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}neo/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.

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JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2005, p. 5679–5684
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.11.5679–5684.2005

Vol. 43, No. 11

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

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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.

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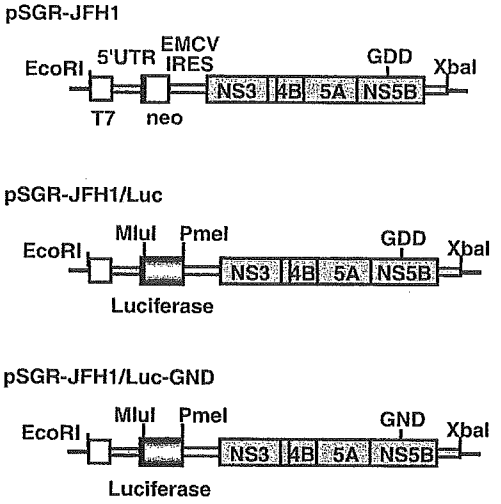

FIG. 1. Structures of the subgenomic and reporter replicon constructs pSGR-JFH1 (top), pSGR-JFH1/Luc (middle), and pSGR-JFH1/Luc-GND (bottom). Open reading frames (thick boxes) are flanked by untranslated regions (thin boxes). EcoRI, XbaI, MluI, and PmeI indicate positions of the respective restriction 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.

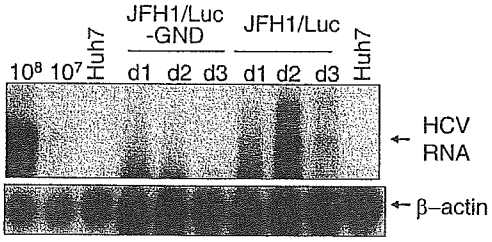

FIG. 2. Detection of reporter replicon RNA by Northern blot analysis. Total RNA from replicon RNA-transfected cells was analyzed by Northern blotting with DNA probes for the NS3-NS5b region of JFH-1 cDNA and β-actin genes. The 10^8 and 10^7 copies of in vitro-synthesized RNA were mixed with cellular 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-

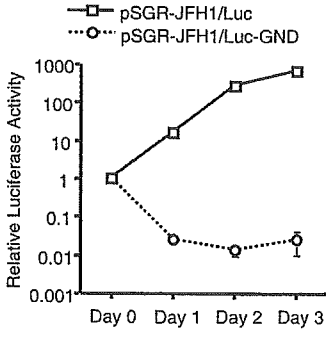

FIG. 3. Exponential replication of reporter replicon in Huh7 cells. Luciferase activity at days 1, 2, and 3 after RNA transfection is presented as multiples of the luciferase activity 4 h after transfection. Experiments were performed at least in triplicate. Data are presented as means and standard deviation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

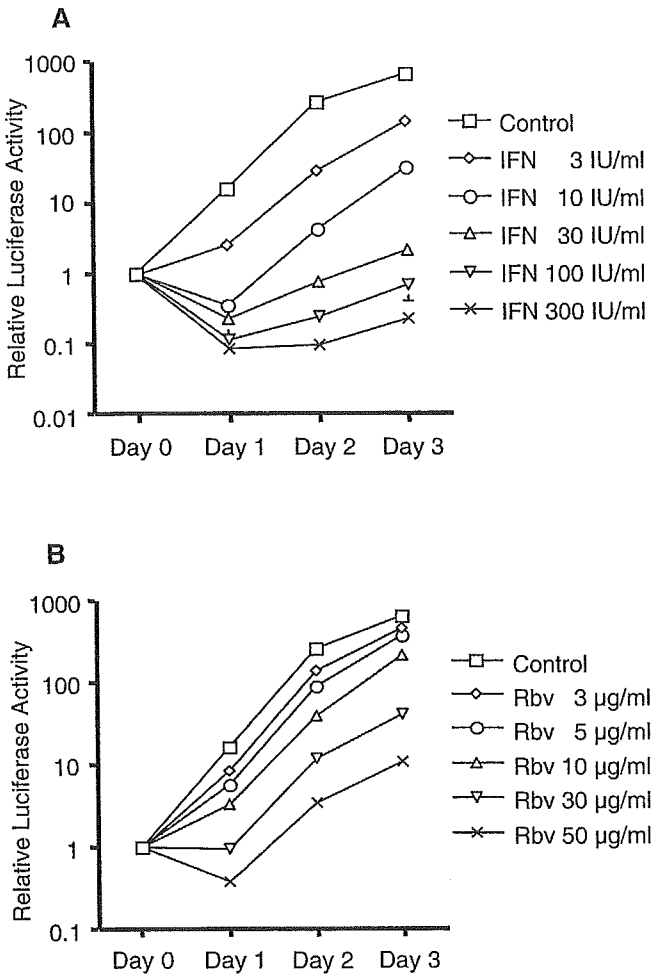

FIG. 4. Dose-dependent suppression of reporter replicon replication by IFN and ribavirin. Transiently transfected Huh7 cells were treated with various doses of IFN and ribavirin after 4 h of transfection. Experiments were performed at least in triplicate. Data are presented as means and standard deviation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

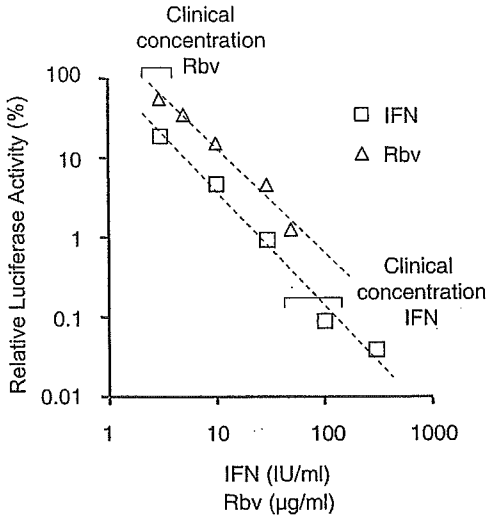

FIG. 5. Log dose-inhibition curve with IFN and ribavirin. Suppression of reporter replicon replication was calculated by comparison with control (without IFN and ribavirin) and presented as the percentage of replication at various concentrations of IFN and ribavirin. Reported clinical concentrations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

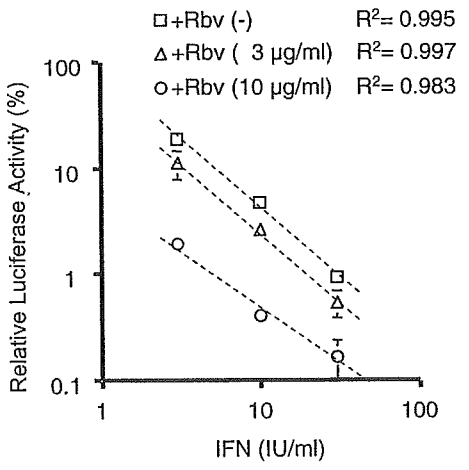

FIG. 6. Log dose-inhibition curve with combination use of IFN and ribavirin. Suppression of reporter replicon replication after combination use of IFN and ribavirin is shown. Rbv, ribavirin.

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.

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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

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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 10^6 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

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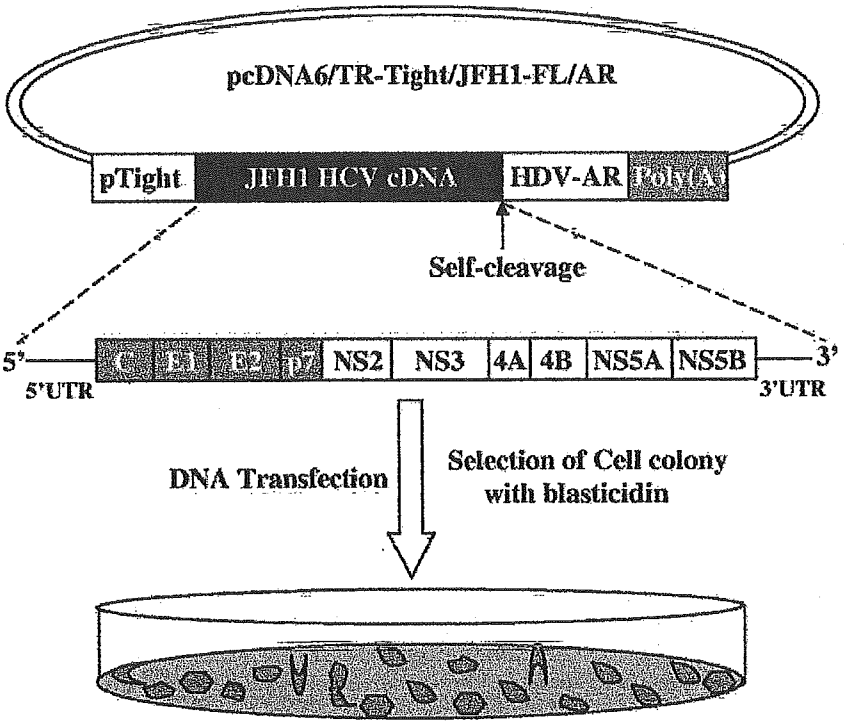

FIG. 1. Diagram of the genetic construction of stable cell lines expressing the HCV RNA genome. The transcriptional expression of the HCV RNA genome is under the control of a minimal CMV promoter (indicated by pTight) and the HDV antigenomic ribozyme (HDV-AR), followed by an SV40 poly(A) signal sequence. 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

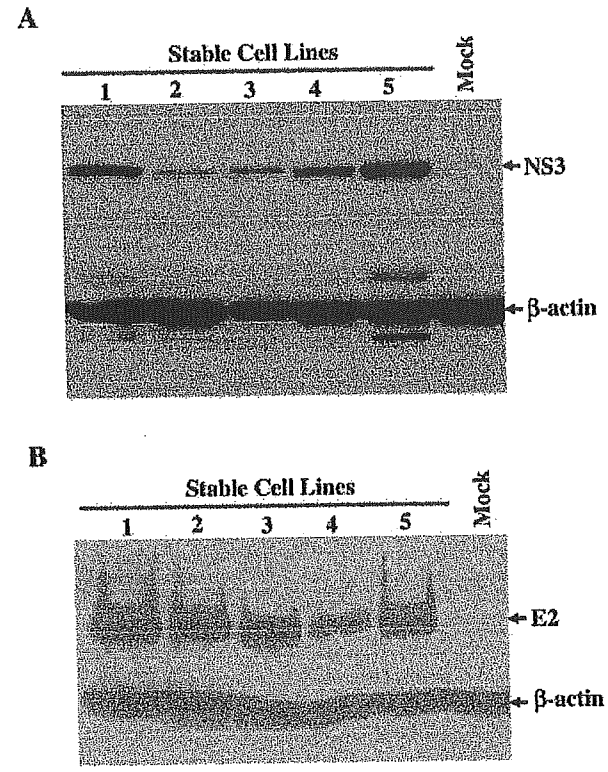

FIG. 2. Determination of the NS3 and E2 proteins in the stable cell lines by Western blotting. A total of 25 μg of cell extract was analyzed in a 10% sodium dodecyl sulfate-polyacrylamide gel. The NS3 and E2 proteins were detected by Western blotting using monoclonal antibodies against NS3 and E2 proteins (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.

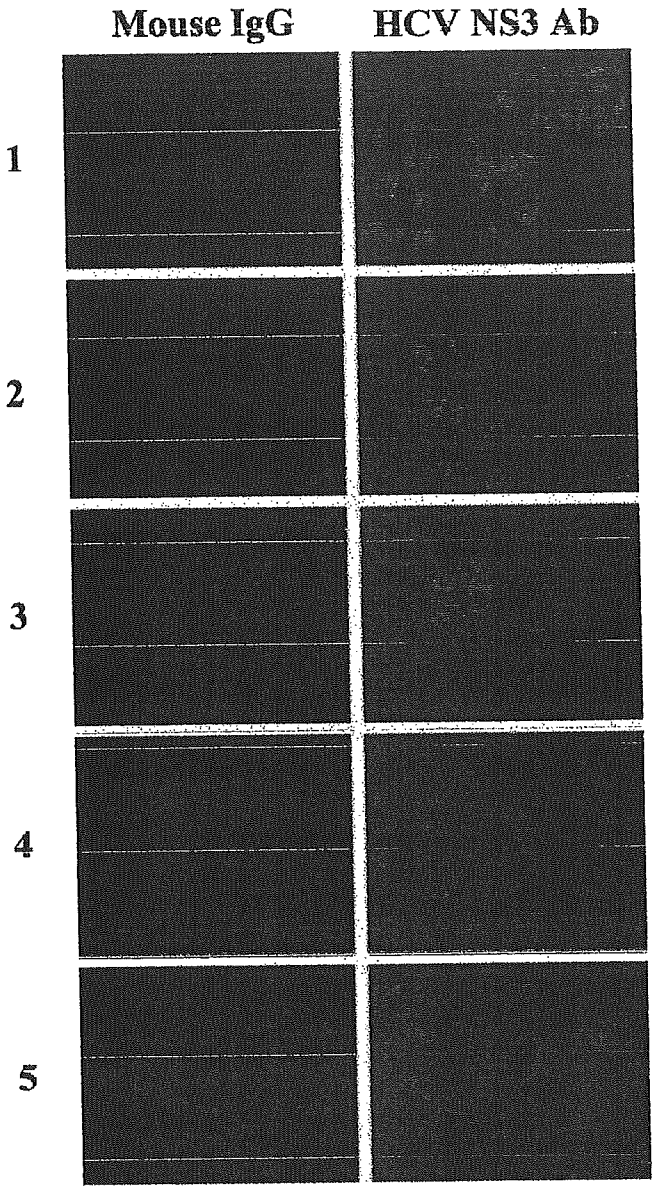

FIG. 3. IFA of the NS3 protein in stable cell lines. The stable cell lines were grown on coverslips. After a 24-h incubation, cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% bovine serum albumin and 1% donkey serum, and incubated with an anti-NS3 monoclonal 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

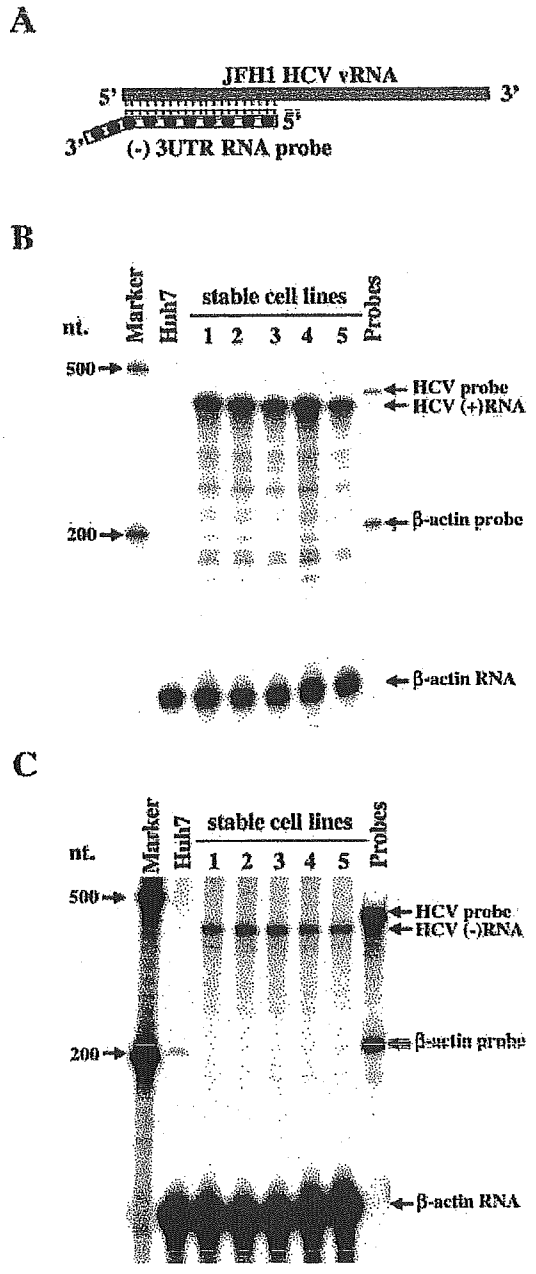

FIG. 4. (A) Schematic of the RPA. A radiolabeled RNA probe contains 377 nucleotides (nt) complementary to either the positive-strand 5′ UTR or negative-strand 3′ UTR of the JFH1 HCV (genotype 2a) and an additional 40 unpaired nucleotides derived from the vector. After hybridization and digestion with 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

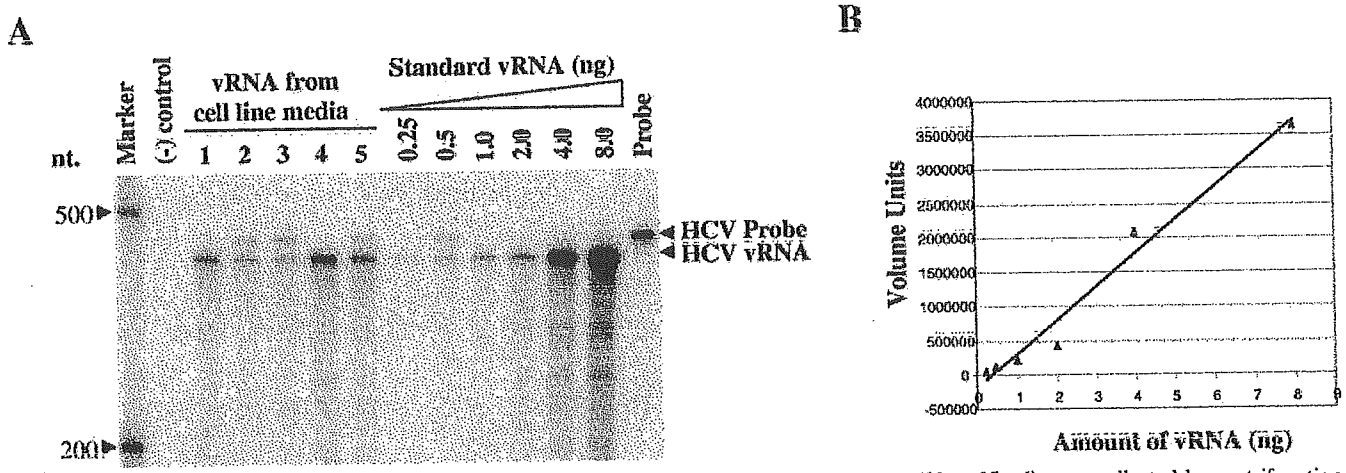

FIG. 5. (A) Determination of the HCV vRNA by RPA. HCV virions in the culture medium (20 to 25 ml) were collected by centrifugation through a 20% sucrose cushion. The vRNA was then extracted with Trizol reagent and dissolved in 50 μl of RNase-free water. A total of 20 μl of vRNA was used to hybridize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

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

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

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

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

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

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

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

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

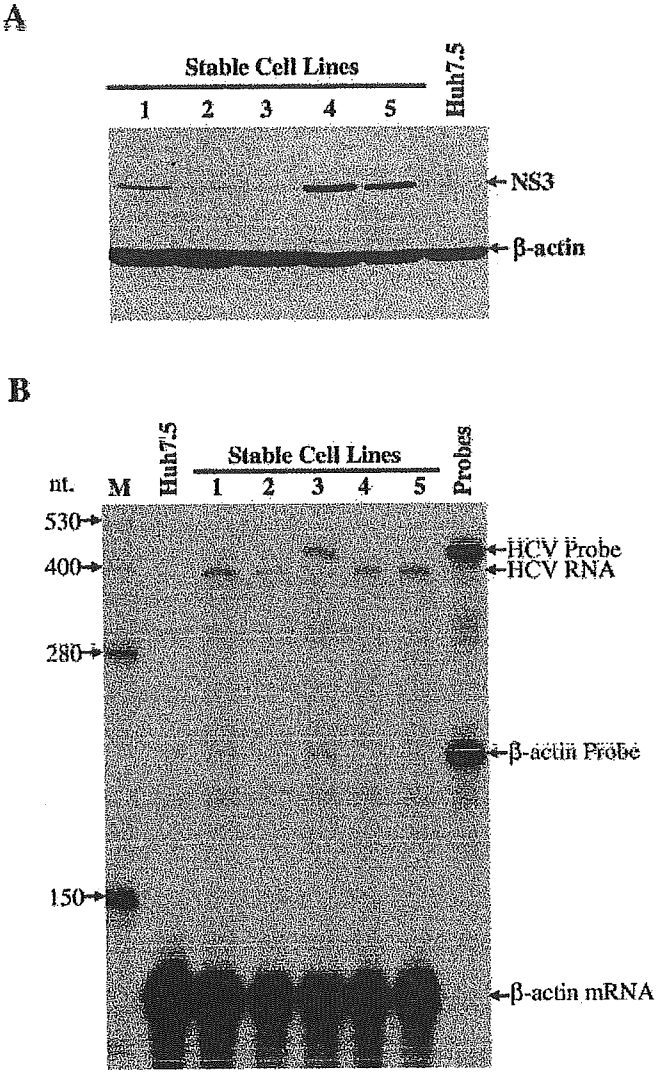

FIG. 6. Determination of the infectivity of the HCV virions secreted by the stable cell lines. The naïve Huh7.5 cells in a six-well plate were incubated with 1 ml of culture medium of each cell line. At 3 h p.i., the HCV-containing medium was replaced with 2 ml of fresh DMEM, and the cells were incubated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

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

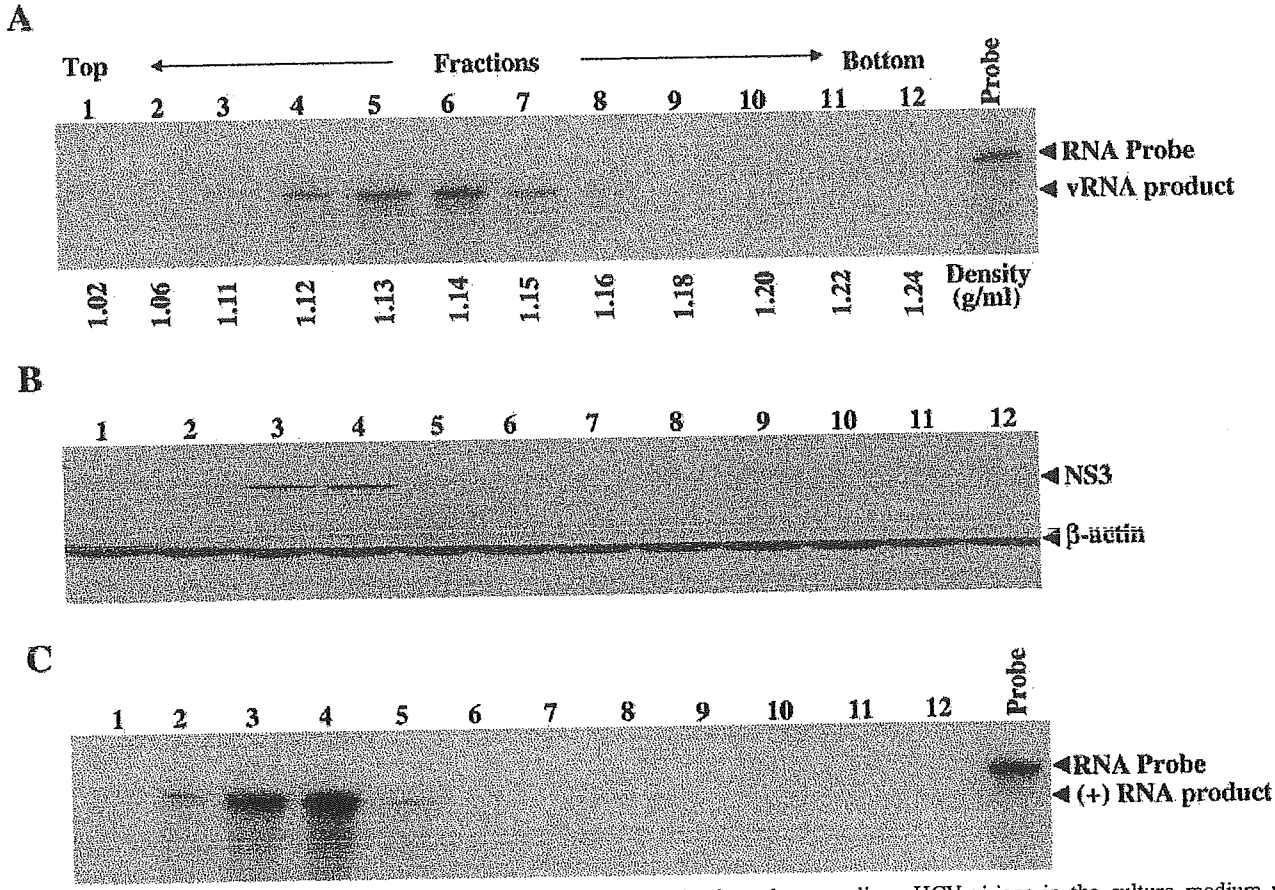

FIG. 7. (A) Sucrose gradient sedimentation analysis of HCV virions in the culture medium. HCV virions in the culture medium were concentrated by centrifugation through 20% sucrose cushion and then fractionated through 20 to 60% continuous sucrose gradient. Fractions (1 ml each) were collected from the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

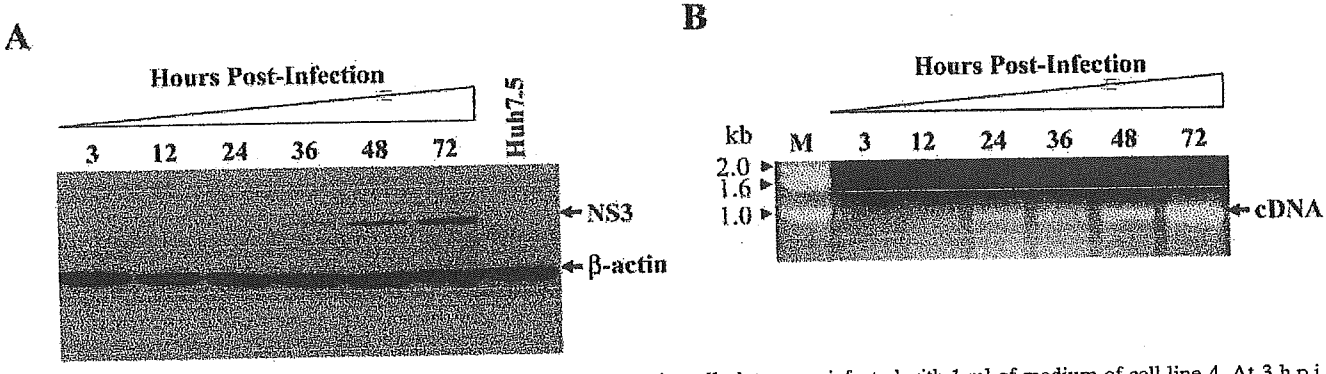

FIG. 8. Time course study of the HCV infection. Huh7.5 cells in a six-well plate were infected with 1 ml of medium of cell line 4. At 3 h p.i., the virus was removed, and cells were washed with PBS and then incubated with 2 ml of DMEM containing 10% FBS. The HCV-infected cells and culture medium were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

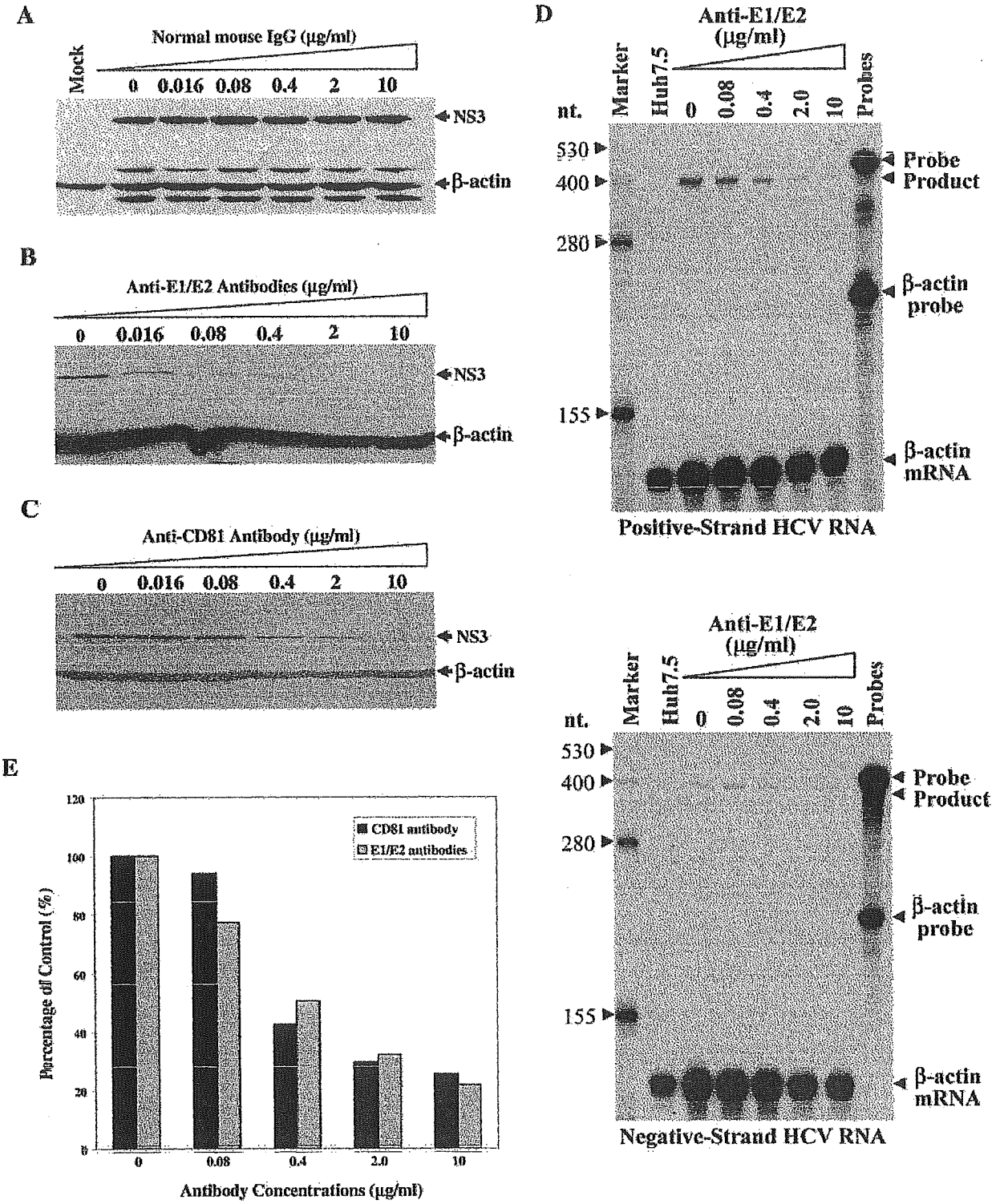

FIG. 9. (A) Effect of normal mouse IgG1 on HCV infection. Huh7.5 cells in a six-well plate were infected with 1 ml of culture medium in the presence of increasing concentrations (μg/ml) of normal mouse IgG1. At 3 h p.i., the HCV-containing medium was removed, and the cells were washed with PBS and incubated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

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

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

DISCUSSION

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

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

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

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

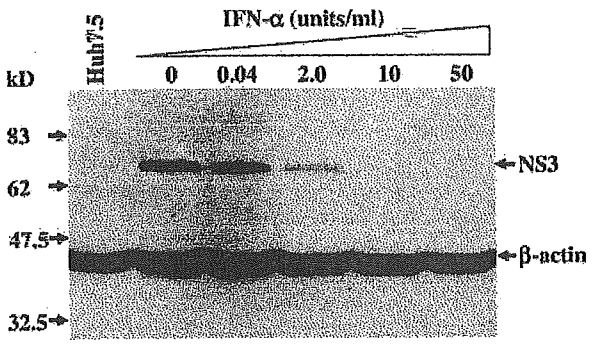

FIG. 10. Inhibition of HCV RNA replication by IFN-α. At 3 h p.i., cells were incubated with DMEM containing increasing concentrations of IFN-α for 3 days. The levels of the NS3 protein were determined by Western blotting. The concentrations of IFN-α are shown at the top.

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

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

ACKNOWLEDGMENTS

We are grateful to Charles M. Rice (Rockefeller University) for providing the Huh7.5 cell line, Ralf Bartenschlager (University of Heidelberg) for the Huh7 cell line, and Harry Greenberg (Stanford University) and Arvind Patel (MRC, Glasgow) for the E1 and E2 antibodies.

This work was supported by National Institute of Health grants (CA093712 and AI51592) and in part by the Kentucky Science and Engineering Foundation (KSEF-148–502-02-18) to G. Luo and in part by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases, NIH (T.J.L.). T.W. was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency (PMDA) and by the Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation.

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Robust hepatitis C virus infection *in vitro*

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Contributed by Francis V. Chisari, May 1, 2005

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

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

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

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

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

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

Materials and Methods

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

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

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

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

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

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‖Wakita, T., Kato, T., Date, T. & Miyamoto, M. 11th International Symposium on Hepatitis C and Related Viruses, 2004, Oct. 3–7, 2004, Heidelberg, Germany.

www.pnas.org/cgi/doi/10.1073/pnas.0503596102