RNA was detectable in fraction 3, most was present in fractions 5 to 6 (Fig. 3*A*). These results were confirmed by real-time quantitative RT-PCR assays targeting a small, conserved segment of the 5′ nontranslated RNA (Fig. 3 *B* and *C*). When fractions were inoculated onto naïve Huh-7.5 cells, H77-S infectivity was found only in fractions 4 and 5, whereas the maximum JFH-1 infectivity was present in fraction 5 (Fig. 3*B*). No infectious virus was present in supernatant fluids from the H77-S/ΔE1p7 transfected cells. The intensity of core antigen staining in cells infected with the H77-S and JFH-1 virus from these gradients showed the characteristic difference in staining intensity described above (Fig. *3*, compare *D* and *E*). These results indicate that infectious H77-S virus has a buoyant density similar to that of JFH-1 virus: ≈1.13–1.14 g/cm^3.

We estimated the specific infectivity of the JFH-1 and H77-S viruses banding at this density by comparing the abundance of viral RNA in these fractions (determined by real-time RT-PCR) with the infectious titer. These results suggested a specific infectivity of 140 ± 13 S.E. RNA copies per FFU for the JFH-1 virus, compared with 54,000 ± 18,000 copies per FFU for the H77 virus (based on analysis of three fractions in two independent experiments). Thus, the JFH-1 virus has ≈400-fold greater specific infectivity than the H77-S virus in these fractions. The magnitude of this difference cannot be explained by differences in the ability of the RT-PCR assay to detect JFH-1 vs. H77-S RNA, nor is it likely to be due to contamination of the H77-S virus in fractions 4 and 5 with the RNA peaking in fraction 2 of the gradient that was not associated with infectious virus (Fig. 3*B*).

Because synthetic RNA transcripts banded at a higher density than infectious virus in these gradients (1.15–1.17 g/cm^3; data not shown), it is likely that the noninfectious H77-S RNA in fractions 2–3 is present in membranous complexes. The possibility that this material might be derived from viral replicase complexes is suggested by the presence of NS3 and NS5B within these fractions (see Fig. 6, which is published as supporting information on the PNAS web site). The abundance of E2 and core protein was not sufficient in fractions containing infectious virus for reproducible detection by immunoblot.

To characterize further the infectious particles produced in H77-S transfected cells, we carried out neutralization assays using serum samples collected prospectively from injection drug users who had experienced acute genotype 1 HCV infection (17). Dilutions of each serum sample were mixed with a fixed amount of H77-S, incubated for 60 min, then inoculated onto naïve Huh-7.5 cells. A >50% reduction in FFU was considered indicative of virus neutralizing Abs. We tested paired sera from three subjects, collected before and 8–14 months after initial serologic evidence of HCV infection. As shown in Fig. 4*A Upper*), none of the preinfection sera neutralized >50% of the H77-S inoculum, at any dilution, whereas a 1:10 dilution of each of the postinfection sera resulted in an 87–97% reduction in FFU relative to either the paired preinfection specimen, or a no serum control (98 ± 7 FFU). Fifty percent neutralization endpoints for the postinfection sera ranged from 1:700 to >1:1250. The specific neutralization of H77-S infectivity with postinfection human sera confirms that the infectious particles produced by H77-S transfected cells are antigenically related to those produced during human infections with wild-type virus. We also tested these sera for the ability to neutralize JFH-1 virus (Fig. 4*A Lower*). Only one of the three postinfection sera (patient no. 455) was capable of neutralizing JFH-1 virus (titer = 1:40), indicating substantial serologic differences between these two viruses.

CD81 is expressed on the surface of many cells and has been shown to interact with the E2 protein of HCV (18). Abs to CD81 block the infection of Huh7 cells with HCV pseudotyped retroviral particles, as well as the genotype 2a JFH-1 virus (2, 3, 19). Moreover, a soluble CD81 fragment blocks infection of hepatoma cells with the FL-J6/JFH chimera (4). We found that

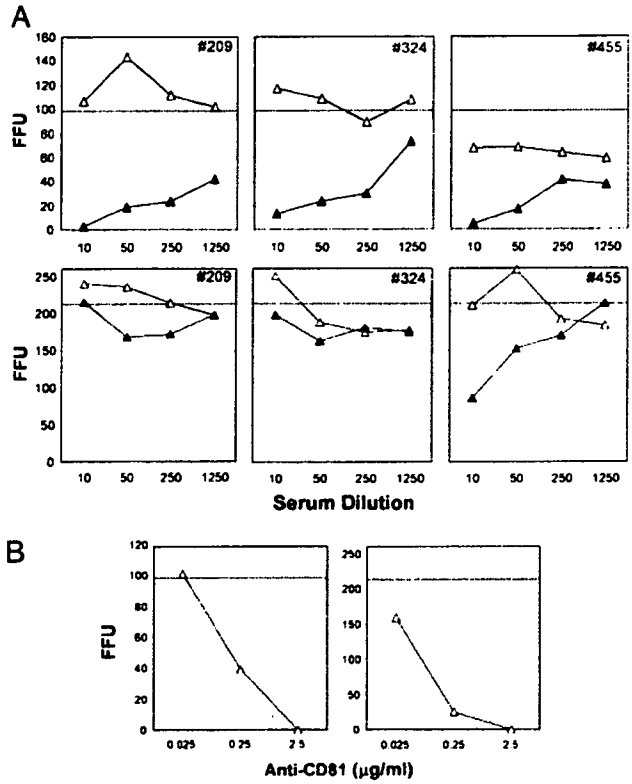

Fig. 4. Neutralization of cell culture-produced virus infectivity by antibody to HCV or CD81. (*A*) Neutralization of H77-S (*Upper*) and JFH-1 (*Lower*) viruses by paired preinfection (△) or after seroconversion (▲) sera from three individuals sustaining infection with genotype 1a HCV. The horizontal lines and shaded zones indicate the mean and range of infectious foci obtained with each viral inoculum in the absence of any serum. (*B*) Anti-CD81 Ab, added to virus before its inoculation onto Huh-7.5 cells, prevents infection with either H77-S (*Left*) or JFH-1 (*Right*) inocula.

anti-CD81 Ab efficiently blocked the infection of Huh-7.5 cells with H77-S virus (Fig. 4*B Left*). These data suggest that H77-S and JFH-1 particles bind to and enter Huh-7.5 cells by similar mechanisms, most likely involving virus recognition of the CD81 molecule on the cell surface.

Discussion

Until recently, efforts to study HCV and its interactions with host cells have been impeded by the absence of cell culture systems that are capable of supporting all stages of the virus life cycle. The development of efficiently replicating subgenomic RNA replicons and genome-length selectable RNAs has been helpful in providing model systems that recapitulate events in viral polyprotein expression, processing, replicase assembly, and viral RNA synthesis (20–22). However, these experimental systems are not capable of providing insights into interactions of the virus with host-cell receptors, the process of viral entry, or assembly and release from the cell. The recent recognition that the genotype 2a virus, JFH-1, is capable of very efficient RNA replication as well as the production of fully infectious virus particles in transfected cells thus represents a major breakthrough for the hepatitis C field.

Our demonstration here of the ability of the H77-S virus to undergo the complete viral life cycle in Huh7.5 cells represents another important step in the development of useful cell culture systems for HCV. Unlike the genotype 2a JFH-1 virus, the genotype 1a H77-S virus is representative of the most prevalent

HCV genotypes causing liver disease within the United States, as well as many other countries (5, 6). It carries five defined cell culture-adaptive mutations that distinguish it from the prototype Hutchinson strain (H77C) virus that is highly infectious for chimpanzees and that has been used in many early studies characterizing HCV (10, 12, 23). The adaptive mutations in H77-S that promote efficient viral RNA replication are located within the NS3/4A protease complex and the NS5A protein, a nonstructural phosphoprotein (12). Both of these proteins appear to be essential components of the viral RNA replicase, but both proteins also play important roles in confounding innate cellular antiviral defenses (24, 25). How these five adaptive mutations modulate these viral functions to promote HCV RNA replication remains unknown, as is their impact, if any, on viral assembly and release. It will be interesting to determine whether these mutations reduce the ability of the virus to infect chimpanzees; previous studies with a genotype 1b virus suggest mutations that promote RNA replication in cultured cells reduce the ability of the virus to infect chimpanzees (26).

Although the lower quantities of infectious H77-S virus released from transfected cells correlates well with the lower abundance of viral proteins expressed, compared with JFH-1 transfected cells (Fig. 1*C*), the production of infectious virus does not appear to be determined only by the cellular abundance of HCV RNA and/or its proteins. In other studies, we could not detect release of infectious virus from cells transfected with a highly cell culture-adapted, genotype 1b RNA derived from HCV-N (22), despite the expression of viral RNA and proteins roughly comparable with that observed with H77-S (M.Y. and S.M.L., unpublished data).

Quantitatively more viral RNA was released from H77-S transfected cells than JFH-1 transfected cells, but most of this RNA banded at a very low density in iodixanol gradients (≈1.03–1.07 g/ml) (Fig. 3*B*). This RNA was not naked viral RNA, which possesses a significantly higher density (1.15–1.17 g/ml) (data not shown). The nature of the low-density RNA is uncertain. It may represent only membrane-bound RNA associated with replication complexes released from dying cells, as suggested by the presence of NS3 and NS5B in these fractions (Fig. 6). It is interesting to note, however, that some circulating HCV RNA molecules present in human sera are found at a density of ≈1.06 gm/cm^3 after equilibrium ultracentrifugation (27), suggesting that the low-density RNA released from H77-S transfected cells may have possible physiologic relevance.

The much lower specific infectivity of the H77-S particles banding at 1.13–1.14 gm/cm^3, compared with JFH-1 particles with the same density, also remains to be explained. Both viral RNAs appear to replicate efficiently in transfected cells (Fig. 1*B*), but the structural and nonstructural proteins accumulate more slowly in H77-S transfected cells (Fig. 1*C*). To document the presence of core antigen in H77-S infected cells, we were required to incubate cells for 96 h after inoculation with virus. In contrast, abundant core antigen was present in JFH-1 infected cells by 48 h. It is tempting to speculate that this difference might explain, at least in part, the 400-fold difference we observed in the specific infectivity of JFH-1 and H77-S particles. However, a specific defect in virus entry or uncoating cannot be excluded. Widely different cell entry efficiencies have been observed with pseudotyped retroviruses bearing envelope glycoproteins from different HCV strains (19).

Although further work will be required to answer these and many other questions, the availability of a genotype 1a virus that is capable of undergoing the complete viral cycle in cultured cells should be a major asset to the hepatitis C field. The widely divergent neutralizing Ab activities we found against genotype 1a and 2a viruses in human sera (Fig. 4*A*) suggest that these viruses may represent distinct serotypes, an observation that has significant implications for vaccine development.

Materials and Methods

Plasmids. The H77-S virus was derived from the chimpanzee-infectious genotype 1a pCV-H77C cDNA clone (GenBank accession no. AF011751) (10). It contains five cell culture-adaptive mutations, two within NS3 (Q1067R, V1651I), one in NS4A (K1691R), and two in NS5A (K2040R, S2204I) (12). Construction of pH77-S, formerly called pH77c/QR/VI/KR/KR^{5A}/SI, as well as the related replication-defective NS5B mutant, H77-S/AAG, has been described (12). pH77-S/ΔE1p7 contains an in-frame deletion spanning the E1-p7 coding (see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site). The JFH-1, JFH-1/GND, and JFH-1/ΔE1E2 plasmids are described in ref. 3.

Cells. The Huh7 cell subline, Huh-7.5, was kindly provided by Charles Rice (Rockefeller University, New York) (13). Cells were cultured as described in ref. 12.

Abs. H53 mAb to E2 was a gift from J. Dubuisson (Institut de Biologie de Lille/Institut Pasteur de Lille, Lille, France) (28); the AP33 mAb was kindly provided by A. Patel (Medical Research Council Virology Unit, University of Glasgow, Glasgow, U.K.) (29). Human sera were provided by D. Netski (The Johns Hopkins University, Baltimore) (17). Commercial Abs included anti-core C7–50 (Affinity BioReagents, Golden, CO), anti-NS3 BDI371 (BioDesign, Saco, ME), and anti-CD81 JS-81 (BD Pharmingen).

HCV RNA Transfection and Virus Production. HCV RNAs were transcribed *in vitro* and electroporated into cells as described in ref. 12. In brief, 10 μg of *in vitro* synthesized HCV RNA was mixed with 5×10^6 Huh-7.5 cells in a 2-mm cuvette and pulsed twice at 1.4 kV and 25 μF. Cells were seeded into 12-well plates for RNA analysis or 6-well plates for protein analysis. For virus production, transfected cells were seeded into 75 cm^2 flasks and fed with medium containing 10% FCS. These cells were passaged with a 3:1 split at 3–4 days after transfection. Twenty-four hours later, the medium was replaced with serum-free medium, which was collected 24 h later as the virus harvest. Virus harvests were clarified by low-speed centrifugation and, where indicated, passed through a 0.2-μm filter before stabilization by addition of 20% FCS and freezing at −80°C.

Infectivity Assays. Huh-7.5 cells were seeded at 2×10^4 cells/well in 8-well chamber slides (Nalge Nunc, Rochester, NY) 24 h before inoculation with 80–100 μl of culture medium or gradient fractions (see below). Cells were tested for the presence of intracellular core antigen by immunofluorescence 96 h later (48 h for JFH-1 virus), as described below. Clusters of infected cells identified by staining for core antigen were considered to constitute a single infectious focus, and virus titers were calculated accordingly in terms of FFU/ml.

Immunofluorescence Detection of Intracellular HCV Antigen. Cells were fixed in methanol:acetone (1:1) at room temperature for 9 min, then stained with mAb C7–50 to the core protein diluted 1:300, followed by extensive washing and staining with FITC-conjugated goat anti-mouse IgG Ab (1010-02, Southern Biotech, Birmingham, AL) at a 1:100 dilution. Nuclei were counterstained with Bisbenzimide H (Hoechst, Frankfurt am Main, Germany), and slides were examined with a Zeiss LSM 510 laser scanning confocal microscope.

Neutralization Assay. Virus stock containing ≈2×10^3 FFU/ml virus was mixed with an equal volume of serial dilutions of heat-inactivated (56°C for 30 min) human sera and incubated at 37°C for 1 h before inoculation onto Huh-7.5 cells in 8-well chamber slides, as described above. After incubation of the

cultures for 48 (JFH-1) or 96 (H77-S) h, cells were fixed and stained for the presence of HCV core antigen by indirect immunofluorescence (see above), and the foci of antigen-positive cells were enumerated. A >50% reduction in FFU (compared with virus incubated with no serum) was considered indicative of neutralizing Ab; endpoint 50% neutralization titers were estimated by using the least-squares method.

Equilibrium Ultracentrifugation. Filtered supernatant fluids collected from transfected cell cultures (no FCS) were concentrated 20- to 50-fold by using a Centricon PBHK Centrifugal Plus-20 Filter Unit with Ultracel PL membrane (100-kDa exclusion) (Millipore), then layered on top of a preformed continuous 10–40% iodixanol (OptiPrep, Sigma-Aldrich) gradient in Hanks' balanced salt solution (HBSS; Invitrogen). Gradients were centrifuged in a SW60 rotor (Beckman Coulter) at 45,000 rpm for 16 h at 4°C, and fractions (500 μl each) were collected from the top of the tube. The density of each fraction was estimated by weighing a 100-μl drop from fractions of a gradient run in parallel but loaded with HBSS.

Quantitation of HCV RNA. Both semiquantitative and quantitative real-time RT-PCR assays were used to determine the abundance of viral RNA in transfected cells and virus harvests. For details, see *Supporting Materials and Methods*.

Immunoblot Analysis. Blots were incubated with Abs to core (C7–50, 1:30,000) or NS3 (BDI371, 1:20,000), followed by horseradish peroxidase-conjugated anti-mouse IgG (1030-05, Southern Biotech) (1:30,000). Proteins were visualized by chemiluminesence using reagents provided with the ECL Advance kit (Amersham Pharmacia Biosciences).

We thank Robert Purcell and Jens Bukh (both of the National Institutes of Health, Bethesda) for the pCV-H77C plasmid; Charles Rice for Huh7.5 cells; and Dale Netski, Jean Dubuisson, and Arvind Patel for human sera and mAbs. We thank Vsevolod Popov for technical advice, Jeremy Yates and Francis Bodola for excellent technical assistance, and Annette Martin for critically reviewing the manuscript. This work was supported by National Institute of Allergy and Infectious Diseases Grants U19-AI40035, R21-AI063451, and N01-AI25488.

1. Chisari, F. V. (2005) *Nature* **436,** 930–932.
2. Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & Chisari, F. V. (2005) *Proc. Natl. Acad. Sci. USA* **102,** 9294–9299.
3. Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krauslich, H.-G., Mizokami, M., *et al.* (2005) *Nat. Med.* **11,** 791–796.
4. Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R., McKeating, J. A., *et al.* (2005) *Science* **309,** 623–626.
5. Zein, N. N. (2000) *Clin. Microbiol. Rev.* **13,** 223–235.
6. Simmonds, P., Bukh, J., Combet, C., Deleage, G., Enomoto, N., Feinstone, S., Halfon, P., Inchauspe, G., Kuiken, C., Maertens, G., *et al.* (2005) *Hepatology* **42,** 962–973.
7. Zanotto, P. M., Gould, E. A., Gao, G. F., Harvey, P. H. & Holmes, E. C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 548–553.
8. Logvinoff, C., Major, M. E., Oldach, D., Heyward, S., Talal, A., Balfe, P., Feinstone, S. M., Alter, H., Rice, C. M. & McKeating, J. A. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 10149–10154.
9. National Institutes of Health Consensus Development Panel (2002) *NIH Consensus Statement on Management of Hepatitis C,* NIH Consensus State Science Statements (Natl. Inst. of Health, Bethesda), Vol. 19, pp. 1–46.
10. Yanagi, M., Purcell, R. H., Emerson, S. U. & Bukh, J. (1997) *Proc. Natl. Acad. Sci. USA* **97,** 8738–8743.
11. Blight, K. J., McKeating, J. A., Marcotrigiano, J. & Rice, C. M. (2003) *J. Virol.* **77,** 3181–3190.
12. Yi, M. & Lemon, S. M. (2004) *J. Virol.* **78,** 7904–7915.
13. Blight, K. J., McKeating, J. A. & Rice, C. M. (2002) *J. Virol.* **76,** 13001–13014.
14. Sumpter, R., Jr., Loo, M. Y., Foy, E., Li, K., Yoneyama, M., Fujita, T., Lemon, S. M. & Gale, M. J., Jr. (2005) *J. Virol.* **79,** 2689–2699.
15. Pietschmann, T., Lohmann, V., Rutter, G., Kurpanek, K. & Bartenschlager, R. (2001) *J. Virol.* **75,** 1252–1264.
16. Scholle, F., Li, K., Bodola, F., Ikeda, M., Luxon, B. A. & Lemon, S. M. (2004) *J. Virol.* **78,** 1513–1524.
17. Netski, D. M., Mosbruger, T., Depla, E., Maertens, G., Ray, S. C., Hamilton, R. G., Roundtree, S., Thomas, D. L., McKeating, J. & Cox, A. (2005) *Clin. Infect. Dis.* **41,** 667–675.
18. Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D., Grandi, G., *et al.* (1998) *Science* **282,** 938–941.
19. McKeating, J. A., Zhang, L. Q., Logvinoff, C., Flint, M., Zhang, J., Yu, J., Butera, D., Ho, D. D., Dustin, L. B., Rice, C. M., *et al.* (2004) *J. Virol.* **78,** 8496–8505.
20. Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999) *Science* **285,** 110–113.
21. Blight, K. J., Kolykhalov, A. A. & Rice, C. M. (2000) *Science* **290,** 1972–1974.
22. Ikeda, M., Yi, M., Li, K. & Lemon, S. M. (2002) *J. Virol.* **76,** 2997–3006.
23. Feinstone, S. M., Alter, H. J., Dienes, H. P., Shimizu, Y., Popper, H., Blackmore, D., Sly, D., London, W. T. & Purcell, R. H. (1981) *J. Infect. Dis.* **144,** 588–598.
24. Lindenbach, B. D. & Rice, C. M. (2005) *Nature* **436,** 933–938.
25. Gale, M., Jr., & Foy, E. M. (2005) *Nature* **436,** 939–945.
26. Bukh, J., Pietschmann, T., Lohmann, V., Krieger, N., Faulk, K., Engle, R. E., Govindarajan, S., Shapiro, M., St Claire, M. & Bartenschlager, R. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 14416–14421.
27. Hijikata, M., Shimizu, Y. K., Kato, H., Iwamoto, A., Shih, J. W., Alter, H. J., Purcell, R. H. & Yoshikura, H. (1993) *J. Virol.* **67,** 1953–1958.
28. Op De Beeck, A., Voisset, C., Bartosch, B., Ciczora, Y., Cocquerel, L., Keck, Z., Foung, S., Cosset, F. L. & Dubuisson, J. (2004) *J. Virol.* **78,** 2994–3002.
29. Owsianka, A., Tarr, A. W., Juttla, V. S., Lavillette, D., Bartosch, B., Cosset, F. L., Ball, J. K. & Patel, A. H. (2005) *J. Virol.* **79,** 11095–11104.

Pathophysiology

Intervirology

Intervirology 2006;49:37–43
DOI: 10.1159/000087261

Comparison between Subgenomic Replicons of Hepatitis C Virus Genotypes 2a (JFH-1) and 1b (Con1 NK5.1)

Michiko Miyamoto[a] Takanobu Kato[a, b] Tomoko Date[a] Masashi Mizokami[b] Takaji Wakita[a]

[a]Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, and
[b]Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Key Words
Genotypes · Hepatitis C virus · Interferon · Replicon

Abstract
Although replicon systems for hepatitis C virus (HCV) recently developed have enabled the replication of HCV in cultured cells, limited genotypes are available for them. We have isolated HCV cDNA of genotype 2a (JFH-1 strain) from serum of a patient with fulminant hepatitis. A subgenomic replicon of JFH-1 was constructed and compared with the HCV replicon of genotype 1b (Con1 NK5.1) which possessed adaptive mutations. Huh7 cells transfected with replicon RNAs that had been transcribed in vitro were cultured in the presence of neomycin sulfate (G418), and selected colonies were isolated and expanded. Then, growth rates and replication of HCV RNA were evaluated on isolated cells hosting replicons. Saturation densities were lower for cells propagating JFH-1 than Con1 NK5.1 or untransfected Huh7 cells, and the mean doubling time was longer for JFH-1 than for Huh7 cells. Levels of HCV RNA replication in isolated clones were similar between JFH-1 and Con1 NK5.1 cells. Replication of RNA decreased reciprocally with cell densities in both JFH-1 and Con1 NK5.1 cells. The replication of HCV RNA was more resistant to interferon-α in JFH-1 than in Con1 NK5.1 cells based on the comparison of an inhibitory concentration of 50%. In conclusion, we found differences between HCV replicon clones of genotypes 1b and 2a. However, these differences may result from strain-specific characteristics, such as the source of HCV, rather than characteristics of distinct genotypes. Therefore, further investigation may be needed on more HCV isolates of diverse genotypes.

Introduction

Hepatitis C virus (HCV) is a positive-strand RNA virus categorized in a flavivirus by structural similarities. The genomic RNA of HCV comprises approximately 9,600 nucleotides encoding a single open reading frame encoding about 3,000 amino acid residues [1–4]. HCV displays marked genetic heterogeneity and is currently classified into six major genotypes which are broken down into many subgroups [5]. Some HCV genotypes have regional distribution, and of those, genotypes 1 and 2 occur worldwide. In Japan, genotype 1b is the most frequent genotype, followed by genotype 2a [6]. Differences be-

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0300–5526/06/0492–0037$23.50/0

Accessible online at:
www.karger.com/int

Takaji Wakita, MD
Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience
2-6 Musashidai, Fuchu-shi
Tokyo 183-8526 (Japan)
Tel. +81 42 325 3881, ext. 4605, Fax +81 42 321 8678, E-Mail wakita@tmin.ac.jp

tween these two HCV genotypes have been noted in clinical profiles, severity of liver disease, and most importantly, response to antiviral therapies [7]. These clinical differences may be due to viral characteristics, and systems to deepen the understanding of molecular mechanisms underlying such differences have been proposed.

Lohmann et al. [8] reported subgenomic replicons of HCV RNA selectable by neomycin sulfate (G418) which can autonomously replicate in Huh7 cells. This system provides a novel powerful tool for studying mechanisms of HCV replication and selecting potential antiviral agents. Despite big advantages of this system, replication-competent replicons have been engineered only from HCV isolates of genotype 1 [8–11]. Recently, we established a subgenomic HCV replicon derived from the JFH-1 clone of genotype 2a, which had been isolated from a patient with fulminant hepatitis [12]. The JFH-1 replicon system has high efficiency in colony formation and robust RNA replication not only in Huh7 cells, but also in other liver-derived cell lines such as HepG2 and IMY-N9 [13], as well as nonhepatic cell lines like HeLa and 293 cells [14]. The availability of this replicon has enabled comparison in replication between replicons of distinct genotypes in cultured cells. The aim of the present study is to compare JFH-1 (genotype 2a) and Con1 NK5.1 (genotype 1b) [8, 15] replicons with respect to cellular growth, HCV RNA titer of replicons in culture and sensitivity to interferon (IFN).

Materials and Methods

Cell Culture System and IFN

Huh7 cells were cultured and maintained in conditions described previously [12]. Recombinant human IFN-α_{2a} (Roferon-A) was obtained from Nippon Roche, Tokyo, Japan.

Constructs of Subgenomic HCV Replicons

The construct of a subgenomic HCV replicon of genotype 1b, pFK-I_{389}neo/NS3-3′/NK5.1 (pFK/Con1 NK5.1) [8, 15], was a generous gift from Dr. Ralf Bartenschlager (University of Heidelberg, Heidelberg, Germany). The construct of a subgenomic HCV clone of genotype 2a, pSGR-JFH1 (accession number AB114136), was prepared using the JFH-1 strain isolated from a patient with fulminant hepatitis [12].

RNA Synthesis

pFK/Con1 NK5.1 was linearized by treatment with *ScaI*. pSGR-JFH1 was digested with *XbaI* and then treated with a mung bean nuclease (New England Biolabs, Beverly, Mass., USA) as described elsewhere [12]. Linearized plasmid DNAs were purified and used as templates for RNA synthesis. Subgenomic HCV RNA was synthesized in vitro with the use of a MEGAscript™ T7 kit (Ambion, Austin, Tex., USA). Synthesized RNA was treated with DNase I (RQ1™ RNase-free DNase, Promega, Madison, Wisc., USA) and extracted with acid phenol to remove any remaining template DNA.

Transfection with RNA

Synthesized replicon RNAs were transfected into Huh7 cells by electroporation as described [12]. Briefly, synthesized RNA (0.1 ng to 1 μg) was mixed with suspension of trypsinized Huh7 cells (400 μl; 7.5 × 10^6 cells/ml). Cells were then pulsed at 260 V and 950 μF in a Gene Pulser II™ apparatus (Bio-Rad, Hercules, Calif., USA). Transfected cells were immediately transferred to culture dishes of 10 cm in diameter, each containing 8 ml of culture medium. G418 (Nacalai Tesque, Kyoto, Japan) in a concentration of 1.0 mg/ml was added to culture medium at 16–24 h after transfection. Culture medium supplemented with G418 was replaced twice weekly. Three weeks after the transfection, cells were fixed with buffered formalin and stained with crystal violet, and some grown colonies were cloned.

Isolation and Analysis of G418-Resistant Cells

G418-resistant colonies were independently isolated using a cloning cylinder (Asahi Techno Glass Co., Tokyo, Japan) and cultured until approximately 90% confluence in 10-cm dishes (Corning Inc., N.Y., USA). Total RNA was isolated from cells using the Isogen reagent (Nippon Gene, Tokyo, Japan). Isolated clones and parental Huh7 cell were seeded at 1 × 10^5 cells/well in 6-well plates (Corning Inc.). In some experiments, replicon cells were seeded at 2 × 10^5 cells/well in 6-well plates. Cells were harvested daily for determining cell growth and HCV RNA titers. At 24 h after G418 had been removed from the culture medium, serial dilutions of IFN-α (Nippon Roche) were added to wells, and cells were harvested after culture for 72 h.

Northern Blot Analysis

Isolated RNA (2.5 or 4 μg) was separated by electrophoresis on 1% (weight/vol) agarose gel containing formaldehyde, then transferred to a positively charged nylon membrane (Hybond-N+, Amersham Pharmacia, Bucks., UK) and immobilized with the use of the Stratalinker UV cross linker (Stratagene, La Jolla, Calif., USA). Hybridization was performed using a DNA probe labeled with [α-^{32}P]dCTP in Rapid-Hyb buffer (Amersham Pharmacia). The DNA probe was deduced from *neo*^{r} and encephalomyocarditits virus internal ribosome entry site genes and synthesized in the Megaprime DNA labeling system (Amersham Pharmacia).

Quantification of HCV RNA by Real-Time Reverse Transcrpition Polymerase Chain Reaction

Copy numbers of HCV RNA were determined by the real-time detection reverse transcription polymerase chain reaction (RTD-PCR) described previously [16] in the ABI Prism 7700 sequence detector system (Applied Biosystems Japan, Tokyo, Japan). Data were standardized by the concentration of intracellular glyceraldehyde-3-phosphate dehydrogenase.

Statistics

Statistical analysis was performed by Student's t test, Welch's t test or the Mann-Whitney U test. Differences with a p value <0.05 were considered statistically significant.

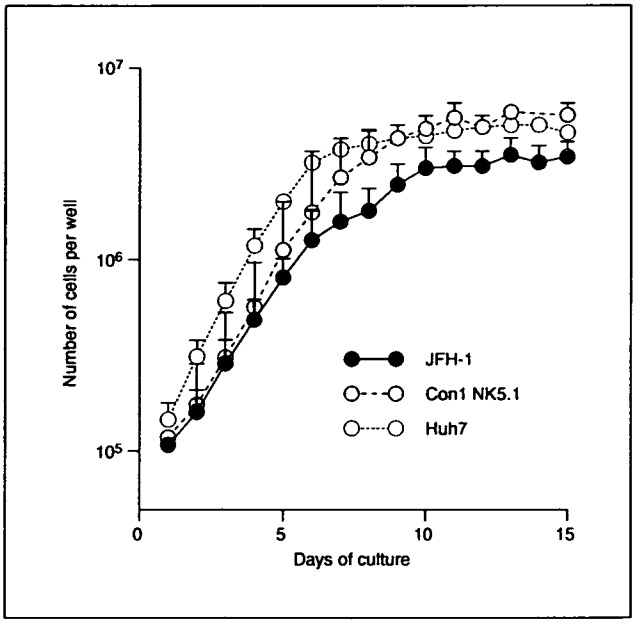

Fig. 1. Rate of cell growth. JFH-1 replicon clones, Con1 NK5.1 replicon clones and Huh7 cells were grown in 6-well culture plates and counted daily. Results are expressed by the mean ± SD cell number/well.

Table 1. HCV RNA copies detected in isolated clones carrying HCV replicon

Replicon clone	Number of isolated clones	HCV RNA copies/μg, RNA[a]
JFH-1	19	$2.73 \pm 2.16 \times 10^7$
Con1 NK5.1	6	$2.38 \pm 2.04 \times 10^7$

[a] Mean ± SD.

Table 2. Growth rates and cell densities (mean ± SD) of Huh7 and other cell lines harboring HCV replicon

Cell line	Mean doubling time, h	Saturation density $\times 10^5$ cells/cm^2
Huh7	27.4 ± 3.5[b]	5.17 ± 0.33[c]
JFH-1[a]	31.2 ± 2.7	3.41 ± 0.48
Con1 NK5.1[a]	28.9 ± 2.9	5.56 ± 0.34[c]

[a] Ten clones of the JFH-1 replicon and 4 clones of Con1 NK5.1 were used in this experiment.
[b] $p < 0.05$ versus JFH-1.
[c] $p < 0.001$ versus JFH-1.

Results

Replication of HCV RNA in Clones Transfected with JFH-1 or Con1 NK5.1 Subgenomic HCV RNA

To determine the replicative ability of the JFH-1 clone, synthetic RNA transcribed from linearized pSGR-JFH1 or pFK/Con1 NK5.1 was transfected into Huh7 cells by electroporation, as described previously [12]. Cells transfected with the Con1 NK5.1 replicon became confluent more rapidly than those transfected with the JFH-1 replicon, until they started to die because of G418; transfected cells were cultured for 3 weeks in the presence of 1 mg/ml G418. Efficiencies of colony formation were 53,200 and 909 colony forming units/μg RNA for JFH-1 and Con1 NK5.1 replicons, respectively [12].

A total of 19 clones were isolated for the JFH-1 replicon and 6 clones for the Con1 NK5.1 replicon. Each clone had been cultured until approximately 80–90% confluence was achieved on the culture dish, whereupon it was harvested. Total RNA was extracted from each replicon clone, and the replication of RNA was confirmed using Northern blot analysis (data not shown). Mean replication levels of HCV RNA determined by RTD-PCR were similar for both replicon clones, $2.73 \pm 2.16 \times 10^7$ copies/μg RNA for JFH-1 and $2.38 \pm 2.04 \times 10^7$ copies/μg RNA for Con1 NK5.1 (table 1). Expressions of HCV proteins were confirmed in all replicon clones by Western blot and immunofluorescence assay (data not shown).

Growth Curves of Replicon Cells

Rates of cellular growth were determined on parental Huh7 cells, 10 cloned cells harboring JFH-1 replicon and 4 cloned cells supporting Con1 NK5.1. Each cell line was seeded at 1.0×10^5 cells/well in 6-well plates, harvested daily and counted. Figure 1 depicts mean rates of cell growth for the JFH-1 replicon, the Con1 NK5.1 replicon and Huh7 cells. The mean doubling time during the logarithmic growing phase was 31.2 ± 2.7 h for JFH-1, 28.9 ± 2.9 h for Con1 NK5.1 and 27.4 ± 3.5 h for Huh7 (JFH-1 vs. Huh7, $p < 0.05$) (table 2). Saturation densities determined by cell counts after they had reached the confluence were $3.41 \pm 0.48 \times 10^5$ cells/cm^2 for JFH-1, $5.56 \pm 0.34\ 010^5$ cells/cm^2 for Con1 NK5.1 and $5.17 \pm 0.33 \times 10^5$ cells/cm^2 for Huh7 (JFH-1 vs. Con1 NK5.1 or Huh7, $p < 0.001$) (table 2). Thus, JFH-1 replicon cells had slower cell growth and lower cell density than parental Huh7 cells.

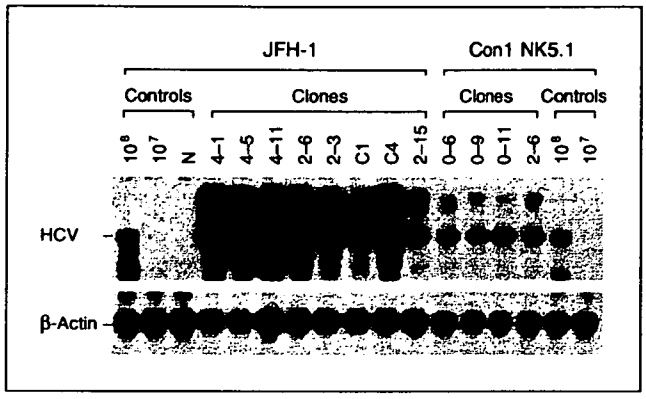

Fig. 2. Northern blot analysis of replicon RNA 1 day after the cell passage. Total cellular RNAs (2.5 μg) isolated from JFH-1 and Con1 NK5.1 replicon cells at the next day of passage were loaded onto agarose gel. After electrophoresis, RNAs of HCV or β-actin were detected by the Northern blot with random-primed DNA probes specific to encephalomyocarditits virus internal ribosome entry site and *neo*^{r} or β-actin sequences, respectively.

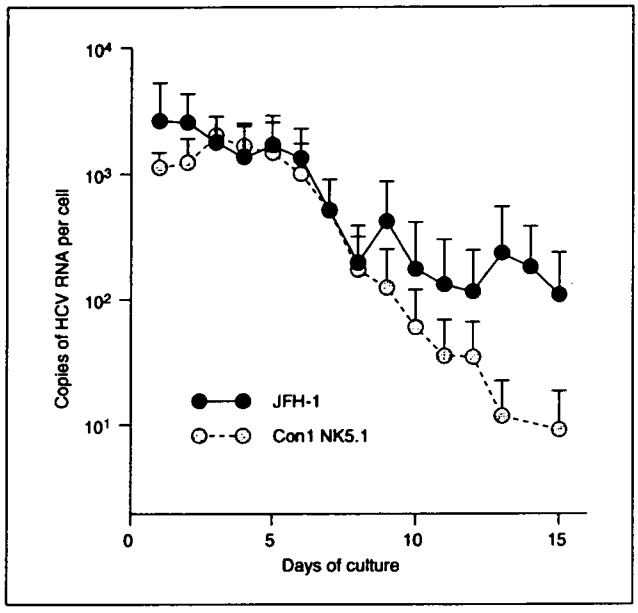

Fig. 3. Replication levels of HCV RNA during cell growth. HCV RNA replicating in JFH-1 or Con1 NK5.1 replicon cells were determined by the quantitative RTD-PCR.

Pietschmann et al. [17] reported that the replication level of the Con1 NK5.1 replicon is inversely related to cell growth. Therefore, we compared the number of HCV RNA copies determined by RTD-PCR between JFH-1 and Con1 NK5.1 replicon clones that had been examined for the cell growth. Mean titers of HCV RNA at 1 day after seeding were 2.65 ± 2.36 × 10^3 copies/cell for JFH-1 and 1.14 ± 0.28 × 10^3 copies/cell for Con1 NK5.1 ($p = 0.396$). However, most JFH-1 replicon clones emitted HCV RNA signals stronger than those of Con1 NK5.1 replicon clones in Northern blot analysis (fig. 2). HCV RNA levels of both JFH-1 and Con1 NK5.1 replicon cells decreased gradually and became comparable during days 3–8 (fig. 3). Both replicon cells became confluent around day 7–9. However, the mean RNA titer of JFH-1 clones remained around 100 copies/cell, while that of Con1 clones decreased to some 10 copies/cell during days 9–15. When all replicon cells were expanded after cloning, they were 80–90% confluent with HCV RNA titers comparable between JFH-1 and Con1 NK5.1 (table 1). The mean levels of replicon RNA at day 15 were 9.84 ± 12.58 × 10^1 copies/cell for JFH-1 and 0.93 ± 0.86 × 10^1 copies/cell for Con1 NK5.1 ($p = 0.0897$). During this experiment, culture medium was replaced on days 4, 8 and 12. The mean level of replicon RNA in JFH-1 clones increased the day after replacement of culture medium. However, RNA levels of Con1 NK5.1 clones were not affected by the replacement of culture medium.

Effects of IFN on the Replication of Subgenomic RNA

HCV replicon clones of genotype 1b are reportedly sensitive to IFN-α [10, 18]. However, in the clinical setting, HCV genotype 1b is generally considered resistant to IFN-α, in contrast to HCV genotype 2a which is more sensitive to it. To compare sensitivities of JFH-1 and Con1 NK5.1 replicons to IFN, serially diluted IFN-α was added to cultures of these replicons. After cultivation for 72 h, replicon cells were harvested and cellular RNA was extracted from each culture. RNAs isolated from cloned replicon cells, JFH-1/4–1 and Con1 NK5.1/0–11, were subjected to Northern blot analysis (fig. 4). The copy number of replicon RNA was also determined by RTD-PCR. IFN-α at a concentration of 10 U/ml clearly reduced the replication of Con1 NK5.1/0-11 replicon RNA, whereas JFH-1/4-1 replicon RNA did not decrease with 10 U/ml IFN-α (fig. 4). Copy numbers of HCV RNA were determined by RTD-PCR in 4 clones each for JFH-1 and Con1 NK5.1 replicons. Changes in the copy number of HCV RNA are expressed by the percentage of that cultured without IFN-α shown in figure 5. Inhibitory concentration of 50% (IC_{50}) thus calculated was 30.61 ± 15.19 U/ml for JFH-1 replicons and 2.94 ± 1.59 U/ml for Con1 NK5.1 replicons ($p < 0.05$). Although there is a

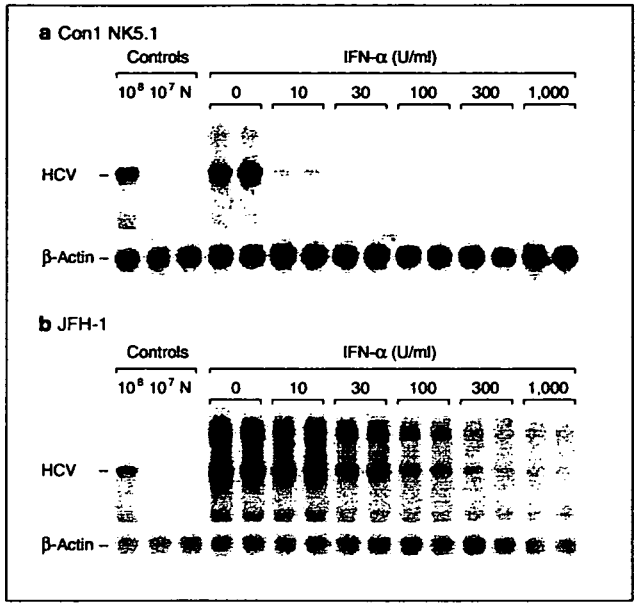

Fig. 4. Northern blot analysis of HCV RNAs in Con1 NK5.1/0-11 (**a**) and JFH-1/4-1 (**b**) replicon clones having undergone treatment with IFN-α. Cells were cultured for 72 h in the absence (lanes 4–5) or presence (lanes 6–15) of 10–1,000 U/ml IFN-α.

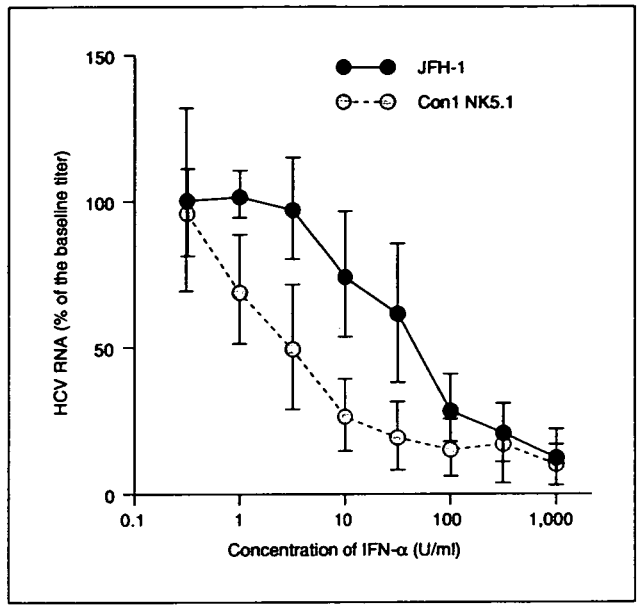

Fig. 5. Inhibition of HCV replicons by IFN-α. Four clones each of JFH-1 and Con1 NK5.1 replicons were cultured for 72 h in the absence or presence of 0.3–1,000 U/ml IFN-α. HCV RNA titers in replicon cells were determined by the quantitative RTD-PCR. HCV RNA levels from control cells (without IFN) were set at 100%. The percentage of HCV RNA levels of the JFH-1 or Con1 NK5.1 replicon was calculated and plotted against the concentration of IFN-α. Data were normalized based on the concentration of an internal control (glyceraldehyde-3-phosphate dehydrogenase).

significant difference in IC_{50} between replicons of genotypes 1b and 2a, the mean copy number of HCV RNA extracted from cells in the absence of IFN was different, namely $5.77 \pm 3.7 \times 10^7$ copies/μg RNA for JFH-1 and $5.56 \pm 6.21 \times 10^6$ copies/μg RNA for Con1 NK5.1. Thus, the difference in IC_{50} between genotypes may be due to different replication capacities of the clones tested rather than a distinct response to IFN-α.

Discussion

Since the discovery of HCV, numerous sequences of HCV isolates have been reported. Comparison of these sequences has highlighted a marked genetic heterogeneity of the HCV genome, by which HCV isolates have been classified into six genotypes. Among them, genotypes 1 and 2 are distributed worldwide [6]. Increasing lines of evidence indicate that patients infected with HCV of different genotypes may present with distinct clinical profiles, in terms of severity of liver disease and response to antiviral therapies [7]. For example, genotype 1b is known to be resistant to IFN, whereas genotype 2a is sensitive to it. However, because of its ubiquity, investigations of viral characteristics have been mostly conducted on HCV isolates of genotype 1b. The newly established subgenomic replicon of genotype 2a [12] has provided opportunities for evaluating differences in viral characteristics between genotypes 1 and 2 to improve our understanding on mechanisms of viral replication and persistence.

In this study, we compared subgenomic HCV replicons of genotype 1b with genotype 2a, using Con1 NK5.1and JFH-1 strains, respectively. As reported previously [12], the efficiency of colony formation is higher for JFH-1 than for Con1 NK5.1. However, HCV RNA titers of both replicons did not differ when harvested after cloning procedures (table 1). The efficiency in colony formation may reflect the replication capacity of the original HCV strain rather than HCV RNA titers of replicon cells, since levels of HCV RNA replication in the selected clones may have been enhanced through G418 selection and cloning procedure. However, a high replication efficiency of the JFH-1 replicon has been demonstrated in a transient replication of JFH-1 in Huh7 cells in the ab-

sence of G418 selection [12], in contrast to Con1 NK5.1 that did not efficiently replicate in conditions of transient transfection (data not shown). It would have been necessary for HCV RNA titers of replicon clones to remain at levels high enough to overcome the selection pressure of G418. In support of this view, the Con1 NK5.1 replicon has acquired adaptive mutations to maintain efficient replication in Huh7 cells [15]. However, adaptive mutations are not always necessary to maintain the replication of HCV RNA, since JFH-1 replicates in Huh7, HepG2, IMY-N9 and other cell lines as well [12–14].

The present study has shown the mean doubling time and saturation densities of JFH-1 replicon clones significantly lower than those of parent Huh7 cells. However, Con1 NK5.1 replicon clones did not show such decreases, in consistence with the original report [17]. Hence, RNA replication and protein expression of the JFH-1 replicon may suppress the cell growth. Otherwise, slower growing Huh7 subclones may have been preferentially selected for the JFH-1 replicon. Several HCV proteins are reported to suppress cell growth when expressed stably or by induction [19–21]. The core protein of HCV has a relatively weak effect on the cell growth [22], and conditional expression of structural HCV proteins (core, E1 and E2) and some nonstructural proteins (NS2 and NS3) completely suppresses it [23]. In contrast, nonstructural proteins such as NS5A may accelerate the rate of cell growth [24]. The viral protein responsible for a phenotype for slower growth of the JFH-1 replicon should therefore be sought for.

HCV titers of JFH-1 and Con1 NK5.1 replicon clones were monitored in analysis for cell growth (fig. 2). After cells reached the confluence, HCV copy numbers of JFH-1 replicon clones remained at around 100 copies/cell contrasting with those of Con1 NK5.1 that decreased to some 10 copies/cell. Furthermore, copy numbers of JFH-1 clones increased by the exchange of culture medium, whereas Con1 NK5.1 clones did not respond to it (fig. 2). This result may suggest that JFH-1 and Con1 HCV replicons replicate in Huh7 cells with the use of different cellular factors or by utilizing the same factors in distinct manners.

Clinical experiences have indicated that infection with HCV genotype 1b is resistant to IFN, while that with genotype 2a is usually sensitive to it [7]. In the present study, the JFH-1 replicon of genotype 2a exhibited a phenotype more resistant to IFN than to the Con1 NK5.1 replicon of genotype 1b. Previous reports have described efficient responses of HCV and Con1 NK5.1 replicons, both of genotype 1, to type I IFN [10, 18]. At present, it is unclear whether or not the response to IFN, which is different between these replicons of genotypes 1b and 2a, contradicts distinct characteristics of genotypes in the clinical setting. However, infections with HCV genotype 2a are not always sensitive to IFN, and the JFH-1 strain may represent a rare IFN-resistant genotype 2a clone. Certainly, more strains need to be isolated for experiments with replicons to compare the phenotype between genotypes 1b and 2a. However, a higher replicative capacity of JFH-1 may depend on IFN-resistant characteristics, especially under the experimental conditions used in this study.

In summary, we compared HCV RNA replication, cell growth and sensitivity to IFN between subgenomic replicons of genotypes 1b and 2a transfecting Huh7 cells. JFH-1, representing genotype 2a, exhibited a higher efficiency in colony formation than Con1 NK5.1 of genotype 1, even though copy numbers of replicating RNA did not significantly differ between them. Of note, JFH-1 was found to be more resistant to IFN than Con1 NK5.1, at least in experiments with subgenomic replicons.

Acknowledgments

We thank Dr. Ralf Bartenschlager for providing the pFK-I_{389}nco/NS3-3'/NK5.1. We are also grateful to Dr. Satoshi Koike and Dr. Kotaro Yasui for their helpful discussions. This work is supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, a grant from Toray Industries, Inc., the Program for Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency (PMDA), as well as the Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation and the Japanese Society of Gastroenterology.

References

1 Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K: Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 1990;87:9524–9528.

2 Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby R, Barr PJ: Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci USA 1991;88:2451–2455.

3 Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, Onishi E, Andoh T, Yoshida I, Okayama H: Structure and organization of the hepatitis C virus genome isolated from human carriers. J Virol 1991;65:1105–1113.

4 Reed KE, Rice CM: Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. Curr Top Microbiol Immunol 2000;242:55–84.

5 Bukh J, Purcell RH, Miller RH: Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc Natl Acad Sci USA 1994;91:8239–8243.

6 Ohno O, Mizokami M, Wu RR, Saleh MG, Ohba K, Orito E, Mukaide M, Williams R, Lau JY: New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. J Clin Microbiol 1997;35:201–207.

7 Yoshioka K, Kakumu S, Wakita T, Ishikawa T, Itoh Y, Takayanagi M, Higashi Y, Shibata M, Morishima T: Detection of hepatitis C virus by polymerase chain reaction and response to interferon-alpha therapy: Relationship to genotypes of hepatitis C virus. Hepatology 1992;16:293–299.

8 Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R: Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110–113.

9 Blight KJ, Kolykhalov AA, Rice CM: Efficient initiation of HCV RNA replication in cell culture. Science 2000;290:1972–1974.

10 Guo JT, Bichko VV, Seeger C: Effect of alpha interferon on the hepatitis C virus replicon. J Virol 2001;75:8516–8523.

11 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.

12 Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, Wakita T: Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 2003;125:1808–1817.

13 Date T, Kato T, Miyamoto M, Zhao Z, Yasui K, Mizokami M, Wakita T: Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells. J Biol Chem 2004;279:22371–22376.

14 Kato T, Date T, Miyamoto M, Zhao Z, Mizokami M, Wakita T: Nonhepatic cell lines HeLa and 293 cells support efficient replication of hepatitis C virus genotype 2a subgenomic replicon. J Virol 2005;79:592–596.

15 Krieger N, Lohmann V, Bartenschlager R: Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J Virol 2001;75:4614–4624.

16 Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-Kohara K, Kawaguchi R, Tanaka S, Kohara M: Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636–642.

17 Pietschmann T, Lohmann V, Rutter G, Kurpanek K, Bartenschlager R: Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J Virol 2001;75:1252–1264.

18 Frese M, Barth K, Kaul A, Lohmann V, Schwarzle V, Bartenschlager R: Hepatitis C virus RNA replication is resistant to tumour necrosis factor-alpha. J Gen Virol 2003;84:1253–1259.

19 Aizaki H, Harada T, Otsuka M, Seki N, Matsuda M, Li YW, Kawakami H, Matsuura Y, Miyamura T, Suzuki T: Expression profiling of liver cell lines expressing entire or parts of hepatitis C virus open reading frame. Hepatology 2002;36:1431–1438.

20 Moradpour D, Kary P, Rice CM, Blum HE: Continuous human cell lines inducibly expressing hepatitis C virus structural and nonstructural proteins. Hepatology 1998;28:192–201.

21 Arima N, Kao CY, Licht T, Padmanabhan R, Sasaguri Y: Modulation of cell growth by the hepatitis C virus nonstructural protein NS5A. J Biol Chem 2001;276:12675–12684.

22 Moradpour D, Englert C, Wakita T, Wands JR: Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. Virology 1996;222:51–63.

23 Moradpour D, Wakita T, Wands JR, Blum HE: Tightly regulated expression of the entire hepatitis C virus structural region in continuous human cell lines. Biochem Biophys Res Commun 1998;246:920–924.

24 Ghosh AK, Steele R, Meyer K, Ray R, Ray RB: Hepatitis C virus NS5A protein modulates cell cycle regulatory genes and promotes cell growth. J Gen Virol 1999;80:1179–1183.

JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2005, p. 5679–5684 Vol. 43, No. 11
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.11.5679–5684.2005

Detection of Anti-Hepatitis C Virus Effects of Interferon and Ribavirin by a Sensitive Replicon System

Takanobu Kato,[1,2] Tomoko Date,[2] Michiko Miyamoto,[2] Masaya Sugiyama,[1] Yasuhito Tanaka,[1] Etsuro Orito,[3] Tomoyoshi Ohno,[3] Kanji Sugihara,[3] Izumi Hasegawa,[3] Kei Fujiwara,[3] Kiyoaki Ito,[3] Atsushi Ozasa,[3] Masashi Mizokami,[1] and Takaji Wakita[2]*

Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya[1]; Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo[2]; and Department of Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya,[3] Japan

Received 8 July 2005/Returned for modification 17 August 2005/Accepted 31 August 2005

Although combination therapy with interferon and ribavirin has improved the treatment for chronic hepatitis C virus (HCV) infection, the detailed anti-HCV effect of ribavirin in clinical concentrations remains uncertain. To detect the anti-HCV effect of ribavirin in lower concentrations, a sensitive and accurate assay system was developed using the reporter replicon system with an HCV genotype 2a subgenomic replicon (clone JFH-1) that exhibits robust replication in various cell lines. This reporter replicon was generated by introducing the luciferase reporter gene (instead of the neomycin resistance gene) into the subgenomic JFH-1 replicon. To assess the replication of this reporter replicon, luciferase activity was measured serially up to day 3 after transient transfection of Huh7 cells. The luciferase activity increased exponentially over the time course of the experiment. After adjustment for transfection efficiency and transfected cell viability, the impacts of interferon and ribavirin were determined. The administration of interferon and ribavirin resulted in dose-dependent suppression of replicon RNA replications. The 50% inhibitory concentration of interferon and ribavirin was 1.80 IU/ml and 3.70 μg/ml, respectively. In clinical concentrations, replications were reduced to 0.09% and 53.74% by interferon (100 IU/ml) and ribavirin (3 μg/ml), respectively. Combination use of ribavirin and interferon enhanced the anti-HCV effect of interferon by 1.46- to 1.62-fold. In conclusion, we developed an accurate and sensitive replicon system, and the antivirus effect of interferon and ribavirin was easily detected within their clinical concentrations by this replicon system. This system will provide a powerful tool for screening new antiviral compounds against HCV.

Hepatitis C virus (HCV) is a major public health problem, infecting an estimated 170 million people worldwide. HCV causes chronic liver diseases, including cirrhosis and hepatocellular carcinoma, because most patients fail to clear the virus and the persistent infection that follows (1, 11, 20). Current therapy for HCV-related chronic hepatitis is based on the use of interferon (IFN). However, virus clearance rates are limited to approximately 10 to 20% of cases treated with IFN only (9, 23, 26). Combination therapy with IFN and ribavirin improves the HCV clearance rate, although the molecular mechanism responsible for this improvement is not yet fully understood (23, 25, 26). However, some direct antiviral mechanisms of ribavirin have been proposed (19). One possible mechanism is the direct inhibition of HCV RNA-dependent RNA polymerase, and another possibility is the RNA mutagen effect that drives a rapidly mutating RNA virus over the threshold to "error catastrophe." The detection of these direct anti-HCV effects has been hampered by the lack of an appropriate sensitive system for evaluating HCV replication.

Although HCV belongs to the *Flaviviridae* family and has a genome structure similar to those of the other flaviviruses (3, 27), efficient cell culture systems and small animal infection models for HCV have not yet been established. This disadvantage not only hampers the understanding of the life cycle of this virus but also prevents the development of adequate antiviral compounds against HCV infection. As an important step toward overcoming this disadvantage, a subgenomic HCV RNA replicon system has been developed and enabled the assessment of HCV replication in cultured cells (22). Although this represents a powerful tool in the study of HCV replication mechanisms and the search for potential antiviral agents, functional replicons have previously been reported only for genotype 1, and efficient replications of these replicons have been accomplished only in limited human hepatocyte-derived cell lines and with some adaptive mutations. To overcome these limitations, we developed an HCV genotype 2a subgenomic replicon system using a clone isolated from a patient with fulminant hepatitis (14, 15). This replicon system provides higher colony formation efficiency and robust replication not only in hepatocyte-derived cell lines but also in non-hepatocyte-derived cell lines, and adaptive mutations are not necessary for replication (6, 16). Recently, the culture cell-generated HCV particles of this clone have been demonstrated to be infectious for both Huh7 cells and a chimpanzee (21, 30, 32). This is the only clone which can produce infectious particles in Huh7 cells, and the replication of this clone in Huh7 cells is closely related to producing infectious particles. In the present study, we used the robust replicable subgenomic replicon of this clone to develop a sensitive and accurate assay system for anti-HCV effects, and we detected the suppression effect of both IFN and ribavirin in clinical concentrations.

* Corresponding author. Mailing address: Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu, Tokyo 183-8526, Japan. Phone: 81-423-25-3881. Fax: 81-423-21-8678. E-mail: wakita@tmin.ac.jp.

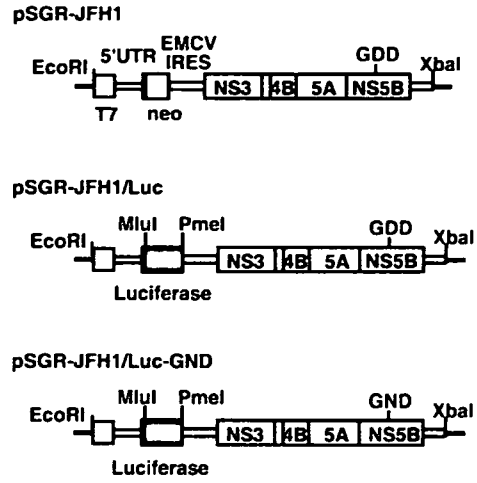

FIG. 1. Structures of the subgenomic and reporter replicon constructs pSGR-JFH1 (top), pSGR-JFH1/Luc (middle), and pSGR-JFH1/Luc-GND (bottom). Open reading frames (thick boxes) are flanked by untranslated regions (thin boxes). EcoRI, XbaI, MluI, and PmeI indicate positions of the respective restriction sites. In pSGR-JFH1/Luc and pSGR-JFH1/Luc-GND, the neomycin resistance gene was replaced by the luciferase reporter gene. GDD is the motif of HCV NS5B, RNA-dependent RNA polymerase. pSGR-JFH1/Luc-GND was constructed as a negative control by a point mutation altering GDD to GND. UTR, untranslated region; EMCV IRES, encephalomyocarditis virus internal ribosome entry site.

MATERIALS AND METHODS

Cell culture system. Huh7 cells were cultured at 37°C in 5% CO_2. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, as previously described (15).

IFN and ribavirin. Recombinant human alpha 2a IFN was obtained from Nippon Roche (Roferon-A; Tokyo, Japan). Ribavirin was purchased from Sigma-Aldrich (St. Louis, MO).

Reporter replicon constructs and RNA synthesis. The reporter replicon construct pSGR-JFH1/Luc was developed by rearrangement with pSGR-JFH1 (DDBJ/EMBL/GenBank accession number AB114136) that was constructed with the HCV genotype 2a clone JFH-1, which was isolated from a patient with fulminant hepatitis (14, 15). A DNA fragment encoding firefly luciferase was fused with the T7 promoter sequence and 5′ untranslated region of HCV clone JFH-1 by PCR and digested with EcoRI and PmeI (these restriction enzyme recognition sequences were artificially introduced in the primer site) and replaced the neomycin resistance gene of pSGR-JFH1 (Fig. 1). The construct of replication-deficient reporter replicon pSGR-JFH1/Luc-GND was also developed by introducing a point mutation at the GDD motif of RNA-dependent RNA polymerase to abolish this enzyme activity (Fig. 1).

The XbaI-digested pSGR-JFH1/Luc and pSGR-JFH1/Luc-GND were purified and used as templates for RNA synthesis. The subgenomic reporter replicon RNAs were synthesized in vitro using the MEGAscript T7 kit (Ambion, Austin, TX). Synthesized RNA was treated with DNase I followed by acid phenol extraction to remove any remaining template DNA.

RNA transfection. The RNAs transcribed from pSGR-JFH1/Luc and pSGR-JFH1/Luc-GND were transfected into Huh7 cells by electroporation as follows. Trypsinized cells were washed with Opti-MEM I reduced-serum medium (Invitrogen, Carlsbad, CA), and 2.0×10^6 cells were resuspended in 400 μl of Cytomix buffer. Three micrograms of synthesized replicon RNA was mixed with the cell suspension. These cells were transferred to an electroporation cuvette (Precision Universal Cuvettes; Thermo Hybrid, Middlesex, United Kingdom) and pulsed at 260 V and 950 μF with the Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred to 10 ml of culture medium and seeded into 12-well culture plates. Four hours after transfection, cells in a portion of the plates were harvested as a control for transfection efficacy, and a portion of the cells in the remaining plates received IFN or ribavirin in various doses. After administration of these agents, cells were harvested serially at 28 (day 1), 52 (day 2), and 76 (day 3) h after transfection.

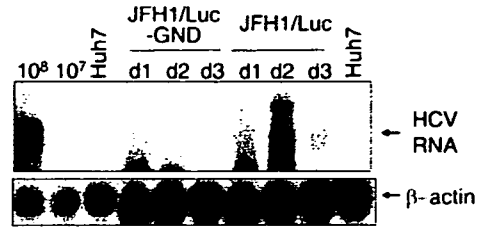

FIG. 2. Detection of reporter replicon RNA by Northern blot analysis. Total RNA from replicon RNA-transfected cells was analyzed by Northern blotting with DNA probes for the NS3-NS5b region of JFH-1 cDNA and β-actin genes. The 10^8 and 10^7 copies of in vitro-synthesized RNA were mixed with cellular RNA from untransfected Huh7 cells and used as positive controls. Arrows indicate target positions of reporter replicon RNA and β-actin. The Huh7 lanes contain cellular RNA from untransfected Huh7 cells as negative control.

MTS and luciferase assay. To adjust the number of viable cells, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay was performed with the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega), according to the manufacturer's instructions. Then, in the same well, luciferase activities were quantified with LUMAT LB9507 (EG&G Berthold, Bad Wildbad, Germany) and the luciferase assay system (Promega). Briefly, cells were lysed with 150 μl of cell culture lysis reagent (Promega), centrifuged, and mixed with luciferase assay reagent. Assays were performed at least in triplicate, and the results were expressed as luciferase activity relative to the luciferase activity at 4 h after transfection.

Northern blot analysis. Isolated RNA (4 μg) was separated in a 1% agarose gel containing formaldehyde, transferred to a positively charged nylon membrane (Hybond-N^+; Amersham Pharmacia, Buckinghamshire, United Kingdom), and immobilized with a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). Hybridization was performed with an [α-^{32}P]dCTP-labeled DNA probe using Rapid-Hyb buffer (Amersham Pharmacia). The DNA probe was synthesized from the nonstructural (NS) 3-NS5b region of JFH-1 cDNA using the Megaprime DNA labeling system (Amersham Pharmacia). A DNA probe for β-actin was also synthesized as a control.

Reverse transcription-PCR and sequencing analysis. The cDNAs of the reporter replicon were synthesized from total RNA that was isolated from replicon RNA-transfected Huh7 cells with a primer in the 3′X region. A part of the reporter replicon cDNA fragment was amplified by nested PCR with DNA polymerase (TaKaRa LA *Taq*; Takara Bio Inc., Shiga, Japan) and primers as follows: 6764S-IH, 5′-AAGCCGTTTTTCCGGGATGAGGTCTCGTTC-3′, and 9382R-IH, 5′-GAGTAATGAGCGGGGTCGGGCGCGCGACAC-3′, for first-round PCR and 8717S-IH, 5′-GGTGATCCCCCCAGACCGGAATAT GACCTG-3′, and 9367R-IH, 5′-CACAGCGTGTCGCGCGCCCGACCCCGC TCA-3′, for second-round PCR. These primers were designed to amplify the approximately 650-bp cDNA fragment in the NS5b region. This fragment con-

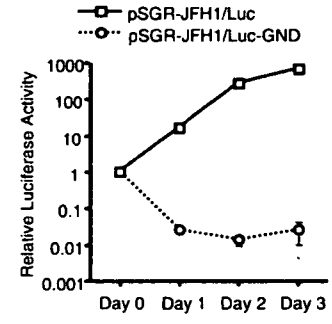

FIG. 3. Exponential replication of reporter replicon in Huh7 cells. Luciferase activity at days 1, 2, and 3 after RNA transfection is presented as multiples of the luciferase activity 4 h after transfection. Experiments were performed at least in triplicate. Data are presented as means and standard deviation bars.

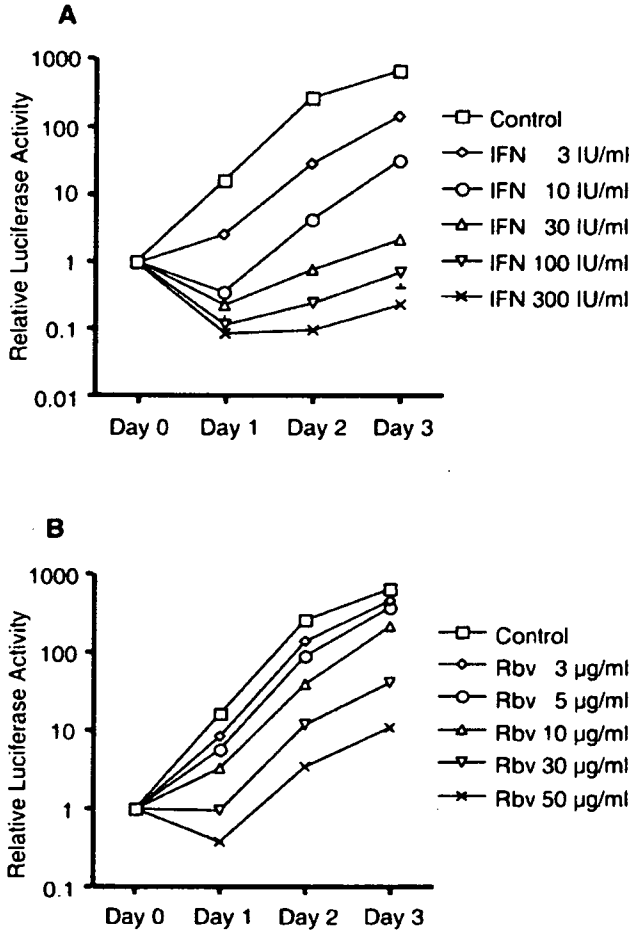

FIG. 4. Dose-dependent suppression of reporter replicon replication by IFN and ribavirin. Transiently transfected Huh7 cells were treated with various doses of IFN and ribavirin after 4 h of transfection. Experiments were performed at least in triplicate. Data are presented as means and standard deviation bars. Rbv, ribavirin.

tains the amino acid position that was identified as being associated with resistance to ribavirin by Young et al. (31). Amplified fragments were cloned into the pCR-TOPO vector (Invitrogen Corp., Carlsbad, CA), and at least 22 isolated clones were sequenced with the ABI 3100 automatic DNA sequencer (Applied Biosystems Japan, Tokyo, Japan) to determine the population of reporter replicons in Huh7 cells.

Computer analysis. To calculate the genetic distances between isolated clones, sequences were aligned by use of Clustal W software (version 1.8; DDBJ), and the numbers of nucleotide substitutions per site were determined with MEGA software (version 2.1) (17).

Statistical analysis. The Student *t* test was used to analyze data. *P* values less than 0.05 were considered statistically significant.

RESULTS

Monitoring the reporter replicon replication with Northern blot analysis and luciferase assays. To determine the transient replication ability of the SGR-JFH1/Luc reporter replicon, Northern blot analysis was performed with total cellular RNA extracted from SGR-JFH1/Luc replicon RNA- and SGR-JFH1/Luc-GND replicon RNA-transfected cells. The correct size of reporter replicon RNA was detected only in SGR-JFH1/Luc replicon RNA-transfected cells (Fig. 2). Signal intensity peaked on day 2 and decreased on day 3. The luciferase activity in lysates of transfected cells was monitored at four time points: day 0 (4 h), day 1 (28 h), day 2 (52 h), and day 3 (76 h). The relative luciferase activity was calculated by adjusting the luciferase activity to be a multiple of the luciferase activity 4 h after transfection. In the case of the SGR-JFH1/Luc replicon, the relative luciferase activity increased exponentially over the time course of the experiment. However, in the case of the replication-deficient replicon, SGR-JFH1/Luc-GND, the relative luciferase activity showed no increases (Fig. 3).

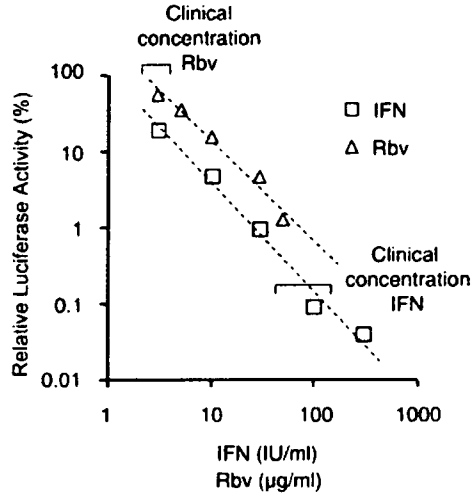

FIG. 5. Log dose-inhibition curve with IFN and ribavirin. Suppression of reporter replicon replication was calculated by comparison with control (without IFN and ribavirin) and presented as the percentage of replication at various concentrations of IFN and ribavirin. Reported clinical concentrations of IFN and ribavirin in serum are indicated. Rbv, ribavirin.

Anti-HCV effects of IFN and ribavirin. To detect the anti-HCV effect of IFN, IFN was added to the culture medium at various doses 4 h after transfection. The luciferase activity was serially monitored every 24 h for 3 days. To adjust for the transfection efficiency, the relative luciferase activity was calculated as a multiple of the luciferase activity 4 h after transfection. To exclude the cytotoxic effects caused by the added agents and the variations in cell seeding, the number of viable cells in each well was normalized by MTS assay.

The administration of IFN at various doses resulted in a dose-dependent suppression of reporter replicon replication (Fig. 4A). When the same experiment was conducted with ribavirin, reporter replicon replication was also suppressed in a dose-dependent manner; but the suppression was substantially weaker than that mediated by IFN (Fig. 4B).

To assess the linear dose dependency of the antiviral effects of both agents, the percentages of relative luciferase activity at day 2 were plotted for each concentration. Both IFN and ribavirin showed linearly correlated dose dependency, and R^2 was 0.987 and 0.976, respectively (Fig. 5). The 50% inhibitory concentration of IFN and ribavirin was 1.80 IU/ml and 3.70 μg/ml, respectively. Next, we compared the antiviral effects of these two agents in clinical concentrations. In a previous report, clinical concentrations of IFN and ribavirin in se-

TABLE 1. Effect of IFN and ribavirin on HCV replication at day 2 after treatment[a]

IFN (IU/ml)	Ribavirin (μg/ml)		
	(−)	3	10
(−)	100 ± 4.78	66.47 ± 0.33	22.78 ± 1.16
3	19.17 ± 2.04	11.06 ± 3.31[b]	1.92 ± 0.31[b]
10	4.82 ± 0.26	2.60 ± 0.39[b]	0.41 ± 0.06[b]
30	0.96 ± 0.06	0.54 ± 0.16[b]	0.17 ± 0.07[b]

[a] Data are represented as percentages of luciferase activity relative to the IFN (−) and ribavirin (−) controls and expressed as means ± standard deviations.
[b] $P < 0.05$ in comparison with ribavirin (−) by Student's t test.

TABLE 2. Numbers of mutations and genetic divergence among replicon populations

Population	No. of clones	No. of mutations[a]		Mean genetic divergence[b]
		Nucleotide	Amino acid	
Control	23	2.9 ± 0.5	4.2 ± 1.1	5.7 ± 0.9
IFN, 10 IU/ml	24	2.4 ± 0.4	5.7 ± 1.1	4.6 ± 0.8
Rbv,[c] 3 μg/ml	22	1.9 ± 0.4	4.2 ± 0.9	3.9 ± 0.7
IFN, 10 IU/ml + Rbv, 3 μg/ml	23	2.1 ± 0.4	5.1 ± 0.9	3.8 ± 0.7
Total	92	2.3 ± 0.2	4.8 ± 0.5	4.5 ± 0.4

[a] Data are expressed as means ± standard errors (10^3/site).
[b] Data are expressed as means ± standard errors (10^3 substitutions/site).
[c] Rbv, ribavirin.

rum were found to be 40.2 to 116.0 IU/ml and 2.2 to 4.3 μg/ml, respectively (29). We found that the reporter replicon replication was suppressed to 0.09% by 100 IU/ml of IFN and to 53.74% by 3 μg/ml of ribavirin (Fig. 5). Thus, the antiviral effect of IFN was much greater than that of ribavirin in clinical concentrations.

To elucidate the effect of IFN and ribavirin combined, these agents were administered simultaneously and the relative luciferase activity was measured 2 days after transfection. IFN was administered in three concentrations, 3, 10, and 30 IU/ml, and ribavirin was administered in two concentrations, 3 and 10 μg/ml. The addition of 3 μg/ml of ribavirin to various concentrations of IFN suppressed the relative luciferase activity by 54 to 66% (Table 1). Likewise, the addition of 10 μg/ml of ribavirin suppressed the relative luciferase activity by 10 to 20% (Table 1). The suppression of reporter replicon replication by IFN was presented as a linear regression ($R^2 = 0.995$; Fig. 6). The additional administration of ribavirin in two concentrations, 3 and 10 μg/ml, shifted the dose-dependent inhibition curves to the left with conserved linear regression ($R^2 = 0.997$ and 0.983, respectively; Fig. 6). The additional effect of ribavirin added to IFN was calculated to be 1.46- to 1.62-fold with 3 μg/ml of ribavirin and 3.94- to 6.14-fold with 10 μg/ml of ribavirin.

Effect of IFN and ribavirin on the mutation induction of the reporter replicon. The mutagen effect of ribavirin has been previously described (4, 5, 13, 28). To assess the mutagen effect of ribavirin in this system, sequences of replicating reporter replicon RNAs in Huh7 cells were determined after treatment with IFN (10 IU/ml), ribavirin (3 μg/ml), or both. These sequences were then compared with an untreated control. Total RNA was isolated 2 days after administration, and cDNA fragments were amplified by reverse transcription-PCR using primers covering the NS5B region. These amplified fragments were inserted into a cloning vector, and 22 to 24 clones in each treated well were sequenced. In the untreated control, the mutation induction rate was $(2.9 \pm 0.5) \times 10^{-3}$/site in nucleotides and $(4.2 \pm 1.1) \times 10^{-3}$ in amino acids. By administration of IFN, ribavirin, or both, the mutation induction rates were not statistically different from the untreated control (Table 2). To evaluate the complexity of quasispecies, mean genetic divergences between all possible isolated clone pairs were compared; there was no significant difference between the untreated control and treatment groups. Thus, the mutagen effect of ribavirin was not detected with this experimental system (Table 2). In addition, conserved amino acid mutations that indicate adaptation by use of IFN and ribavirin alone or in combination were not observed in the part of NS5b that was investigated (data not shown).

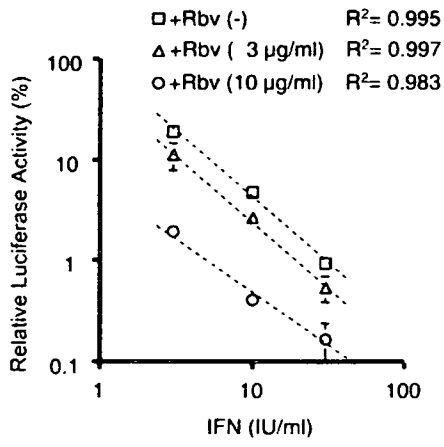

FIG. 6. Log dose-inhibition curve with combination use of IFN and ribavirin. Suppression of reporter replicon replication after combination use of IFN and ribavirin is shown. Rbv, ribavirin.

DISCUSSION

The development of an HCV replicon system has enabled the study of mechanisms for HCV replication and anti-HCV effects. Using this replicon system, the anti-HCV effects of IFN and ribavirin have been evaluated (13, 18, 28, 33). However, a number of these previous studies could not observe the anti-HCV effect of ribavirin in lower concentrations. In this report, we were able to identify the anti-HCV effect of ribavirin in clinical concentrations, because our replicon system has several advantages over the system used in previous reports. First, the HCV genotype 2a clone used in this system had potent replication activity in Huh7 cells (15). Previously reported replicons showed no exponential increment of replicon titer in the time course using normal Huh7 cells. By using a clone that efficiently replicates, the reporter replicon had an exponential increase in luciferase activity over time (Fig. 3). Additionally, this reporter replicon could replicate in a G418-free environment, although some of the previous replicons needed G418 selection during preparation or assessment for antiviral activities. G418 selection may alter the cellular characteristics of

anti-HCV status or modify the sensitivity to anti-HCV agents. Thus, the robust replication of this reporter replicon may be essential to detect the anti-HCV effect with higher sensitivity and accuracy. Second, in this reporter system, we did not use the established replicon-hosting cells. Instead, we used the transient-transfection method. Replicon-hosting cells were selected to be sufficiently permissive for replicon replication (2). These cells, known as permissive cells, were expected to have disruptions in their antiviral systems, such as IFN signaling pathways. Thus, the study of established replicon-hosting cells to detect antiviral activities may lead to false conclusions. Besides, cell-derived ribavirin resistance was identified in Huh7 cells recently (7). Long-term cultivation with ribavirin may select the cells with these characteristics. To overcome this disadvantage, we used the transient-transfection assay for the reporter replicon system and used normal Huh7 cells. Third, we used a luciferase assay to quantify the reporter replicon replication in this study. Some of the previous data regarding anti-HCV effects with replicon systems were determined by colony-forming efficiency or replicon titer that was quantified by real-time detection PCR. Drawbacks of the assay for colony-forming efficiency are that it is affected by the condition of transfected cells and it consumes a lot of time. A disadvantage of real-time detection PCR is that it might be affected by degraded RNA fragments. We introduced the luciferase gene (instead of a neomycin resistance gene) into the replicon construct (Fig. 1); this allowed us to estimate the replicon RNA replication by measuring the luciferase activity. This reorganized replicon construct not only improved the accuracy and sensitivity of this system but also made replicon replication easy to measure. Finally, in this study, the luciferase activity data were adjusted by the luciferase activity 4 h after transfection and by the viable cell count (determined by MTS assay). Thus, we obtained more accurate and reliable data by avoiding the variations caused by transfection efficiency and cell seeding or the cytotoxic effects of anti-HCV agents and could detect the intracellular anti-HCV effects of IFN and ribavirin.

In some clinical studies, ribavirin monotherapy did not improve the clearance rate of HCV or reduce the viral load (8, 10, 12). This clinical observation may appear to conflict with our data that ribavirin has an antiviral effect against HCV. In our study, ribavirin in clinical concentrations certainly suppressed the replicon replication, but the suppression ratio was around 50%. Although ribavirin suppressed the HCV replication by half in vivo, it may be difficult to detect this suppression in the viral titer of circulating blood. However, combined with IFN, ribavirin enhanced the IFN effect by 1.46- to 1.62-fold; this boost may be crucial to clear the virus and may improve the efficacy of IFN therapy.

Several previous reports have described increases in mutation frequencies induced by ribavirin (4, 13, 18, 28). However, this mutagen effect of ribavirin was not detected in this study. Neither the observed mutation rate nor the mean genetic divergences between isolated clones treated with ribavirin alone or combined with IFN differed from those of the control or clones treated with IFN alone (Table 2). This discrepancy with previous data may be caused by differences in the ribavirin concentration and duration of administration. We used 3 μg/ml of ribavirin as a clinical concentration; this concentration is lower than that used in the previous reports. Moreover, the observation period in this study was only 48 h. This short duration was long enough to detect the antiviral effect of ribavirin but may be too short to detect the mutagen effect. Thus, the observed antiviral effect of ribavirin in this study does not appear to be the result of accumulated mutations. However, the mutagen effect of ribavirin administered for a longer time may not be negligible in clinical studies. Another possible explanation for the lack of ribavirin-induced mutations in this study is the characteristics of the HCV clone used in this system. The clone used in this system is JFH-1, which was isolated from a fulminant hepatitis patient (14). JFH-1 has exhibited efficient replication without adaptive mutations in various cell lines (6, 15, 16). According to ribavirin resistance-related mutations in NS5a reported by Pfeiffer et al., JFH-1 has Glu instead of Gly (amino acid 404 in NS5a) and Thr instead of Glu (amino acid 442 in NS5a), although the association of these mutations with ribavirin resistance is still obscure (25a). This clone may be more resistant against the mutagen effect of ribavirin than previously reported clones. Thus, it may be necessary to form the basis for resistance genotyping or phenotyping of patient HCV isolates using this new replicon system. The observed anti-HCV effect of ribavirin in this study cannot be attributed to the error-prone characteristics but may be the direct replication inhibition that has been reported for other viruses. Recently, a new model of HCV dynamics has been proposed (24). This model was based on the assumption that ribavirin reduces the infectious virion production and could explain the synergic effect of ribavirin and interferon. Unfortunately, our system cannot assess the infectivity of HCV, because it uses subgenomic replicons. Thus, a new system will be necessary to assess the HCV virion production and infectivity with the JFH-1 clone in order to verify this hypothesis.

In summary, we developed an accurate and sensitive replicon system using a luciferase reporter gene and JFH-1 HCV cDNA. The anti-hepatitis C virus effect of IFN and ribavirin was easily detected within their clinical concentrations by this replicon system. This system will provide a powerful tool for screening the newer antiviral compounds against HCV.

ACKNOWLEDGMENTS

This work was partially supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and from the Ministry of Health, Labor and Welfare of Japan; by a grant from Toray Industries, Inc.; by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO); and by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation.

REFERENCES

1. **Alter, H. J., and L. B. Seeff.** 2000. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. Semin. Liver Dis. 20:17–35.
2. **Blight, K. J., J. A. McKeating, J. Marcotrigiano, and C. M. Rice.** 2003. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J. Virol. 77:3181–3190.
3. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362.
4. **Contreras, A. M., Y. Hiasa, W. He, A. Terella, E. V. Schmidt, and R. T. Chung.** 2002. Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replication system. J. Virol. 76: 8505–8517.
5. **Crotty, S., C. E. Cameron, and R. Andino.** 2001. RNA virus error catastrophe: direct molecular test by using ribavirin. Proc. Natl. Acad. Sci. USA 98:6895–6900.

6. **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.
7. **Di Bisceglie, A. M., H. S. Conjeevaram, M. W. Fried, R. Sallie, Y. Park, C. Yurdaydin, M. Swain, D. E. Kleiner, K. Mahaney, and J. H. Hoofnagle.** 1995. Ribavirin as therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. Ann. Intern. Med. **123**:897–903.
8. **Di Bisceglie, A. M., M. Shindo, T. L. Fong, M. W. Fried, M. G. Swain, N. V. Bergasa, C. A. Axiotis, J. G. Waggoner, Y. Park, and J. H. Hoofnagle.** 1992. A pilot study of ribavirin therapy for chronic hepatitis C. Hepatology **16**: 649–654.
9. **Di Bisceglie, A. M., and J. H. Hoofnagle.** 2002. Optimal therapy of hepatitis C. Hepatology **36**:S121–S127.
10. **Dixit, N. M., J. E. Layden-Almer, T. J. Layden, and A. S. Perelson.** 2004. Modelling how ribavirin improves interferon response rates in hepatitis C virus infection. Nature **432**:922–924.
11. **Hoofnagle, J. H.** 2002. Course and outcome of hepatitis C. Hepatology **36**:S21–S29.
12. **Kakumu, S., K. Yoshioka, T. Wakita, T. Ishikawa, M. Takayanagi, and Y. Higashi.** 1993. A pilot study of ribavirin and interferon beta for the treatment of chronic hepatitis C. Gastroenterology **105**:507–512.
13. **Kanda, T., O. Yokosuka, F. Imazeki, M. Tanaka, Y. Shino, H. Shimada, T. Tomonaga, F. Nomura, K. Nagao, T. Ochiai, and H. Saisho.** 2004. Inhibition of subgenomic hepatitis C virus RNA in Huh-7 cells: ribavirin induces mutagenesis in HCV RNA. J. Viral Hepat. **11**:479–487.
14. **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.
15. **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.
16. **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.
17. **Kumar, S., K. Tamura, and M. Nei.** 1994. MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. Comput. Appl. Biosci. **10**: 189–191.
18. **Lanford, R. E., B. Guerra, H. Lee, D. R. Averett, B. Pfeiffer, D. Chavez, L. Notvall, and C. Bigger.** 2003. Antiviral effect and virus-host interactions in response to alpha interferon, gamma interferon, poly(I)-poly(C), tumor necrosis factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. J. Virol. **77**:1092–1104.
19. **Lau, J. Y., R. C. Tam, T. J. Liang, and Z. Hong.** 2002. Mechanism of action of ribavirin in the combination treatment of chronic HCV infection. Hepatology **35**:1002–1009.
20. **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.
21. **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. (First published 9 June 2005; 10.1126/science1114016.)
22. **Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285**:110–113.
23. **McHutchison, J. G., S. C. Gordon, E. R. Schiff, M. L. Shiffman, W. M. Lee, V. K. Rustgi, Z. D. Goodman, M. H. Ling, S. Cort, J. K. Albrecht, et al.** 1998. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N. Engl. J. Med. **339**:1485–1492.
24. **Murray, E. M., J. A. Grobler, E. J. Markel, M. F. Pagnoni, G. Paonessa, A. J. Simon, and O. A. Flores.** 2003. Persistent replication of hepatitis C virus replicons expressing the beta-lactamase reporter in subpopulations of highly permissive Huh7 cells. J. Virol. **77**:2928–2935.
25. **Pawlotsky, J. M., H. Dahari, A. U. Neumann, C. Hezode, G. Germanidis, I. Lonjon, L. Castera, and D. Dhumeaux.** 2004. Antiviral action of ribavirin in chronic hepatitis C. Gastroenterology **126**:703–714.
25a. **Pfeiffer, J. K., and K. Kirkegaard.** 2005. Ribavirin resistance in hepatitis C virus replicon-containing cell lines conferred by changes in the cell line or mutations in the replicon RNA. J. Virol. **79**:2346–2355.
26. **Poynard, T., P. Marcellin, S. S. Lee, C. Niederau, G. S. Minuk, G. Ideo, V. Bain, J. Heathcote, S. Zeuzem, C. Trepo, J. Albrecht, et al.** 1998. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. Lancet **352**:1426–1432.
27. **Takamizawa, A., C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, and H. Okayama.** 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. J. Virol. **65**:1105–1113.
28. **Tanabe, Y., N. Sakamoto, N. Enomoto, M. Kurosaki, E. Ueda, S. Maekawa, T. Yamashiro, M. Nakagawa, C. H. Chen, N. Kanazawa, S. Kakinuma, and M. Watanabe.** 2004. Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. J. Infect. Dis. **189**:1129–1139.
29. **Tsubota, A., N. Akuta, F. Suzuki, Y. Suzuki, T. Someya, M. Kobayashi, Y. Arase, S. Saitoh, K. Ikeda, and H. Kumada.** 2002. Viral dynamics and pharmacokinetics in combined interferon alfa-2b and ribavirin therapy for patients infected with hepatitis C virus of genotype 1b and high pretreatment viral load. Intervirology **45**:33–42.
30. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Kräusslich, 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.
31. **Young, K. C., K. L. Lindsay, K. J. Lee, W. C. Liu, J. W. He, S. L. Milstein, and M. M. Lai.** 2003. Identification of a ribavirin-resistant NS5B mutation of hepatitis C virus during ribavirin monotherapy. Hepatology **38**:869–878.
32. **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.
33. **Zhou, S., R. Liu, B. M. Baroudy, B. A. Malcolm, and G. R. Reyes.** 2003. The effect of ribavirin and IMPDH inhibitors on hepatitis C virus subgenomic replicon RNA. Virology **310**:333–342.

JOURNAL OF VIROLOGY, Nov. 2005, p. 13963–13973 Vol. 79, No. 22
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.22.13963–13973.2005

Robust Production of Infectious Hepatitis C Virus (HCV) from Stably HCV cDNA-Transfected Human Hepatoma Cells

Zhaohui Cai,[1] Chen Zhang,[1] Kyung-Soo Chang,[1] Jieyun Jiang,[1] Byung-Chul Ahn,[1] Takaji Wakita,[2] T. Jake Liang,[3] and Guangxiang Luo[1]*

Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky College of Medicine, 800 Rose Street, Lexington, Kentucky 40536-0298[1]; Department of Microbiology and Immunology, Tokyo Metropolitan Institute for Neuroscience, Tokyo 183–8526, Japan[2]; and Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892[3]

Received 3 June 2005/Accepted 22 August 2005

Hepatitis C virus (HCV) chronically infects approximately 170 million people worldwide, with an increased risk of developing cirrhosis and hepatocellular carcinoma. The study of HCV replication and pathogenesis has been hampered by the lack of an efficient stable cell culture system and small-animal models of HCV infection and propagation. In an effort to develop a robust HCV infection system, we constructed stable human hepatoma cell lines that contain a chromosomally integrated genotype 2a HCV cDNA and constitutively produce infectious virus. Transcriptional expression of the full-length HCV RNA genome is under the control of a cellular Pol II polymerase promoter at the 5′ end and a hepatitis delta virus ribozyme at the 3′ end. The resulting HCV RNA was expressed and replicated efficiently, as shown by the presence of high levels of HCV proteins as well as both positive- and negative-strand RNAs in the stable Huh7 cell lines. Stable cell lines robustly produce HCV virions with up to 10^8 copies of HCV viral RNA per milliliter (ml) of the culture medium. Subsequent infection of naïve Huh7.5 cells with HCV released from the stable cell lines resulted in high levels of HCV proteins and RNAs. Additionally, HCV infection was inhibited by monoclonal antibodies specific to CD81 and the HCV envelope glycoproteins E1 and E2, and HCV replication was suppressed by alpha interferon. Collectively, these results demonstrate the establishment of a stable HCV culture system that robustly produces infectious virus, which will allow the study of each aspect of the entire HCV life cycle.

Discovered in 1989 by molecular cloning (10), hepatitis C virus (HCV) has been recognized as a major cause of viral hepatitis in humans. HCV infection is characterized by the establishment of chronic infection in the majority (up to 85%) of individuals exposed to HCV. It is estimated that approximately 4 million people in the United States and 170 million people worldwide are persistently infected (9, 38). The chronic HCV infection carries an increased risk of developing fatal liver diseases such as cirrhosis, liver failure, and hepatocellular carcinoma. HCV is a single-stranded positive-sense RNA virus belonging to the *Hepacivirus* genus of the family *Flaviviridae* (30). The 9.6-kb RNA genome encodes a single polyprotein that is cleaved by cellular and viral proteases into at least 10 structural (C, E1, E2, and probably p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins that play important roles in virus entry, replication, assembly, and pathogenesis (24, 29). The sequence and structures of the untranslated regions (UTR) at both the 5′ and 3′ ends of the HCV RNA genome, which contain *cis*-acting RNA elements required for HCV RNA translation and replication, are highly conserved (7, 13, 20, 24, 25, 35, 40, 41).

A great deal of progress has been made with respect to the HCV genome organization, properties and roles of viral proteins and conserved RNA sequence/structures, virus-host interactions, and mechanisms of HCV replication since the discovery of HCV (1, 24, 29, 36). A number of studies demonstrated that cDNA-derived HCV RNAs were infectious in chimpanzees upon intrahepatic inoculation (6, 19, 39). The chimpanzee model of HCV infection has played a central role in understanding HCV replication, disease progression, and host immune response (6, 32). A breakthrough advance was the development of a cell-based replication system in which HCV RNAs were efficiently replicated (4, 23). The HCV replicons have made it possible to determine the roles of viral proteins and RNA sequence and structures in HCV RNA replication (24). However, our ability to study the entire life cycle of HCV propagation and to perform genetic analysis and manipulation of HCV has been hampered until recently by the lack of a robust cell culture system of HCV infection and propagation. For the first time, it has recently been shown that transient transfection of a DNA vector expressing a full-length genotype 1b HCV RNA into Huh7 cells resulted in high levels of HCV virion production (15).

In an effort to develop a robust cell culture system for HCV production and infection, we have constructed stable human hepatoma cell lines that contain a chromosomally integrated cDNA of the genotype 2a HCV genome and robustly produce and secrete infectious HCV into the culture medium. The infectivity of the released HCV from the stable cell lines was demonstrated by the detection of high levels of HCV proteins and RNA in the HCV-infected Huh7.5 cells. Additionally, the HCV infectivity was efficiently neutralized by monoclonal antibodies against the HCV E1 and E2 proteins and CD81 in a

* Corresponding author. Mailing address: Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky College of Medicine, 800 Rose Street, Lexington, KY 40536-0298. Phone: (859) 257-5577. Fax: (859) 257-8994. E-mail: gluo0@uky.edu.

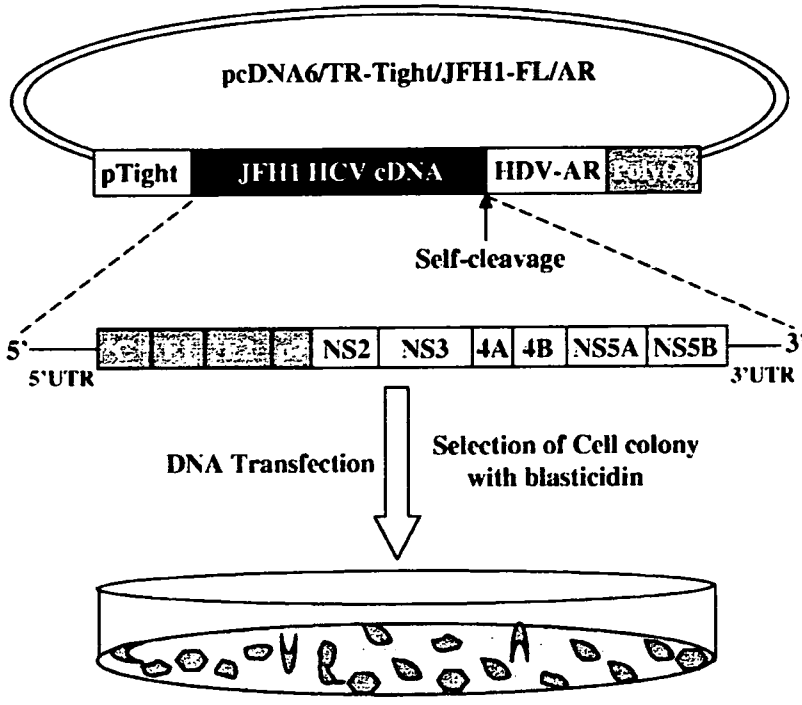

FIG. 1. Diagram of the genetic construction of stable cell lines expressing the HCV RNA genome. The transcriptional expression of the HCV RNA genome is under the control of a minimal CMV promoter (indicated by pTight) and the HDV antigenomic ribozyme (HDV-AR), followed by an SV40 poly(A) signal sequence. The JFH1 HCV genome organization and proteins are highlighted by letters and numbers. The plasmid DNA is introduced into Huh7 cells with DMRIE-C reagent, and stable cell colonies are selected by incubation with 5 μg/ml of blasticidin.

dose-dependent manner. The HCV replication was also inhibited by treatment with alpha interferon (IFN-α). Collectively, our findings demonstrate the establishment of a stable HCV culture system that robustly produces infectious virus, which will allow the study of each aspect of the entire HCV life cycle.

MATERIALS AND METHODS

Cell culture and HCV infection. A human hepatoma cell line, Huh7, was generously provided by Ralf Bartenschlager and was maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, nonessential amino acids, and 10% fetal bovine serum (FBS) (Invitrogen) (8). Huh7.5, an Huh7 variant cell line that is highly permissive to HCV RNA replication (5), was kindly provided by Charles M. Rice. Stable Huh7 cell lines containing a chromosomally integrated HCV genotype 2a cDNA were selected, amplified, and maintained in DMEM with 10% FBS and 5 μg/ml of blasticidin (A. G. Scientific).

DNA construction. The sequence of the full-length genotype 2a HCV (JFH1) cDNA was described previously (18). The vector pSGR-JFH1, which contains a subgenomic JFH1 cDNA (16), was used for construction of the full-length JFH1 HCV cDNA. The structural genes C, E1, E2, and p7 as well as the nonstructural gene NS2 were amplified by multiple rounds of PCR using synthetic oligonucleotides (Sigma-Genosys), as described previously (4, 25), based on the reported sequence (18). The synthetic C to NS2 genes were then cloned into the pBR322 vector between the EcoRI and NheI sites, and mutations that occurred during PCR were corrected by site-directed mutagenesis (Stratagene). The resulting C-NS2 genes were combined with the JFH1 subgenomic replicon cDNA, resulting in a full-length cDNA of the JFH1 HCV RNA, designated pSGR-JFH1-FL. A hepatitis delta virus (HDV) antigenomic ribozyme was placed at the immediate 3' end of the HCV RNA, followed by a simian virus 40 (SV40) poly(A) sequence, resulting in a vector designated pSGR/JFH1-FL/AR/pA. The T7 promoter at the 5' end of the pSGR/JFH1-FL/AR/pA vector was replaced with a minimal cytomegalovirus (CMV) promoter (pTight) derived from the pTRE-Tight vector (BD Biosciences) (26), resulting in a DNA construct named pSGR/Tight-JFH1-FL/AR/pA. For construction of stable cell lines, the pcDNA6/TR containing a blasticidin resistance gene was used as a vector, which was modified by introduction of unique restriction enzyme sites EcoR I and Sbf I. A short DNA fragment formed with oligonucleotides Linker-S (5'-AATTGAATTCGG TACCGCGGCCGCACTAGTCCTGCAGGT-3') and Linker-AS (5'-CCGGAC CTGCAGGACTAGTGCGGCCGCGGTACCGAATTC-3') was inserted into pcDNA6/TR between the restriction enzyme sites MfeI and AgeI. The DNA fragment containing the pTight promoter to SV40 poly(A) sequence was excised from the pSGR/Tight-JFH1-FL/AR/pA vector by EcoRI and SbfI digestion and then inserted into the modified pcDNA6/TR vector. The resulting DNA construct was designated pcDNA6/TR-Tight/JFH1-FL/AR.

DNA transfection and stable cell line selection. The pcDNA6/TR-Tight/JFH1-FL/AR DNA was transfected into Huh7 cells in a six-well cell culture plate by a lipofection method. Briefly, 2 μg of DNA was mixed with 5 μl of DMRIE-C reagent in Opti-MEM (Invitrogen) and then transferred onto Huh7 cells. At 24 h posttransfection, cells were split into 100-mm cell culture dishes at various cell densities. Cell colonies were selected by incubation with DMEM containing 10% FBS and 5 μg/ml of blasticidin for approximately 2 weeks. Stable cell lines were picked up and amplified. The expression of HCV proteins was detected by Western blotting and immunofluorescence assays using an NS3-specific monoclonal antibody (11), while the levels of the positive- and negative-strand HCV RNAs were determined by an RNase protection assay (RPA) using HCV strand-specific and radiolabeled RNA probes (25).

Western blot analysis. The HCV cDNA-harboring Huh7 cells or the HCV-infected Huh7.5 cells were lysed in a radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1% Nonidet P40, 0.5% sodium deoxycholate) containing a cocktail of proteinase inhibitors (Roche). The protein concentration of cell extracts was determined by using a protein assay reagent (Bio-Rad). Twenty-five micrograms of total protein for each sample was electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membrane was blocked by incubation with 5% skim milk. The levels of HCV NS3 and E2 proteins were determined by using monoclonal antibodies specific to NS3 and E2 proteins. To raise NS3-specific monoclonal antibodies, the HCV NS3 helicase domain with a six-His tag (NS3H) was expressed in *Escherichia coli* and purified by a nickel column chromatograph method (42). The purified recombinant NS3H was used

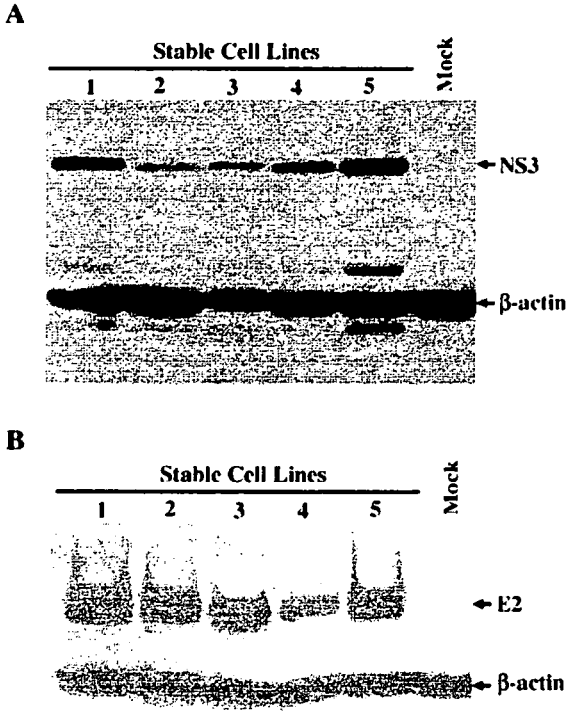

FIG. 2. Determination of the NS3 and E2 proteins in the stable cell lines by Western blotting. A total of 25 μg of cell extract was analyzed in a 10% sodium dodecyl sulfate-polyacrylamide gel. The NS3 and E2 proteins were detected by Western blotting using monoclonal antibodies against NS3 and E2 proteins (see Materials and Methods). The stable cell lines are numbered on the top. The naïve parental cell extract (Mock) was used as a negative control, and the β-actin protein was used as an internal control to normalize the amounts of proteins.

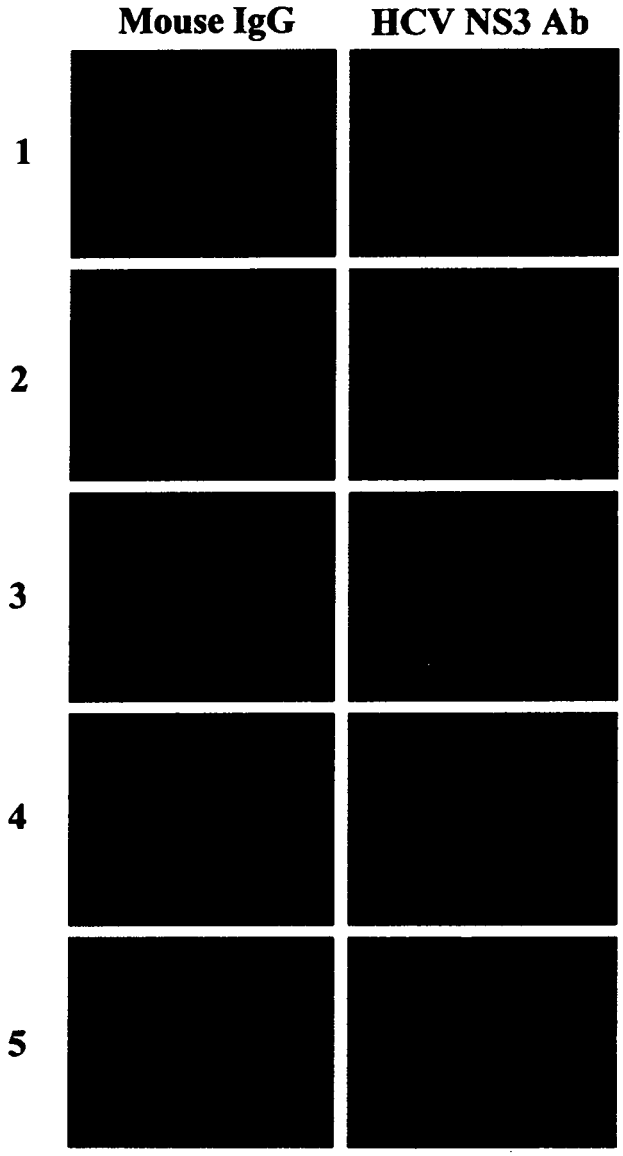

FIG. 3. IFA of the NS3 protein in stable cell lines. The stable cell lines were grown on coverslips. After a 24-h incubation, cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% bovine serum albumin and 1% donkey serum, and incubated with an anti-NS3 monoclonal antibody. The NS3 protein in the cell was then visualized with a donkey anti-mouse IgG conjugated with the Alexa Fluor 594 fluorescein (1:1,000 dilution). Each cell line had a negative control using normal mouse IgG as primary antibody. The stable cell lines are numbered at the left.

as an antigen to immunize mice, and hybridoma cell lines producing NS3 monoclonal antibodies were selected and identified by screening with the recombinant NS3H protein (K. S. Chang et al., unpublished data). The E1 (E1A4) and E2 (AP33) monoclonal antibodies have been described previously (15). The HCV NS3 and E2 proteins were subsequently visualized by using a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; Pierce) and staining with a chemiluminescence substrate (Pierce). The β-actin protein used as an internal control was detected by using an anti-β-actin monoclonal antibody (Sigma).

Immunofluorescence assay (IFA). Stable cell lines were grown overnight on coverslips in a 24-well culture plate. Cells were washed with 1× phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde solution, and permeabilized with 0.1% Triton X-100 (Sigma), as described previously (11). Subsequently, fixed cells were blocked with 1% bovine serum albumin and 1% donkey serum in PBS. The HCV NS3 and E2 proteins in cells were then detected by incubation with NS3- and E2-specific monoclonal antibodies and visualized with the secondary donkey anti-mouse IgG conjugated with Alexa Fluor 594 fluorescein (1:1,000 dilution) (Molecular Probes) (11). As a negative control, purified normal mouse IgG1 (Santa Cruz Biotechnology) was used as a primary antibody. Coverslips were then mounted onto slides, and the HCV proteins were visualized with a Zeiss Axioplan 2 fluorescence microscope.

RNA preparation and RPA. The full-length genotype 2a HCV RNA was transcribed in vitro by a T7 RNA polymerase from the pSGR-JFH1-FL/AR DNA linearized with the restriction enzyme XbaI (NEB) using an RNA transcription kit (Promega). After extensive treatment with RNase-free DNase I, the T7 RNA transcripts were purified by using an RNeasy RNA purification kit (QIAGEN). Total cellular RNA was extracted from stable Huh7 cell lines using an RNeasy RNA isolation kit (QIAGEN) or from the HCV-infected Huh7.5 cells with Trizol reagent (Invitrogen). The RNA concentration was determined by spectrophotometry. The levels of positive- and negative-strand HCV RNAs in the stable cell lines or HCV-infected Huh7.5 cells were determined by RPA using [α-^{32}P]UTP-labeled HCV-specific RNA probes, as described previously (8, 25). Briefly, 10 μg of total RNA was used in the RPA for hybridization with 4×10^4 cpm of [α-^{32}P]UTP-labeled β-actin probe and 10^5 cpm of either HCV (−)3′ untranslated region (UTR) or (+)5′ UTR RNA probe (8, 25). RPA was performed by using an RPA III kit following the manufacturer's procedures (Ambion). RNA products were analyzed by electrophoresis in a 6% polyacrylamide–7.7 M urea gel. The levels of RNAs were quantified with phosphorimager analysis.

Virus purification and sucrose gradient sedimentation. The culture medium (20 to 25 ml) of each stable cell line in a 162-cm^2 tissue culture flask was collected

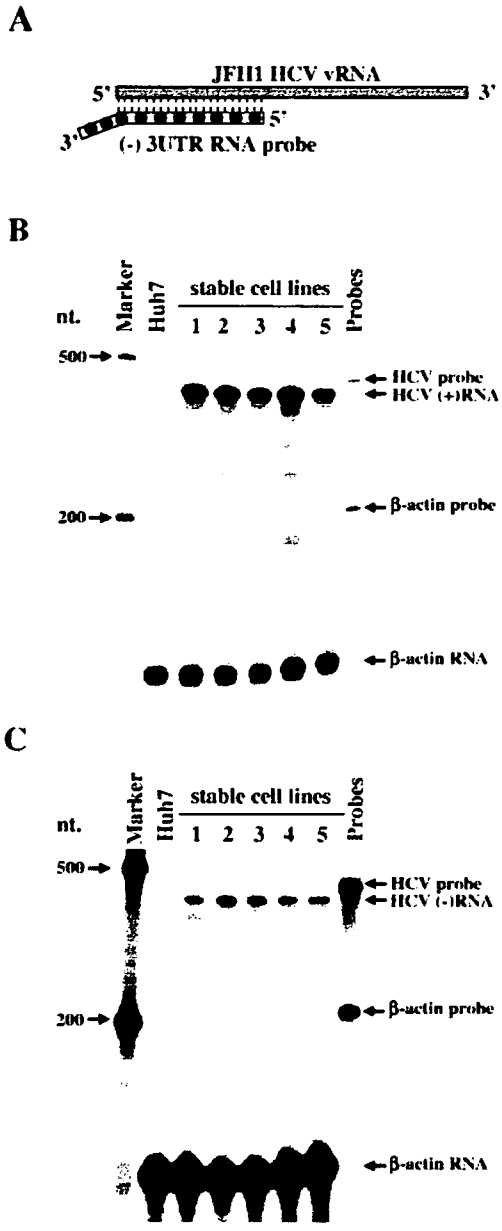

FIG. 4. (A) Schematic of the RPA. A radiolabeled RNA probe contains 377 nucleotides (nt) complementary to either the positive-strand 5′ UTR or negative-strand 3′ UTR of the JFH1 HCV (genotype 2a) and an additional 40 unpaired nucleotides derived from the vector. After hybridization and digestion with RNase A/T1, the region (377 nucleotides) protected from RNase digestion migrates faster than the incompletely digested RNA probe (417 nucleotides). (B) Determination of the levels of positive-strand HCV RNA present in the stable cell lines by RPA. A total of 10 μg of total cellular RNA extracted from stable cell lines was hybridized with 10^5 cpm of the [^{32}P]UTP-labeled negative-strand 3′ UTR RNA probe and 4×10^4 cpm of human β-actin RNA probe. After RNase digestion, the RNA products were analyzed in a 6% polyacrylamide–7.7M urea gel, autoradiographed, and quantified with a phosphorimager. (C) Determination of the levels of negative-strand HCV RNA by RPA. RPA is done in the same way as described in panel B except that the HCV strand-specific RNA probe was the positive-strand 5′ UTR RNA, which is complementary to the negative-strand HCV 3′ UTR region (25).

and clarified by centrifugation at 2,000 rpm for 10 min in a tabletop centrifuge to remove any cell debris. The medium was then loaded onto a 20% sucrose cushion in an ultracentrifuge tube. The HCV virions were purified by centrifugation at 27,000 rpm for 4 h at 4°C in a Beckman SW28 rotor. For Western blotting to detect the envelope glycoprotein E2, the virus pellet was lysed in a radioimmunoprecipitation assay buffer. For determination of the HCV virion RNA (vRNA), the virus pellet was resuspended in 250 μl of a TNE buffer (100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA). The HCV vRNA was extracted with a Trizol-SP reagent (Invitrogen) following the manufacturer's instructions and collected by ethanol precipitation. The vRNA was dissolved in 50 μl of RNase-free water. For sucrose density gradient sedimentation analysis of HCV virions, the above-concentrated HCV pellet was loaded onto the top of a continuous 20 to 60% sucrose gradient, followed by centrifugation at 40,000 rpm and 4°C for 16 h in a SW41 rotor (Beckman). Fractions of 1 ml each were collected from the top to the bottom of the sucrose gradient. The vRNA in each fraction (0.25 ml) was extracted with Trizol reagent and quantified by RPA. The infectivity of HCV virions in each fraction was determined by detection of the NS3 protein and positive-strand RNA in Huh7.5 cells infected with 0.1 ml of each fraction.

HCV infection and infectivity inhibition and neutralization. The naïve Huh7.5 cells in a 6-well tissue culture plate or 24-well plate (IFA) were infected with 1 ml of culture medium. At 3 h postinfection (p.i.), the HCV-containing medium was replaced with 2 ml of DMEM containing 10% FBS, and the cells were incubated at 37°C for 3 days prior to protein and RNA analyses. To determine the infectious titer of HCV, the culture medium was serially diluted 10-fold and used to infect Huh7.5 cells on coverslips in a 24-well culture plate. At 3 days p.i., the HCV infectivity was determined by IFA for NS3 using an NS3-specific monoclonal antibody, as described previously (44). For infectivity neutralization experiments, monoclonal antibodies specific to HCV E1 and E2 proteins (a mixture of two antibodies in equal amounts) and CD81 (Santa Cruz Biotechnology) were diluted with the HCV-containing culture medium. As a negative control, normal mouse IgG1 (Santa Cruz Biotechnology) was used in the neutralization experiments. Huh7.5 cells were infected with the virus-containing medium in the presence of increasing concentrations of normal mouse IgG1, E1/E2 monoclonal antibodies, or monoclonal CD81 antibody. After a 3-h incubation, the virus-containing medium was removed, and the cells were washed with PBS and incubated with fresh DMEM with 10% FBS. For IFN-α inhibition experiments, the cells were infected with HCV at 37°C for 3 h and then incubated with DMEM containing increasing concentrations of human IFN-α (Sigma) for 3 days. The effects of E1/E2 and CD81 monoclonal antibodies and IFN-α on HCV infectivity and replication were determined by the levels of NS3 (Western blotting) protein or HCV RNAs (RPA).

RT-PCR. The vRNA in the culture medium at different time points after HCV infection was extracted with Trizol reagent. The vRNA was then determined by a Titan one-tube reverse-transcription PCR (RT-PCR) system using the synthetic oligonucleotides 2a/SfiI (5′-TCCTCAAATGTGTCTGTGGCGTTGG-3′) and 2a/3′ UTR (5′-TCTAGACATGATCTGCAGAGAGACCAGT-3′) as primers. The RT-PCR DNA was analyzed in 1% agarose gel and photographed.

RESULTS

Genetic construction of stable human hepatoma cell lines expressing HCV RNA genome. The study of hepatitis B virus (HBV) replication and anti-HBV drug discovery have benefited enormously from the availability of a stable cell line, HepG2.2.15, which constitutively secretes infectious HBV into the culture medium (31). In this study, our goal was to genetically engineer stable human hepatoma cell lines to robustly produce infectious HCV from the chromosomally integrated HCV cDNA. To this end, we chose the JFH1 genotype 2a HCV since the JFH1 HCV RNA was isolated from a fulminant hepatitis C patient and was shown to replicate efficiently in human hepatic as well as nonhepatic cells (16–18). Additionally, we found that the JFH1 HCV RNA was able to replicate efficiently in mouse cells, which will be described elsewhere. To produce HCV RNA in the cell, we constructed a plasmid DNA in which transcription of the full-length HCV RNA genome is under the control of a 5′ minimal CMV promoter, and the 3′ terminus of the transcript is processed by an HDV ribozyme