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CD81 Expression Is Important for the Permissiveness of Huh7 Cell Clones for Heterogeneous Hepatitis C Virus Infection[▽]

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Huh7 cells constitute a permissive cell line for cell culture of hepatitis C virus (HCV) particles. However, our Huh7 line shows limited permissiveness for HCV. Thus, in this study we set out to determine which host factors are important for conferring permissiveness. To analyze the limited permissiveness of our Huh7 cells, 70 clones were obtained after single-cell cloning of parental Huh7 cells. The cloned Huh7 cells exhibited various levels of HCV pseudoparticles and JFH-1 virus infection efficiency, and some clones were not permissive. A subgenomic replicon was then transfected into the cloned Huh7 cells. While the replication efficiencies differed among the cloned Huh7 cells, these efficiencies did not correlate with infectious permissibility. Flow cytometry showed that CD81, scavenger receptor class B type I, and low-density-lipoprotein receptor expression on the cell surfaces of the Huh7 clones differed among the clones. Interestingly, we found that all of the permissive cell clones expressed CD81 while the nonpermissive cell clones did not. To confirm the importance of CD81 expression for HCV permissiveness, CD81 was then transiently and stably expressed on a nonpermissive Huh7 cell clone, which was consequently restored to HCV infection permissiveness. Furthermore, permissiveness was down-regulated upon transfection of CD81 silencing RNA into a CD81-positive cell clone. In conclusion, CD81 expression is an important determinant of HCV permissiveness of Huh7 cell clones harboring different characteristics.

Hepatitis C virus (HCV) is a worldwide human pathogen, and most infected patients progress to chronic liver disease. The primary therapy for HCV is treatment with pegylated interferon and ribavirin; however, these agents do not cause a marked decline in the virus titers of all treated patients. Thus, the elucidation of native virus-host interactions is necessary to develop new, more effective therapies. However, the lack of a robust cell culture system to produce infectious virions has hampered research. That said, in a great boon to HCV research, a cell culture system that allows the whole life cycle of HCV to be investigated was recently developed (22, 34, 41).

HCV is an enveloped virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family (23). Cell attachment of flaviviruses generally leads to endocytosis of bound virions. Several molecules have been proposed as cell entry receptors of HCV; most of these have been identified based on binding with soluble recombinant E2 protein or HCV-like particles (2, 3, 12, 25, 30, 31). Putative HCV receptors include CD81 (30), low-density-lipoprotein (LDL) receptor (1), scavenger receptor class B type I (SR-BI) (31), and several molecules that induce concentration of viral particles at the cell surface. Infectious HCV pseudoparticles (HCVpp) harboring E1E2 glycoproteins (5, 11) have substantiated the functional roles of the candidate receptors CD81 and SR-BI in HCV entry (5, 6, 15). The importance of CD81 for HCV entry was recently confirmed using cell-cultured HCV particles (22, 34). Furthermore, CD81 is important for postattachment of HCV particles on Huh7 cells (19, 28).

Huh7 and its interferon-cured cells are considered permissive cell lines for HCV particles (22, 34, 41), but our Huh7 cell line shows limited permissiveness. In the present study, we performed single-cell cloning of Huh7 cells and then analyzed heterogeneity. To investigate the host factors important for HCV infection, the Huh7 cell clones were then transiently infected with JFH-1 virus and comparisons of efficiency of replication and expression of candidate receptors were performed.

MATERIALS AND METHODS

Cell culture and single-cell cloning. Parental Huh7 cells, Huh7.5.1 cells (41) (a generous gift from Francis V. Chisari), and Huh7 cell clones 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 described previously (18). Parental Huh7 cells were diluted with medium and seeded into 96-well plates at 0.3 cells per well. Seventy single-cell-derived clones were then selected, and after 3 weeks their cells were passaged. The resultant Huh7 cell clones were stored at −80°C until use.

Plasmids. pJFH-1 (34), pSGR-JFH1/Luc, pSGR-JFH1/Luc-GND (17), and pFGR-JFH1 (10) were generated as previously reported. pFGR-J6/N2X-JFH1 was generated by replacement of the JFH-1 structural region (a core coding region to the BclI site) with pJ6CF (35) (a generous gift from Jens Bukh). pFGR-JFH1/EGFP and pFGR-J6/N2X-JFH1/EGFP were generated by replacement of the neomycin-resistant gene of pFGR-JFH1 and that of pFGR-J6/N2X-JFH1 with the enhanced green fluorescent protein (EGFP) gene from pEGFP-N3 (Clontech, Mountain View, CA). pcDNA3.1-CD81 and the vesicular stomatitis virus (VSV) G protein-expressing construct pCAG-VSVG (27) were

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kind gifts from Yoshiharu Matsuura (Osaka University, Suita, Japan). The JFH-1 E1E2 expression construct pcDNAdeltaC-E1-E2(JFH1) was a kind gift from Thomas Pietschmann (University of Heidelberg, Heidelberg, Germany), while the murine leukemia virus packaging construct and the luciferase-based transfer vector construct have been described previously (5).

RNA synthesis. RNA synthesis was performed as described previously (17, 34). Briefly, the plasmids pJFH-1, pSGR-JFH1/Luc, pSGR-JFH1/Luc-GND, pFGR-JFH1/EGFP, and pFGR-J6/N2X-JFH1/EGFP were digested with XbaI and treated with mung bean nuclease (New England Biolabs, Beverly, MA). Digested plasmid DNA fragments were then purified and used as templates for RNA synthesis. HCV RNA was synthesized in vitro by use of a MEGAscript T7 kit (Ambion, Austin, TX). Synthesized RNA was treated with DNase I, followed by acid phenol extraction to remove any remaining template DNA.

Replication assay of JFH-1 subgenomic replicon. Replication of a JFH-1 subgenomic replicon (SGR-JFH1) in Huh7 cells was detected as described previously (17). Briefly, 2 μg of reporter replicon RNA transcribed from pSGR-JFH1/Luc and pSGR-JFH1/Luc-GND was transfected into 2×10^6 Huh7 cells by electroporation. Transfected cells were immediately transferred to culture medium and seeded into six-well culture plates.

Cells were harvested serially at 4 h (day 0), 24 h (day 1), and 48 h (day 2) after transfection and lysed with 200 μl of cell culture lysis reagent (Promega, Madison, WI). Debris was then removed by centrifugation. Luciferase activities were quantified by use of LUMAT LB9507 (EG & G Berthold, Bad Wildbad, Germany) and a luciferase assay system (Promega). Assays were performed in duplicate, and the results at 24 and 48 h after transfection were normalized and expressed as the relative luciferase activities (RLA) compared to the luciferase activity at 4 h after transfection. The replication efficiency of each cell is indicated in Fig. 3 and Table 1 as follows: −, RLA below 1; ±, RLA between 1 and 10; +, RLA between 10 and 50; ++, RLA between 50 and 100; +++, RLA over 100.

Production of infectious HCV particles. HCV particles derived from JFH-1 were prepared as described previously (34). Briefly, in vitro-synthesized RNA was transfected into Huh7 cells by electroporation. Cell culture supernatants were collected 72 h after transfection and passed through a 0.45-μm filter. Filtrated culture medium was then pooled and concentrated using Amicon Ultra-15 (100,000 molecular weight cutoff; Millipore, Bedford, MA). The infectious titer was 1.6×10^4 focus-forming units (ffu) per ml and was determined by immunofluorescence detection of infected foci following infection of naïve parental Huh7 cells. RNA quantification was performed by real-time detection reverse transcription-PCR analysis, as described previously (32), using an ABI Prism 7700 sequence detector system (Applied Biosystems Japan, Tokyo, Japan). The titer was determined to be 4.3×10^8 RNA copies/ml. Concentrated culture medium samples were stored at −80°C until use.

HCV particle infection and immunofluorescence. Parental Huh7 cells and Huh7 cell clones were seeded at 1×10^4 cells/well in poly-D-lysine-coated 96-well plates (Corning, Inc., Corning, NY), cultured overnight, and then inoculated with serially diluted culture medium containing infectious HCV particles. At 48 h after inoculation, the cells were fixed in methanol for 15 min at −20°C, and the infected foci were visualized by immunofluorescence as described below.

Cells were permeabilized and blocked for 1 h with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) containing 0.3% (vol/vol) Triton X-100. The cells were then washed five times with phosphate-buffered saline (PBS), and anticore monoclonal antibody 2H9 (34) was added at 50 μg/ml in BlockAce. After incubation for 1 h at room temperature, the cells were washed and incubated with a 1:400 dilution of AlexaFluor 488-conjugated or AlexaFluor 546-conjugated anti-mouse immunoglobulin G (IgG) (Molecular Probes, Eugene, OR) with BlockAce. The cells were then washed and examined by fluorescence microscopy (Olympus, Tokyo, Japan). Quantification of infectivity was performed by counting the infected foci, and the assay was performed in triplicate. The infectivity of each clone is indicated in Fig. 3 and Table 1 as follows: −, no infected foci; ±, between 1 and 5 foci; +, between 5 and 10 foci; ++, between 10 and 50 foci; +++, over 50 foci.

Stable and transient expression of CD81. A trypsinized CD81-negative clone (clone 25) was washed with Opti-MEM I (Invitrogen, Carlsbad, CA) and resuspended at 5×10^6 cells/ml in Cytomix buffer (18). pcDNA3.1-CD81 plasmid DNA (75 μg) was mixed with 400 μl of cell suspension and the mix then transferred to an electroporation cuvette (Precision universal cuvette; Thermo Hybrid, Middlesex, United Kingdom). The cells were then pulsed at 220 V and 950 μF with a Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred to a 75-cm² flask (Corning) and incubated at 37°C/5% CO_2. After 3 days, the cells were passaged and seeded into 10-cm dishes, and G418 (0.8 mg/ml) (Nacalai Tesque, Kyoto, Japan) was then added to the culture medium. Culture medium supplemented with G418 was replaced twice a week. Three weeks later, the colonies were observed and then the cells were trypsinized. CD81-positive cells were obtained and confirmed by the same method as for single-cell cloning of parental Huh7 cells. We obtained a clone, Huh7-25-CD81, in which CD81 was stably expressed.

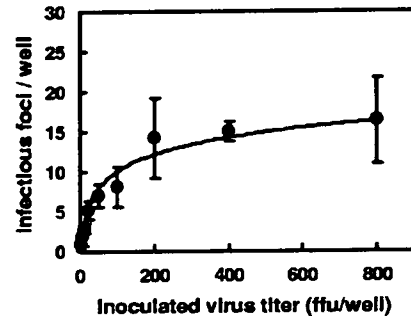

FIG. 1. Infectivity of JFH-1 virus in parental Huh7 cell. Parental Huh7 cells were seeded at 1×10^4 cells/well and infected with JFH-1 virus at 3 to 800 ffu/well for 48 h in 96-well plates. Infected cells were visualized with immunofluorescence using anticore antibody (2H9), and the foci were counted. All experiments were performed in triplicate, and the data are shown as means ± standard deviations.

For transient CD81 expression, 6 μg of pcDNA3.1-CD81 plasmid was transfected into Huh7 clone 25 (Huh7-25) cells (2.5×10^6) by using FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis, IN). After 24 h, cells were passaged and an aliquot used for flow cytometric analysis and virus infection. As controls, Huh7-25 and Huh7-25-CD81 cells were similarly treated by only FuGENE6 and used for flow cytometric analysis and virus infection.

Pseudotype production and infection. Murine leukemia virus pseudotypes were generated according to methods described previously (5). Briefly, the Gag-Pol packaging construct (3.1 μg), the transfer vector construct (3.1 μg), and the JFH-1 glycoprotein or the VSV-G protein-expressing construct (1 μg) DNAs were transfected into 2.5×10^6 293T cells seeded the day before in 10-cm dishes by use of FuGENE6 transfection reagent (Roche Diagnostics). For the negative control, the constructs (except for the glycoprotein-expressing construct) were similarly transfected. The medium (8 ml/dish) was replaced 6 h after transfection. Supernatants containing the pseudotypes were collected 48 h later and passed through a 0.45-μm filter. The supernatants were stored at −80°C until use.

Target cells were seeded into 48-well plates at a density of 2×10^4 cells/well and incubated overnight at 37°C. A 100-μl aliquot of each diluted supernatant containing pseudotypes was added to each well and incubated for 3 h. The supernatants were removed, and the cells were incubated in regular medium for 72 h at 37°C. Cells were washed once with PBS and lysed with 40 μl/well of cell culture lysis reagent. Luciferase activities were quantified using a luciferase assay system (Promega) as described above. Assays were performed in triplicate. All Huh7 cell clones showed infectivity by the VSV-G pseudoparticle, and infectivity by HCVpp was indicated by the luciferase activity (relative luciferase units [RLU]), determined by subtraction from the activity of the nonenveloped control. The infectivity of each clone is indicated in Table 1 as follows: −, luciferase activity below 1 RLU; ±, activity between 1 and 5,000 RLU; +, activity between 5,000 and 30,000 RLU; ++, activity between 30,000 and 100,000 RLU; +++, activity over 100,000 RLU.

RNA interference. A 40-pmol amount of silencing RNA (siRNA) duplex for CD81 (Santa Cruz, Inc., Santa Cruz, CA) was electroporated into 2.5×10^6 Huh7 clone 54 (Huh7-54) cells (260 V, 950 μF). Control irrelevant siRNA (siIRR) was designed as described previously (37) and transfected, as was the siRNA of CD81. Cells were then propagated and tested for CD81 expression and JFH-1 virus infection.

Flow cytometric analysis. Cells were seeded in 10-cm dishes (Corning) and cultured overnight. Then, subconfluent cells were harvested either by trypsinization or by treatment with 0.05% EDTA in PBS. Parental Huh7 cells and Huh7 cell clones (1×10^6) were incubated with or without 1 μg mouse anti-CD81 antibody (JS-81; Pharmingen, Franklin Lakes, NJ) for 30 min at 4°C and washed with PBS. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (Cappel, Durham, NC) at 1:100 for 30 min at 4°C, washed repeatedly, and resuspended in PBS containing 1% (vol/vol) formaldehyde. SR-BI expression of each cell was tested using rat anti-human SR-BI antiserum (1:50) and FITC-conjugated anti-rat IgG secondary antibody (Cappel) by the same method as for CD81 (2). Rat preimmune serum

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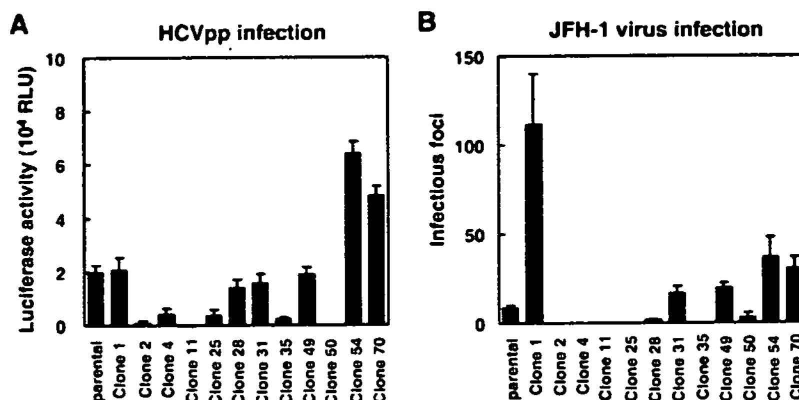

FIG. 2. Infectivity of HCVpp and JFH-1 virus in Huh7 cell clones. (A) Target cells (2×10^4 cells) were inoculated with supernatant containing HCVpp for 3 h in 48-well plates. After 72 h, cells were harvested and the luciferase activities quantified. All experiments were performed in triplicate, and infectivity is indicated as the RLU minus the activity of the nonenveloped negative control. (B) Target cells (1×10^4 cells) were infected with the same titer of JFH-1 virus for 48 h in 96-well plates. Infected cells were visualized with immunofluorescence using anticore antibody (2H9), and the foci were counted. All experiments were performed in triplicate, and the data are shown as means ± standard deviations.

was used as the negative-control primary antibody. LDL receptor expression was tested using rabbit anti-LDL receptor antibody (BP5014 at 1:10; Acris Antibodies GmbH, Hiddenhausen, Germany) and FITC-conjugated anti-rabbit IgG secondary antibody (Cappel). Analyses were performed using an EPICS ALTRA MultiCOMP unit (Beckman Coulter, Fullerton, CA) and FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). The expression levels are indicated in Table 1 as follows: −, mean fluorescence intensity (MFI) in relative units was below 1; +, MFI in relative units was between 1 and 3; ++, MFI in relative units was between 3 and 6; +++, MFI in relative units was over 6.

Analysis of cell surface CD81 expression levels and HCV infection. For EGFP virus production, in vitro-synthesized RNA was transfected into Huh7.5.1 cells by electroporation. Cell culture supernatants of transfected cells were collected and concentrated as described above. The infectious titer was also determined as described above. Huh7-70 and Huh7-25-CD81 cells were seeded in six-well plates at 1×10^5 cells/well 24 h before virus inoculation. Cells were inoculated with EGFP virus (multiplicity of infection, 2) for 4 h, followed by a PBS wash. The inoculated cells were cultured in complete medium and then harvested at 24, 48, 72, and 96 h after inoculation. CD81 expression and GFP-positive cells were analyzed by FACSCalibur as described above, using allophycocyanin-conjugated anti-mouse IgG (R&D Systems, Minneapolis, MN) as a secondary antibody at 1:10. All experiments were performed in triplicate, and analysis was performed using CellQuest Pro software (Becton Dickinson). CD81 expression on uninfected and infected cells was calculated from the geometric MFI of the each quadrant plot. The results are given as MFIs ± standard deviations.

RESULTS

Parental Huh7 cell shows limited permissiveness for JFH-1 virus. A parental Huh7 cell was infected with JFH-1 virus and infectivity assessed by counting the infected foci in anticore immunofluorescence. The number of infected foci increased linearly with lower doses of virus infection (<50 ffu/well). However, the number of infected foci did not increase with higher doses of infection (>200 ffu/well) (Fig. 1). Furthermore, when the parental Huh7 cell infected with JFH-1 was passaged, the infected cells did

TABLE 1. Permissiveness to infection and expression of candidate receptors[a]

Cell type	Replication efficiency (RLA)[b]	CD81 expression (MFI)[c]	SR-BI expression (MFI)[d]	LDLr expression (MFI)[e]	HCVpp infectivity[f]	JFH-1 infectivity[g]
Huh7 (parental)	+++	++	++	+	+	+
Clone 1	++	+	++	+	+	+++
Clone 2	+	−	+	+	±	−
Clone 4	−	−	+	+	±	−
Clone 11	+++	−	++	+	−	−
Clone 25	+++	−	++	+	±	−
Clone 28	±	+++	++	++	+	±
Clone 31	±	+	++	+	+	++
Clone 35	+++	−	++	++	±	−
Clone 49	±	++	+	++	+	++
Clone 50	−	++	+	+	+	±
Clone 54	+	+	++	++	++	++
Clone 70	±	++	++	+	++	++
Huh7-25-CD81	+++	+++	++	+	+++	+++

[a] See Materials and Methods for definitions of symbols.
[b] Detection of replication of the JFH-1 subgenomic replicon on Huh7 clones at 48 h after transfection of SGR-JFH1/Luc RNA.
[c] Detection of CD81 by flow cytometry using JS-81 antibodies on the surfaces of the indicated cells.
[d] Detection of SR-BI by flow cytometry using rat anti-SR-BI antiserum on the surfaces of the indicated cells.
[e] Detection of LDL receptor (LDLr) by flow cytometry using rabbit anti-LDLr antibody (BP5014) on the surfaces of the indicated cells.
[f] Detection of luciferase activities by subtraction from the activity of the nonenveloped control.
[g] Infected foci were detected by immunofluorescence using HCV anticore antibody (2H9).

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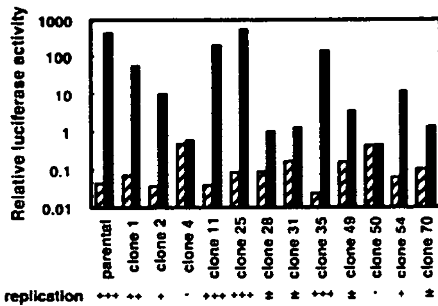

FIG. 3. Replication of JFH-1 subgenomic replicon in Huh7 cell clones. Reporter replicon RNA was transfected into Huh7 cell clones, and luciferase activities at 4 and 48 h after RNA transfection were then detected. The SGR-JFH1/Luc-GND RNA was used as the negative control. All data indicate the RLA compared to the luciferase activities at 4 h after transfection. The RLA of SGR-JFH1/Luc (solid bars) and SGR-JFH1/Luc-GND (hatched bars) are also shown. All experiments were performed in duplicate, and the data are shown as the means.

not expand (data not shown). Thus, the original parental Huh7 cell had limited permissiveness for JFH-1 infection.

Huh7 cell clones have different levels of permissiveness for HCVpp and JFH-1 virus infections. The parental Huh7 cell did not show dose-dependent permissiveness for JFH-1 infection. Thus, single-cell cloning of this Huh7 cell was performed by limiting dilution, and the efficiency of virus infection for each clone was then investigated. Seventy clones were obtained. Among the isolated cloned cells, 65 clones were first screened with JFH-1 virus infection, and the other 5 clones were not tested, because of their slower growth. We found different numbers of focus formation in the clones (46 positive and 19 negative). Among them, we selected seven positive (clones 1, 28, 31, 49, 50, 54, and 70) and five negative (clones 2, 4, 11, 25, and 35) clones for further analysis. Next, these 12 cell clones were infected with HCVpp and JFH-1 virus. Interestingly, the efficiencies of both virus infections of the Huh7 cell clones differed among the clones (Fig. 2; Table 1). Furthermore, some differences were observed between HCVpp and JFH-1 virus permissiveness. Four clones that were not permissive for JFH-1 virus showed slight permissiveness for HCVpp (clones 2, 4, 25, and 35). In addition, three clones were more permissive for both HCVpp and JFH-1 virus than for the parental Huh7 cell (clones 1, 54, and 70). However, clone 11 was not permissive at all for either virus infection. Thus, the parental Huh7 cell population was most likely heterogeneous and included cells with different characteristics.

Replication of JFH-1 subgenomic replicon in Huh7 cell clones does not correlate with the efficiency of virus infection. In a previous study, a subgenomic replicon of JFH-1 was shown to efficiently replicate in Huh7 cells (18). Thus, to investigate whether replication efficiency correlated with infectious efficiency, we transiently transfected SGR-JFH1/Luc RNA into each Huh7 cell clone and measured the resultant RLA. At 48 h posttransfection, levels of replication of the replicon in the Huh7 cell clones differed among the clones (Fig. 3), but the efficiency did not correlate with JFH-1 virus infectivity (Table 1).

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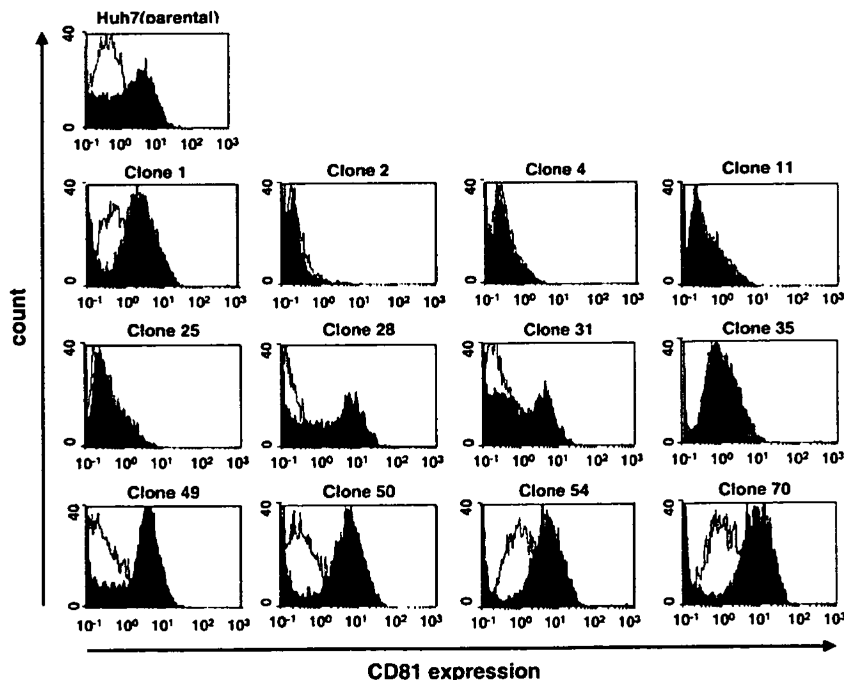

FIG. 4. CD81 expression on the surfaces of Huh7 cell clones. Huh7 cell clones were seeded in 10-cm dishes and cultured overnight. Then, subconfluent cells were harvested either by trypsinization or by treatment with 0.05% EDTA in PBS. Cells (1×10^6) were incubated with 1 μg of mouse anti-CD81 monoclonal antibody (JS-81) and subsequently stained with FITC-conjugated goat anti-mouse IgG. The negative control represents cells incubated with only secondary antibody. The analysis was performed by EPICS ALTRA MultiCOMP (Beckman Coulter). The *x* and *y* axes show fluorescence intensity and relative number of stained cells, respectively.

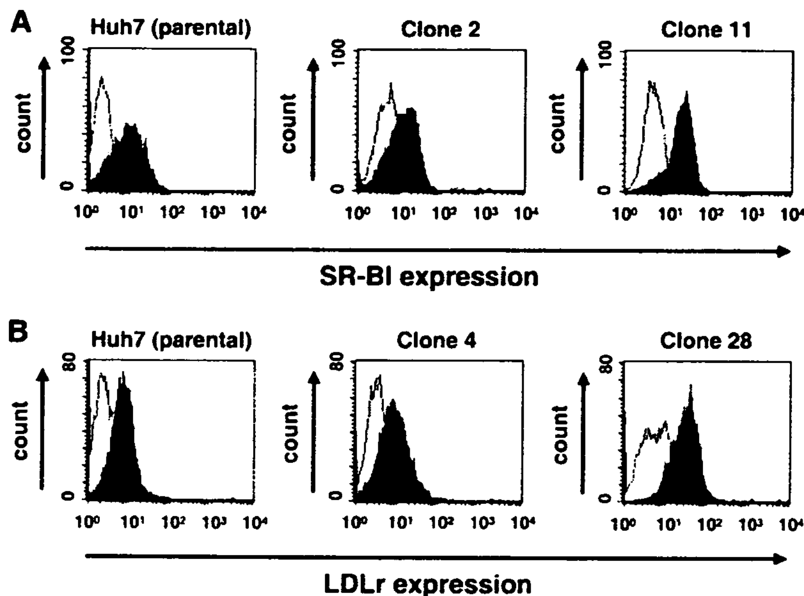

FIG. 5. SR-BI and LDL receptor on the surfaces of Huh7 cell clones. Huh7 cell clones (1×10^6 cells) were incubated with rat anti-SR-BI antiserum or rabbit anti-LDL receptor antibody and subsequently stained with FITC-conjugated secondary antibody. The negative control represents cells incubated with rat preimmune serum (SR-BI) or only secondary antibody (LDL receptor [LDLr]). The analysis was performed by FACSCalibur (Becton Dickinson). The x and y axes show fluorescence intensity and relative number of stained cells, respectively. (A) Expression of SR-BI on parental Huh7 cells, clone 2 (the cell clone with the lowest expression level), and clone 11 (the cell clone with the highest expression level). (B) Expression of LDL receptor on parental Huh7 cells, clone 4 (the cell clone with the lowest expression level), and clone 28 (the cell clone with the highest expression level).

Expression of CD81 on Huh7 cell clones is correlated with HCV permissiveness. The Huh7 cell clones displayed differing levels of permissiveness for HCVpp and JFH-1 virus infections, and replication of the replicon did not correlate with infectivity. Thus, the difference in infectivity was most likely due to host factors related to the initial phase of infection. Previous studies using pseudotype particles bearing envelope proteins of HCV have shown that CD81 is a candidate receptor of HCV (5, 6, 9, 15, 38). Therefore, we investigated CD81 expression on our Huh7 cell clones by flow cytometry using an anti-CD81 antibody. We found that the levels of CD81 expression on the Huh7 cells differed among the clones (Fig. 4). All of the CD81-negative clones (clones 2, 4, 11, 25, and 35) were also negative for JFH-1 virus infectivity. However, CD81-positive clones (clones 1, 28, 31, 49, 50, 54, and 70) showed HCV permissiveness at different levels (Table 1). Interestingly, clones 28 and 50 expressed relatively high levels of CD81 but low levels of permissiveness. This may have been due to a lower replication efficiency of these clones, although clones 49 and 70 also had low replication capacities but were permissive of HCV infection. Thus, the low permissiveness of clones 28 and 50 is most likely due to other, as yet unknown mechanisms (Table 1). To confirm that the different expression levels of CD81 among the clones were not due to the cell-harvesting conditions, we harvested using two different techniques, namely, trypsinization and EDTA treatment. Neither method affected the results. This finding suggests that CD81 expression is highly correlated with HCV infectivity, although the level of CD81 expression did not necessarily correlate with JFH-1 virus infectivity among these Huh7 cell clones (Table 1).

Expression levels of SR-BI and the LDL receptor on Huh7 cell clones do not correlate with permissiveness. CD81 expression correlated highly with infectivity of Huh7 cell clones. In a previous report, CD81 expression level determined permissiveness, as shown by a transient-transfection experiment (40). However, the levels of CD81 expression among the clones from this study did not correlate with virus infectivity (Table 1), suggesting that multiple factors determine the level of infectivity. As previous studies have suggested that SR-BI and the LDL receptor play important roles in HCV infection (4, 20, 33), we next investigated the expression of these molecules on the surfaces of our Huh7 cell clones. The expression of these molecules was also detected by flow cytometry. Small differences in the expression levels of SR-BI and the LDL receptor compared to that of CD81 were observed (Fig. 5; Table 1). In terms of virus permissiveness, SR-BI and LDL receptor expression did not display the high correlation seen with CD81 (Fig. 5; Table 1).

CD81 expression restores HCV infection permissiveness in a nonpermissive Huh7 cell clone. To confirm the importance of CD81 expression for HCV permissiveness, CD81 was expressed on a nonpermissive, non-CD81-expressing Huh7 cell clone (Huh7-25) (Fig. 4; Table 1) and the cells were then infected with JFH-1 virus. When CD81 was transiently and stably expressed on Huh7-25 cells, these cells were restored to permissiveness. The CD81-positive cells of transiently and stably transfected cells were 45.1 and 80.6%, respectively, and infectivities (infected foci) were 58.0 ± 7.9 and 257.7 ± 14.6, respectively, with inoculation of the same titer of JFH-1 virus (Fig. 6A). A clone in which CD81 was stably expressed (Huh7-

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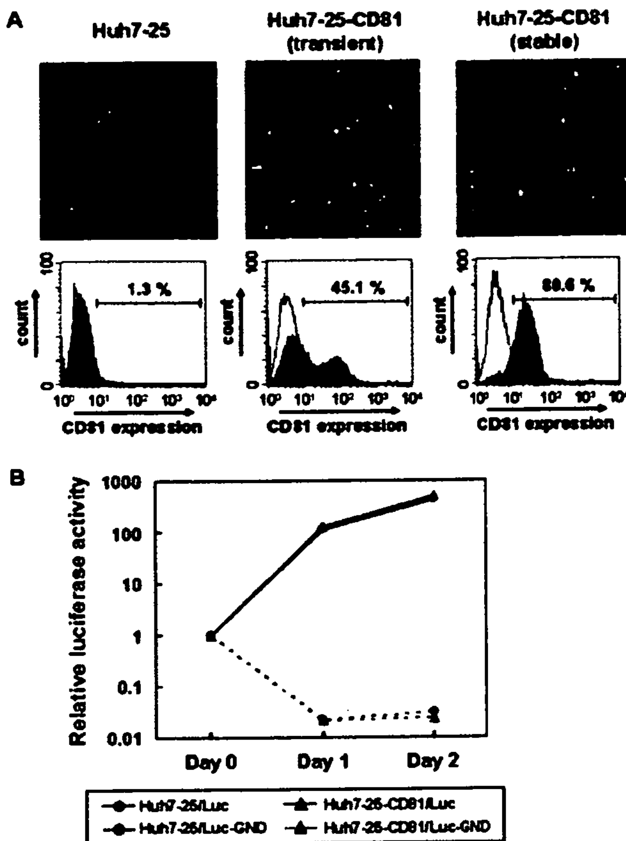

FIG. 6. Infectivity in CD81-transfected Huh7 cells. (A) CD81 was transiently and stably expressed on a Huh7 nonpermissive clone (Huh7-25). The expression of CD81 on the cell surface was examined by flow cytometry. Bars indicate the populations of the CD81-positive cells. Cells were inoculated with JFH-1 virus (1×10^6 copies of HCV RNA) for 3 h. Virus was then removed and cells incubated at 37°C for 48 h. Infected cells were detected by immunofluorescence using anti-core antibody. (B) Replication of SGR-JFH1 in Huh7-25 cells and Huh7-25 cells in which CD81 was stably expressed (Huh7-25-CD81 cells).

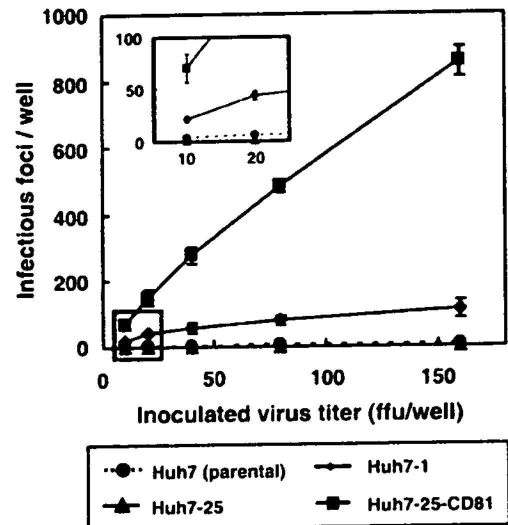

FIG. 7. Dependency of permissiveness on virus concentration in Huh7-25-CD81 cells. Huh7-25-CD81 cells (1×10^4) were infected with JFH-1 virus at 10 to 160 ffu/well in 96-well plates. Infected cells were visualized and assayed as described in the legend for Fig. 1. All experiments were performed in triplicate, and the data are shown as means ± standard deviations. The upper-left panel shows a magnified version of the area in the square at the lower left.

25-CD81) had high infectivity for JFH-1 virus (Fig. 6A), although the replication efficiency was equal to that of Huh7-25 cells (Fig. 6B). In addition, the infected foci indicated a linear, dose-dependent increase in virus dose (Fig. 7). Thus, ectopic expression of CD81 in a nonpermissive Huh7 cell clone restored HCV infection permissiveness. Furthermore, the Huh7-25-CD81 cell clone supported the highest permissiveness for HCV infection.

Knockdown of CD81 expression reduced HCV infection permissiveness. A permissive and CD81-expressing Huh7 cell clone (Huh7-54) was transfected with siRNA for CD81. At 48 h after transfection, the expression of CD81 on the cell surface declined by about 60% compared to that for the cell transfected with siIRR (Fig. 8A). The CD81 knockdown cell was then infected with JFH-1 virus, and infectivity was determined. Infectivity of the CD81 knockdown cell declined by about 80% compared to that for the siIRR-transfected cell or mock-transfected cells (Fig. 8B). Thus, CD81 expression was shown to be an important factor in HCV infection permissiveness. Taken together, the level of CD81 expression is also a determinant of the level of HCV permissiveness in a cell clone.

CD81 expression levels followed by HCV infection. CD81 expression on the cell surface may alter with culture conditions or HCV infections. To test this, we produced infectious HCV particles harboring the EGFP gene. J6/JFH1 EGFP virus was derived from pFGR-J6/N2X-JFH1/EGFP, and JFH-1 EGFP virus was from pFGR-JFH1/EGFP. J6/JFH1 EGFP virus was produced more efficiently from synthetic RNA-transfected cells than wild-type JFH-1 EGFP virus (data not shown). We thus used J6/JFH1 EGFP virus to inoculate Huh7-25-CD81 and Huh7-70 cells. Cell surface CD81 expression and HCV infection were detected simultaneously by flow cytometry. In Fig. 9A, results of the fluorescence-activated cell sorting analysis are given as quadrant plots of Huh7-25-CD81 cells inoculated with J6/JFH1 EGFP virus at 96 h after inoculation. The infected cells were observed by a shift to positive GFP fluorescence, and CD81 expression was detected by an anti-CD81 antibody. Under this experimental condition, HCV infections were detected in both cell types at 24 h after inoculation (Fig. 9B) (0.5% and 0.6% for Huh7-25-CD81 and Huh7-70 cells, respectively). The ratio of infected cells increased substantially in Huh7-25-CD81 cells (Fig. 9B) (27.7% at 96 h postinoculation); however, the ratio of infected cells was not increased in Huh7-70 cells (Fig. 9B) (0.9% at 96 h). On the other hand, the CD81 expression level of Huh7 cells increased until 2 days after cell passages and then declined gradually (data not shown). This kinetic property was also confirmed with mock-infected Huh7-25-CD81 and Huh7-70 cells (Fig. 9C) (incubation was started 24 h after passage). After J6/JFH1 EGFP virus inoculations, CD81 expression levels on the cell surfaces of uninfected and infected cells were also analyzed (Fig. 9C).

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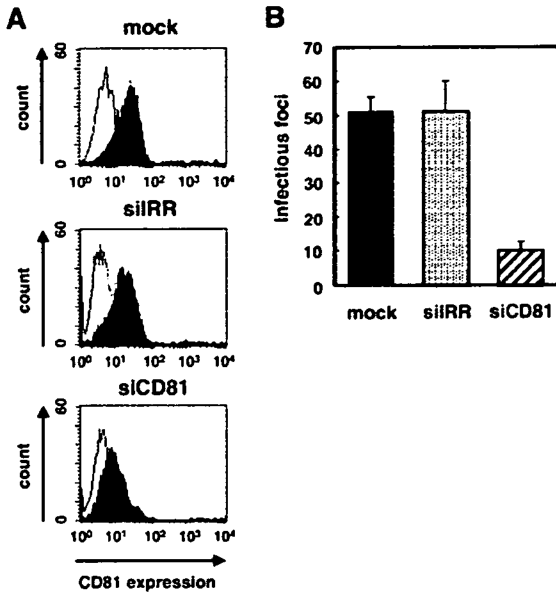

FIG. 8. siRNA silencing of CD81 expression on Huh7-54 cells and JFH-1 virus infection. (A) CD81-positive Huh7-54 cells transfected with either siIRR or siRNA of CD81 (siCD81) were stained with anti-CD81 antibody (JS-81) at 48 h posttransfection and analyzed by flow cytometry. (B) siRNA-transfected Huh-54 cells were inoculated with the same titer of JFH-1 virus (1×10^6 copies HCV RNA) for 3 h. Virus was then removed, and cells were incubated at 37°C for 48 h. Infected cells were visualized and assayed as described in the legend for Fig. 1. All experiments were performed in triplicate, and the data are shown as means ± standard deviations.

CD81 expression levels of uninfected cells were not different from those of mock-infected cells. Interestingly, CD81 expression levels of infected Huh7-25-CD81 cells were higher than those of uninfected cells at 48 and 72 h after virus infection (Fig. 9C, left panel). However, CD81 expression of infected cells also decreased gradually, as observed for uninfected cells. The CD81 expression level of Huh7-70 cells was lower than that of Huh7-25-CD81 cells, and the difference in CD81 expression level between uninfected and infected cells was not clear for Huh7-70 cells (Fig. 9C, right panel).

DISCUSSION

CD81 is a candidate receptor for HCV and plays an important, as yet unknown role in HCV infection. Pileri et al. were the first to demonstrate a relationship between CD81 and HCV when they found that the envelope protein E2 of HCV bound to the large extracellular loop of CD81 (30). However, experiments that use recombinant proteins are limited in that they can only provide information regarding molecular interactions. The development of pseudoparticles with HCV envelope proteins (HCVpp) made possible the investigation of HCV infection and cell entry, and some candidate receptors for HCV have been proposed previously (2, 3, 12, 25, 30, 31). The involvement of CD81 in HCV entry was also ascertained by the HCVpp system. The HCVpp system has shed some light on the role of cell surface molecules involved in the early steps of the HCV life cycle. On the other hand, a cell culture system to produce HCV bearing the genotype 2a genome has recently been established (22, 34, 41). This system enables investigation of the whole HCV life cycle.

The Huh7 cell line is a human hepatoma cell line, established in 1982 (29), that is now recognized as being permissive for HCV particles (34). Since our Huh7 cell showed limited permissiveness, in this study we performed single-cell cloning of a Huh7 cell and found that the parental Huh7 cell produced a heterogeneous population upon culture (Table 1). The heterogeneity of subsequent infectivity may have been due to the limited permissiveness of the parental Huh7 cells. Further analysis of these Huh7 cell clones revealed that CD81 expression determined permissiveness with high correlation (Fig. 4; Table 1). Moreover, given that HCV replication efficiency was not changed by CD81 expression (Fig. 6B), this molecule must be important in the early steps of HCV infection. However, the level of CD81 expression on the Huh7 cell clones did not necessarily correlate with HCVpp and JFH-1 virus infectivity, with some clones displaying high permissiveness but relatively low CD81 expression and vice versa (Table 1). It is thus likely that replication efficiency is related to the appearance of infected foci, since the translation of HCV core protein is affected by HCV RNA replication. For example, Huh7 clone 1 indicated relatively low permissiveness for HCVpp but high permissiveness for JFH-1 virus, while clone 28 indicated the opposite. These differences may have arisen from the postinfection steps of virus infection, as the JFH-1 system depends on HCV infection and replication and the HCVpp system depends on HCV infection and pseudotype gene expression. Nonetheless, multiple factors are predicted to play a role in HCV infection, in addition to CD81.

When the siRNA for CD81 was transfected into a CD81-positive cell clone and expression subsequently down-regulated, the permissiveness for HCVpp (38) and JFH-1 virus was also down-regulated (Fig. 8). On the other hand, when a Huh7 cell line was transfected with serial doses of CD81 expression vector, the transfected cells indicated permissiveness according to the level of CD81 expression (40). Although the MFIs differed between transiently and stably transfected cells, more clonal CD81 expression was observed for stably transfected cells than for transiently transfected cells (Fig. 6A). This may account for the difference in infectivity between transiently and stably transfected cells.

SR-BI and the LDL receptor are other putative molecules thought to be involved in HCV infection (1, 31), and their relationships to HCV have been investigated using recombinant proteins and HCVpp (5, 6, 15). The expression levels of SR-BI and the LDL receptor on our Huh7 cell clones differed slightly among the clones (Fig. 4); however, their expression did not appear to determine HCV permissiveness, unlike with CD81. On the other hand, it was recently reported that CD81 and SR-BI function cooperatively and cholesterol dependently to initiate HCV entry (16). In the present study, JFH-1 virus infection levels varied among the Huh7 cell clones, and thus each molecule may have a threshold expression level that determines HCV permissiveness.

CD81 expression level on the cell surface may be changed with cell culture condition and HCV infection. Therefore, it is important to analyze a dynamic expression of CD81. In fact, higher CD81 expression was observed for infected Huh7-25-

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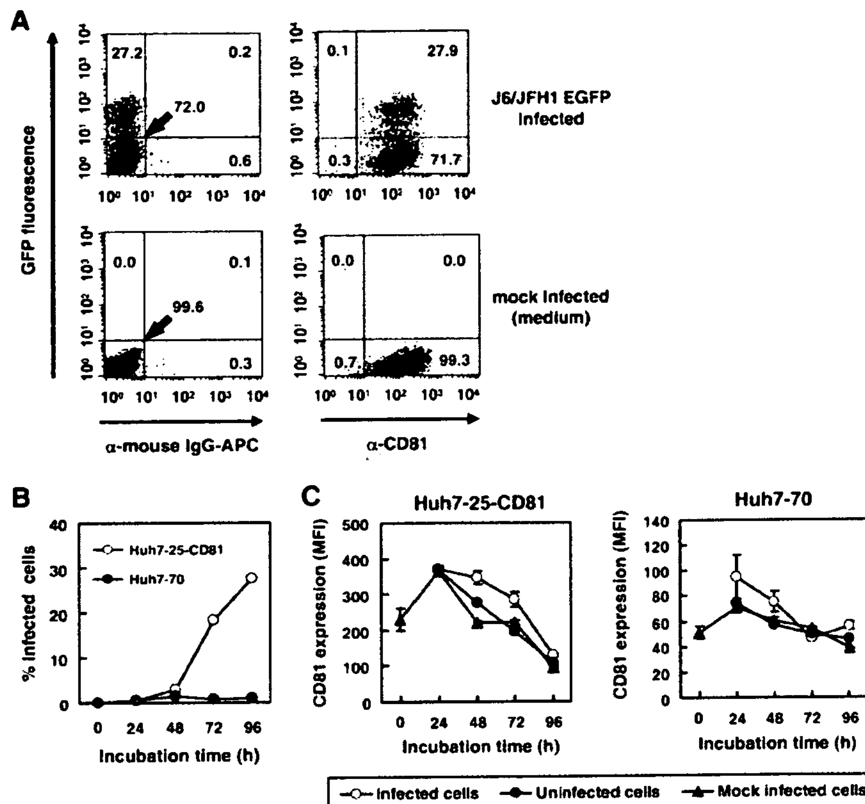

FIG. 9. Kinetics of cell surface CD81 expression and HCV infection. Huh7-25-CD81 and Huh7-70 cells were seeded into six-well plates at a density of 1×10^5 cells/well. Twenty-four hours later, J6/JFH1 EGFP virus (multiplicity of infection, 2) was inoculated. Cells were harvested at 24, 48, 72, and 96 h after inoculation and analyzed for infection and cell surface CD81 expression by FACSCalibur. The experiments were performed as described in the legend for Fig. 4, using anti (α)-mouse IgG–allophycocyanin (APC) as a secondary antibody. (A) Huh7-25-CD81 cells were harvested 96 h after inoculation and analyzed by fluorescence-activated cell sorting. Relative numbers of cells in the respective quadrants are given. (B) The proportion of infected cells was determined at each time point postinoculation and plotted for Huh7-25-CD81 and Huh7-70 cells. Mean values from triplicate experiments are given. (C) The cell surface CD81 expression (MFI) of infected cells, uninfected cells, and mock-infected cells is plotted at each time point after inoculation for Huh7-25-CD81 and Huh7-70 cells. Mean values and standard deviations are given.

CD81 cells than for uninfected cells (Fig. 9C, left panel). However, this difference was not clear with Huh7-70 cells (Fig. 9C, right panel). This discrepancy may be due to the different CD81 expression levels between the cells. Huh7-25-CD81 cells express higher levels of CD81 on the cell surface (Fig. 6 and 9; Table 1), and when such cells are infected with HCV, CD81 molecules might be stabilized to keep higher expression levels on the cell surface. Alternatively, CD81 expression in Huh7-25-CD81 cells may be controlled differently than in other Huh7 cell clones because CD81 is expressed from the transfected vector in Huh7-25-CD81 cells. Taken together, a more detailed analysis will be necessary for a dynamic expression of CD81 and HCV infection.

CD81 is a member of the tetraspanin family, and its functions are unclear. It is known that CD81 is a component of the tetraspanin web on the plasma membrane (21) and that the homologous region shared with CD9 is involved in egg-sperm fusion (42). Thus, CD81 may play an important role in cell-virus fusion through the tetraspanin web. Having said that, it is unclear what kinds of molecules associate with CD81 on human hepatic cells. Since CD81 and other tetraspanins are thought to interact with various molecules (13), including integrins (36), GPR56 (24), 14-3-3 (8), and signaling enzymes (7, 37, 39), various signal transductions through CD81 and multiprotein complexes may be involved in the level of HCV permissiveness. Thus, the tetraspanin-enriched microdomain on permissive cell lines may be necessary for virus-host interaction (14, 26).

On the other hand, the level of CD81 expression on the Huh7 cell clones did not correlate with the level of permissiveness, indicating that CD81-independent molecules were also involved in permissiveness. Recently, our laboratory and others have indicated that heparan sulfate proteoglycan (HSPG) may play an important role in the initial cell surface binding of HCV particles (19, 28). Since HCV particles are thought to be concentrated by HSPG on the surfaces of cells, the differences in infectivity among Huh7 cell clones may be due to differences in the expression levels or types of HSPG. Furthermore, other unknown molecules that harbor affinity with HCV particles may also be important. A more detailed analysis is clearly required.

In conclusion, we investigated HCV permissiveness and host factors by use of cell-cultured infectious particles and a hetero-

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geneous population of Huh7 cells derived from a single cell. We discovered that HCV particle permissiveness is determined by CD81 expression with high correlation. However, the level of permissiveness of each Huh7 cell clone is not explained by only CD81 expression levels, suggesting that another host factor(s) is involved.

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

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

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

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

MATERIALS AND METHODS

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

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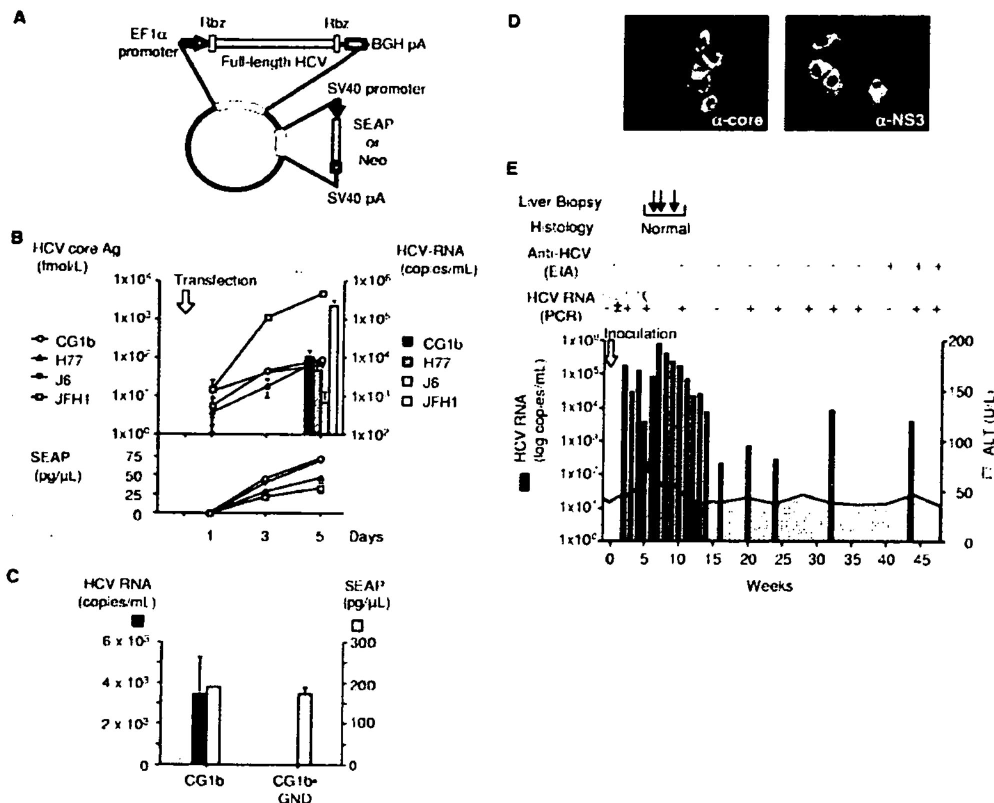

FIG. 1. Production and infection of HCV produced in cell culture. (A) Construction of HCV-expressing plasmid with the HCV-ribozyme system. Two hammerhead ribozyme sequences were engineered at the 5′ and 3′ ends of HCV full-length cDNA. This system was expressed under the control of EF1α promoter. The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

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

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

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

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

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

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

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

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

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

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

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

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

RESULTS

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

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

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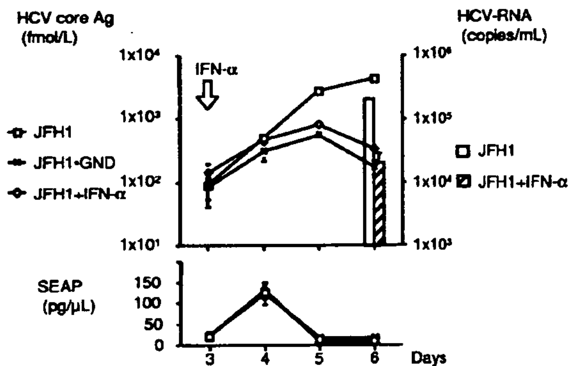

FIG. 2. Inhibition of HCV replication and production by IFN-α. IFN-α (100 IU/ml) was administered to the JFH1-ribozyme-transfected cells 3 days after transfection. The HCV core Ag level, HCV RNA titer, and SEAP activity in the culture supernatants were monitored at the indicated time points. The JFH1–GND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

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

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

Long-term culture of JFH1-expressing cells. To evaluate the continuous production of HCV particles, JFH1-transfected Huh 7.5 cells were cultured and passaged for more than 6 weeks. The production of HCV core Ag and secretion of HCV RNA were maintained during the observed period (Fig. 4). The highest titer of HCV core Ag and level of HCV RNA were

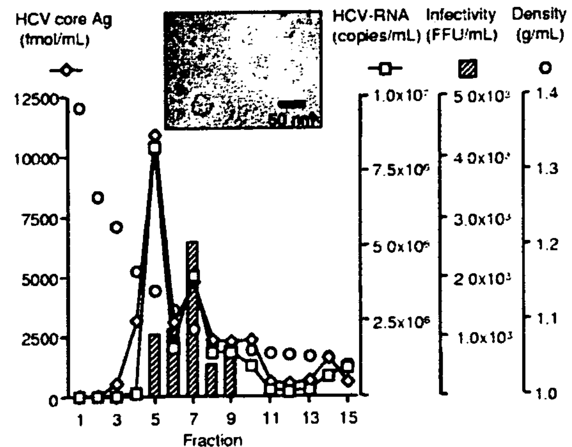

FIG. 3. Iodixanol density gradient analysis of the cell culture-generated JFH-1 HCV. Huh7.5.1 cells were transfected with JFH-1-ribozyme plasmid, and culture medium was collected and analyzed by iodixinol density gradient as described in Materials and Methods. Fractions were collected from the bottom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

observed on day 31 after transfection in this experiment: 6.35×10^4 fmol/liter and 1.48×10^6 copies/ml, respectively. The SEAP production disappeared about 2 weeks after transfection, supporting the continuous replication and production of the JFH-1 virus. The infectivity titers of culture medium were also determined. The peak infectivity titer of 2.50×10^3 FFU/ml was observed at 25 days after transfection, but in general the HCV core Ag, HCV RNA, and infectivity titers correlated reasonably well in this experiment. However, the

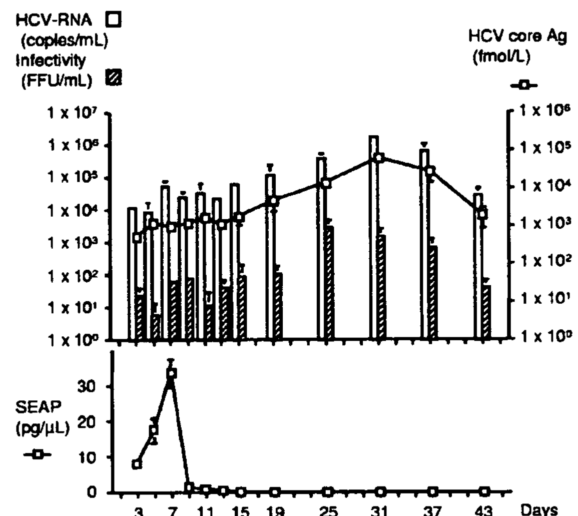

FIG. 4. Long-term culture of JFH1-ribozyme-transfected cells. Huh7.5 cells were transfected with JFH1-ribozyme plasmid as described previously. The transfected cells were passaged 3 to 4 days, and culture medium was collected at various time points. HCV core Ag, HCV RNA, and infectivity titers and SEAP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

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

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

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

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

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

DISCUSSION

The discovery of the JFH-1 strain enabled us to develop a robust system for HCV replication and infection in culture cells (13, 22, 26). However, the JFH-1 strain is unique among the HCV strains and not necessarily representative of HCV biology (7). Studies in chimpanzees suggest that the virus is not particularly infectious in vivo, causing an attenuated and transient infection, which is atypical for the general behavior of HCV (22). It is indeed interesting that this virus was originally isolated from a patient with fulminant hepatitis C (7). It is likely that the fulminant hepatitis is associated more with the clinical setting of the patient than with the virus. Further studies are necessary to resolve the question of whether a viral factor(s) plays a role in the severity of acute HCV infection.

Currently, no other natural HCV strain has yet been shown to replicate efficiently and demonstrate robust infectivity in cell culture without adaptive mutations. Although the establishment of a genotype 1a (H77 strain) infectious system in cell culture is important, introduction of several adaptive mutations is clearly required (24). These adaptive mutations have been shown to confer unusual biological properties to the viral strain in vivo (3). Chimeric viruses containing the structural region of other genotypes and the JFH-1 nonstructural genes have been generated and showed in vitro infectivity (13, 14, 17), but the biological relevance of these chimeric viruses is difficult to assess.

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

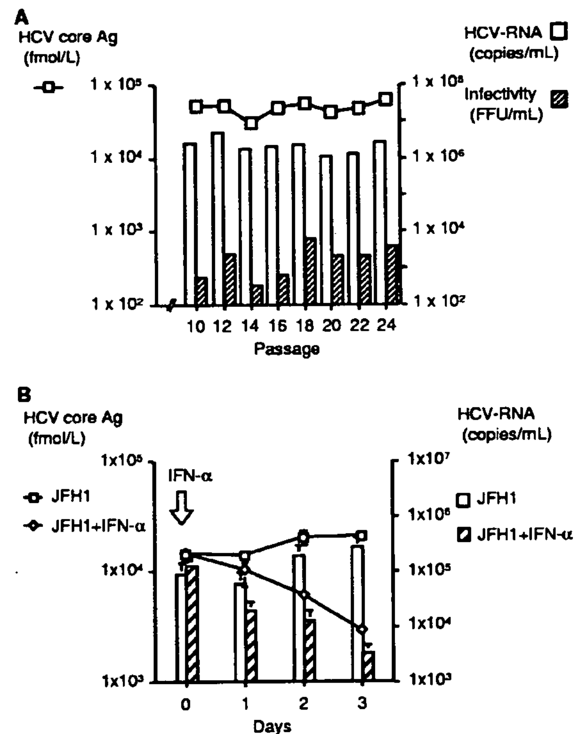

FIG. 5. Production of HCV in the stable JFH1-virus producing cell line. (A) Huh7.5 cells were transfected with the pEF/JFH1-Rz/N plasmid and exposed to 1 mg of G418/ml. Clones were isolated as described in the text. The 7.5-20 clone was passaged every 3 to 4 days and monitored continuously for an extended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

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

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

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

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

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

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

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Upregulation of Indoleamine 2,3-Dioxygenase in Hepatitis C Virus Infection[▽]

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Indoleamine 2,3-dioxygenase (IDO) is induced by proinflammatory cytokines and by CTLA-4-expressing T cells and constitutes an important mediator of peripheral immune tolerance. In chronic hepatitis C, we found upregulation of IDO expression in the liver and an increased serum kynurenine/tryptophan ratio (a reflection of IDO activity). Huh7 cells supporting hepatitis C virus (HCV) replication expressed higher levels of IDO mRNA than noninfected cells when stimulated with gamma interferon or when cocultured with activated T cells. In infected chimpanzees, hepatic IDO expression decreased in animals that cured the infection, while it remained high in those that progressed to chronicity. For both patients and chimpanzees, hepatic expression of IDO and CTLA-4 correlated directly. Induction of IDO may dampen T-cell reactivity to viral antigens in chronic HCV infection.

Indoleamine 2,3-dioxygenase (IDO) mediates conversion of tryptophan to catabolites collectively known as kynurenines (22). This enzyme is expressed by both epithelial and dendritic cells induced by proinflammatory cytokines, including gamma interferon (IFN-γ) and tumor necrosis factor alpha (20, 25). Also, engagement of CTLA-4 with CD80/CD86 on the membrane of dendritic cells stimulates IDO transcriptional expression and activity (4, 9, 19). Increased IDO activity provokes tolerogenicity of antigen-presenting cells and deprives T cells of tryptophan, leading to proliferation arrest and T-cell apoptosis (15). Kynurenine, on the other hand, has been shown to act as an immunoregulatory molecule that mediates immunosuppressive effects in the tissue microenvironment (7, 22, 26). IDO activity contributes to maternal tolerance in pregnancy (21), control of allograft rejection (9), and protection against autoimmunity (8).

Chronic infection caused by hepatitis C virus (HCV) is characterized by weak T-cell responses, recognizing very few epitopes. In contrast, viral clearance after acute infection or after interferon therapy is associated with the presence of a robust and polyclonal T-cell reaction (2, 3, 6, 10, 14, 18, 23, 24). Thus, HCV has developed efficient means to escape T-cell immunity, thus causing a high rate of chronic infections. The molecular mechanisms that are responsible for immune tolerance to HCV antigens remains ill understood. Since IDO activity may dampen T-cell reactivity and can contribute to tolerogenicity of dendritic cells (17), we have analyzed IDO expression by quantitative real-time PCR using β-actin gene expression as an endogenous control (12, 13) (IDO sense primer, TGGCACACGCTATGGAAAAC; antisense, ATGCATCCCAGAACTAGACG; β-actin sense primer, AGCCTCGCCTTTGCCGA; antisense, CTGGTGCCTGGGGCG) in liver samples from patients with chronic hepatitis C (CHC), subjects with sustained virological response (SVR) after interferon therapy, and patients with other forms of chronic liver inflammation (chronic hepatitis B and steatohepatitis) and in normal liver samples (Table 1, cohort 1). IDO mRNA levels were significantly higher in the CHC group than in the other groups. Patients with other forms of liver disease had values higher than those for normal livers but lower than the CHC values (Fig. 1A). Subjects with SVR showed values similar to those for controls.

As an index of IDO activity, we measured the serum kynurenine/tryptophan ratio (KTR) for equivalent groups of patients and for healthy controls (Table 1, cohort 2). KTR was determined by high-performance liquid chromatography (27). We found that KTR was significantly higher for the CHC group than for the other groups, which did not show significant differences among them (Fig. 1B). Since both IDO mRNA levels and serum KTRs are significantly higher for CHC than in other forms of liver disease (see Fig. 1A and B), it seems possible that HCV might be especially efficient at facilitating IDO overexpression in an inflamed milieu.

To determine whether HCV replication may enhance IDO expression in response to proinflammatory cytokines, we stimulated with IFN-γ (100 U/ml; R&D Systems, Minneapolis, MN), for 16, 24, and 40 h, Huh7 cells containing the full-length HCV replicon (Huh7-Core-3′) (12, 16), Huh7 cells producing JFH1-HCV viral particles (28), and control cells. JFH1-Huh7 cells were used at 30 to 35 days postin-

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TABLE 1. Characteristics of patient cohorts

Variable[a]	Value for patient group			
	Normal liver	Chronic hepatitis C	Sustained virological response	Miscellaneous liver diseases
Amt of aspartate aminotransferase (IU/liter)				
Cohort 1	14.7 ± 5	42.1 ± 28	10.8 ± 2	35.4 ± 24
Cohort 2		46.2 ± 28.3	13.8 ± 5	67.0 ± 55
Amt of alanine aminotransferase (IU/liter)				
Cohort 1	19.3 ± 9	78.8 ± 74	10.7 ± 2	53.1 ± 31
Cohort 2		36.5 ± 17.9	15.4 ± 5	50.3 ± 44.5
Viral load (mean, IU/ml)				
Cohort 1		6.5×10^7	0	
Cohort 2		1.1×10^8	0	
No. of samples with viral genotype (1/non-1/not determined)				
Cohort 1		15/5/4		
Cohort 2		12/4/3		
Liver biopsy (Knodel's score) inflammatory activity				
Cohort 1		4.8 ± 1.8	2.3 ± 1.0	5.6 ± 2.5
Cohort 2		5.4 ± 2.2	0.25 ± 0.5	5.4 ± 2.5
Fibrosis score				
Cohort 1		0.5 ± 0.8	0.5 ± 0.5	1.5 ± 1.5
Cohort 2		1.4 ± 1.5	0.25 ± 0.5	1.1 ± 1.2

[a] Cohort 1, liver tissues from normal liver, n = 13 (samples obtained at surgery of liver metastasis or cholelithiasis); miscellaneous liver diseases, n = 23 (of whom 11 were chronic hepatitis B patients and 12 were steatohepatitis patients); chronic hepatitis C, n = 24 (of whom 11 were naive and 13 were nonresponders to pegylated IFN-α2 plus ribavirin); sustained virological response, n = 11. Cohort 2, serum samples from healthy subjects, n = 14; miscellaneous liver diseases, n = 17 (of whom 6 were chronic hepatitis B patients and 11 steatohepatitis patients); chronic hepatitis C, n = 19 (of whom 7 were naive and 12 nonresponders to pegylated IFN-α2 plus ribavirin); sustained virological response, n = 19. Miscellaneous liver diseases and chronic hepatitis C patients did not differ in terms of aspartate aminotransferase/alanine aminotransferase levels and histological grading. The study was approved by the local ethical committee.

fection, when about 50 to 60% of cells were positive for the HCV core protein, as determined by immunofluorescence. As shown in Fig. 2A and B, both Huh7-Core-3′ cells and Huh7 cells producing JFH1 generated increased amounts of IDO mRNA in response to IFN-γ at all time points compared to control Huh7 cells. These findings indicate that HCV replication sensitizes the cells to produce IDO at high levels in response to IFN-γ, a proinflammatory cytokine that is upregulated in the livers of patients with CHC (1). IDO upregulation in response to IFN-γ does not affect the replicative activity of HCV in the infected cells, since treatment of the cells with IFN-γ plus an IDO inhibitor (1-methyl tryptophan) or plus kynurenine did not provoke changes in HCV-RNA levels in the infected cells with respect to those observed with treatment of the cells with IFN-γ alone (data not shown). It seems, therefore, that IDO upregulation may represent a strategy of HCV to escape T-cell immunity rather than a mechanism directly influencing HCV replication.

Our data suggest that one of the strategies used by HCV to

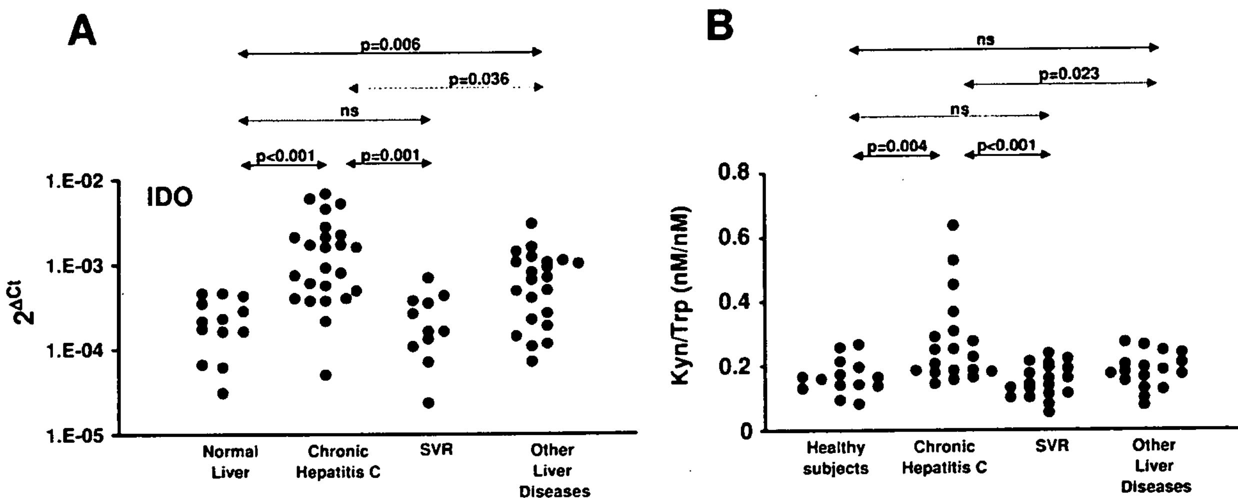

FIG. 1. IDO and HCV infection. (A) Real-time PCR quantitation of IDO mRNA in liver samples from normal livers, from patients with chronic hepatitis C (CHC), from patients with CHC who cleared the virus after interferon therapy (SVR), or from a miscellaneous group of patients with liver disorders unrelated to HCV. Results are normalized with β-actin. (B) Kynurenine/tryptophan ratio in serum samples from individuals belonging to groups equivalent to those shown in panel A. Statistical analyses were performed using nonparametric Kruskal-Wallis and Mann-Whitney U tests. ns, not significant.

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