mutations had a lower HCV RNA

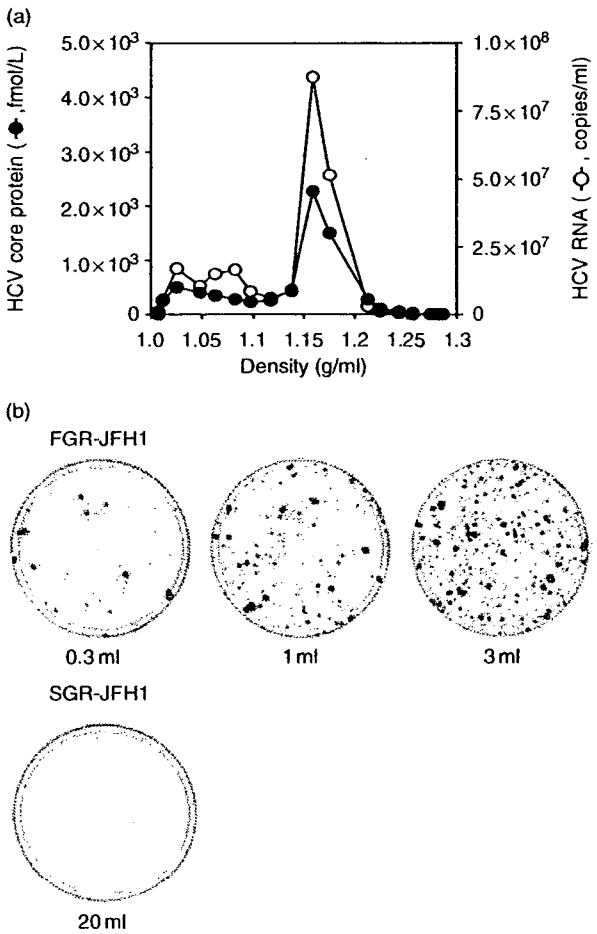

Figure 3 Analysis of culture supernatant from replicon cells. (a) Density gradient analysis. Culture supernatant from full-genome JFH-1 replicon cell clone 3 was filtered and layered on a stepwise sucrose gradient (60% to 10% wt/vol) in centrifugation tubes. After centrifugation, 22 fractions were collected from the bottom of the tubes. Core protein concentration (●) and HCV RNA titer (○) were measured in each fraction. (b) Colony formation by cells inoculated with culture supernatant from JFH-1 replicon cells. Culture supernatants from full-genome FGR-JFH1 replicon cell clone 3 and subgenomic SGR-JFH1 replicon cell clone 4–1 were cleared by centrifugation and filtration. Naïve Huh7 cells were inoculated with the cleared culture supernatant, and the inoculated cells were cultured in medium supplemented with G418 (0.3 mg/mL) for 3 weeks before staining with crystal violet. The figure shows representative staining of Huh7 cells inoculated with 0.3 mL, 1 mL and 3 mL of culture medium from FGR-JFH1 clone 3, and cells inoculated with 20 mL of medium from SGR-JFH1 clone 4–1. Before inoculation, culture media were concentrated by ultrafiltration.

titer than the full-genome replicon cells without mutations ($1.62 \times 10^7 \pm 6.43 \times 10^6$ *vs* $5.58 \times 10^7 \pm 1.35 \times 10^7$ copies/μg RNA, $P < 0.05$); however, when HCV RNA titer per cell was calculated, there was no significant difference ($5.75 \times 10^3 \pm 2.45 \times 10^3$ *vs* $8.90 \times 10^3 \pm 1.29 \times 10^3$ copies/cell, $P = 0.09$). We also determined the colony formation efficiency of replicon clones 1–8 by transfection of cellular RNA isolated from replicon cells as 1.66×10^{-6}, 1.48×10^{-6}, 3.67×10^{-7}, 8.98×10^{-7}, 5.60×10^{-7}, 1.23×10^{-6}, 1.16×10^{-6} and 7.28×10^{-7} c.f.u./RNA copy, respectively. Thus, the mutations that occurred in the full-genome of the JFH-1 replicon genome have no or slight effect of reducing RNA replication efficiency. Studies indicate that certain adaptive mutations in genotype 1 HCV replicon clones significantly increase RNA replication.[24–26] Many adaptive mutations alter the phosphorylation status of NS5A protein, and it has been reported that RNA replication efficiency is associated with NS5A phosphorylation status.[27,28] In the present study, p56 and p58 bands were observed in all the full-genome JFH-1 replicon cell clones (Fig. 2b). Thus, the high replication capacity of JFH-1 and its efficient production of infectious virus may be dependent on mechanisms other than phosphorylation of NS5A.

We previously reported incorporation of the luciferase reporter gene into a JFH-1 replicon construct and detected neutralizing antibody in chronically HCV infected patient sera.[12] In addition, Koutsoudakis *et al.* characterized the early steps of HCV infection using this luciferase reporter virus.[29] The wild-type JFH-1 genome has been shown to replicate efficiently in permissive cell lines.[18] However, an infectious, selectable full-length HCV replicon containing a neomycin-resistant gene is particularly useful for tests of the infectivity of HCV in cells with low permissiveness for HCV infection. It would be also interesting to test cell lines such as HepG2, IMY, HeLa and 293 cells, which support JFH-1 subgenomic replicon replication.[10] Recently, we also found that JFH-1 replicon can replicate in mouse cell lines.[30] These cells were not permissive for HCV infection;[12] however, they might support full-genomic replicon replication and infectious virus production. In particular, replicon cells using HeLa and 293 cells should be useful to analyze the host factors important for virus infection because these cell lines express CD81, SR-BI and LDL receptor, which are potentially important for HCV infection. In preliminary observations, full-genomic replicon could replicate in HepG2, IMY, HeLa and 293 cells, and replicon cells were established (unpublished data).

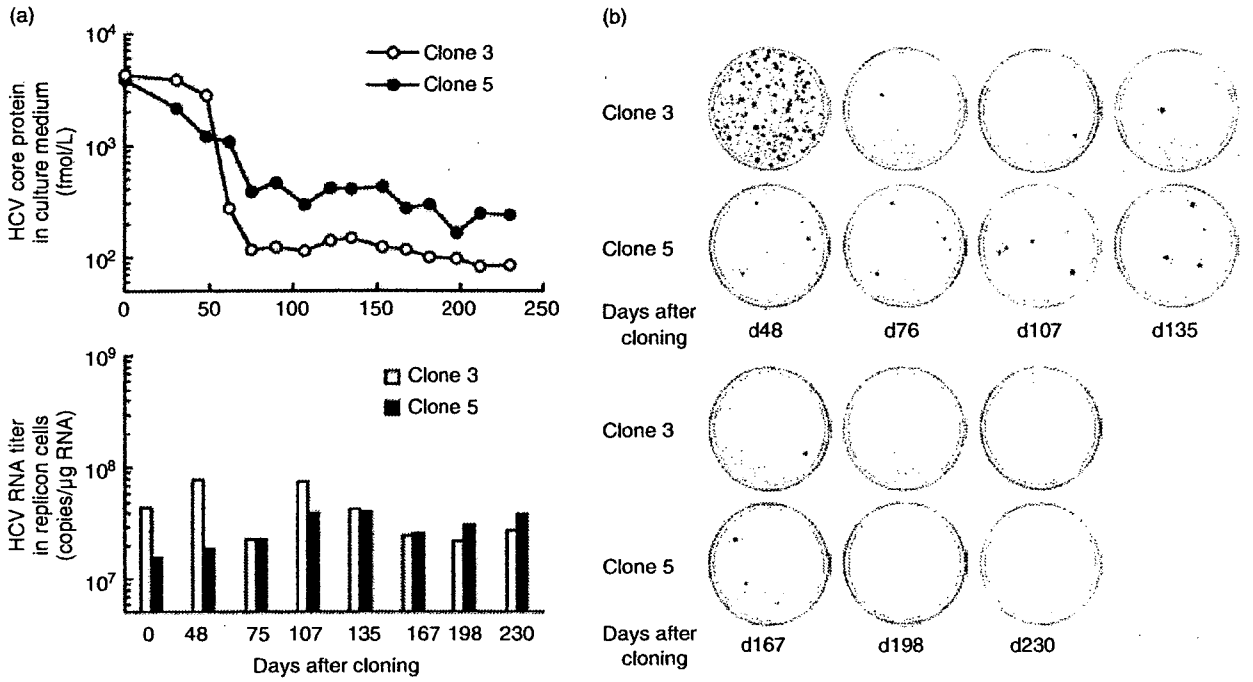

Figure 4 Long-term culture of full-genome replicon cell clones. FGR-JFH1 replicon cell clones 3 and 5 were cultured continuously for 230 days after the clones were transfected. (a) We measured the HCV core protein concentration in the culture supernatant and HCV RNA titer in replicon cells harvested at each passage. (b) We measured colony formation by naïve Huh7 cells inoculated with culture supernatant (harvested at each passage) and cultured in medium supplemented with G418 (0.3 mg/mL) for 3 weeks before staining with crystal violet.

Permissiveness for HCV infection has been shown to vary among Huh7 cell subtypes. Mutant cell lines such as Huh7.5 and Huh7.5.1 exhibit greater permissiveness than standard Huh7 cells,[18,19] whereas other Huh7 subtypes exhibit relatively low permissiveness.[12] In the present study, secretion of core protein into culture media and infectivity of culture supernatant were abolished by long-term culture of replicon cells (Fig. 4). However, long-term repeated infection of secreted replicon virus increased core protein secretion and infectivity of secreted virus, suggesting that some viruses become adapted to naïve Huh7 cells, resulting in increased secretion of infectious replicon virus (Fig. 5a, transfection 2). It is also interesting that virus replication levels were not significantly changed by repeated virus infection, which has been demonstrated to decrease the infectivity and virus secretion of some virus strains (Fig. 5a, transfection 1). Further study is needed to determine whether these differences are dependent on mutations in the virus genome or selection within infected cells. In future studies, we plan to examine mechanisms of virus adaptation to Huh7 cells and adaptive mechanisms of host cell lines.[31]

In the present study, colony formation efficiency after inoculation with culture supernatant was partly dependent on the core protein concentration of the supernatant. Colony formation efficiency for culture supernatant from clone 3 was 133.3 c.f.u./mL. The cells used in the present study were standard Huh7 cells, which are not highly permissive for HCV.[12,18] Use of cured cells such as Huh7.5 cells may increase the infection efficiency of replicon culture supernatants.[19] However, the present low infection efficiency of the replicon virus may also be due to its genomic length. The present replicon genome is about 1.5 kb longer than the wild-type HCV genome. The colony formation efficiency of the present full-genome replicon was significantly lower than that of the subgenomic replicon. The ability of viral particles to incorporate a longer genome than the wild-type genome may allow us to add other genes to the viral replicon genome, and to test expression of those genes in the infected cells.

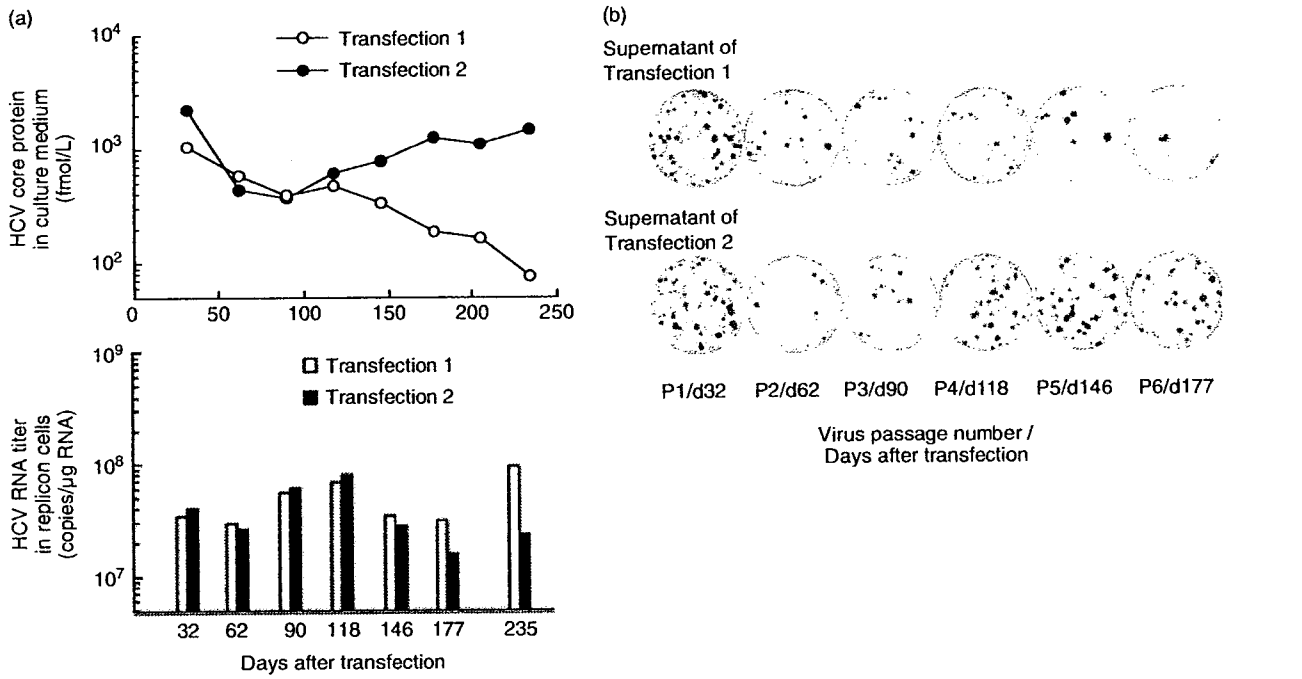

Figure 5 Repeated infection of cells with culture supernatant harvested from full-genome replicon cells. Naïve Huh7 cells were transfected with FGR-JFH1 RNA. Transfected cells were cultured for 4 weeks in medium supplemented with G418 (1 mg/mL), and culture supernatant was then harvested. New naïve Huh7 cells were inoculated with the harvested supernatant, and were then cultured in medium supplemented with G418 (0.3 mg/mL) for 4 weeks. Culture supernatant was harvested at the end of the 4 weeks, and was used to inoculate new Huh7 cells. This harvesting of supernatant, inoculation of new Huh7 cells, and incubation of the inoculated cells was repeated every 4 weeks for 235 days after transfection. Six independent experiments were performed and two representative results are shown. (a) We measured the HCV core protein concentration in culture supernatant (upper panel) and HCV RNA titer in infected cells (lower panel) harvested at each passage. (b) We measured colony formation by naïve Huh7 cells inoculated with culture supernatant (harvested at each passage) and cultured in medium supplemented with G418 (0.3 mg/mL) for 4 weeks before staining with crystal violet.

ACKNOWLEDGMENTS

THIS WORK WAS partly supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, grants from the Ministry of Health, Labour and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Toray Industries Inc., and Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation.

REFERENCES

1 Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A non-B viral hepatitis genome. *Science* 1989; 244: 359–62.

2 Kuo G, Choo QL, Alter HJ *et al.* An assay for circulating antibodies to a major etiologic virus of human non-A non-B hepatitis. *Science* 1989; 244: 362–4.

3 Kiyosawa K, Sodeyama T, Tanaka E *et al.* Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; 12: 671–5.

4 Bartenschlager R, Lohmann V. Replication of hepatitis C virus. *J Gen Virol* 2000; 81: 1631–48.

5 Lohmann V, Korner F, Koch J *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999; 285: 110–13.

6 Ikeda M, Yi M, Li K, Lemon SM. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. *J Virol* 2002; 76: 2997–3006.

7 Pietschmann T, Lohmann V, Kaul A *et al.* Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J Virol* 2002; 76: 4008–21.
8 Bukh J, Purcell RH, Miller RH. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. *Proc Natl Acad Sci USA* 1994; 91: 8239–43.
9 Kato T, Furusaka A, Miyamoto M *et al.* Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. *J Med Virol* 2001; 64: 334–9.
10 Kato T, Date T, Miyamoto M *et al.* Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* 2003; 125: 1808–17.
11 Date T, Kato T, Miyamoto M *et al.* Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells. *J Biol Chem* 2004; 279: 22371–6.
12 Wakita T, Pietschmann T, Kato T *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; 11: 791–6.
13 Aoyagi K, Ohue C, Iida K *et al.* Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. *J Clin Microbiol* 1999; 37: 1802–8.
14 Takeuchi T, Katsume A, Tanaka T *et al.* Real-time detection system for quantification of hepatitis C virus genome. *Gastroenterology* 1999; 116: 636–42.
15 Kolykhalov AA, Agapov EV, Blight KJ, Mihalik K, Feinstone SM, Rice CM. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* 1997; 277: 570–4.
16 Yanagi M, Purcell RH, Emerson SU, Bukh J. Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc Natl Acad Sci USA* 1997; 94: 8738–43.
17 Kato T, Date T, Miyamoto M, Zhao Z, Mizokami M, Wakita T. Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon. *J Virol* 2005; 79: 592–6.
18 Zhong J, Gastaminza P, Cheng G *et al.* Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005; 102: 9294–9.
19 Lindenbach BD, Evans MJ, Syder AJ *et al.* Complete replication of hepatitis C virus in cell culture. *Science* 2005; 309: 623–6.
20 Pietschmann T, Kaul A, Koutsoudakis G *et al.* Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci USA* 2006; 103: 7408–13.
21 Blight KJ, McKeating JA, Rice CM. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* 2002; 76: 13001–14.
22 Blight KJ, McKeating JA, Marcotrigiano J, Rice CM. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. *J Virol* 2003; 77: 3181–90.
23 Yi M, Villanueva RA, Thomas DL, Wakita T, Lemon SM. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc Natl Acad Sci USA* 2006; 103: 2310–15.
24 Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. *Science* 2000; 290: 1972–4.
25 Lohmann V, Korner F, Dobierzewska A, Bartenschlager R. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J Virol* 2001; 75: 1437–49.
26 Krieger N, Lohmann V, Bartenschlager R. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. *J Virol* 2001; 75: 4614–24.
27 Evans MJ, Rice CM, Goff SP. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc Natl Acad Sci USA* 2004; 101: 13038–43.
28 Appel N, Pietschmann T, Bartenschlager R. Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. *J Virol* 2005; 79: 3187–94.
29 Koutsoudakis G, Kaul A, Steinmann E *et al.* Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *J Virol* 2006; 80: 5308–20.
30 Uprichard SL, Chung J, Chisari FV, Wakita T. Replication of a hepatitis C virus replicon clone in mouse cells. *Virol J* 2006; 3: 89.
31 Bartenschlager R, Pietschmann T. Efficient hepatitis C virus cell culture system: what a difference the host cell makes. *Proc Natl Acad Sci USA* 2005; 102: 9739–40.

JOURNAL OF VIROLOGY, May 2007, p. 4405–4411 Vol. 81, No. 9
0022-538X/07/$08.00+0 doi:10.1128/JVI.02334-06

Production of Infectious Hepatitis C Virus of Various Genotypes in Cell Cultures[▽]

Takanobu Kato,[1] Takuya Matsumura,[1] Theo Heller,[1] Satoru Saito,[1] Ronda K. Sapp,[1] Krishna Murthy,[2] Takaji Wakita,[3]† and T. Jake Liang[1]*

Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892[1]; Southwest Foundation for Biomedical Research, San Antonio, Texas 78227[2]; and Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan[3]

Received 24 October 2006/Accepted 7 February 2007

A unique hepatitis C virus (HCV) strain JFH-1 has been shown to replicate efficiently in cell culture with production of infectious HCV. We previously developed a DNA expression system containing HCV cDNA flanked by two self-cleaving ribozymes to generate HCV particles in cell culture. In this study, we produced HCV particles of various genotypes, including 1a (H77), 1b (CG1b), and 2a (J6 and JFH-1), in the HCV-ribozyme system. The constructs also contain the secreted alkaline phosphatase gene to control for transfection efficiency and the effects of culture conditions. After transfection into the Huh7-derived cell line Huh7.5.1, continuous HCV replication and secretion were confirmed by the detection of HCV RNA and core antigen in the culture medium. HCV replication levels of strains H77, CG1b, and J6 were comparable, whereas the JFH-1 strain replicates at a substantially higher level than the other strains. To evaluate the infectivity in vitro, the culture medium of JFH-1-transfected cells was inoculated into naive Huh7.5.1 cells. HCV proteins were detected by immunofluorescence 3 days after inoculation. To evaluate the infectivity in vivo, the culture medium from HCV genotype 1b-transfected cells was inoculated into a chimpanzee and caused a typical course of HCV infection. The HCV 1b propagated in vitro and in vivo had sequences identical to those of the HCV genomic cDNA used for cell culture transfection. The development of culture systems for production of various HCV genotypes provides a valuable tool not only to study the replication and pathogenesis of HCV but also to screen for antivirals.

Hepatitis C virus (HCV) is a major public health problem and infects about 200 million people worldwide (12, 18). The majority of HCV-infected patients fail to clear the virus, and many develop chronic liver diseases, including cirrhosis and hepatocellular carcinoma. HCV does not replicate efficiently in cultured cells, and robust model systems for HCV infection have been difficult to develop. Recently, we identified a unique HCV genotype 2a strain JFH1 that can replicate and produce viral particles efficiently in cell culture and established an HCV infection model system with cell culture generated JFH-1 virus that is infectious both in vitro and in vivo (5, 7–10, 16, 22, 26).

Like other RNA viruses, HCV displays marked genetic heterogeneity and is currently classified into six major genotypes (19). Among these genotypes, genotypes 1 and 2 have worldwide distribution and are known to be associated with different clinical profiles and therapeutic responses (25). These differences in clinical features are likely to be a result of viral characteristics. Study of the molecular mechanisms underlying such differences would provide valuable information regarding the pathogenesis and therapy of hepatitis C in humans. Despite the development of the JFH1 infectious cell culture system, similar systems with other HCV strains have been difficult to establish. Recent studies have shown the production of infectious 1a strain in vitro, but multiple adaptive mutations must be introduced to confer a high level of replication (24). Therefore, a more general system that can be applied to various HCV genotypes and to antiviral testing is urgently needed. Previously, we reported a DNA expression system for efficient HCV particle production system by expressing a genomic-length HCV genotype 1b cDNA with self-cleaving ribozymes (6). This system supported HCV replication and produced and secreted HCV particles into the culture medium. In the present study, we applied this HCV-ribozyme system to various HCV genotypes. These HCV expression plasmids also contain the secreted alkaline phosphatase (SEAP) gene to control for transfection efficiency and the effects of culture conditions. Using this system, we could generate various genotypes of HCV and confirmed the infection of generated virus both in vitro (strain JFH-1) and in vivo (strain CG1b and H77). We also established permanent cell lines continuously expressing replicating HCV by transfecting the HCV-ribozyme construct with a selection marker.

* Corresponding author. Mailing address: Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 10 Center Dr., Rm. 9B16, Bethesda, MD 20892. Phone: (301) 496-1721. Fax: (301) 402-0491. E-mail: JakeL@bdg10.niddk.nih.gov.

† Present address: Department of Virology II, National Institute of Infectious Diseases, Shinjuku, Tokyo 162-8640, Japan.

▽ Published ahead of print on 14 February 2007.

MATERIALS AND METHODS

HCV expression plasmids. Various HCV strains, H77 (genotype 1a, accession no. AF009606 [11]), CG1b (genotype 1b, accession no. AF333324 [21]), J6 (genotype 2a, accession no. AF177036 [23]), and JFH1 (genotype 2a, accession no. AB047639 [7]) were used. The HCV plasmid (pTHr) containing the HCV CG1b genomic cDNA with ribozymes was reported previously (6). As a strategy for further construction, a PmeI site was introduced in 3′ untranslated region (3′UTR) of the CG1b genome. To generate SEAP-expressing vector, the frag-

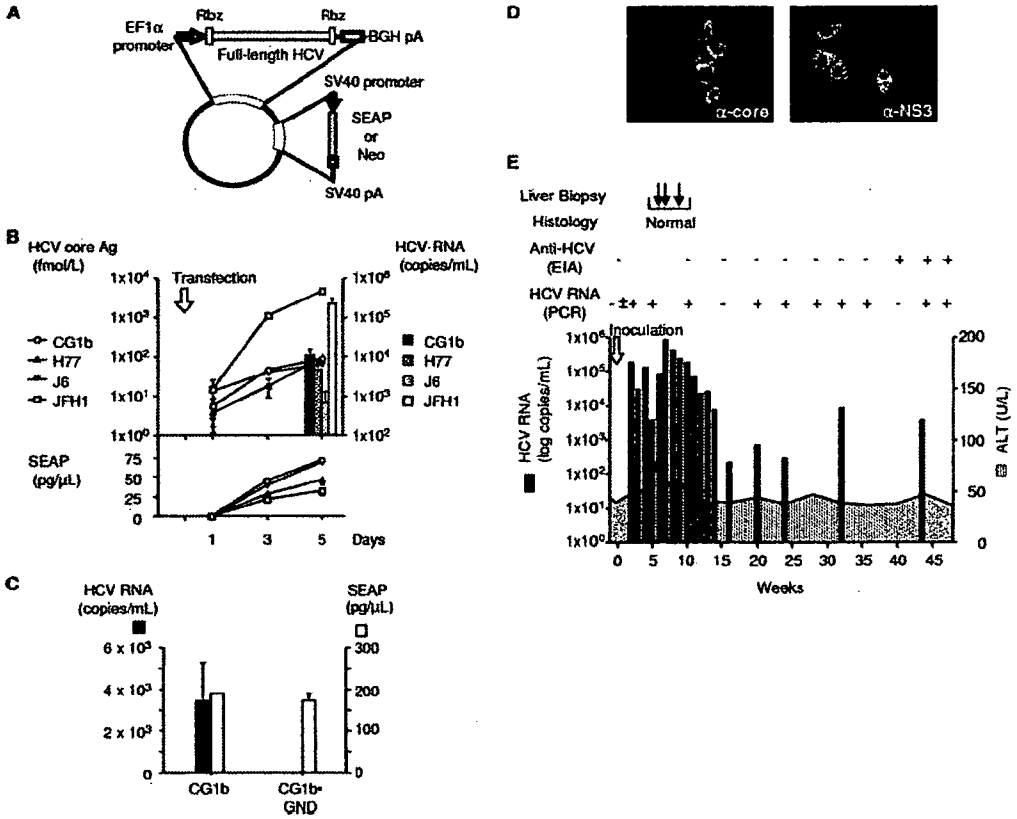

FIG. 1. Production and infection of HCV produced in cell culture. (A) Construction of HCV-expressing plasmid with the HCV-ribozyme system. Two hammerhead ribozyme sequences were engineered at the 5' and 3' ends of HCV full-length cDNA. This system was expressed under the control of EF1α promoter. The SEAP reporter gene was included under the control of the SV40 promoter. (B) Production of HCV various strains after transfection of HCV-ribozyme plasmids. Various HCV-ribozyme plasmids were transfected into Huh7.5.1 cells. HCV core Ag levels and SEAP activities in the culture medium were measured at the indicated time points. HCV RNA titers in culture medium were measured at the end of the follow-up time point. (C) Comparison of HCV RNA titers of CG1b and CG1b–GND plasmid-transfected cells. HCV RNA titers and SEAP activities in the culture medium were measured 5 days after transfection. (D) Infection of naive Huh7.5.1 cells by cell culture-generated JFH-1 virus. Culture medium form JFH1-ribozyme-transfected cells were inoculated into naive Huh7.5.1 cells. The expression of HCV core and NS3 proteins was detected by immunofluorescence with appropriate antibodies. (E) Infectivity of cell culture-generated CG1b virus in a naive chimpanzee. Chimpanzee X0140 was inoculated with culture medium of CG1b plasmid-transfected cells containing 2.3×10^3 copies of HCV RNA.

ment encompassing the SEAP gene was amplified by PCR from pSEAP-control vector (Clontech Laboratories, Inc., Mountain View, CA). The fragment was inserted into the pEF1/Myc-His plasmid (Invitrogen, Carlsbad, CA) in place of the neomycin-resistant gene (*neo*) as the pEF/S plasmid. The fragment containing the CG1b-ribozyme sequence was digested with EcoRI and XbaI and cloned into the pEF/S vector as pEF/CG1b-Rz/S. This plasmid comprises the full-length CG1b genome and flanking ribozymes directed by the EF1α promoter and the SEAP gene by the simian virus 40 (SV40) promoter (Fig. 1A). The replication-deficient mutant of CG1b strain that had been reported previously (6) was also cloned into the pEF/S vector as pEF/CG1b–GND-Rz/S.

For JFH1-ribozyme construction, the ribozyme sequences were introduced into the 5' and 3' ends of the JFH1 genomic cDNA (pJFH1) by PCR (22). The ribozyme-containing 5'UTR of JFH1 was introduced into the JFH1 genomic cDNA by cloning via the EcoRI and AgeI sites and similarly with the ribozyme-containing 3'UTR via the AscI and XbaI sites. The fragment containing the JFH1 genomic cDNA and ribozymes was inserted into the EcoRI and XbaI sites of the pEF/S vector to generate the pEF/JFH1-Rz/S. This fragment was also transferred to the pEF1/Myc-His plasmid to generate the *neo*-containing construct as the pEF/JFH1-Rz/N for the establishment of stable JFH1 virus-producing cells (Fig. 1A). The replication-deficient clone of JFH1 was generated by introducing a point mutation at the GDD motif of the NS5b to abolish the RNA-dependent RNA polymerase activity as the pEF/JFH1–GND-Rz/S (22).

For the H77 and J6-ribozyme construction, both full-length HCV cDNAs were cloned into the pEF/CG1b-Rz/S plasmid via the AgeI (5'UTR) and PmeI (3'UTR) sites. The ribozyme-containing 5'UTR fragment of H77 was generated by PCR and replaced the ribozyme-5'UTR sequence of the CG1b via the EcoRI and AgeI sites to generate the pEF/H77-Rz/S. The pEF/J6-Rz/S construct was similarly generated by using the ribozyme-containing 5'UTR fragment of JFH1.

Cell culture and DNA transfection. The Huh7 derivative cell lines Huh7.5 and Huh7.5.1 were provided by C. Rice (Rockefeller University, New York, NY) and F. Chisari (Scripps Research Institute, La Jolla, CA), respectively (2, 25). Huh7 cells or these derivative cell lines were maintained at 37°C in 5% CO_2 in Dulbecco modified Eagle medium containing 10% fetal bovine serum.

A 10-μg portion of HCV-ribozyme plasmids was transfected into Huh7 cells or its derivative cells in a 10-cm dish (2×10^6/dish) by using Lipofectamine and Lipofectamine Plus reagents (Invitrogen) according to the manufacturer's instructions. Alpha interferon (IFN-α) was purchased from Fitzgerald (Concord, MA).

Quantification of HCV core Ag, HCV RNA, and SEAP activity. To assess HCV replication, the HCV core antigen (Ag) in culture supernatant was quantified by a highly sensitive enzyme inunuoassay (Ortho HCV antigen ELISA kit; Ortho Clinical Diagnostics, Tokyo, Japan) (1). To determine the amount of HCV RNA in culture supernatant, RNA in the culture medium was extracted from 100 to 250 μl of culture medium by TRIzol LS reagent (Invitrogen) and treated with DNase (TURBO DNase; Ambion, Austin, TX) at 37°C for 1 h. Extracted RNA was purified by using an RNeasy minikit, which includes another step of RNase-free DNase digestion (QIAGEN, Valencia, CA). Copy numbers of HCV RNA were determined by real-time quantitative reverse transcription-PCR as described previously (20). The detection limit was estimated as 300 copies/ml. When necessary, the culture medium was concentrated by ultrafiltration concentrator (Vivaspin 20, molecular weight cutoff of 100,000; Vivascience, Hannover,

Germany). SEAP activity in the culture medium was detected by using a Great EscAPe SEAP detection kit (Clontech Laboratories, Inc.).

Titration of HCV infectivity. Huh7.5.1 cells were seeded at 10^4 cells/well in 96-well flat bottom plate 24 h before inoculation. A total of 100 μl of serially 10-fold diluted culture media or gradient fractions were inoculated to the cells in the 96-well plate. After 4 h of incubation, the inocula were replaced with fresh media, and the cells were incubated for 72 h. Intracellular expression of HCV core protein were assayed by indirect immunofluorescence with α-core C1 antibody (provided by H. Greenberg, Stanford Medical School, Palo Alto, CA) and Alexa Flour 488-conjugated goat anti-mouse immunoglobulin G (Invitrogen). Clusters of core protein-positive cells were counted as a single infection focus, and infectivity titers were represented as focus-forming units (FFU). Infection of the generated virus in culture medium was also confirmed by the use of α-NS3 antibody (provided from G. Luo, University of Kentucky College of Medicine, Lexington).

Density gradient analysis. A total of 50 ml of culture medium harvested 5 days after transfection and passed through a 0.45-μm-pore-size filter was precipitated with one-fourth volume of 40% (wt/vol) polyethylene glycol 8000 in phosphate-buffered saline by overnight incubation at 4°C. Virus precipitates were collected by centrifugation, resuspended with HEPES-NaCl buffer (10 mM HEPES [pH 7.55], 0.85% NaCl, 0.02% bovine serum albumin), and purified by 20% (wt/vol) iodixanol (Optiprep; Axis-Shield, Oslo, Norway) cushion by centrifugation for 6 h at 40,000 rpm at 4°C in an SW41 Ti rotor. Purified virus was layered on top of 10 to 40% iodixanol gradient and centrifuged for 16 h at the same condition for iodixanol cushion. Fractions were collected from the bottom of gradient. The HCV RNA titer of each fraction was measured after DNase treatment and RNA extraction as described above. The HCV core Ag level and HCV infectivity titer of each fraction were assayed. Negative stain electron microscope was performed on each fraction.

Infection study in chimpanzee. The chimpanzee experiment, approved by the Institutional Animal Care and Use Committee and the NIH Interagency Animal Models Committee, was conducted in the Southwest Foundation for Biomedical Research, an American Association of Laboratory Animal Care-accredited animal facility. A naive chimpanzee (X0140) was first inoculated with culture medium from mock-transfected cells that were exposed to the pEF/CG1b-Rz/S plasmid without transfection reagent. This inoculation serves as a control for the potential infectivity of any residual plasmid DNA in the culture medium. After inoculation of this control medium, the chimpanzee was observed for 8 weeks. The chimpanzee was then inoculated with culture medium from pEF/CG1b-Rz/S plasmid-transfected cells. After inoculation, the chimpanzee was monitored weekly with blood samples for HCV RNA (Roche Amplicor Monitor II with a lower limit of detection of 200 copies/ml), anti-HCV (Bayer Anti-HCV EIA II), and alanine aminotransferase (ALT). Liver biopsy was performed for histology after the demonstration of infection. To evaluate the in vivo infectivity of cell culture generated H77 virus, the culture medium from pEF/H77-Rz/S plasmid-transfected cells was also inoculated into another chimpanzee (X0199) who had previously recovered from HCV CG1b infection.

RT-PCR and sequencing. The cDNA of the CG1b virus in chimpanzee was synthesized from RNA extracted from serum at 4 weeks after inoculation using reverse primer at the 3'UTR or 3' X region. The cDNA was subsequently amplified with DNA polymerase (TaKaRa LA *Taq*; Takara Mirus Bio, Madison, WI). Four separate PCR primer sets were used to amplify the fragments of nucleotides [nt] 152 to 2777, nt 2743 to 5098, nt 4923 to 7670, and nt 7611 to 9390 covering the entire open reading frame and part of 5'UTR and 3'UTR of the CG1b strain. The sequence of each amplified fragment was determined.

Statistical analysis. Data from repeated experiments were averaged and are expressed as means ± the standard deviations. Statistical analysis was performed by using the Student *t* test, Welch's *t* test, or one-factor analysis of variance. *P* values of <0.05 were considered statistically significant.

RESULTS

Production of various HCV genotypes in cell culture. Various HCV-ribozyme expression plasmids (pEF/H77-Rz/S, pEF/CG1b-Rz/S, pEF/J6-Rz/S, and pEF/JFH1-Rz/S) were transfected into the Huh7.5.1 cells. Culture media were harvested from days 1, 3, and 5 after transfection and assayed for HCV core Ag level and SEAP activity. HCV RNA titers were measured in the culture medium on day 5. The HCV core Ag levels of strains H77, CG1b, and J6 on day 5 were 97.1 ± 28.7, 89.8 × 10.6, and 67.3 ± 5.1 fmol/liter, respectively. JFH-1 produced a much higher HCV core Ag level (4686.6 ± 287.1 fmol/liter on day 5) than the other strains ($P < 0.05$). Likewise, the HCV RNA titer in culture supernatant of strain JFH1 transfected cell was $2.10 \times 10^5 \pm 7.55 \times 10^4$ copies/ml on day 5, significantly higher than for strains H77, CG1b, and J6 ($4.28 \times 10^3 \pm 3.00 \times 10^3$, $9.94 \times 10^3 \pm 4.64 \times 10^3$, and $6.29 \times 10^2 \pm 5.76 \times 10^2$ copies/ml, respectively; $P < 0.05$) (Fig. 1B). The SEAP activities in the culture supernatants were comparable among all strains, indicating similar transfection efficiencies. These data indicate that the JFH-1 strain replicates and produces viral particles more efficiently than the other strains in the HCV-ribozyme system. This observation is not unexpected because of the higher replication potential of JFH-1 in the HCV subgenomic replicon system (9). To confirm CG1b virus replication in this system, the CG1b-ribozyme expression plasmid (pEF/CG1b-Rz/S) and its replication-deficient mutant expression plasmid (pEF/CG1b–GND-Rz/S) were transfected into the Huh7.5.1 cells. At 5 days after transfection, the HCV RNA titer in the culture supernatant of the CG1b-transfected cells was $3.42 \times 10^3 \pm 1.84 \times 10^3$ copies/ml. The HCV RNA titer in the culture supernatant of CG1b–GND-transfected cells was undetectable, although the SEAP activities in both culture supernatants were comparable (Fig. 1C). To test the infectivity of the cell culture-produced HCV, culture supernatants were inoculated into naive Huh7.5.1 cells. By immunofluorescence microscopy, HCV core and NS3 proteins were detected in JFH-1 supernatant-infected cells. The HCV core protein showed spotty perinuclear and cytoplasmic distribution, and NS3 protein showed cytoplasmic distribution (Fig. 1D). However, no HCV-positive cells were detected in naive cells incubated with culture medium from H77-, CG1b-, and J6-transfected cells.

Infection of cell culture-generated CG1b virus in a chimpanzee. JFH-1 virus generated in a cell culture was shown previously to be infectious in a chimpanzee (22). To assess the in vivo infection of cell culture-produced CG1b virus, a naive chimpanzee was inoculated with culture medium first from mock-transfected cells (control medium) and then from pEF/CG1b-Rz/S plasmid-transfected cells (2.3×10^3 copies of HCV RNA). After inoculation of the control medium, the chimpanzee showed no signs of infection for 8 weeks. However, 2 weeks after the inoculation of culture medium from CG1b plasmid-transfected cells, HCV RNA became positive in the chimpanzee serum and persisted for up to 48 weeks after inoculation (Fig. 1E). The highest virus titer was 7.5×10^5 copies/ml, and there was a transient mild elevation in ALT. Anti-HCV was detected 40 weeks after inoculation. To demonstrate that the virus generated in cell culture did not acquire adaptive mutations in cultured cells, the entire HCV open reading frame from serum at 4 weeks after inoculation was sequenced. The sequence was completely identical to that of the CG1b strain used for transfection. Culture medium of pEF/H77-Rz/S-transfected cells (1.4×10^4 copies of HCV RNA) was also inoculated into a chimpanzee who had previously recovered from HCV CG1b infection. The chimpanzee developed viremia but quickly resolved the infection (data not shown). This attenuated infection was typically observed in rechallenge experiments of chimpanzees that had recovered from a previous HCV infection (15).

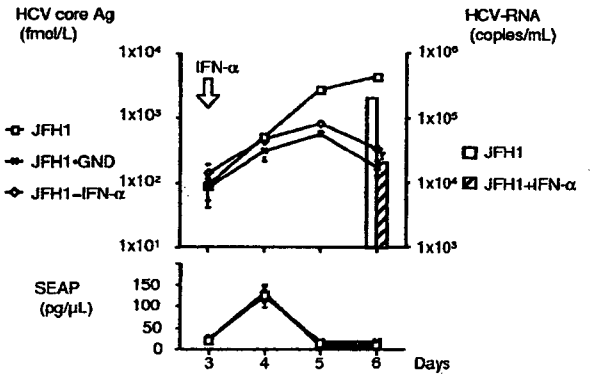

FIG. 2. Inhibition of HCV replication and production by IFN-α. IFN-α (100 IU/ml) was administered to the JFH1-ribozyme-transfected cells 3 days after transfection. The HCV core Ag level, HCV RNA titer, and SEAP activity in the culture supernatants were monitored at the indicated time points. The JFH1–GND mutant was used as a replication-deficient control.

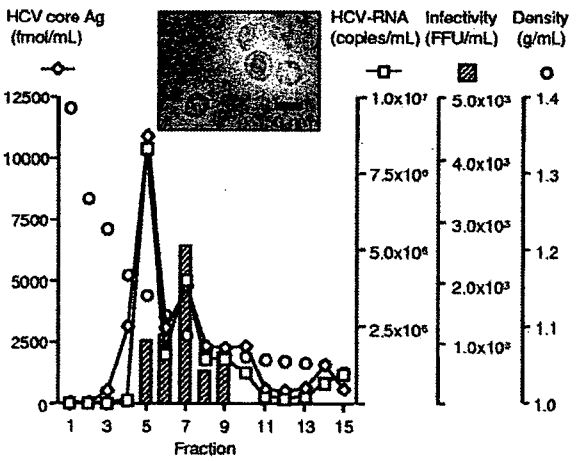

FIG. 3. Iodixanol density gradient analysis of the cell culture-generated JFH-1 HCV. Huh7.5.1 cells were transfected with JFH-1-ribozyme plasmid, and culture medium was collected and analyzed by iodixinol density gradient as described in Materials and Methods. Fractions were collected from the bottom of the gradient, and the HCV core Ag, HCV RNA, and infectivity titers were determined. The HCV particles visualized by negative-stain electron microscopy in the peak fraction of infectivity titer is shown in the inset.

Suppression of JFH-1 replication by IFN-α. To test the sensitivity of HCV production to antiviral in this system, JFH1-ribozyme plasmid-transfected cells were treated with IFN-α (100 IU/ml) 3 days after transfection, and HCV core Ag and HCV RNA were measured in the culture supernatants (Fig. 2). JFH1-GND mutant that has an inactivating mutation in the NS5b RdRp site was used as a replication deficient control. IFN-α significantly suppressed the HCV core Ag level in the supernatant (4050.3 ± 310.8 to 331.3 ± 14.1 fmol/liter, $P < 0.01$) to a level that is similar to that of JFH1-GND-transfected cells (171.5 ± 42.0 fmol/liter). The HCV RNA titer of the supernatant was also suppressed by IFN-α ($1.92 \times 10^5 \pm 1.69 \times 10^4$ to $1.93 \times 10^4 \pm 8.25 \times 10^3$ copies/ml, $P < 0.0001$). The HCV RNA titer of JFH1–GND mutant transfected was under the detection limit. The SEAP levels were not significantly affected by IFN-α.

Density gradient analysis. To confirm HCV particle production of this system, the culture medium of JFH1 expression plasmid-transfected cells was concentrated, purified, and subjected to iodixanol density gradient centrifugation. Distributions of HCV core Ag and HCV RNA showed similar profiles and peaked in the fraction with a density of 1.14 g/ml (Fig. 3). The infectivity titer of each fraction to naive Huh7.5.1 cells was also evaluated. The peak infectivity titer located in the fraction at a density of 1.09 g/ml. By negative-stain electron microscopy, the HCV particle was observed as a spherical structure measuring about 50 nm in diameter (Fig. 3, inset). The presence of the particles was detected primarily in the peak fraction of the infectivity. We have previously reported the production and secretion of HCV particles in CG1b-ribozyme plasmid-transfected cells (6). The density gradient analysis and electron microscopic morphology of the particles are similar to those of the JFH1 particles described here.

Long-term culture of JFH1-expressing cells. To evaluate the continuous production of HCV particles, JFH1-transfected Huh 7.5 cells were cultured and passaged for more than 6 weeks. The production of HCV core Ag and secretion of HCV RNA were maintained during the observed period (Fig. 4). The highest titer of HCV core Ag and level of HCV RNA were observed on day 31 after transfection in this experiment: 6.35×10^4 fmol/liter and 1.48×10^6 copies/ml, respectively. The SEAP production disappeared about 2 weeks after transfection, supporting the continuous replication and production of the JFH-1 virus. The infectivity titers of culture medium were also determined. The peak infectivity titer of 2.50×10^3 FFU/ml was observed at 25 days after transfection, but in general the HCV core Ag, HCV RNA, and infectivity titers correlated reasonably well in this experiment. However, the

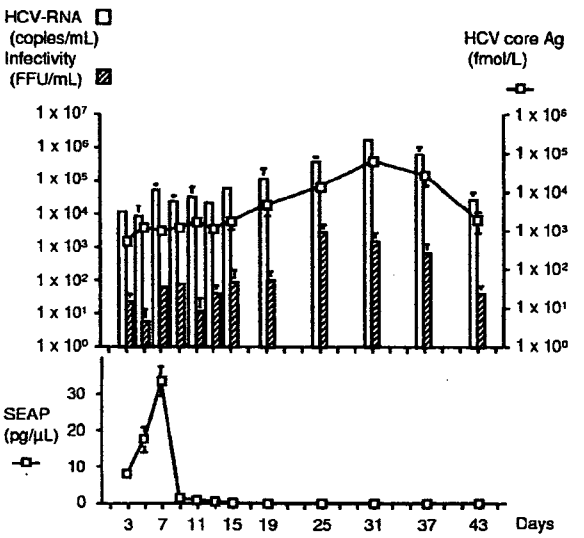

FIG. 4. Long-term culture of JFH1-ribozyme-transfected cells. Huh7.5 cells were transfected with JFH1-ribozyme plasmid as described previously. The transfected cells were passaged 3 to 4 days, and culture medium was collected at various time points. HCV core Ag, HCV RNA, and infectivity titers and SEAP activities in the culture medium were monitored for up to 43 days after transfection.

TABLE 1. Established clones of stable JFH1 virus-producing cells

Clone	Origin	HCV core Ag (fmol/liter)	HCV RNA (copies/ml)	Infectivity (FFU/ml)
7-09	Huh7	112	$<3.00 \times 10^{2a}$	$<1.00 \times 10^{1b}$
7-10	Huh7	5,053	1.78×10^{5}	1.50×10^{2}
7-19	Huh7	4,438	9.72×10^{4}	3.00×10^{1}
7-20	Huh7	1,025	4.32×10^{4}	2.00×10^{1}
7.5-01	Huh7.5	354	$<3.00 \times 10^{2}$	$<1.00 \times 10^{1}$
7.5-15	Huh7.5	158	$<3.00 \times 10^{2}$	$<1.00 \times 10^{1}$
7.5-20	Huh7.5	51,397	2.32×10^{6}	5.30×10^{2}

[a] Under the detection limit of quantitative reverse transcription-PCR.
[b] Under the detection limit of indirect immunofluorescence.

transfected cells gradually lost the ability to support HCV replication and production beyond this time period.

Establishment of cell lines stably producing JFH-1 virus. To establish stable JFH-1 virus-producing cells, pEF/JFH1-Rz/N plasmid was transfected into Huh7 and Huh7.5 cells. After 3 weeks of culture with G418 at a concentration of 1.0 mg/ml, visible colonies were identified in both transfected cell lines. A total of 23 Huh7 colonies and 26 Huh7.5 colonies were selected and screened by HCV core Ag production and indirect immunofluorescence with α-core and α-NS3 antibodies. Four Huh7-derived clones and three Huh7.5-derived clones were identified to produce HCV proteins (Table 1). Three of four Huh7 clones and one of three Huh7.5 clones were found to produce high levels of HCV core Ag and detectable HCV RNA and infectivity titers in culture medium. Among these clones, one Huh 7.5-derived clone (clone 7.5-20) with the highest HCV production was monitored for an extended period of time under drug selection. This clone showed continuous and stable production of HCV core Ag (2.96×10^4 to 6.20×10^4) and HCV RNA (1.03×10^6 to 4.73×10^6) in the medium for up to 24 passages (Fig. 5A). The infectivity titers of culture medium were also determined in this period and ranged from 3.25×10^2 to 6.00×10^3 FFU/ml. To assess the sensitivity of HCV production to IFN-α, 7.5-20 cells were treated with IFN-α (100 IU/ml), and HCV core Ag and HCV RNA were measured in the culture supernatants (Fig. 5B). IFN-α significantly suppressed the HCV core Ag ($2.04 \times 10^4 \pm 1.85 \times 10^3$ to $2.90 \times 10^3 \pm 1.51 \times 10^2$ fmol/liter, $P < 0.005$) and HCV RNA titer ($2.52 \times 10^5 \pm 1.25 \times 10^5$ to $3.13 \times 10^3 \pm 1.26 \times 10^3$ copies/ml, $P < 0.05$) in the supernatant.

DISCUSSION

The discovery of the JFH-1 strain enabled us to develop a robust system for HCV replication and infection in culture cells (13, 22, 26). However, the JFH-1 strain is unique among the HCV strains and not necessarily representative of HCV biology (7). Studies in chimpanzees suggest that the virus is not particularly infectious in vivo, causing an attenuated and transient infection, which is atypical for the general behavior of HCV (22). It is indeed interesting that this virus was originally isolated from a patient with fulminant hepatitis C (7). It is likely that the fulminant hepatitis is associated more with the clinical setting of the patient than with the virus. Further studies are necessary to resolve the question of whether a viral factor(s) plays a role in the severity of acute HCV infection. Currently, no other natural HCV strain has yet been shown to replicate efficiently and demonstrate robust infectivity in cell culture without adaptive mutations. Although the establishment of a genotype 1a (H77 strain) infectious system in cell culture is important, introduction of several adaptive mutations is clearly required (24). These adaptive mutations have been shown to confer unusual biological properties to the viral strain in vivo (3). Chimeric viruses containing the structural region of other genotypes and the JFH-1 nonstructural genes have been generated and showed in vitro infectivity (13, 14, 17), but the biological relevance of these chimeric viruses is difficult to assess.

In the present study, we established an HCV particle production system with various HCV genotypes by exploiting a recently established DNA transfection cell culture system (6). By engineering two hammerhead ribozyme sequences at the both 5′ and 3′ ends of the HCV genomic cDNA, DNA expression plasmids of multiple HCV strains could be constructed. These plasmids also contain the SEAP gene under the control of the SV40 promoter. By measuring the SEAP activity in the culture medium, the transfection efficiency and the effect of the culture conditions, such as antiviral treatment, could be mon-

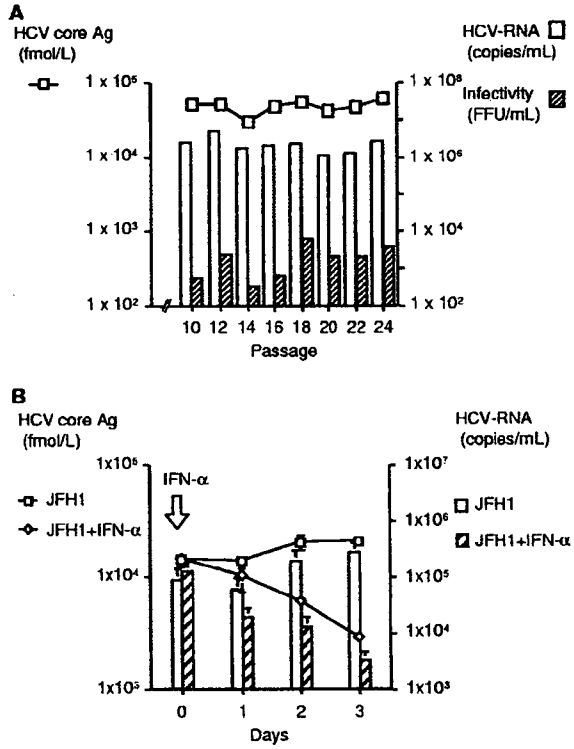

FIG. 5. Production of HCV in the stable JFH1-virus producing cell line. (A) Huh7.5 cells were transfected with the pEF/JFH1-Rz/N plasmid and exposed to 1 mg of G418/ml. Clones were isolated as described in the text. The 7.5-20 clone was passaged every 3 to 4 days and monitored continuously for an extended period of time. The HCV core Ag, HCV RNA, and infectivity titers in the culture medium at various time points were analyzed and are shown. (B) Suppression of HCV replication and production in the stable JFH1 virus-producing cell line (clone 7.5-20) by IFN-α. The production of HCV core Ag and HCV RNA in the culture medium was assessed at various time points after the administration of IFN-α (100 IU/ml).

itored. Using this system, we demonstrated that the HCV particle production of H77 strain (genotype 1a), CG1b strain (genotype 1b), and J6 strain (genotype 2a) were comparable. Viral particles resembling HCV virions were visualized by electron microscopy in CG1b- and JFH1-transfected cells (6) (Fig. 3). JFH-1 strain showed substantially higher virus production; the HCV core Ag was 50 times and HCV RNA production was 1 to 2 logs higher than those of the other strains. JFH-1 virus released into the culture medium was infectious in naive Huh7 cells. Consistent with previous reports, the infectivity titer (in FFU/ml) was about 3 logs lower than the HCV RNA titer (4, 13, 22, 26). The infectivity of the culture medium of other strain-transfected cells was also assessed. However, evidence of in vitro infection of these strains by immunofluorescence was not detected. The absence of infection is likely a result of the sensitivity limit of the detection method and a much lower replication efficiency of the other strains compared to the JFH-1 strain. The JFH-1 replicates about 1 to 2 logs more than the other strains, and the infectivity titer of the JFH-1 was about 10^2 FFU/ml in this experiment. Therefore, it is not surprising that we could not detect infection by the other strains. Perhaps by optimizing the culture and infection conditions, using more sensitive methods to detect infection, and concentrating the virus produced in the culture medium, we could detect infection by other genotypes.

To demonstrate the infectivity of strains other than JFH-1, the cell culture-generated CG1b virus was inoculated into a naive chimpanzee and caused a typical course of infection as the infectious CG1b RNA or serum (21). Similarly, H77 virus generated in cell culture was also infectious in vivo. The sequence of CG1b virus replicating in the chimpanzee was completely identical to that of the CG1b strain used for transfection. CG1b virus seems to be capable of replicating more efficiently than the JFH-1 virus in chimpanzees without adaptive mutations, although it has a lower replication efficiency in vitro. This observation is consistent with the contrasting effects of the described adaptive mutations on in vitro replication and in vivo productive infection (3). This discrepancy may be explained by the possibility that lower replication efficiency in vitro may be essential for productive infection and persistence in vivo.

In the iodixanol density gradient analysis, we demonstrated the colocalization of HCV RNA and core Ag proteins. Their peaks were in the identical fraction at a density of about 1.14 g/ml, which is consistent with our previous reports (6, 22). However, the peak of infectivity titer was at a less dense fraction (a density of 1.09 g/ml). This discrepancy has also been reported previously (4, 13). We could detect HCV particles in the peak infectivity fraction; about 50 nm of spherical structures resembling putative HCV were observed by electron microscopy. Thus, infection-competent HCV particles probably exist mainly in the peak fraction of the infectivity titer but not in the peak fraction of the HCV RNA and HCV core Ag. This observation explains the difference between HCV infectivity and the RNA titer of cell culture-generated HCV (an ~1,000-fold difference). The forms of the viral RNA and protein in the peak fraction are unknown. It is possible that they represent defective viral particles and/or nucleocapsids. Further studies are needed to clarify this point. It is also interesting that the ratio of HCV RNA titer to HCV core Ag level is higher in these gradient fractions than in the unfractionated culture medium. This could be explained by the presence of nonparticulate core protein or empty nucleocapsid in the medium.

This system has the advantage in that it is based on DNA expression plasmids, it is much easier to manipulate, and it contains a reporter gene to monitor various culture conditions. This approach can be extended to various HCV genotypes and strains as well as to the generation of stable cell lines expressing replicating HCV. In the transient-transfection system, robust HCV replication could be observed for up to 6 weeks posttransfection (Fig. 4). It is interesting that the transfected cells eventually lost their ability to support HCV replication. It is possible that some major alterations in the biology of the infected cultured cells, such as the activation of endogenous antiviral mechanisms and/or genetic or epigenetic changes, eventually occurred to shut off the viral replication. However, using a selectable marker such as the *neo* gene, we could develop stable JFH-1 virus-producing cells. We have generated one clone that supports continuous and stable high-level viral replication and production with multiple passages for an extended period of time. A recent study reported the application of a similar DNA expression system in generating a JFH-1-producing cell line (4). The system used the HDV ribozyme sequence at the 3′ end without any ribozyme sequence at the 5′ end of the JFH-1 genomic cDNA. It is not clear whether the 5′ end of the resulting HCV RNA contains the correct sequence. Furthermore, we also tested the HDV ribozyme sequence in our DNA expression system and found it to be not as efficient in generating the cleaved product as the hammerhead ribozyme sequence we have designed (unpublished data).

This system is also applicable to antiviral testing. In both transient-transfection and stable cell lines, HCV production was substantially suppressed by IFN-α (Fig. 2 and 5B). Both HCV core Ag and HCV RNA titers were suppressed to the baseline level of the JFH1–GND-transfected cells. The absence of a significant change in SEAP activity in IFN-α-treated cells indicates that the effect is not of a general toxicity but specific to HCV replication. Because this system represents the complete replication cycle of HCV, it could prove very useful for high-throughput antiviral screening.

In summary, we have established an HCV production system with various genotypes by using DNA expression plasmids with HCV genomic cDNA flanked by self-cleaving ribozymes. HCV generated in this system showed infection both in vitro and in vivo. This system provides a valuable tool not only to study the replication and pathogenesis of HCV but also to screen for antivirals against multiple HCV strains.

ACKNOWLEDGMENTS

We thank Z. Hong Zhou and his coworkers at the University of Texas, Houston, for performing the electron microscopy, and Charles Rice, Francis Chisari, Harry Greenberg, Robert Purcell, Jens Bukh, and Guangxiang George Luo for providing various valuable reagents.

This study was supported in part by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. T. W. is supported by grants from the Japan Society for the Promotion of Science; the Ministry of Health, Labor, and Welfare of Japan; and the Research on Health Sciences Focusing on Drug Innovation program of the Japan Health Sciences Foundation.

REFERENCES

1. **Aoyagi, K., C. Ohue, K. Iida, T. Kimura, E. Tanaka, K. Kiyosawa, and S. Yagi.** 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J. Clin. Microbiol. **37**:1802–1808.
2. **Blight, K. J., J. A. McKeating, and C. M. Rice.** 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. **76**:13001–13014.
3. **Bukh, J., T. Pietschmann, V. Lohmann, N. Krieger, K. Faulk, R. E. Engle, S. Govindarajan, M. Shapiro, M. St Claire, and R. Bartenschlager.** 2002. Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. Proc. Natl. Acad. Sci. USA **99**:14416–14421.
4. **Cai, Z., C. Zhang, K. S. Chang, J. Jiang, B. C. Ahn, T. Wakita, T. J. Liang, and G. Luo.** 2005. Robust production of infectious hepatitis C virus (HCV) from stably HCV cDNA-transfected human hepatoma cells. J. Virol. **79**: 13963–13973.
5. **Date, T., T. Kato, M. Miyamoto, Z. Zhao, K. Yasui, M. Mizokami, and T. Wakita.** 2004. Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells. J. Biol. Chem. **279**:22371–22376.
6. **Heller, T., S. Saito, J. Auerbach, T. Williams, T. R. Moreen, A. Jazwinski, B. Cruz, N. Jeurkar, R. Sapp, G. Luo, and T. J. Liang.** 2005. An in vitro model of hepatitis C virion production. Proc. Natl. Acad. Sci. USA **102**:2579–2583.
7. **Kato, T., A. Furusaka, M. Miyamoto, T. Date, K. Yasui, J. Hiramoto, K. Nagayama, T. Tanaka, and T. Wakita.** 2001. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J. Med. Virol. **64**:334–339.
8. **Kato, T., M. Miyamoto, A. Furusaka, T. Date, K. Yasui, J. Kato, S. Matsushima, T. Komatsu, and T. Wakita.** 2003. Processing of hepatitis C virus core protein is regulated by its C-terminal sequence. J. Med. Virol. **69**:357–366.
9. **Kato, T., T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, and T. Wakita.** 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology **125**:1808–1817.
10. **Kato, T., T. Date, M. Miyamoto, Z. Zhao, M. Mizokami, and T. Wakita.** 2005. Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon. J. Virol. **79**:592–596.
11. **Kolykhalov, A. A., E. V. Agapov, K. J. Blight, K. Mihalik, S. M. Feinstone, and C. M. Rice.** 1997. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science **277**:570–574.
12. **Liang, T. J., B. Rehermann, L. B. Seeff, and J. H. Hoofnagle.** 2000. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann. Intern. Med. **132**:296–305.
13. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309**:623–626.
14. **Lindenbach, B. D., P. Meuleman, A. Ploss, T. Vanwolleghem, A. J. Syder, J. A. McKeating, R. E. Lanford, S. M. Feinstone, M. E. Major, G. Leroux-Roels, and C. M. Rice.** 2006. Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. Proc. Natl. Acad. Sci. USA **103**:3805–3809.
15. **Major, M. E., K. Mihalik, M. Puig, B. Rehermann, M. Nascimbeni, C. M. Rice, and S. M. Feinstone.** 2002. Previously infected and recovered chimpanzees exhibit rapid responses that control hepatitis C virus replication upon rechallenge. J. Virol. **76**:6586–6595.
16. **Miyamoto, M., T. Kato, T. Date, M. Mizokami, and T. Wakita.** 2006. Comparison between subgenomic replicons of hepatitis C virus genotypes 2a (JFH-1) and 1b (Con1 NK5.1). Intervirology **49**:37–43.
17. **Pietschmann, T., A. Kaul, G. Koutsoudakis, A. Shavinskaya, S. Kallis, E. Steinmann, K. Abid, F. Negro, M. Dreux, F. L. Cosset, and R. Bartenschlager.** 2006. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc. Natl. Acad. Sci. USA **103**:7408–7413.
18. **Seeff, L. B., and J. H. Hoofnagle.** 2003. Appendix: the National Institutes of Health Consensus Development Conference Management of Hepatitis C 2002. Clin. Liver Dis. **7**:261–287.
19. **Simmonds, P., J. Bukh, C. Combet, G. Deleage, N. Enomoto, S. Feinstone, P. Halfon, G. Inchauspe, C. Kuiken, G. Maertens, M. Mizokami, D. G. Murphy, H. Okamoto, J. M. Pawlotsky, F. Penin, E. Sablon, T. Shin-I, L. J. Stuyver, H. J. Thiel, S. Viazov, A. J. Weiner, and A. Widell.** 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology **42**:962–973.
20. **Takeuchi, T., A. Katsume, T. Tanaka, A. Abe, K. Inoue, K. Tsukiyama-Kohara, R. Kawaguchi, S. Tanaka, and M. Kohara.** 1999. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology **116**:636–642.
21. **Thomson, M., M. Nascimbeni, S. Gonzales, K. K. Murthy, B. Rehermann, and T. J. Liang.** 2001. Emergence of a distinct pattern of viral mutations in chimpanzees infected with a homogeneous inoculum of hepatitis C virus. Gastroenterology **121**:1226–1233.
22. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11**:791–796.
23. **Yanagi, M., R. H. Purcell, S. U. Emerson, and J. Bukh.** 1999. Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. Virology **262**:250–263.
24. **Yi, M., R. A. Villanueva, D. L. Thomas, T. Wakita, and S. M. Lemon.** 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. USA **103**:2310–2315.
25. **Zein, N. N.** 2000. Clinical significance of hepatitis C virus genotypes. Clin. Microbiol. Rev. **13**:223–235.
26. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA **102**:9294–9299.