

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 μ g 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 μ l 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; \pm , 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- μ m filter. Filtrate culture medium was then pooled and concentrated using Amicon Ultra-15 (100,000 MWCO; Millipore, Bedford, MA). The infectious titer was 1.6×10^8 focus-forming units (ffu) per ml and was determined by immunofluorescence detection of infected foci following infection of naive 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 μ g/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; \pm , 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 μ g) was mixed with 400 μ l 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 μ F with a Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred to a 75-cm² flask (Corning) and incubated at $37^\circ\text{C}/5\% \text{CO}_2$. 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

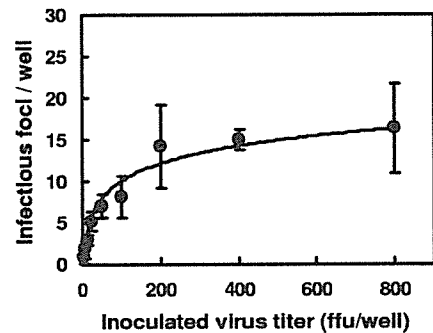


FIG. 1. Infectivity of JFH-1 virus in parental Huh7 cell. A parental Huh7 cell (1×10^4 cells) was infected with JFH-1 virus at 3 to 800 ffu/well for 48 h in 96-well plates. Infected cells were visualized with immunofluorescence using anticore antibody (2H9), and the foci were counted. All experiments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

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.

For transient CD81 expression, 6 μ g of pcDNA3.1-CD81 plasmid was transfected into 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 μ g), the transfer vector construct (3.1 μ g), and the JFH-1 glycoprotein or the VSV-G protein-expressing construct (1 μ g) 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- μ m 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- μ l 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 μ l/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 nonenveloped control. The infectivity of each clone is indicated in Table 1 as follows: -, luciferase activity below 1 RLU; \pm , 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 μ F). 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 μ g 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

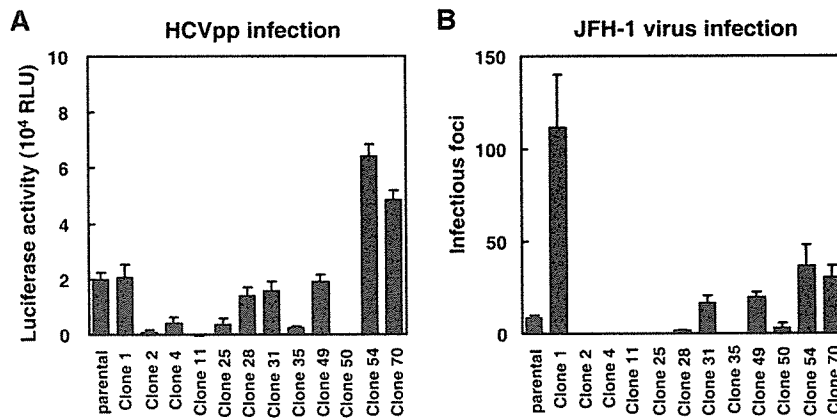


FIG. 2. Infectivity of HCVpp and JFH-1 virus in Huh7 cell clones. (A) Target cells (2×10^4 cells) were inoculated with supernatant containing HCVpp for 3 h in 48-well plates. After 72 h, cells were harvested and the luciferase activities quantified. All experiments were performed in triplicate, and infectivity 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 \pm 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 \pm 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 not expand (data not shown). Thus, the original parental Huh7 cell had limited permissiveness for JFH-1 infection.

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	JFH-1 infectivity ^f
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 Infected foci were detected by immunofluorescence using HCV anticore antibody (2H9).

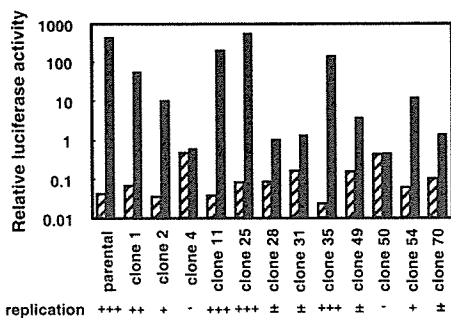


FIG. 3. Replication of JFH-1 subgenomic replicon in Huh7 cell clones. Reporter replicon RNA was transfected into Huh7 cell clones, and luciferase activities at 4 and 48 h after RNA transfection were then detected. The SGR-JFH1/Luc-GND RNA was used as the negative control. All data indicate the RLA compared 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.

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).

Expression of CD81 on Huh7 cell clones is correlated with HCV permissiveness. The Huh7 cell clones displayed differing

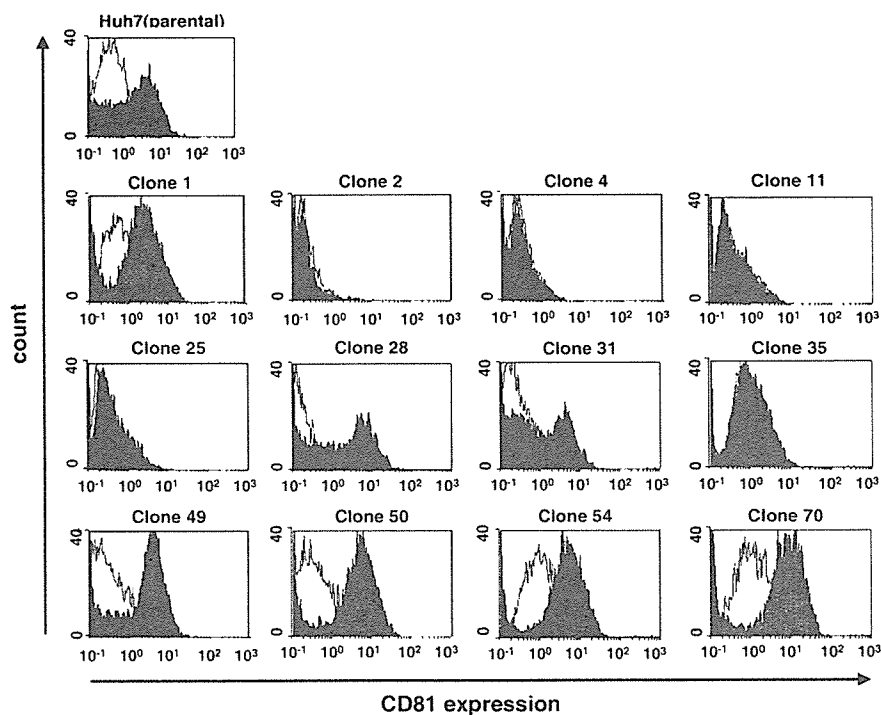


FIG. 4. CD81 expression on the surfaces of Huh7 cell clones. Huh7 cell clones were seeded in 10-cm dishes and cultured overnight. Then, subconfluent cells were harvested either by trypsinization or by treatment with 0.05% EDTA in PBS. Cells (1×10^6) were incubated with $1 \mu\text{g}$ of mouse anti-CD81 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.

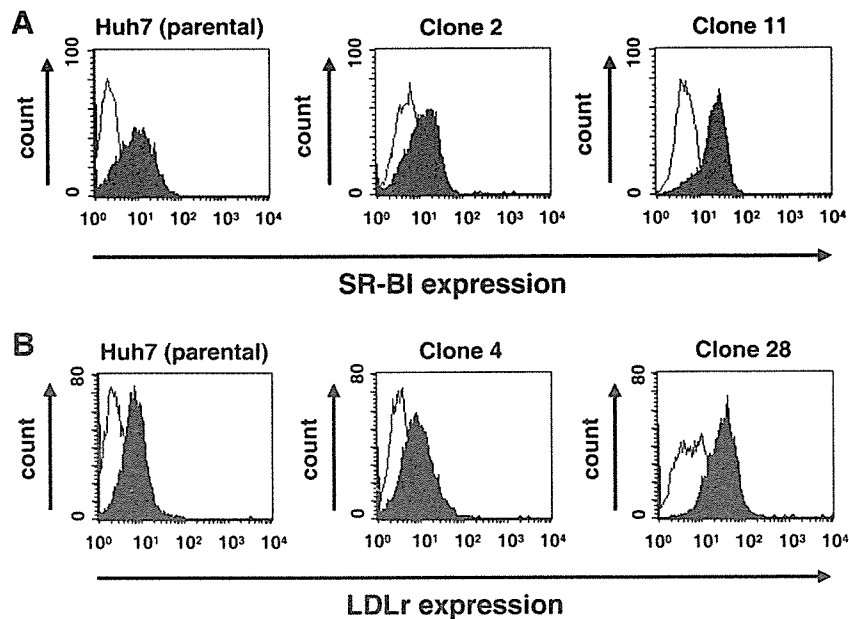


FIG. 5. SR-BI and LDL receptor on the surfaces of Huh7 cell clones. Huh7 cell clones (1×10^6 cells) were incubated with rat anti-SR-BI antiserum or rabbit anti-LDL receptor antibody and subsequently stained with FITC-conjugated secondary antibody. The negative control represents cells incubated with 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).

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 expres-

sion 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, 32), 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 (Huh7-25-CD81) had high infectivity for JFH-1 virus (Fig. 6A), although the replication efficiency was equal to that of Huh7-25

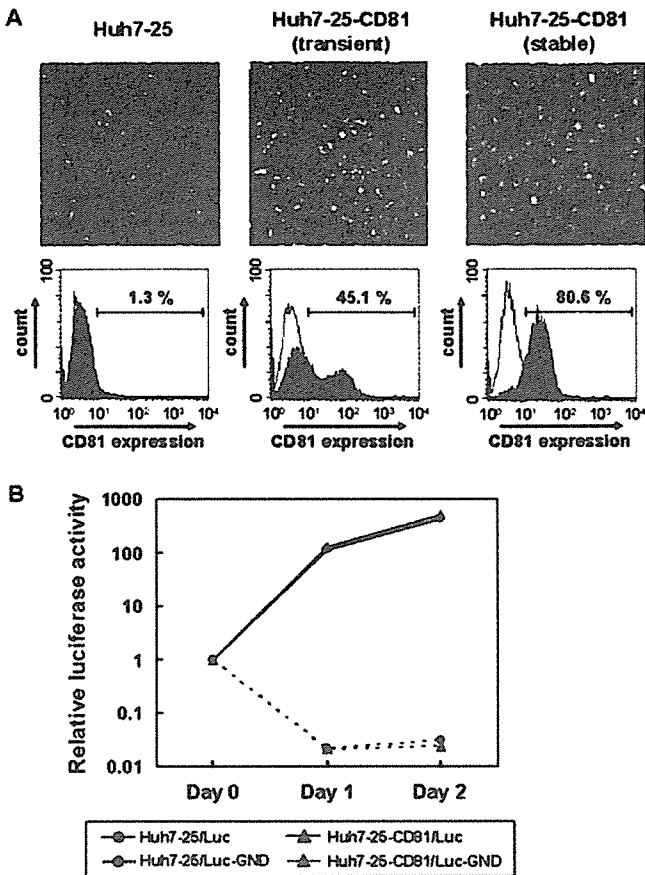


FIG. 6. Infectivity in CD81-transfected Huh7 cells. (A) CD81 was transiently and stably expressed on a Huh7 nonpermissive clone (Huh7-25). The expression of CD81 on the cell surface was examined by flow cytometry. Bars indicate the populations of the CD81-positive cells. Cells were inoculated with JFH-1 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).

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 siRR (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 siRR-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

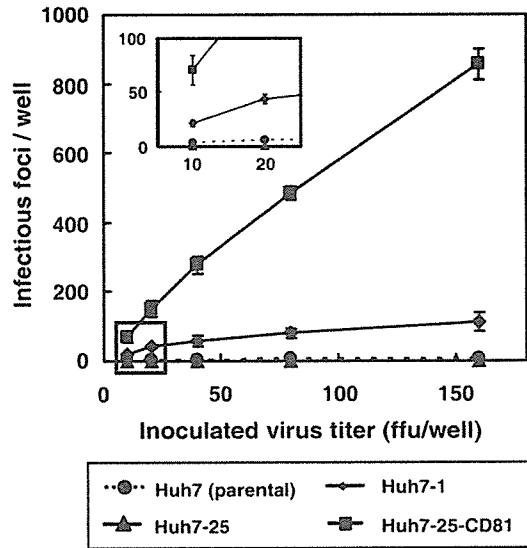


FIG. 7. Dependency of permissiveness on virus concentration in Huh7-25-CD81 cells. Huh7-25-CD81 cells (1×10^4) were infected with JFH-1 virus at 10 to 160 ffu/well in 96-well plates. Infected cells were visualized and assayed as described in the legend for Fig. 1. All experiments were performed in triplicate,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

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). CD81 expression levels of uninfected cells were not different

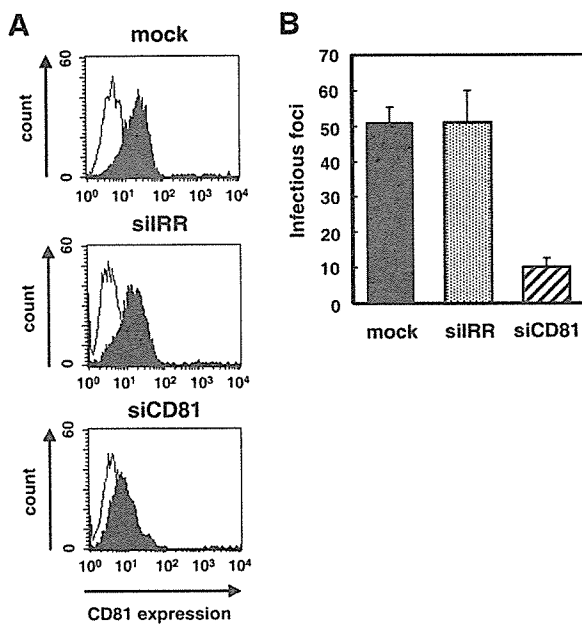


FIG. 8. siRNA silencing of CD81 expression on Huh7-54 cells and JFH-1 virus infection. CD81-positive Huh7-54 cells transfected with either siIRR or siRNA of CD81 (siCD81) were stained with anti-CD81 antibody (JS-81) at 48 h posttransfection and analyzed by flow cytometry. (B) siRNA-transfected Huh-54 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 \pm standard deviations.

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-CD81 cells than for uninfected cells (Fig. 9C, left panel). How-

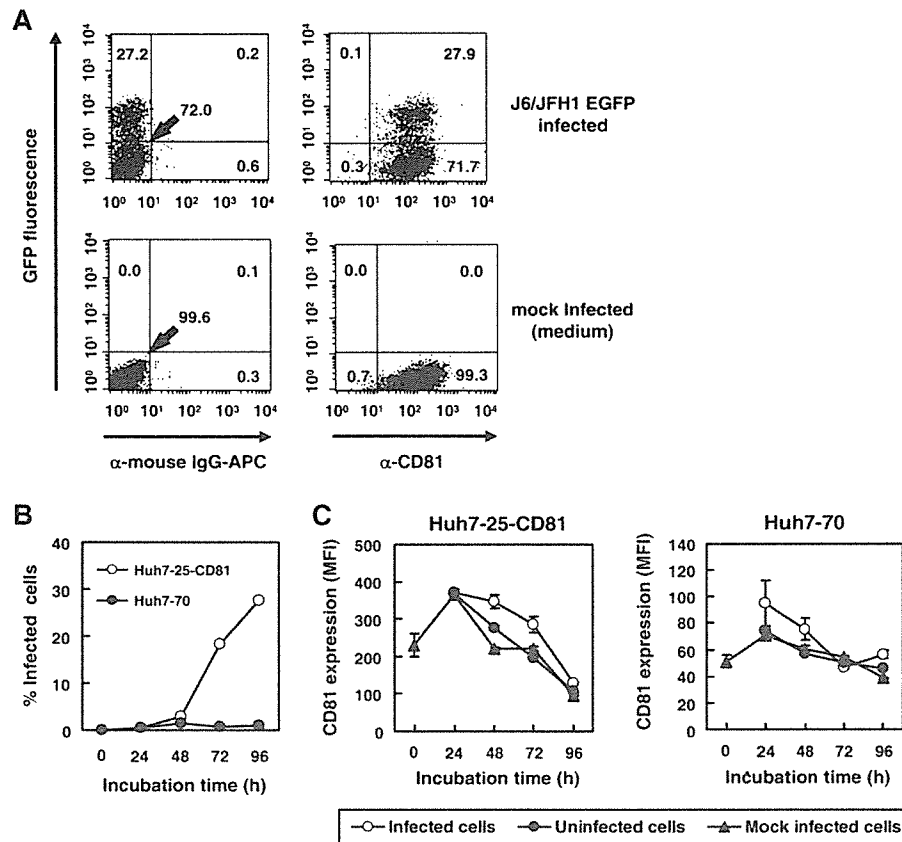


FIG. 9. Kinetics of cell surface CD81 expression and HCV infection. Huh7-25-CD81 and Huh7-70 cells were seeded into six-well plates at a density of 1×10^5 cells/well. Twenty-four hours later, J6/JFH1 EGFP virus (multiplicity of infection, 2) was inoculated. Cells were harvested at 24, 48, 72, and 96 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.

ever, 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 in-

tegrins (36), GPR56 (24), 14-3-3 (8), and signaling enzymes (7, 37, 39), various signal transductions through CD81 and multi-protein 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-

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.

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 the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO); and by Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation.

Huh7.5.1 cells were a kind gift from Francis V. Chisari. pJ6CF plasmid DNA was a kind gift from Jens Bukh. pcDNA3.1-CD81 plasmid DNA and pCAG-VSVG plasmid DNA were kind gifts from Yoshiharu Matsuura. pcDNAdeltaC-E1-E2(JFH1) was a kind gift from Thomas Pietschmann.

REFERENCES

- Agnello, V., G. Abel, M. Elfahal, G. B. Knight, and Q. X. Zhang. 1999. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc. Natl. Acad. Sci. USA* 96:12766–12771.
- Barth, H., R. Cerino, M. Arcuri, M. Hoffmann, P. Schürmann, M. I. Adah, B. Gissler, X. Zhao, V. Ghisetti, B. Lavezzo, H. E. Blum, F. von Weizsäcker, A. Vitelli, E. Scarselli, and T. F. Baumert. 2005. Scavenger receptor class B type I and hepatitis C virus infection of primary *Tupaia* hepatocytes. *J. Virol.* 79:5774–5785.
- Barth, H., C. Schäfer, M. I. Adah, F. Zhang, R. J. Linhardt, H. Toyoda, A. Kinoshita-Toyoda, T. Toida, T. H. Van Kuppevelt, E. Depla, F. Von Weizsäcker, H. E. Blum, and T. F. Baumert. 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J. Biol. Chem.* 278:41003–41012.
- Bartosch, B., G. Verney, M. Dreux, P. Donot, Y. Morice, F. Penin, J. M. Pawlotsky, D. Lavillette, and F. L. Cosset. 2005. An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. *J. Virol.* 79:8217–8229.
- Bartosch, B., J. Dubuisson, and F. L. Cosset. 2003. Infectious hepatitis C virus pseudoparticles containing functional E1–E2 envelope protein complexes. *J. Exp. Med.* 197:633–642.
- Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F. L. Cosset. 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J. Biol. Chem.* 278:41624–41630.
- Berditchevski, F., K. F. Toliai, K. Wong, C. L. Carpenter, and M. E. Hemler. 1997. A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J. Biol. Chem.* 272:2595–2598.
- Clark, K. L., A. Oelke, M. E. Johnson, K. D. Eilert, P. C. Simpson, and S. C. Todd. 2004. CD81 associates with 14-3-3 in a redox-regulated palmitoylation-dependent manner. *J. Biol. Chem.* 279:19401–19406.
- Cormier, E. G., F. Tsamis, F. Kajumo, R. J. Durso, J. P. Gardner, and T. Dragic. 2004. CD81 is an entry coreceptor for hepatitis C virus. *Proc. Natl. Acad. Sci. USA* 101:7270–7274.
- Date, T., M. Miyamoto, T. Kato, K. Morikawa, A. Murayama, D. Akazawa, J. Tanabe, S. Sone, M. Mizokami, and T. Wakita. An infectious and selectable full-length replicon system with hepatitis C virus JFH-1 strain. *Hepatol. Res.*, in press.
- Drummer, H. E., A. Maerz, and P. Pombourios. 2003. Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins. *FEBS Lett.* 546:385–390.
- Gardner, J. P., R. J. Durso, R. R. Arrigale, G. P. Donovan, P. J. Maddon, T. Dragic, and W. C. Olson. 2003. L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. *Proc. Natl. Acad. Sci. USA* 100:4498–4503.
- Hemler, M. E. 2005. Tetraspanin functions and associated microdomains. *Nat. Rev. Mol. Cell Biol.* 6:801–811.
- Hemler, M. E. 2003. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. *Annu. Rev. Cell Dev. Biol.* 19:397–422.
- Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice, and J. A. McKeating. 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc. Natl. Acad. Sci. USA* 100:7271–7276.
- Kapadia, S. B., H. Barth, T. Baumert, J. A. McKeating, and F. V. Chisari. 2007. Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. *J. Virol.* 81:374–383.
- Kato, T., T. Date, M. Miyamoto, M. Sugiyama, Y. Tanaka, E. Orito, T. Ohno, K. Sugihara, I. Hasegawa, K. Fujiwara, K. Ito, A. Ozasa, M. Mizokami, and T. Wakita. 2005. Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. *J. Clin. Microbiol.* 43:5679–5684.
- Kato, T., T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, and T. Wakita. 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* 125:1808–1817.
- Koutsoudakis, G., A. Kaul, E. Steinmann, S. Kallis, V. Lohmann, T. Pietschmann, and R. Bartenschlager. 2006. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *J. Virol.* 80:5308–5320.
- Lavillette, D., A. W. Tarr, C. Voisset, P. Donot, B. Bartosch, C. Bain, A. H. Patel, J. Dubuisson, J. K. Ball, and F. L. Cosset. 2005. Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. *Hepatology* 41:265–274.
- Levy, S., S. C. Todd, and H. T. Maecker. 1998. CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu. Rev. Immunol.* 16:89–109.
- Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wölk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice. 2005. Complete replication of hepatitis C virus in cell culture. *Science* 309:623–626.
- Lindenbach, B. D., and C. M. Rice. 2001. *Flaviviridae*: the viruses and their replication, p. 991–1042. In D. M. Knipe and P. M. Howley (ed.), *Fields virology*, 4th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, PA.
- Little, K. D., M. E. Hemler, and C. S. Stipp. 2004. Dynamic regulation of a GPCR-tetraspanin-G protein complex on intact cells: central role of CD81 in facilitating GPR56-G_q/11 association. *Mol. Biol. Cell* 15:2375–2387.
- Lozach, P. Y., H. Lortat-Jacob, A. de Lacroix de Lavalette, I. Staropoli, S. Foug, A. Amara, C. Houles, F. Fieschi, O. Schwartz, J. L. Virelizier, F. Arenzana-Seisdedos, and R. Altmeyer. 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. *J. Biol. Chem.* 278:20358–20366.
- Martin, F., D. M. Roth, D. A. Jans, C. W. Pouton, L. J. Partridge, P. N. Monk, and G. W. Moseley. 2005. Tetraspanins in viral infections: a fundamental role in viral biology? *J. Virol.* 79:10839–10851.
- Matsuura, Y., H. Tani, K. Suzuki, T. Kimura-Someya, R. Suzuki, H. Aizaki, K. Ishii, K. Moriishi, C. S. Robison, M. A. Whitt, and T. Miyamura. 2001. Characterization of pseudotype VSV possessing HCV envelope proteins. *Virology* 286:263–275.
- Morikawa, K., Z. Zhao, T. Date, M. Miyamoto, A. Murayama, D. Akazawa, J. Tanabe, S. Sone, and T. Wakita. Infectious hepatitis C virus particle binding to the Huh7 cell surface is mediated by glycosaminoglycans and its internalization by CD81. *J. Med. Virol.*, in press.
- Nakabayashi, H., K. Taketa, K. Miyano, T. Yamane, and J. Sato. 1982. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. *Cancer Res.* 42:3858–3863.
- Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani. 1998. Binding of hepatitis C virus to CD81. *Science* 282:938–941.
- Scarselli, E., H. Ansuini, R. Cerino, R. M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, and A. Vitelli. 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J.* 21:5017–5025.
- Takeuchi, T., A. Katsume, T. Tanaka, A. Abe, K. Inoue, K. Tsukiyama-Kohara, R. Kawaguchi, S. Tanaka, and M. Kohara. 1999. Real-time detection system for quantification of hepatitis C virus genome. *Gastroenterology* 116:636–642.
- Voisset, C., N. Callens, E. Blanchard, A. Op De Beeck, J. Dubuisson, and N. Yu-Dac. 2005. High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I. *J. Biol. Chem.* 280:7793–7799.
- Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11:791–796.
- Yanagi, M., R. H. Purcell, S. U. Emerson, and J. Bukh. 1999. Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. *Virology* 262:250–263.
- Yang, X., O. V. Kovalenko, W. Tang, C. Claas, C. S. Stipp, and M. E. Hemler. 2004. Palmitoylation supports assembly and function of integrin-tetraspanin complexes. *J. Cell Biol.* 167:1231–1240.
- Yauch, R. L., F. Berditchevski, M. B. Harler, J. Reichner, and M. E. Hemler. 1998. Highly stoichiometric, stable, and specific association of integrin alpha3beta1 with CD151 provides a major link to phosphatidylinositol 4-kinase, and may regulate cell migration. *Mol. Biol. Cell* 9:2751–2765.
- Zhang, J., G. Randall, A. Higginbottom, P. Monk, C. M. Rice, and J. A. McKeating. 2004. CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. *J. Virol.* 78:1448–1455.

39. Zhang, X. A., A. L. Bontrager, and M. E. Hemler. 2001. Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific beta(1) integrins. *J. Biol. Chem.* **276**:25005–25013.
40. Zhong, J., P. Gastaminza, J. Chung, Z. Stamataki, M. Isogawa, G. Cheng, J. A. McKeating, and F. V. Chisari. 2006. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. *J. Virol.* **80**:11082–11093.
41. Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari. 2005. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. USA* **102**:9294–9299.
42. Zhu, G. Z., B. J. Miller, C. Boucheix, E. Rubinstein, C. C. Liu, R. O. Hynes, D. G. Myles, and P. Primakoff. 2002. Residues SFQ (173–175) in the large extracellular loop of CD9 are required for gamete fusion. *Development* **129**:1995–2002.

Production of Infectious Hepatitis C Virus of Various Genotypes in Cell Culture

Running Title; Infectious HCV Production in Cell Culture

Takanobu Kato¹, Takuya Matsumura¹, Theo Heller¹, Satoru Saito¹,
Ronda K. Sapp¹, Krishna Murthy², Takaji Wakita^{3§}, T. Jake Liang^{1*}.

¹Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney
Diseases, National Institutes of Health, Bethesda, MD, 20892,

²Southwest Foundation for Biomedical Research, San Antonio, TX, 78227, USA.

³Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo,
Japan.

*To whom correspondence should be addressed.

Dr. T. Jake Liang

TEL: (301) 496-1721

FAX: (301) 402-0491

E-mail: JakeL@bdg10.niddk.nih.gov

§Current address:

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

Word Count;

Abstract (249 words)

Abstract

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 effects of culture conditions. After transfection into Huh7-derived cell line Huh7.5.1, continuous HCV replication and secretion were confirmed by 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 were inoculated to 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 identical sequences as 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.

Introduction

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 a HCV infection model system with cell culture generated JFH-1 virus that is infectious both *in vitro* and *in vivo* (5, 7, 8, 9, 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 a result of viral characteristics. To study 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 this 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 effects of culture conditions. Using this system, we could generate various genotypes of HCV and confirmed 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, ref. 11), CG1b (genotype 1b, accession no.; AF333324, ref. 21), J6 (genotype 2a, accession no.; AF177036, ref. 23), and JFH1 (genotype 2a, accession no.; AB047639, ref. 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 (UTR) of the CG1b genome. To generate SEAP-expressing vector, the fragment 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 EF1 α promoter and the SEAP gene by the 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. Huh7 derivative cell lines, Huh7.5 and Huh7.5.1, were provided by C. Rice (Rockefeller University, New York, NY) and Dr. F. Chisari (Scripps Research Institute, La Jolla, CA), respectively (2, 25). Huh7 cells or these derivative cell lines were maintained at 37°C, 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum.

Ten µg of HCV-ribozyme plasmids were transfected into Huh7 cells or its derivative cells in 10 cm dish (2 x 10⁶ / dish) using Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's instructions. IFN-α was purchased from Fitzgerald (Concord, MA).

Quantification of HCV core antigen, HCV RNA and SEAP activity. To assess HCV replication, HCV core antigen (Ag) in culture supernatant was quantified by 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 culture medium was extracted from 100-250 μ L of culture medium by TRIzol LS reagent (Invitrogen) and treated with DNase (TURBO DNase; Ambion, Austin, TX) at 37 °C for 1h. Extracted RNA was purified by RNeasy Mini Kit, which includes another step of RNase-Free DNase digestion (Qiagen, Valencia, CA). Copy numbers of HCV RNA were determined by real-time quantitative RT-PCR (RT-qPCR) as described previously (20). The detection limit was estimated as 300 copies/mL. When necessary, culture medium was concentrated by ultra-filtration concentrator (Vivaspin 20, cut off; 100k MW, Vivascience, Hannover, Germany). SEAP activity in the culture medium was detected by Great EscAPe SEAP Detection Kit (Clontech Laboratories, Inc.).

Titration of HCV infectivity. Huh7.5.1 cells were seeded at 1×10^4 cells / well in 96 well flat bottom plate 24 h before inoculation. 100 μ L of serially 10-fold diluted culture media or gradient fractions were inoculated to the cells in the 96-well plate. After 4 h incubation, inoculums were replaced with fresh media and 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 IgG

(Invitrogen). Clusters of core protein positive cells were counted as a single infection focus, and infectivity titers were represented as a focus-forming unit (FFU). Infection of the generated virus in culture medium was also confirmed by the use of α -NS3 antibody (provided from Dr. G. Luo, University of Kentucky College of Medicine, Lexington, KY).

Density gradient analysis. 50 mL of culture medium harvested 5 days after transfection and passed through a 0.45 μ m filter was precipitated with one-fourth volume of 40% (w/v) polyethylene glycol 8000 in PBS 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% BSA), and purified by 20% (w/v) iodixanol (Optiprep; Axis-Shield, Oslo, Norway) cushion by centrifugation for 6 h at 40,000 rpm, 4°C in an SW-41 Ti rotor. Purified virus was layered on top of 10 – 40% iodixanol gradient, and centrifuged for 16 h at the same condition for iodixanol cushion. Fractions were collected from the bottom of gradient. HCV RNA titer of each fraction was measured after DNase treatment and RNA extraction as described above. 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 AALAC (American Association of Laboratory Animal Care)-accredited animal facility. A naïve chimpanzee (X140) 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. Next the chimpanzee was 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 (X199) 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 nt 152 - 2777, nt 2743 - 5098, nt 4923 - 7670, and nt 7611- 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 expressed as mean \pm standard deviation. Statistical analysis was performed using the Student's t-test, Welch's t-test or One-factor ANOVA. *P* values of less than 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/L, respectively. JFH1 produced a much higher HCV core Ag level (4686.6 ± 287.1 fmol/L on day 5) than the other strains ($p < 0.05$). Likewise, 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 those of strains H77, CG1b and J6 ($4.28 \times 10^3 \pm 3.00 \times 10^3$, $9.94 \times 10^3 \pm 4.64 \times 10^3$, $6.29 \times 10^2 \pm 5.76 \times 10^2$ copies/mL, respectively; $p < 0.05$) (Fig. 1B). SEAP activities in the culture supernatants were comparable among all strains indicating similar transfection efficiencies. These data indicate that the JFH1 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 JFH1 in the HCV subgenomic replicon system (9). To confirm CG1b virus replication in this system, 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. Five days after transfection, HCV RNA titer in culture supernatant of CG1b-transfected cell was $3.42 \times 10^3 \pm 1.84 \times 10^3$ copies/mL. HCV RNA titer in culture supernatant of