ment with sphingomyelin, and JEV and JEVrv were coprecipitated with the ceramide beads, suggesting that the interaction of ceramide with the JEV E protein plays a crucial role in the early steps of infection. Ceramide is known to bind to the ceramide transport protein (CERT), which transports ceramide from the ER to the Golgi apparatus (12), and thus, it might be feasible to speculate that CERT participates in the translocation or maturation of the JEV E protein. Further studies are needed to clarify the interaction among ceramide, CERT, and the JEV E protein. Recently Aizaki et al. reported that the infectivity of HCV particles was decreased by treatment with MβCD or SMase, suggesting that cholesterols or sphingolipids incorporated into the virions are important for the infectivity of HCV (1). In this study, SMase treatment of HCVpv particles but not of JEVpv particles reduced infectivity, suggesting that incorporation of cholesterols and sphingolipids into the viral particles was different among flaviviruses.

The discrepancy between the drastic increase in the production of infectious particles of JEVrv and the marginal increase in that for JEV induced by SMase treatment in ceramide-enriched cells may indicate that ceramide enrichment enhances the entry and egress steps but negatively regulates genomic replication of JEV. Previously it was reported that digestion of sphingomyelin by SMase induces cholesterol redistribution (32), an increase in intracellular cholesterol esterification (4), and a decrease in cholesterol biosynthesis (39). Furthermore, ceramide has been shown to selectively displace cholesterol from lipid rafts and decrease the association of the cholesterol binding protein caveolin-1 (28, 50). Although we have not determined the cholesterol composition of the membranes of cells treated with SMase, cholesterol depletion induced by SMase treatment may also participate in the enhancement of JEV entry.

JEV initiates infection by interacting with receptor and/or coreceptor molecule(s), probably in cooperation with ceramide located in the ceramide-enriched platforms. The ceramide-enriched membrane domains facilitate signal transduction through reorganization and clustering of cell surface receptor molecules. Although the entry receptor(s) of JEV has not been well characterized yet, modification of the distribution, organization, and steric conformation of the receptor molecule(s) by treatment with SMase may facilitate entry of JEV. Generation of ceramide by SMase treatment has been shown to promote vesicular fusion processes and fusion of phagosomes, thereby engulfing bacteria with late endosomes and resulting in efficient intracellular bacterial killing (46).

In conclusion, we have demonstrated that the entry and egress processes of JEV were enhanced by treatment with SMase by using pseudotype and recombinant VSVs. The interaction of cellular ceramide and the E glycoproteins facilitates infection and propagation of JEV. Modification of sphingolipids on the plasma membrane of the target cells might be a novel target for the development of antivirals against JEV infection.

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Production of Infectious Hepatitis C Virus by Using RNA Polymerase I-Mediated Transcription▽

Takahiro Masaki,[1]† Ryosuke Suzuki,[1]† Mohsan Saeed,[1,4] Ken-ichi Mori,[2] Mami Matsuda,[1] Hideki Aizaki,[1] Koji Ishii,[1] Noboru Maki,[2] Tatsuo Miyamura,[1] Yoshiharu Matsuura,[3] Takaji Wakita,[1] and Tetsuro Suzuki[1]*

Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan[1]; Advanced Life Science Institute, Wako, Saitama 351-0112, Japan[2]; Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Suita-shi, Osaka 565-0871, Japan[3]; and Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan[4]

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In this study, we used an RNA polymerase I (Pol I) transcription system for development of a reverse genetics protocol to produce hepatitis C virus (HCV), which is an uncapped positive-strand RNA virus. Transfection with a plasmid harboring HCV JFH-1 full-length cDNA flanked by a Pol I promoter and Pol I terminator yielded an unspliced RNA with no additional sequences at either end, resulting in efficient RNA replication within the cytoplasm and subsequent production of infectious virions. Using this technology, we developed a simple replicon *trans*-packaging system, in which transient transfection of two plasmids enables examination of viral genome replication and virion assembly as two separate steps. In addition, we established a stable cell line that constitutively produces HCV with a low mutation frequency of the viral genome. The effects of inhibitors of N-linked glycosylation on HCV production were evaluated using this cell line, and the results suggest that certain step(s), such as virion assembly, intracellular trafficking, and secretion, are potentially up- and downregulated according to modifications of HCV envelope protein glycans. This Pol I-based HCV expression system will be beneficial for a high-throughput antiviral screening and vaccine discovery programs.

Over 170 million people worldwide have been infected with hepatitis C virus (HCV) (22, 33, 37), and persistence of HCV infection is one of the leading causes of liver diseases, such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (16, 25, 38). The HCV genome is an uncapped 9.6-kb positive-strand RNA sequence consisting of a 5′ untranslated region (UTR), an open reading frame encoding at least 10 viral proteins (Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and a 3′UTR (46). The structural proteins (Core, E1, and E2) reside in the N-terminal region.

The best available treatment for HCV infection, which is pegylated alpha interferon (IFN-α) combined with ribavirin, is effective in only about half of patients and is often difficult to tolerate (25). To date, a prophylactic or therapeutic vaccine is not available. There is an urgent need to develop more effective and better tolerated therapies for HCV infection. Recently, a robust system for HCV production and infection in cultured cells has been developed. The discovery that some HCV isolates can replicate in cell cultures and release infectious particles has allowed the complete viral life cycle to be studied (23, 49, 53). The most robust system for HCV production involves transfection of Huh-7 cells with genomic HCV RNA of the JFH-1 strain by electroporation. However, using this RNA transfection system, the amount of secreted infectious viruses often fluctuate and mutations emerge in HCV genome with multiple passages for an extended period of time (54), which limits its usefulness for antiviral screening and vaccine development.

DNA-based expression systems for HCV replication and virion production have also been examined (5, 15, 21). With DNA-based expression systems, transcriptional expression of functional full-length HCV RNA is controlled by an RNA polymerase II (Pol II) promoter and a self-cleaving ribozyme(s). DNA expression systems using RNA polymerase I (Pol I) have been utilized in reverse genetics approaches to replicate negative-strand RNA viruses, including influenza virus (12, 29), Uukuniemi virus (11), Crimean-Congo hemorrhagic fever virus (10), and Ebola virus (13). Pol I is a cellular enzyme that is abundantly expressed in growing cells and transcribes rRNA lacking both a 5′ cap and a 3′ poly(A) tail. Thus, viral RNA synthesized in cells transfected with Pol I-driven plasmids containing viral genomic cDNA has no additional sequences at the 5′- or 3′ end even in the absence of a ribozyme sequence (28). The advantages of DNA-based expression systems are that DNA expression plasmids are easier to manipulate and generate stable cell lines that constitutively express the viral genome.

We developed here a new HCV expression system based on transfection of an expression plasmid containing a JFH-1 cDNA clone flanked by Pol I promoter and terminator sequences to generate infectious HCV particles from transfected cells. The technology presented here has strong potential to be the basis for *trans*-encapsidation system by transient transfection of two plasmids and for the establishment of an efficient and reliable screening system for potential antivirals.

MATERIALS AND METHODS

DNA construction. To generate HCV-expressing plasmids containing full-length JFH1 cDNA embedded between Pol I promoter and terminator se-

* Corresponding author. Present address: Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan. Phone: 81-53-435-2336. Fax: 81-53-435-2337. E-mail: tesuzuki@hama-med.ac.jp.

† T.M. and R.S. contributed equally to this study.

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quences, part of the 5′UTR region and part of the NS5B to the 3′UTR region of full-length JFH-1 cDNA were amplified by PCR using primers containing BsmBI sites. Each amplification product was then cloned into a pGEM-T Easy vector (Promega, Madison, WI) and verified by DNA sequencing. Both fragments were excised by digestion with NotI and BsmBI, after which they were cloned into the BsmBI site of the pHH21 vector (a gift from Yoshihiro Kawaoka, School of Veterinary Medicine, University of Wisconsin-Madison [29]), which contains a human Pol I promoter and a mouse Pol I terminator. The resultant plasmid was digested by AgeI and EcoRV and ligated to JFH-1 cDNA digested by AgeI and EcoRV to produce pHHJFH1. pHHJFH1/GND having a point mutation at the GDD motif in NS5B to abolish RNA-dependent RNA polymerase activity and pHHJFH1/R783A/R785A carrying double Arg-to-Ala substitutions in the cytoplasmic loop of p7 were constructed by oligonucleotide-directed mutagenesis. To generate pHHJFH1/ΔE carrying in-frame deletions of parts of the E1 and E2 regions (amino acids [aa] 256 to 567), pHHJFH1 was digested with NcoI and AscI, followed by Klenow enzyme treatment and self-ligation. To generate pHH/SGR-Luc carrying the bicistronic subgenomic HCV reporter replicon and its replication-defective mutant, pHH/SGR-Luc/GND, AgeI-SpeI fragments of pHHJFH1 and pHHJFH1/GND were replaced with an AgeI-SpeI fragment of pSGR-JFH1/Luc (20). In order to construct pCAG/C-NS2 and pCAG/C-p7, PCR-amplified cDNA for C-NS2 and C-p7 regions of the JFH-1 strain were inserted into the EcoRI sites of pCAGGS (30). In order to construct stable cell lines, a DNA fragment containing a Zeocin resistance gene excised from pSV2/Zeo2 (Invitrogen, Carlsbad, CA) was inserted into pHH21 (pHHZeo). Full-length JFH-1 cDNA was then inserted into the BsmBI sites of pHHZeo. The resultant construct was designated pHHJFH1/Zeo.

Cells and compounds. The human hepatoma cell line, Huh-7, and its derivative cell line, Huh7.5.1 (a gift from Francis V. Chisari, The Scripps Research Institute), were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with nonessential amino acids, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. *N*-Nonyl-deoxynojirimycin (*N*N-DNJ) and kifunensine (KIF) were purchased from Toronto Research Chemicals (Ontario, Canada), castanospermine (CST) and 1,4-dideoxy-1,4-imino-D-mannitol hydrochloride (DIM) were from Sigma-Aldrich (St. Louis, MO), 1-deoxymannojirimycin (DMJ) and swainsonine (SWN) were from Alexis Corp. (Lausen, Switzerland), and *N*-butyl-deoxynojirimycin (*N*B-DNJ) was purchased from Wako Chemicals (Osaka, Japan). BILN 2061 was a gift from Boehringer Ingelheim (Canada), Ltd. These compounds were dissolved in dimethyl sulfoxide and used for the experiments. IFN-α was purchased from Dainippon-Sumitomo (Osaka, Japan).

DNA transfection and selection of stable cell lines. DNA transfection was performed by using FuGENE 6 transfection reagent (Roche, Mannheim, German) in accordance with the manufacturer's instructions. To establish stable cell lines constitutively producing HCV particles, pHHJFH1/Zeo was transfected into Huh7.5.1 cells within 35-mm dishes. At 24 h posttransfection (p.t.), the cells were then divided into 100-mm dishes at various cell densities and incubated with DMEM containing 0.4 mg of zeocin/ml for approximately 3 weeks. Selected cell colonies were picked up and amplified. The expression of HCV proteins was confirmed by measuring secreted core proteins. The stable cell line established was designated H751JFH1/Zeo.

***In vitro* synthesis of HCV RNA and RNA transfection.** RNA synthesis and transfection were performed as previously described (26, 49).

RNA preparation, Northern blotting, and RNase protection assay (RPA). Total cellular RNA was extracted with a TRIzol reagent (Invitrogen), and HCV RNA was isolated from filtered culture supernatant by using the QIAamp viral RNA minikit (Qiagen, Valencia, CA). Extracted cellular RNA was treated with DNase (TURBO DNase; Ambion, Austin, TX) and cleaned up by using an RNeasy minikit, which includes another step of RNase-free DNase digestion (Qiagen). The cellular RNA (4 μg) was separated on 1% agarose gels containing formaldehyde and transferred to a positively charged nylon membrane (GE Healthcare, Piscataway, NJ). After drying and cross-linking by UV irradiation, hybridization was performed with [α-^{32}P]dCTP-labeled DNA using Rapid-Hyb buffer (GE Healthcare). The DNA probe was synthesized from full-length JFH-1 cDNA using the Megaprime DNA labeling system (GE Healthcare). Quantification of positive- and negative-strand HCV RNA was performed using the RPA with biotin-16-uridine-5′-triphosphate (UTP)-labeled HCV-specific RNA probes, which contain 265 nucleotides (nt) complementary to the positive-strand (+) 5′UTR and 248 nt complementary to the negative-strand (−) 3′UTR. Human β-actin RNA probes labeled with biotin-16-UTP were used as a control to normalize the amount of total RNA in each sample. The RPA was carried out using an RPA III kit (Ambion) according to the manufacturer's procedures. Briefly, 15 μg of total cellular RNA was used for hybridization with 0.3 ng of the β-actin probe and 0.6 ng of either the HCV (+) 5′UTR or (−) 3′UTR RNA probe. After digestion with RNase A/T1, the RNA products were analyzed by electrophoresis in a 6% polyacrylamide–8 M urea gel and visualized by using a chemiluminescent nucleic acid detection module (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions.

Reverse transcriptase PCR (RT-PCR), sequencing, and rapid amplification of cDNA ends (RACE). Aliquots (5 μl) of RNA solution extracted from filtered culture supernatant were subjected to reverse transcription with random hexamer and Superscript II reverse transcriptase (Invitrogen). Four fragments of HCV cDNA (nt 129 to 2367, nt 2285 to 4665, nt 4574 to 7002, and nt 6949 to 9634), which covers most of the HCV genome, were amplified by nested PCR. Portions (1 or 2 μl) of each cDNA sample were subjected to PCR with TaKaRa LA *Taq* polymerase (Takara, Shiga, Japan). The PCR conditions consisted of an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 3 min. The amplified products were separated by agarose gel electrophoresis and used for direct DNA sequencing. To establish the 5′ ends of the HCV transcripts from pHHJFH1, a synthetic 45-nt RNA adapter (Table 1) was ligated to RNA extracted from the transfected cells 1 day p.t. using T4 RNA ligase (Takara). The viral RNA sequences were then reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) with a primer, RT (Table 1). The resultant cDNA sequences were subsequently amplified by PCR with 5′RACEouter-S and 5′RACEouter-R primers, followed by a second cycle of PCR using 5′RACEinner-S and 5′RACEinner-R primers (Table 1). To establish the terminal 3′-end sequences, extracted RNA sequences were polyadenylated using a poly(A) polymerase (Takara), reverse transcribed with CAC-T35 primer (Table 1), and amplified with the primers 3X-10S (Table 1) and CAC-T35. The amplified 5′ and 3′ cDNA sequences were then separated by agarose gel electrophoresis, cloned into the pGEM-T Easy vector (Promega), and sequenced.

Western blotting. The proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA) after separation by SDS-PAGE. After blocking, the membranes were probed with a mouse monoclonal anti-HCV core antibody (2H9) (49), a rabbit polyclonal anti-NS5B antibody, or a mouse monoclonal GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (Chemicon, Temecula, CA), followed by incubation with a peroxidase-conjugated secondary antibody and visualization with an ECL Plus Western blotting detection system (Amersham, Buckinghamshire, United Kingdom).

Quantification of HCV core protein. HCV core protein was quantified by using a highly sensitive enzyme immunoassay (Ortho HCV antigen ELISA kit; Ortho Clinical Diagnostics, Tokyo, Japan) in accordance with the manufacturer's instructions.

Sucrose density gradient analysis. Samples of cell culture supernatant were processed by low-speed centrifugation and passage through a 0.45-μm-pore-size filter. The filtrated supernatant was then concentrated ~30-fold by ultrafiltration by using an Amicon Ultra-15 filter device with a cutoff molecular mass of 100,000 kDa (Millipore), after which it was layered on top of a continuous 10 to 60% (wt/vol) sucrose gradient, followed by centrifugation at 35,000 rpm at 4°C for 14 h with an SW41 rotor (Beckman Coulter, Fullerton, CA). Fractions of 1 ml were collected from the bottom of the gradient. The core level and infectivity of HCV in each fraction were determined.

Quantification of HCV infectivity. Infectious virus titration was performed by a 50% tissue culture infectious dose ($TCID_{50}$) assay, as previously described (23, 26). Briefly, naive Huh7.5.1 cells were seeded at a density of 10^4 cells/well in a 96-well flat-bottom plate 24 h prior to infection. Five serial dilutions were performed, and the samples were used to infect the seeded cells (six wells per dilution). At 72 h after infection, the inoculated cells were fixed and immunostained with a rabbit polyclonal anti-NS5A antibody (14), followed by an Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen).

Labeling of *de novo*-synthesized viral RNA and immunofluorescence staining. Labeling of *de novo*-synthesized viral RNA was performed as previously described with some modifications (40). Briefly, cells were plated onto an eight-well chamber slide at a density of 5×10^4 cells/well. One day later, the cells were incubated with actinomycin D at a final concentration of 10 μg/ml for 1 h and washed twice with HEPES-saline buffer. Bromouridine triphosphate (BrUTP) at 2 mM was subsequently transfected into the cells using FuGENE 6 transfection reagent, after which the cells were incubated for 15 min on ice. After the cells were washed twice with phosphate-buffered saline (PBS), they were incubated in fresh DMEM supplemented with 10% FBS at 37°C for 4 h. The cells were then fixed with 4% paraformaldehyde for 20 min and permeabilized with PBS containing 0.1% Triton X-100 for 15 min at room temperature. Immunofluorescence staining of NS5A and *de novo*-synthesized HCV RNA was performed as previously described (26, 40). The nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole) solution (Sigma-Aldrich). Confocal microscopy was performed

TABLE 1. Oligonucleotides used for RT-PCR and RACE of the JFH-1 genome

Method or segment	Oligonucleotide	Sequences (5′–3′)
5′RACE	RT	GTACCCCATGAGGTCGGCAAAG
	45-nt RNA adapter	GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA
	5′RACEouter-S	GCTGATGGCGATGAATGAACACTG
	5′RACEouter-R	GACCGCTCCGAAGTTTTCCTTG
	5′RACEinner-S	GAACACTGCGTTTGCTGGCTTTGATG
	5′RACEinner-R	CGCCCTATCAGGCAGTACCACAAG
3′RACE	CAC-T35	CACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
	3X-10S	ATCTTAGCCCTAGTCACGGC
nt 129-2367	44S (1st PCR)	CTGTGAGGAACTACTGTCTT
	2445R	TCCACGATGTTCTGGTGAAG
	17S (2nd PCR)	CGGGAGAGCCATAGTGG
	2367R	CATTCCGTGGTAGAGTGCA
nt 2285-4665	2099S (1st PCR)	ACGGACTGTTTTAGGAAGCA
	4706R	TTGCAGTCGATCACGGAGTC
	2285S (2nd PCR)	AACTTCACTCGTGGGGATCG
	4665R	TCGGTGGCGACGACCAC
nt 4574-7002	4547S (1st PCR)	AAGTGTGACGAGCTCGCGG
	7027R	CATGAACAGGTTGGCATCCACCAT
	4594S (2nd PCR)	CGGGGTATGGGCTTGAACGC
	7003R	GTGGTGCAGGTGGCTCGCA
nt 6949-9634	6881S (1st PCR)	ATTGATGTCCATGCTAACAG
	3X-75R	TACGGCACTCTCTGCAGTCA
	6950S (2nd PCR)	GAGCTCCTCAGTGAGCCAG
	3X-54R	GCGGCTCACGGACCTTTCAC

using a Zeiss confocal laser scanning microscope LSM 510 (Carl Zeiss, Oberkochen, Germany).

Luciferase assay. Huh7.5.1 cells were seeded onto a 24-well cell culture plate at a density of 3×10^4 cells/well 24 h prior to inoculation with 100 μl of supernatant from the transfected cells. The cells were incubated for 72 h, followed by lysis with 100 μl of lysis buffer. The luciferase activity of the cells was determined by using a luciferase assay system (Promega). All luciferase assays were done at least in triplicate. For the neutralization experiments, a mouse monoclonal anti-CD81 antibody (JS-81; BD Pharmingen, Franklin Lakes, NJ) and a mouse monoclonal anti-FLAG antibody (Sigma-Aldrich) were used.

Flow cytometric analysis. Cells detached by treatment with trypsin were incubated in PBS containing 1% (vol/vol) formaldehyde for 15 min. A total of 5×10^5 cells were resuspended in PBS and treated with or without 0.75 μg of anti-CD81 antibody for 30 min at 4°C. After being washed with PBS, the cells were incubated with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen) at 1:200 for 30 min at 4°C, washed repeatedly, and resuspended in PBS. Analyses were performed by using FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ).

RESULTS

Analysis of the 5′ and 3′ ends of HCV RNA sequences generated from Pol I-driven plasmids. To examine whether the HCV transcripts generated from Pol I-driven plasmids had correct nucleotides at the 5′ and 3′ ends, we extracted RNA from Huh-7 cells transfected with pHHJFH1, which carries a genome-length HCV cDNA with a Pol I promoter/terminator, as well as from the culture supernatants. After this, the nucleotide sequences at both ends were determined using RACE and sequence analysis. A 328-nt fragment corresponding to cDNA from the 5′ end of HCV RNA was detected in the cell samples (Fig. 1A). Cloning of amplified fragments confirmed that the HCV transcripts were initiated from the first position of the viral genome in all of the clones sequenced (Fig. 1B). Similarly, a 127-nt amplification fragment was detected in each sample by 3′RACE (Fig. 1C), and the same 3′-end nucleotide sequence was observed in all clones derived from the culture supernatant (Fig. 1D, left). An additional two nucleotides (CC) were found at the 3′ end of the HCV transcript in a limited number of sequences (1 of 11 clones) derived from the cell sample (Fig. 1D, right), which were possibly derived from the Pol I terminator sequence by incorrect termination. These results indicate that most HCV transcripts generated from the Pol I-based HCV cDNA expression system are faithfully processed, although it is not determined whether the 5′ terminus of the viral RNA generated from Pol I system is triphosphate or monophosphate. It can be speculated that viral RNA lacking modifications at the 5′ and 3′ ends is preferentially packaged and secreted into the culture supernatant.

Production of HCV RNA, proteins, and virions from cells transiently transfected with Pol I-driven plasmids. To examine HCV RNA replication and protein expression in cells transfected with pHHJFH1, pHHJFH1/GND, or virion production-defective mutants, pHHJFH1/ΔE and pHHJFH1/R783A/R785A, which possess an in-frame deletion of E1/E2 region and substitutions in the p7 region, respectively (19, 42, 49), RPA and Western blotting were performed 5 days p.t. (Fig. 2A, B, and D). Positive-strand HCV RNA sequences were more abundant than negative-strand RNA sequences in these cells. Positive-strand RNA, but not negative-strand RNA, was detected in cells transfected with the replication-defective mutant pHHJFH1/GND (Fig. 2A and B). Northern blotting showed that genome-length RNA was generated in pHHJFH1-transfected cells but not in pHHJFH1/GND-transfected cells (Fig. 2C).

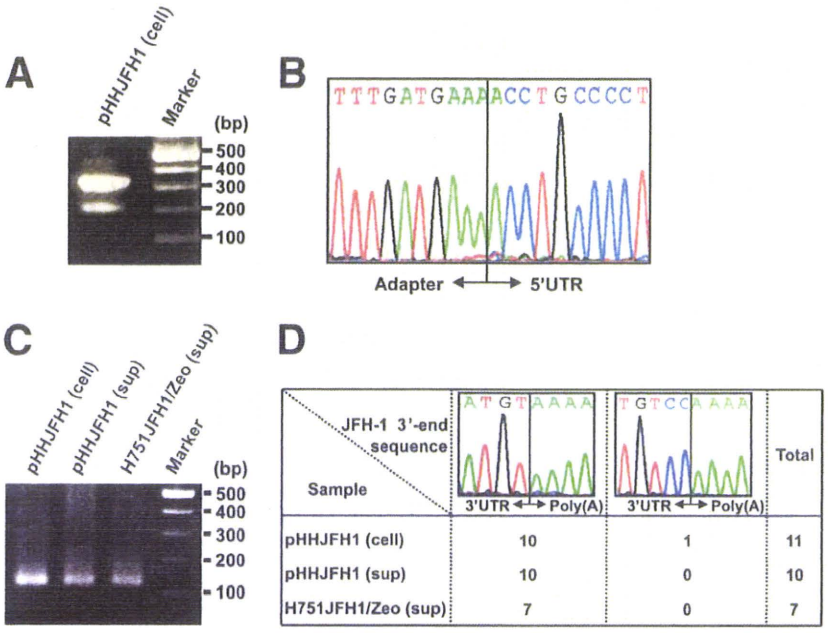

FIG. 1. Determination of the nucleotide sequences at the 5′-and 3′ ends of HCV RNA produced by the Pol I system. (A and B) 5′RACE and sequence analysis. A synthesized RNA adapter was ligated to RNA extracted from cells transfected with pHHJFH1. The positive-strand HCV RNA was reverse transcribed, and the resulting cDNA was amplified by nested PCR. The amplified 5′-end cDNA was separated by agarose gel electrophoresis (A), cloned, and sequenced (B). (C and D) 3′RACE and sequence analysis. RNA extracted from pHHJFH1-transfected cells, the culture supernatant of transfected cells, and the culture supernatant of H751JFH1/Zeo cells were polyadenylated, reverse transcribed, and amplified by PCR. The amplified 3′-end cDNA was separated by agarose gel electrophoresis (C), cloned, and sequenced (D).

As shown in Fig. 2D, the intracellular expression of core and NS5B proteins was comparable among cells transfected with pHHJFH1, pHHJFH1/ΔE, and pHHJFH1/R783A/R785A. Neither viral protein was detected in pHHJFH1/GND-transfected cells, suggesting that the level of viral RNA generated transiently from the DNA plasmid does not produce enough HCV proteins for detection and that ongoing amplification of the HCV RNA by the HCV NS5B polymerase allows a high enough level of viral RNA to produce detectable levels of HCV proteins.

To assess the release of HCV particles from cells transfected with Pol I-driven plasmids, core protein was quantified in culture supernatant by enzyme-linked immunosorbent assay (ELISA) or sucrose density gradient centrifugation. Core protein secreted from pHHJFH1-transfected cells was first detectable 2 days p.t., with levels increasing up to ~4 pmol/liter on day 6 (Fig. 3A). This core protein level was 4- to 6-fold higher than that in the culture supernatant of pHHJFH1/ΔE- or pHHJFH1/R783A/R785A-transfected cells, despite comparable intracellular core protein levels (Fig. 2D). Core protein was not secreted from cells transfected with pHHJFH1/GND (Fig. 3A). In another experiment, a plasmid expressing the secreted form of human placental alkaline phosphatase (SEAP) was cotransfected with each Pol I-driven plasmid. SEAP activity in culture supernatant was similar among all transfection groups, indicating comparable efficiencies of transfection (data not shown). Sucrose density gradient analysis of the concentrated supernatant of pHHJFH1-transfected cells indicated that the distribution of core protein levels peaked in the fraction of 1.17 g/ml density, while the peak of infectious titer was observed in the fraction of 1.12 g/ml density (Fig. 3B), which is consistent with the results of previous studies based on JFH-1-RNA transfection (23).

We next compared the kinetics of HCV particle secretion in the Pol I-driven system and RNA transfection system. Huh-7 cells, which have limited permissiveness for HCV infection (2), were transfected with either pHHJFH1 or JFH-1 RNA, and then cultured by passaging every 2 or 3 days. As shown in Fig. 3C, both methods of transfection demonstrated similar kinetics of core protein levels until 9 days p.t., after which levels gradually fell. However, significantly greater levels of core protein were detected in the culture of pHHJFH1-transfected cells compared to the RNA-transfected cells on day 12 and 15 p.t. This is likely due to an ongoing production of positive-strand viral RNA from transfected plasmids since RNA degradation generally occurs more quickly than that of circular DNA.

Establishment of stable cell lines constitutively producing HCV virion. To establish cell lines with constitutive HCV production, pHHJFH1/Zeo carrying HCV genomic cDNA and the Zeocin resistance gene were transfected into Huh7.5.1 cells. After approximately 3 weeks of culture with zeocin at a concentration of 0.4 mg/ml, cell colonies producing HCV core protein were screened by ELISA, and three clones were identified that constitutively produced the viral protein (H751JFH1/Zeo cells). Core protein levels within the culture supernatant of selected clones (H751-1, H751-6, and H751-50) were 2.0×10^4, 2.7×10^3, and 1.4×10^3 fmol/liter, respectively. Clone H751-1 was further analyzed. Indirect immunofluorescence with an anti-NS5A antibody showed fluorescent staining of NS5A in the cytoplasm of almost all H751JFH1/

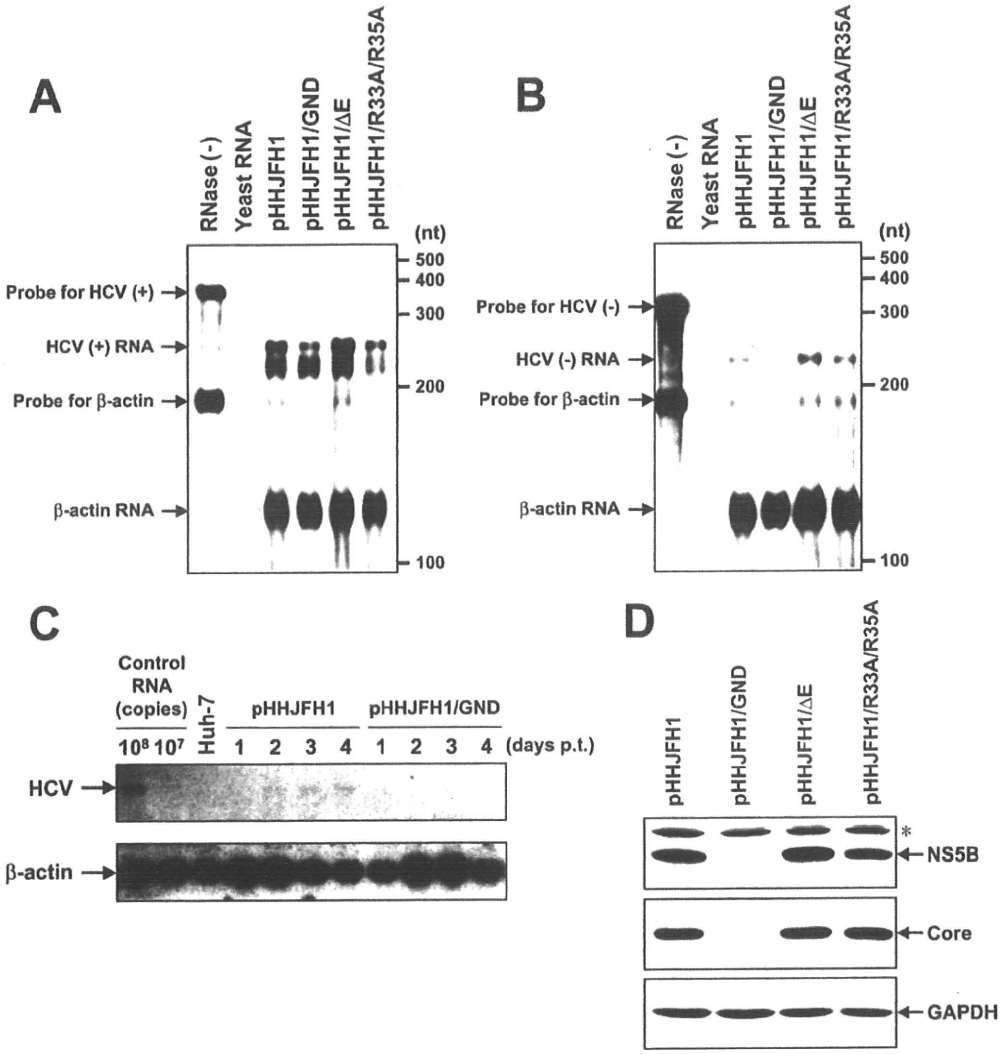

FIG. 2. HCV RNA replication and protein expression in cells transfected with Pol I-driven plasmids. (A and B) Assessment of HCV RNA replication by RPA. Pol I-driven HCV-expression plasmids were transfected into Huh-7 cells. Total RNA was extracted from the cells on day 5 p.t. and positive (A)- and negative (B)-strand HCV RNA levels were determined by RPA as described in Materials and Methods. In the RNase (−) lanes, yeast RNA mixed with RNA probes for HCV and human β-actin were loaded without RNase A/T1 treatment. In the yeast RNA lanes, yeast RNA mixed with RNA probes for HCV and human β-actin were loaded in the presence of RNase A/T1. (C) Northern blotting of total RNAs prepared from the transfected cells. Huh-7 cells transfected with pHHJFH1 or pHHJFH1/GND were harvested for RNA extraction through days 1 to 4 p.t. Control RNA, given numbers of synthetic HCV RNA; Huh-7, RNA extracted from naive cells. Arrows indicate full-length HCV RNA and β-actin RNA. (D) HCV protein expression in the transfected cells. Pol I-driven HCV-expression plasmids were transfected into Huh-7 cells, harvested, and lysed on day 6 p.t. The expression of NS5B, core, and GAPDH was analyzed by Western blotting as described in Materials and Methods. The asterisk indicates nonspecific bands.

Zeo cells (Fig. 4A), whereas no signal was detected in parental Huh7.5.1 cells (Fig. 4B). To determine where HCV RNA replicates in H751JFH1/Zeo cells, labeling of *de novo*-synthesized HCV RNA was performed. After interfering with mRNA production by exposure to actinomycin D, BrUTP-incorporated *de novo*-synthesized HCV RNA was detected in the cytoplasm of H751JFH1/Zeo cells (Fig. 4D) colocalized with NS5A in the perinuclear area (Fig. 4E and F).

Low mutation frequency of the viral genome in a long-term culture of H751JFH1/Zeo cells. The production level of infectious HCV from H751JFH1/Zeo cells at a concentration of $\sim 10^3$ $TCID_{50}$/ml was maintained over 1 year of culture (data not shown). It has been shown that both virus and host cells may adapt during persistent HCV infection in cell cultures, such that cells become resistant to infection due to reduced expression of the viral coreceptor CD81 (54). As shown in Fig. 5, we analyzed the cell surface expression of CD81 on the established cell lines by flow cytometry and observed markedly reduced expression on H751JFH1/Zeo cells compared to parental Huh7.5.1 cells. It is therefore possible that only a small proportion of HCV particles generated from H751JFH1/Zeo cells enter and propagate within the cells. The H751JFH1/Zeo system is thought to result in virtually a single cycle of HCV production from the chromosomally integrated gene and thus

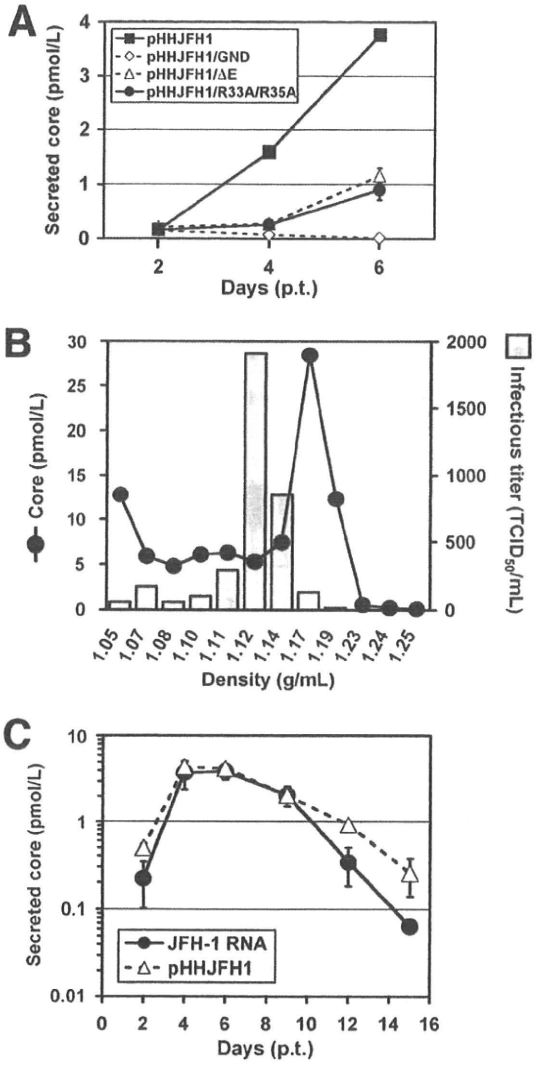

FIG. 3. HCV released from cells transfected with Pol I-driven plasmids. (A) HCV particle secretion from the transfected cells. The culture supernatant of Huh-7 cells transfected with Pol I-driven plasmids containing wild-type or mutated HCV genome were harvested on days 2, 4, and 6 and assayed for HCV core protein levels. The data for each experiment are averages of triplicate values with error bars showing standard deviations. (B) Sucrose density gradient analysis of the culture supernatant of pHHJFH1-transfected cells. Culture supernatant collected on day 5 p.t. was cleared by low-speed centrifugation, passed through a 0.45-μm-pore-size filter, and concentrated ~30-fold by ultrafiltration. After fractionating by sucrose density gradient centrifugation, the core protein level and viral infectious titer of each fraction were measured. (C) Kinetics of core protein secretion from cells transfected with pHHJFH1 or with JFH-1 genomic RNA. A total of 10^6 Huh-7 cells were transfected with 3 μg of pHHJFH1 or the same amount of *in vitro*-transcribed JFH-1 RNA by electroporation. The cells were passaged every 2 to 3 days before reaching confluence. Culture supernatant collected on the indicated days was used for core protein measurement. The level of secreted core protein (pmol/liter) is expressed on a logarithmic scale. The data for each experiment are averages of triplicate values with error bars showing standard deviations.

may yield a virus population with low mutation frequencies. To further examine this, we compared HCV genome mutation rates following production from H751JFH1/Zeo cells compared to cells constitutively infected with HCV after serial passages. RNAs were extracted from the supernatant of H751JFH1/Zeo cells cultured for 120 days, and cDNA sequences were amplified by nested PCR with four sets of primers encompassing almost the entire HCV genome (Table 1). PCR products with expected sizes of 2 to 2.5 kb were obtained [Fig. 6A, RT(+)] and subjected to direct sequencing. No amplified product was detected in samples without reverse transcription [Fig. 6A, RT(−)], suggesting no DNA contamination in culture supernatants or extracted RNA solutions. As shown in Fig. 5B (upper panel), three nucleotide mutations, including two substitutions in the E1 (nt 1218) and E2 (nt 1581) regions, and one deletion in the 3′ UTR (nt 9525) were found within the HCV genome with the mutation rate calculated at 9.6×10^{-4} base substitutions/site/year. These mutations were not detected in the chromosomally integrated HCV cDNA (data not shown). The present results also indicate that no splicing of the viral RNA occurred in the Pol I-based HCV JFH-1 expression system. The HCV genome sequence produced by JFH-1 virus-infected Huh7.5.1 cells was analyzed in the same way using culture supernatant 36 days after RNA transfection. As shown in Fig. 6B (lower panel), 10 mutations, including five substitutions throughout the open reading frame and five deletions in the 3′UTR, were detected, and the mutation rate was calculated at 1.1×10^{-2} base substitutions/site/year.

Effects of glycosylation inhibitors on HCV production. It is known that N-linked glycosylation and oligosaccharide trimming of a variety of viral envelope proteins including HCV E1 and E2 play key roles in the viral maturation and virion production. To evaluate the usefulness of the established cell line for antiviral testing, we determined the effects of glycosylation inhibitors, which have little to no cytotoxicity at the concentrations used, on HCV production in a three day assay using H751JFH1/Zeo cells. The compounds tested are known to inhibit the endoplasmic reticulum (ER), Golgi-resident glucosidases, or mannosidases that trim glucose or mannose residues from N-linked glycans. Some are reported to be involved in proteasome-dependent or -independent degradation of misfolded or unassembled glycoproteins to maintain protein integrity (4, 8, 27, 35).

As shown in Fig. 7A and B, treatment of H751JFH1/Zeo cells with increasing concentrations of *N*N-DNJ, which is an inhibitor of ER α-glucosidases, resulted in a dose-dependent reduction in secreted core protein. *N*N-DNJ was observed to have an IC_{50} (i.e., the concentration inhibiting 50% of core protein secretion) of ~20 μM. In contrast, KIF, which is an ER α-mannosidase inhibitor, resulted in a 1.5- to 2-fold increase in secreted core protein compared to control levels. The other five compounds did not significantly change core protein levels. We further determined the effects of *N*N-DNJ and KIF on the production of infectious HCV (Fig. 7C). As expected, *N*N-DNJ reduced the production of infectious virus in a dose-dependent manner, while production increased in the presence of KIF at 10 to 100 μM. Since *N*N-DNJ and KIF did not significantly influence viral RNA replication, as determined using the subgenomic replicon (data not shown), the present results suggest that some step(s), such as virion assembly, intracellular trafficking, and secretion, may be up- or downregulated depending on glycan modifications of HCV envelope proteins within the ER. Inhibitory effect of *N*N-DNJ was reproducibly observed using the cell line after 1 year of culturing

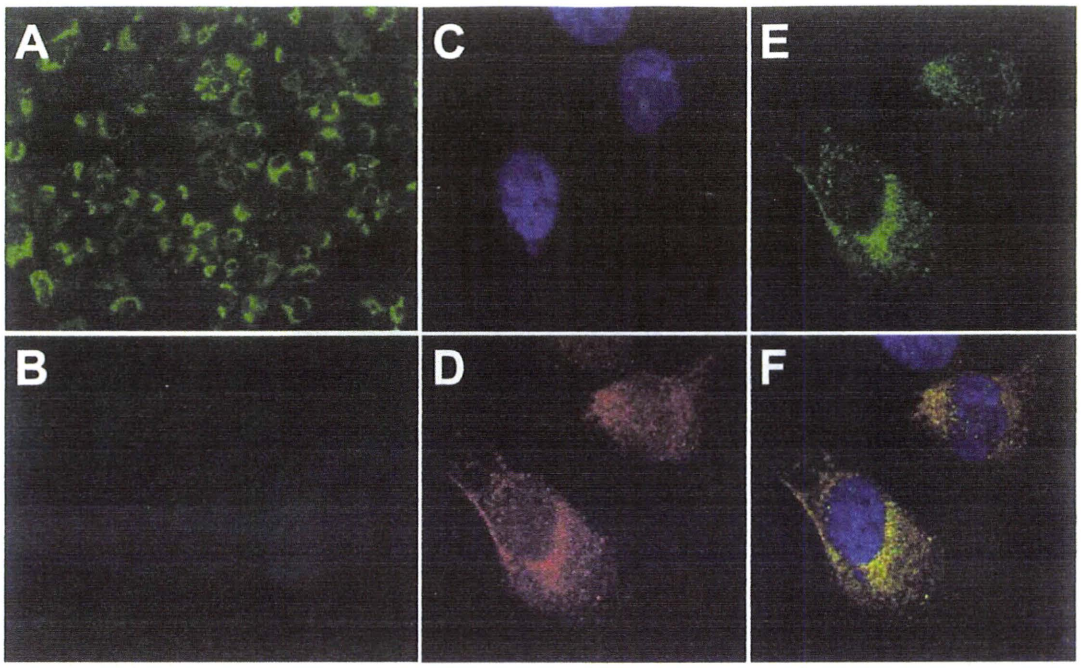

FIG. 4. Indirect immunofluorescence analysis of H751JFH1/Zeo cells. (A and B) H751JFH1/Zeo cells (A) and parental Huh7.5.1 cells (B) were immunostained with an anti-NS5A antibody. (C to F) The subcellular colocalization of *de novo*-synthesized HCV RNA and NS5A in H751JFH1/Zeo cells was analyzed. The cells were stained with DAPI (C), an anti-bromodeoxyuridine antibody (D), and an anti-NS5A antibody (E). The merge panel is shown in panel F.

(Fig. 7D). Under the same condition, the core protein secretion was inhibited by 28 and 58% with 10 and 100 nM BILN 2061, an NS3 protease inhibitor, respectively (Fig. 7D).

Replicon *trans*-packaging system. Recently, ourselves and others have developed a packaging system for HCV subgenomic replicon RNA sequences by providing *trans* viral core-NS2 proteins (1, 17, 41). Since viral structural proteins are not encoded by the subgenomic replicon, progeny virus cannot be produced after transfection. Thus, the single-round infectious HCV-like particle (HCV-LP) generated by this system potentially improves the safety of viral transduction. Here, in order to make the *trans*-packaging system easier to manipulate, we used a Pol I-driven plasmid to develop a transient two-plasmid expression system for the production of HCV-LP. pHH/SGR-Luc, which carries a bicistronic subgenomic reporter replicon with a Pol I promoter/terminator, or its replication-defective mutant, were cotransfected with or without a core-NS2 expression plasmid (Fig. 8A). The culture supernatant was then collected between days 2 and 5 p.t. and used to inoculate naive Huh7.5.1 cells. Reporter luciferase activity, as a quantitative measure of infectious virus production, was assessed in the cells 3 days postinoculation. As shown in Fig. 8B, reporter replication activity was easily detectable in cells inoculated with culture supernatant from cells cotransfected with pHH/

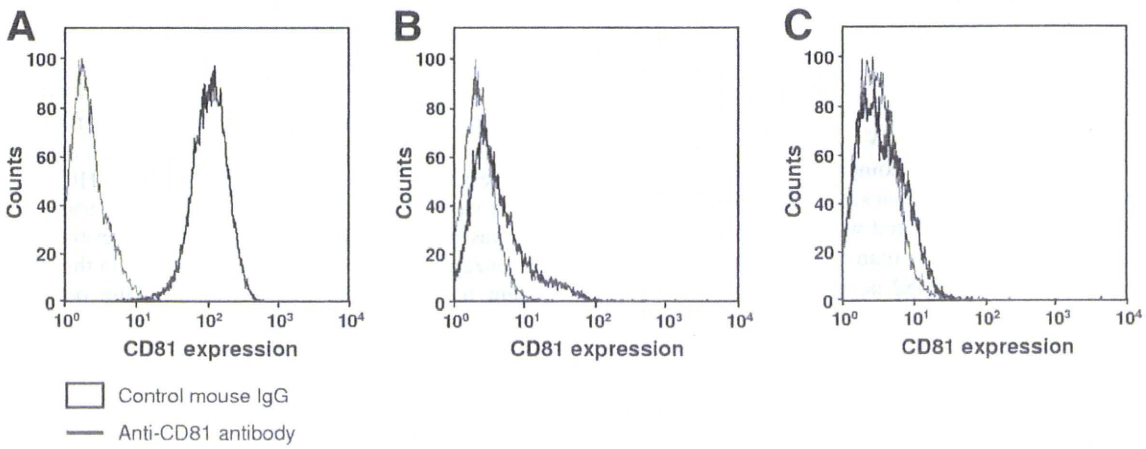

FIG. 5. Loss of CD81 expression in H751JFH1/Zeo cells. The cell surface expression of CD81 on Huh7.5.1 cells (A), H751JFH1/Zeo clone H751-1 (B), and clone H751-50 (C) was analyzed by flow cytometry after being stained with anti-CD81 antibody.

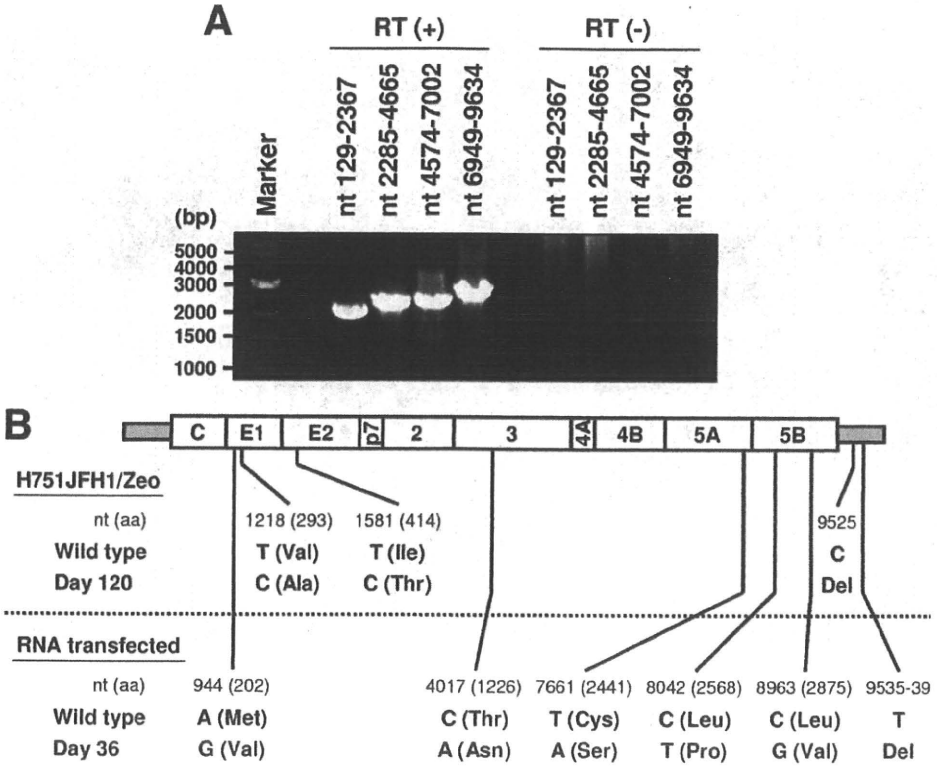

FIG. 6. Genome mutations of HCV secreted from H751JFH1/Zeo cells. (A) RT-PCR of HCV genome extracted from the culture supernatant of H751JFH1/Zeo cells. Viral RNA sequences were reverse transcribed [RT (+)] or not [RT (−)], followed by amplification with primer pairs encompassing the specified HCV genome regions. (B) Comparison of the genome mutations of HCV secreted from H751JFH1/Zeo cells cultured for 120 days (upper panel) and JFH-1 RNA-transfected cells cultured for 36 days (lower panel). The positions of original (wild-type) and mutated (day 120, day 36) nucleotides are indicated under the schematic diagram of the HCV genome. Amino acid residues and their positions are marked in parentheses. Del, deletion.

SGR-Luc and pCAG/C-NS2, with an ~10-fold increase in activity observed at 2 to 5 days p.t. In contrast, luciferase signal in the Huh7.5.1 cells inoculated from supernatant of cells transfected with pHH/SGR-Luc with polymerase-deficient mutation (GND) showed background levels. There was a faint luciferase signal in the cells inoculated from supernatant of cells transfected with pHH/SGR-Luc in the absence of pCAG/C-NS2, suggesting carryover of a low level of cells with the supernatants. Transfer of supernatant from infected cells to naive Huh7.5.1 cells did not result in infection, as judged by undetectable luciferase activity (data not shown). To examine whether NS2 is important for HCV production as previously demonstrated (17–19, 52), we compared the expression of core-NS2 versus core-p7 in the packaged cells (Fig. 8C). The reporter activity in cells inoculated with virus *trans*-packaged by core-p7 was ~100-fold lower than the virus *trans*-packaged by core-NS2, indicating that NS2 needs to be expressed with the structural proteins for efficient assembly and/or infectivity. CD81-dependent infection of HCV-LP was further confirmed by demonstrating reduced reporter activity in the presence of anti-CD81 antibody (Fig. 8D). Thus, we developed a simple *trans*-encapsidation system based on transient two-plasmid transfection, which permits experimental separation of HCV genome replication and virion assembly.

DISCUSSION

Here, we exploited Pol I-derived vectors for expression of the HCV genome, a strategy that generates viral RNAs from the Pol I promoter and terminator. We demonstrated that the HCV JFH-1 RNA produced using this system is unspliced with precise sequences at both ends and that it is replicated in the cytoplasm of transfected cells to produce infectious particles. This approach was used to establish a replicon *trans*-packaging system based on transient two-plasmid transfection and enables the production of a stable cell line capable of constitutive HCV production. The cell line produced using this method can be used to screen a large number of potential antiviral agents by assessing their ability to interfere with HCV replication and/or virion formation. The Pol I-mediated transcription system was originally developed to perform reverse genetics on influenza A viruses (12, 29) which replicate in the nucleus. This system has also been shown useful in the development of reverse genetics for negative-strand RNA viruses having a cytoplasmic replication cycle (3, 10, 11, 31). The results of the present study suggest that the Pol I system can also be used to perform reverse genetics on a cytoplasmically replicating positive-strand RNA virus.

Although viral RNA transfection by electroporation is the most commonly used method to perform reverse genetics on

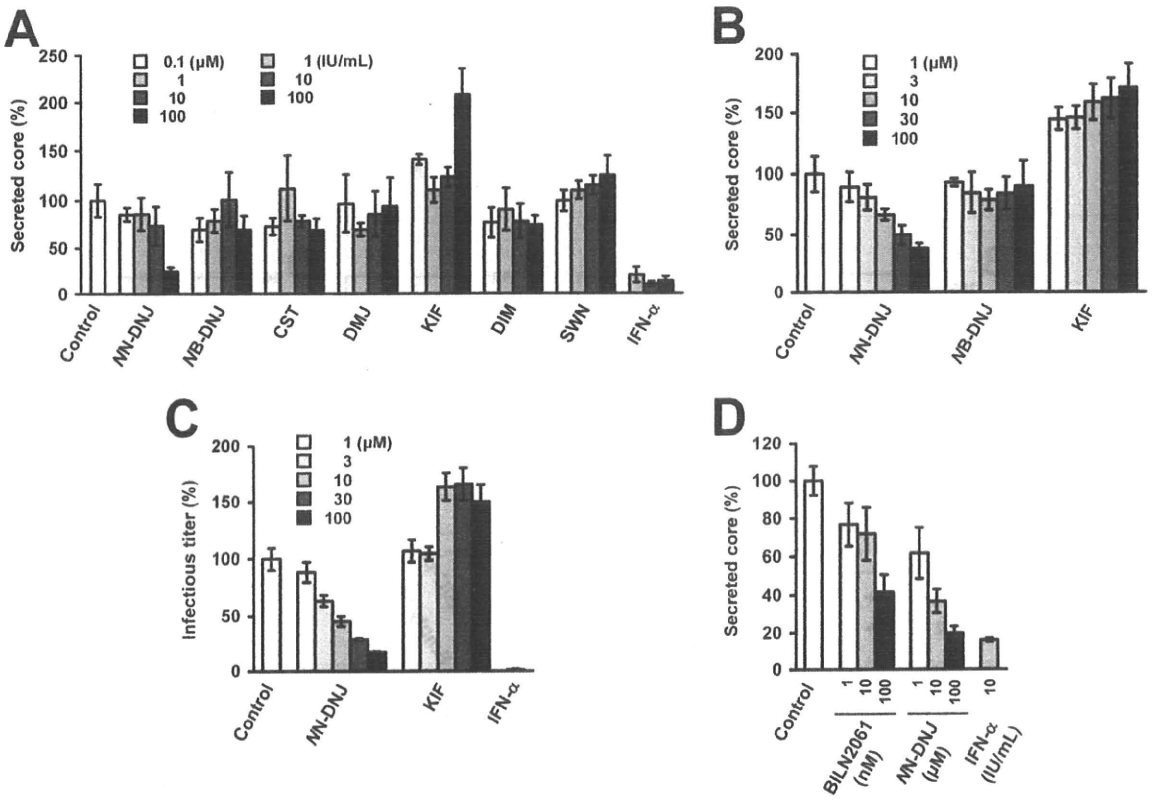

FIG. 7. Effects of glycosylation inhibitors on HCV production from H751JFH1/Zeo cells. (A and B) Effects of glycosylation inhibitors on the secretion of HCV core protein. H751JFH1/Zeo cells were seeded at a density of 1×10^4 cells/well in a 96-well culture plate (A) or 3×10^4 cells/well in a 12-well cell culture plate (B). One day later, each compound was added to the cell culture supernatant at the indicated concentrations. The culture supernatant was collected after a further 3-day culture and processed by core protein-specific ELISA. The control represents an untreated cell culture. The level of secreted core protein was normalized by setting the control value at 100%. The data for each experiment are averages of triplicate values with error bars showing standard deviations. (C) Effects of *N*N-DNJ and KIF on infectious HCV production. The culture supernatant obtained in panel B was used to infect naive Huh7.5.1 cells. At 72 h after infection, the inoculated cells were fixed and immunostained as described in Materials and Methods for titration of virus infectivity. The infectious titer was normalized by setting the control value at 100%. Cells were treated with INF-α at 100 IU/ml as a positive control. The data for each experiment are averages of triplicate values with error bars showing standard deviations. The control represents an untreated cell culture. (D) After 1 year of culturing H751JFH1/Zeo cells, antiviral effects of *N*N-DNJ and BILN 2061 were evaluated. H751JFH1/Zeo cells were seeded at a density of 3×10^4 cells/well in a 12-well cell culture plate. One day later, each compound was added to the cell culture supernatant at the indicated concentrations. The culture supernatant was collected after a further 3-day culture and processed by core protein-specific ELISA. The control represents an untreated cell culture. The level of secreted core protein was normalized by setting the control value at 100%. The data for each experiment are averages of triplicate values with error bars showing standard deviations.

HCV (23, 49, 53), it is comparatively difficult to manipulate. RNA electroporation requires high-quality *in vitro*-synthesized RNA and a large quantity of exponential-growth-phase cells, which may be hard to provide when a number of different RNA constructs are being examined in the same experiment. In addition to the Pol I system, other DNA expression systems have been examined with regard to HCV particle production (5, 15, 21). These systems require ribozyme sequences to be inserted at either end of the HCV genomic cDNA sequence in order to generate appropriately processed viral RNA. However, Heller et al. have reported that the HCV RNA generated by *in vitro* transcription of a HCV-ribozyme plasmid contains uncleaved or prematurely terminated forms of HCV RNA. These authors have also demonstrated that HCV RNA from the culture supernatant of HCV-ribozyme plasmid-transfected cells possesses nucleotide changes at the 5′ and 3′ ends (15), suggesting that the ribozyme is less reliable at generating correct transcripts compared to our Pol I system. In fact, there is evidence to suggest that a mouse Pol I terminator is significantly more effective than an HDV ribozyme in generating precise 3′ ends of RNA, as demonstrated in a plasmid-based influenza virus rescue system (9). Recently, it has been demonstrated that Pol I-catalyzed rRNA transcription is activated in Huh-7 cells following infection with JFH-1 or transfection with a subgenomic HCV replicon (34). HCV NS5A has been shown to upregulate the transcription of Pol I, but not Pol II, through phosphorylation of an upstream binding factor, a Pol I DNA binding transcription factor. These observations indicate that a Pol I-mediated expression system is suitable for efficient production of infectious HCV by DNA transfection.

We established a stable cell line, H751JFH1/Zeo, that constitutively and efficiently produced infectious HCV particles by introducing a Pol I-driven plasmid containing a selection marker into Huh7.5.1 cells. Interestingly, the established cell

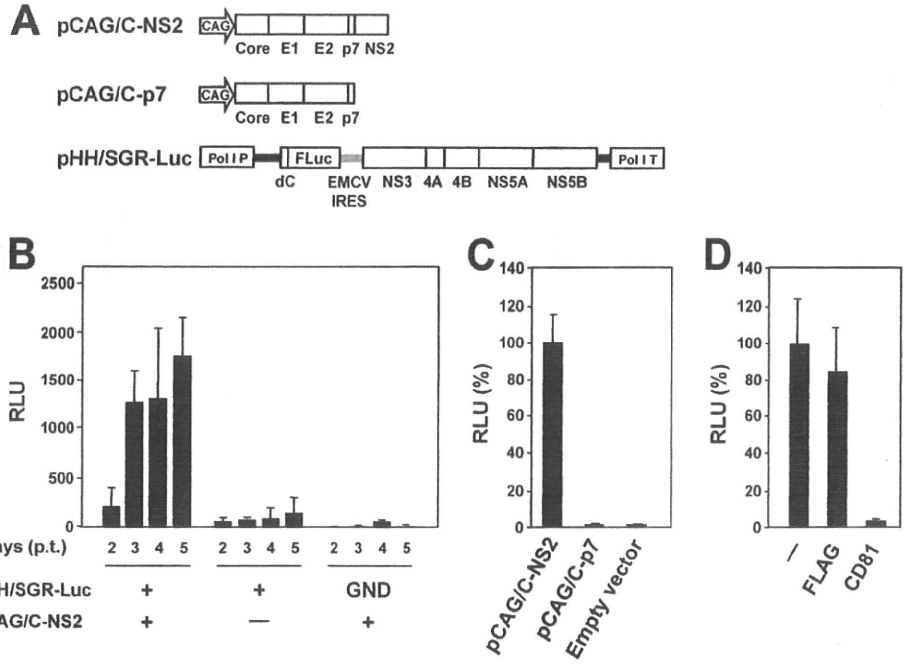

FIG. 8. Establishment of a *trans*-packaging system involving two-plasmid transfection. (A) Schematic representation of the plasmids used for the production of HCV-LP. HCV polyproteins are indicated by the open boxes. Bold lines indicate the HCV UTR. EMCV IRES is denoted by gray bars. The firefly luciferase gene (F Luc) is depicted as a gray box. CAG, CAG promoter; Pol I P, Pol I promoter; dC, 5′ region of Core gene; Pol I T, Pol I terminator. (B) Luciferase activity in Huh7.5.1 cells inoculated with culture supernatant from cells transfected with the indicated plasmids. Luciferase activity is expressed in terms of relative luciferase units (RLU). The data for each experiment are averages of triplicate values with error bars showing standard deviations. (C) Culture supernatant from cells cotransfected with pHH/SGR-Luc and the indicated plasmids were collected 4 days p.t. The luciferase activity in Huh7.5.1 cells inoculated with culture supernatant was determined 3 days postinoculation and expressed as relative luciferase units (RLU). The RLU was normalized according to the luciferase activity observed in the pCAG/C-NS2-transfected sample (C-NS2), which was set at 100%. The data for each experiment are averages of triplicate values with error bars showing standard deviations. (D) Huh7.5.1 cells were inoculated with HCVLP in the absence (−) or presence of 5 μg of anti-CD81 or anti-FLAG antibody/ml. The luciferase activity was determined 72 h postinoculation and is expressed as relative luciferase units (RLU). The RLU was normalized to the level of luciferase activity observed in the antibody-untreated sample (−), which was set at 100%. The data for each experiment are averages of triplicate values with error bars showing standard deviations.

clones exhibited little to no surface expression of CD81, one of the key features of HCV glycoprotein-mediated infection (Fig. 5). Defective expression of receptor molecules might be advantageous in generating stable cell lines for robust production of HCV. HCV-induced cytotoxicity has been reported (7, 45, 54). Persistent HCV infection was established after electroporation of JFH-1 genomic RNA, and a variable cytopathic effect was observed at the peak of acute HCV infection, as well as during the persistent phase of infection (54). A recent study has demonstrated that the cytopathic effect triggered by HCV RNA transfection and viral infection is characterized by massive apoptotic cell death with expression of several ER stress markers, such as GRP78 and phosphorylated eIF2-α (39). Therefore, in the present study, it is likely that selective forces to evade cell death during high levels of HCV replication produced cell populations resistant to virus infection. As a consequence, H751JFH1/Zeo cells maintained robust production of infectious HCV particles over a long period of time without gross cytopathic effects or changes in cell morphology.

Substantial evidence demonstrates that the mutation rate of the HCV genome produced in H751JFH1/Zeo was low (Fig. 6) presumably because of consistent expression of wild-type HCV RNA from the chromosomally integrated gene. Nevertheless, a considerable proportion of the genome was mutated, with two nonsynonymous mutations in the E1 (V293A) and E2 (I414T) regions identified in the culture supernatant of H751JFH1/Zeo cells after 4 months of passages (Fig. 6). A I414T mutation has also been reported after long-term propagation of HCV in culture after JFH-1-RNA transfection (54). This mutation is located between the hypervariable regions 1 and 2 within the N terminus of E2 (51). Adaptive mutations in this region have been shown to enhance virus expansion, presumably by enabling more efficient virus entry (6, 36, 54). A possible CD81-independent mechanism for cell-to-cell transmission of HCV has been proposed (48, 50). However, the mechanisms governing cell-to-cell spread of HCV are not well understood. Further investigation into the importance of envelope protein mutations in HCV transmission independent of CD81 provide a better understanding of the complex interactions required for HCV infection.

In the present study we assessed the effects of N-linked glycosylation inhibitors on HCV production using H751JFH1/Zeo (Fig. 7) and found that an α-glucosidase inhibitor *N*N-DNJ inhibits the production of infectious HCV, which has also been observed in previous studies (43, 47). In contrast, HCV production is increased in the presence of an ER α-mannosidase inhibitor KIF, but not in the presence of the Golgi α-mannosidase inhibitors DMJ, DIM, and SWN. KIF inhibits α-man-

nosidase I, which primarily functions to remove the middle mannose branch from $Man_9GlcNAc_2$ to form $Man_8GlcNAc_2$ after the removal of glucose residues by glucosidases I and II (8, 24). Experiments to elucidate the role of mannose trimming of N-glycans in the HCV life cycle are currently under way.

It has recently been demonstrated that subgenomic replicons or defective genomes of HCV that have the potential of translation and self-replication can be encapsidated into infectious viruslike particles by *trans*-complementation of the viral structural proteins (1, 17, 32, 41, 44). In these studies, the viral RNAs were generally generated by *in vitro* transcription from linearized corresponding plasmids, followed by electroporation into the cells. Structural proteins or Core to NS2 proteins were then provided by DNA or RNA transfection, viral-vector-based transduction, or stable packaging cell lines established. Here, we achieved the replicon *trans*-encapsidation via transient cotransfection with two DNA plasmids. This system, which is apparently easier to manipulate and allows production of *trans*-encapsidated materials more rapidly compared to the systems published, can be applied to the study for understanding phenomenon and biological significance of a variety of naturally occurring HCV subgenomic deletion variants that possibly circulate in hepatitis C patients.

In summary, we have established a Pol I-based reverse-genetics system for the efficient production of infectious HCV. This methodology can be applied to develop (i) a stable HCV-producing cell line with a low mutation frequency of the viral genome and (ii) a simple *trans*-encapsidation system with the flexibility of genome packaging and improved biosafety. This may be useful for antiviral screening and may assist in the development of a live-attenuated HCV vaccine.

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Variants in *IL28B* in Liver Recipients and Donors Correlate With Response to Peg-Interferon and Ribavirin Therapy for Recurrent Hepatitis C

TAKASUKE FUKUHARA,[*,§] AKINOBU TAKETOMI,[*] TAKASHI MOTOMURA,[*] SHINJI OKANO,[‡] AKINORI NINOMIYA,[§] TAKAYUKI ABE,[§] HIDEAKI UCHIYAMA,[*] YUJI SOEJIMA,[*] KEN SHIRABE,[*] YOSHIHARU MATSUURA,[§] and YOSHIHIKO MAEHARA[*]

[*]*Department of Surgery and Science and* [‡]*Division of Pathophysiological and Experimental Pathology, Department of Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; and* [§]*Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*

BACKGROUND & AIMS: Patients with hepatitis C virus (HCV)–related liver disease frequently undergo orthotopic liver transplantation, but recurrent hepatitis C is still a major cause of morbidity. Patients are treated with peg-interferon and ribavirin (PEG-IFN/RBV), which has substantial side effects and is costly. We investigated genetic factors of host, liver donor, and virus that might predict sensitivity of patients with recurrent hepatitis C to PEG-IFN/RBV. **METHODS:** Liver samples were analyzed from 67 HCV-infected recipients and 41 liver donors. Liver recipient and donor DNA samples were screened for single nucleotide polymorphisms near the *IL28B* genes (rs12980275 and rs8099917) that affect sensitivity to PEG-IFN/RBV. HCV RNA was isolated from patients and analyzed for mutations in the core, the IFN sensitivity-determining region, and IFN/RBV resistance-determining regions in nonstructural protein 5A. **RESULTS:** In liver recipients and donors, the *IL28B* single nucleotide polymorphism rs8099917 was significantly associated with a sustained viral response (SVR; $P = 0.003$ and $P = .025$, respectively). Intrahepatic expression of *IL28* messenger RNA was significantly lower in recipients and donors that carried the minor alleles (T/G or T/T) in rs8099917 ($P = .010$ and .009, respectively). Genetic analyses of *IL28B* in patients and donors and of the core and nonstructural protein 5A regions encoded by HCV RNA predicted an SVR with 83% sensitivity and 82% specificity; this was more effective than analysis of any single genetic feature. **CONCLUSIONS: In patients with recurrent HCV infection after orthotopic liver transplantation, combination analyses of single nucleotide polymorphisms of *IL28B* in recipient and donor tissues and mutations in HCV RNA allow prediction of SVR to PEG-IFN/RBV therapy.**

Keywords: ISDR; IRRDR; Genetic Analysis; Genetic Variations.

Hepatitis C virus (HCV) infection affects 170 million people worldwide and can lead to decompensated cirrhosis and hepatocellular carcinoma.[1,2] As a result, HCV-related liver disease is the leading indication for orthotopic liver transplantation (OLT) worldwide.[3,4] However, several reports have shown that post-OLT patient and graft survival are significantly negatively affected by HCV recurrence after OLT.[5,6] This can be mitigated by achievement of a sustained virological response (SVR) with pegylated interferon and ribavirin (PEG-IFN/RBV) therapy.[7] However, many patients cannot tolerate curative doses or do not respond to therapy with PEG-IFN/RBV.[6,8] Because of the substantial cost of therapy, both financial and with regard to side effects, it would be ideal to be able to predict which patients would benefit from PEG-IFN/RBV therapy for recurrent HCV.[9,10]

Many reports have demonstrated that HCV-RNA mutations, including those of amino acid residues 70 and 91 in the Core region,[11] and those in the interferon sensitivity determining region (ISDR)[12] and variable region 3 domain[13] in the nonstructural protein 5A (NS5A), were significantly associated with IFN sensitivity in patients infected with genotype 1 HCV. We previously reported that these genetic mutations have a significant impact on patients' responsiveness to PEG-IFN/RBV therapy for recurrent hepatitis C after OLT.[14] However, in addition to viral factors, host factors can also be used to predict IFN sensitivity. For example, Asahina et al demonstrated that the pretreatment induction level of IFN-stimulated genes (ISGs) was significantly associated with SVR to PEG-IFN/RBV therapy.[15] In addition, it was recently reported that single nucleotide polymorphisms near the IL28B gene on chromosome 19q13, rs12980275, or rs8099917 are significantly associated with the sensitivity of IFN/RBV combination therapy for chronic hepatitis

Abbreviations used in this paper: DW, double-wild; ETR, end of treatment response; HCV, hepatitis C virus; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; ISG, interferon-stimulated gene; mRNA, messenger RNA; NR, nonresponse; NS5A, nonstructural protein 5A; OLT, orthotopic liver transplantation; PCR, polymerase chain reaction; PEG-IFN, pegylated interferon; RBV, ribavirin; SVR, sustained viral response.

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C.[16-21] In the present study, we examined the impact of genetic variations of IL28B in recipients and donors, and of genetic variations in HCV-RNA on the responsiveness to IFN/RBV therapy for recurrent hepatitis C after OLT.

Patients and Methods

Patients

Recipients enrolled in this study underwent OLT for HCV-related liver disease, had normal ejection fraction, lung capacity, and renal function (creatinine clearance >70 mL/min), were treated with PEG-IFN/RBV combination therapy after OLT, were negative for hepatitis B virus and human immunodeficiency virus, and positive for HCV-RNA. At Kyushu University Hospital, 112 liver transplantations were performed between April 1999 and March 2009 on HCV-infected patients. PEG-IFN/RBV therapy was administered to 78 of these recipients. Eleven recipients were excluded from this analysis because of ongoing therapy with PEG-IFN/RBV therapy (n = 5) or because their therapy was discontinued secondary to side effects (n = 6). Therefore, 67 recipients were retrospectively analyzed. A total of 41 of their donors were available for analysis. All OLTs were performed after obtaining informed consent from recipients and donors, and the current study was approved by the Kyushu University ethics committee.

Surgical Technique and Immunosuppression

The surgical procedure for the recipients has been described previously.[22] The choice of resected segments for donation was dictated by the need to obtain a graft volume >35% of the recipient's standard liver volume. Simultaneous splenectomies for 41 recipients (61%) were performed to prevent pancytopenia due to antiviral therapy. The induction and maintenance of immunosuppression was achieved using a calcineurin inhibitor, mycophenolate mofetil, and steroids in most cases. Cyclosporine and tacrolimus were used for 35 and 32 recipients, respectively. The response rate to antiviral therapy was comparable (data not shown). Steroids were administered intraoperatively (methylprednisolone, 1000 mg) and were tapered off by 6 months after transplantation. In 8 cases, steroid-free immunosuppression was performed using basiliximab (Simulect; Novartis Pharma, Tokyo, Japan).

Antiviral Treatment Regimen and Assessment of the Therapeutic Effects

The primary doses of PEG-IFNα-2b (Pegintron; Schering-Plough Inc, Kenilworth, NJ) and RBV (Rebetol; Schering-Plough Inc) were 0.5 μg/kg per week and 200 mg/day and were increased to 1.5 μg/kg per week and 800 mg/day in a stepwise manner according to individual tolerance within the first 12 weeks. The proportion of patients receiving >70% of the full treatment dose (1.0 μg/kg/week) during 80% of the treatment period was 79% (53 of 67). Hematopoietic growth factors including granulocyte colony-stimulating factor and erythropoietin were not used. Antiviral therapy was discontinued in cases with severe depression, renal dysfunction, or autoimmune hepatitis. The viral titers were assessed in all patients using a polymerase chain reaction (PCR)–based quantitative assay (Amplicor Monitor or PCR Cobas TaqMan system; Roche Diagnostics, Mannheim, Germany). SVR was defined as an undetectable level of HCV RNA at 6 months after completion of treatment, while a nonresponse (NR) was defined as a detectable level of HCV RNA at the end of treatment. End of treatment response (ETR) was defined as an undetectable HCV-RNA at the end of treatment.

Analysis of Genetic Variations of IL28B and HCV-RNA

DNA from recipients and donors was extracted from exenterated liver tissue at OLT and biopsied liver tissue after OLT. PCR and direct sequencing were performed using TaKaRa Ex-Taq polymerase (Takara Bio Inc, Tokyo, Japan) and a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc, Tokyo, Japan), respectively. The PCR primers for single nucleotide polymorphisms near the IL28B gene were used as previously described.[18] For the genetic analysis of HCV-RNA in the Core and NS5A regions, reverse transcription using Superscript 3 First-Strand Synthesis SuperMix (Invitrogen, Tokyo, Japan) and nested PCR were performed as described previously.[14] The numbering of amino acids was performed according to the polyprotein of HCV genotype 1b prototype HCV-J (GenBank accession no. D90208). HCV-J was used as the consensus sequences for Core and ISDR. To evaluate the association between the genetic variations in IL28B and HCV-RNA mutations, the patients were divided into 2 groups based on the presence or absence of mutations at amino acid residues 70 and 91 in the Core (a double-wild [DW] group and non-DW group) and on the numbers of mutations in the ISDR (ISDR $\geq$ 2 and ISDR < 2) and interferon/ribavirin resistance-determining region (IRRDR) (IRRDR $\geq$ 6 and IRRDR < 6) regions.

Real-Time PCR of IL28 Messenger RNA

Total RNA was extracted from resected liver tissue derived from the recipient, donated liver tissue derived from the donor, and biopsied liver tissue after OLT using reagents for RNA extraction including ISOGEN and Ethachinmate (Nippon Gene, Tokyo, Japan). The synthesis of first-strand complementary DNA and quantitative reverse transcription PCR were performed using TaqMan EZ RT-PCR Core Reagents (Applied Biosystems) according to manufacturer's protocol. A standard curve was prepared by serial 10-fold dilutions of complementary DNA from Huh7 cell lines stimulated by vesicular stomatitis virus infection. Primers and a TaqMan-probe were designed for the IL28 genes as previously reported.[18]

Table 1. Comparison of the Data Among Patients Showing ETR, SVR, and NR

	ETR (n = 47)	NR (n = 20)	SVR (n = 23)	*P* value
Pretransplantation factor				
Recipient's age (*y*), mean ± SD	55.8 ± 7.3	56.9 ± 7.6	56.0 ± 6.8	NS
Recipient's sex (*male/female*), n	28/19	13/7	15/8	NS
Donor's age (*y*), mean ± SD	34.8 ± 9.6	31.8 ± 10.4	32.1 ± 8.8	NS
Donor's sex (*male/female*), n	30/17	16/4	16/7	NS
HCV genotype (*1/2*), n	40/7	19/1	21/2	NS
MELD score, mean ± SD	12.4 ± 4.7	10.6 ± 4.3	11.7 ± 4.7	NS
Pretransplantation viral load (*logIU/mL*), mean ± SD	5.7 ± 0.8	5.8 ± 0.9	5.7 ± 0.7	NS
History of IFN therapy (*yes/no*), n	14/27	7/11	5/14	NS
Intraoperative factor, mean ± SD				
Intraoperative bleeding (*mL*)	5449 ± 4265	7018 ± 5571	5701 ± 4378	NS
Operation time (*min*)	808 ± 155	877 ± 191	803 ± 174	NS
GV/SLV (*%*)	40.4 ± 6.9	41.7 ± 8.1	39.4 ± 5.8	NS
Post-transplantation factor				
Acute cellular rejection (*yes/no*), n	7/30	2/13	2/18	NS
Bile duct complication (*yes/no*), n	10/37	3/17	4/19	NS
CMV infection (*yes/no*), n	5/32	2/13	3/17	NS
Steroid pulse therapy (*yes/no*), n	4/37	1/16	1/17	NS
Time to antiviral therapy from transplantation (*y*), mean ± SD	1.2 ± 1.3	1.0 ± 1.1	1.3 ± 1.2	NS
Pretreatment viral load (*logIU/mL*), mean ± SD	6.4 ± 0.7	6.5 ± 0.6	6.1 ± 0.8	NS
Pathological activity score, mean ± SD	1.27 ± 0.62	1.32 ± 0.58	1.36 ± 0.58	NS
Pathological fibrosis score, mean ± SD	0.93 ± 1.07	0.74 ± 0.99	1.17 ± 1.16	NS
Pretreatment ALT level (*IU/L*), mean ± SD	53.0 ± 47.0	73.8 ± 48.7	63.7 ± 48.7	NS
Pretreatment WBC level (*per μL*), mean ± SD	4107 ± 1292	4441 ± 2414	3953 ± 1127	NS
Pretreatment Hb level (*mg/dL*), mean ± SD	11.7 ± 1.5	10.9 ± 1.4	11.8 ± 1.5	NS
Pretreatment Plt level (*per μL*), mean ± SD	18.3 ± 9.9	21.7 ± 12.4	18.7 ± 10.0	NS

ALT, alanine aminotransferase; CMV, cytomegalovirus; ETR, end of treatment response; GV, graft volume; Hb, hemoglobin; HCV, hepatitis C virus; IFN, interferon; MELD, model for end-stage liver disease; NR, null response; Plt, platelet; SLV, standard liver volume; SVR, sustained viral response; WBC, white blood cell.

Statistical Analysis

Data are expressed as means ± standard deviation. The statistical analyses were performed using Student *t* test and Fisher's exact probability test. SPSS software (version 15.0, SPSS, Inc, Chicago, IL) was used for all analyses. A difference of $P < .05$ was considered to be significant.

Results

Characteristics of the Patients

The age of the recipients was 56.1 ± 7.4 years; 41 patients were male. The age of the donors was 33.9 ± 9.9 years; 36 patients were male. The viral titers before OLT and PEG-IFN/RBV therapy were 5.7 ± 0.9 log IU/mL and 6.4 ± 0.7 log IU/mL, respectively. Sixty-eight percent of recipients with HCV genotype 1 (40 of 59) and 88% of recipients with genotype 2 (7 of 8) exhibited an ETR. Forty percent of recipients (23 of 57) attained an SVR in the current study. The patient characteristics are presented in Table 1. There were no significant differences in pretransplantation factors among SVR, ETR, and NR patients.

Correlation Between Genetic Variations in IL28B and IFN Sensitivity After OLT

Genetic variations in rs8099917 and rs12970275 of IL28B were evaluated for all recipients and donors, and the match rate of the haplotype between rs8099917 and rs12980275 was 94% (101 of 108; Supplementary Table 1). Of the 67 recipients and 41 donors enrolled in this study, 19 (28%) recipients and 11 (27%) donors had the minor allele (T/G or T/T) in rs8099917. We first examined the correlation of IL28B genetic variation in recipients with the responsiveness to IFN therapy after OLT. With regard to the recipient genotype, the SVR rate was significantly higher in the recipients carrying the major homozygous allele than in those with the minor heterozygous or homozygous allele (54% vs 11%; $P = .003$, Figure 1*A*). Interestingly, the SVR rate was also significantly higher in the recipients transplanted with the liver grafts from donors carrying the major homozygous allele (44% vs 9%; $P = .025$, Figure 1*B*). Combined analyses revealed that the SVR rate was significantly increased when both donors and recipients were major-allele homozygotes (56%; $P = .005$), whereas it was lower in the recipients who carried the major homozygous allele but received a minor heterozygote or homozygote allele transplant, or those carrying the minor allele who received a transplant from a major-allele homozygous donor (10%). SVR was not seen in heterozygote or homozygote minor allele recipients transplanted with liver tissue from heterozygote or homozygote minor allele donors (Figure 1C). The achievement of ETR was also significantly associated with single nucleotide polymorphisms

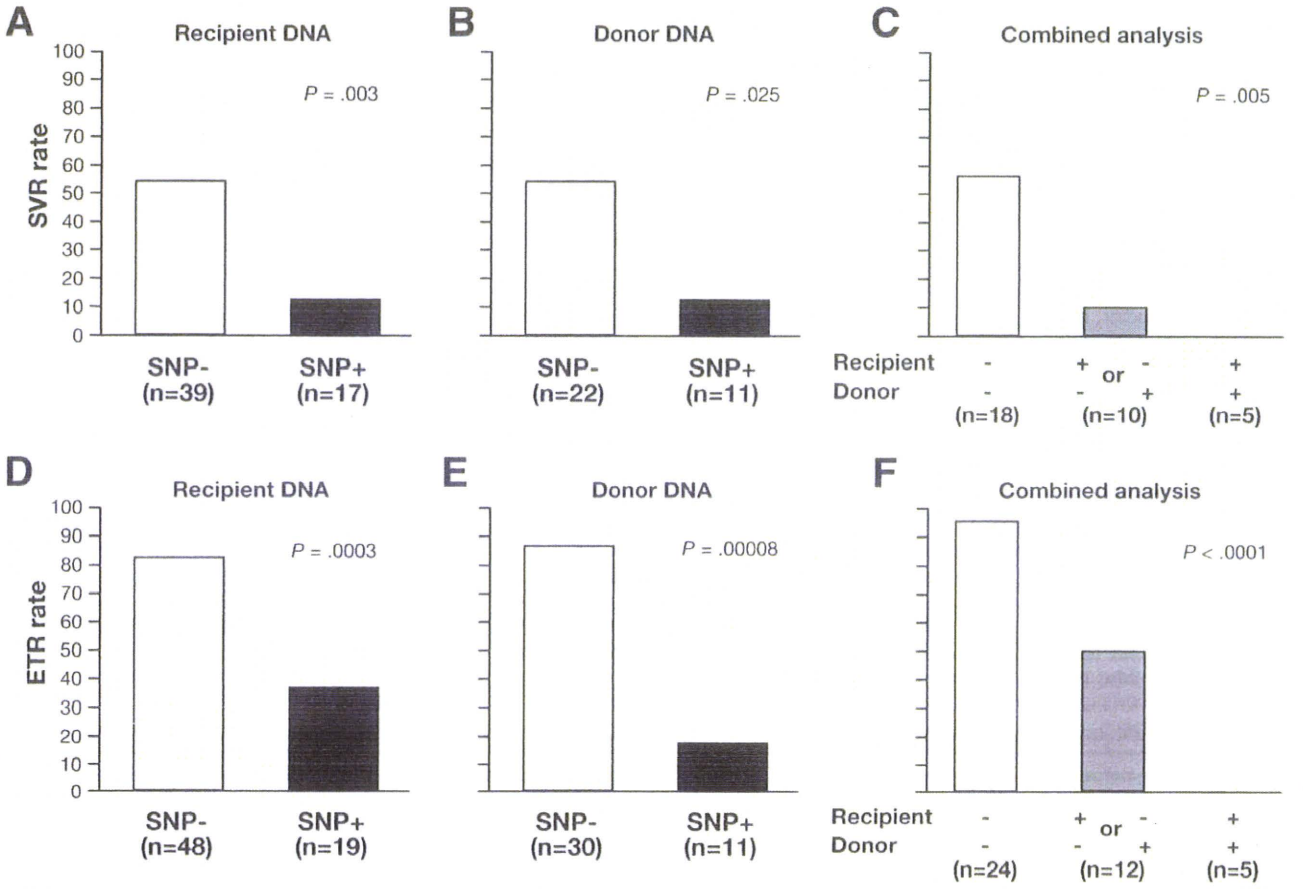

Figure 1. Association of the IL28B genetic variation in rs8099917 with the sensitivity to interferon/rivavirin combination therapy for recurrent hepatitis C virus infection after orthotopic liver transplantation. The sustained viral response (*SVR*) and end of treatment response (*ETR*) ratios in the recipients (*A*, *C*) and donors (*B*, *D*) carrying the major homozygous allele (*white bar*) or the minor allele (*black bar*) are shown. Combined analyses of the factors are shown in (*E*) and (*F*). The SVR and ETR ratios are shown for the recipients carrying the major-allele homozygote who received a transplant from donors carrying the same allele (*white bar*), the major homozygous allele in either the recipients or the donors (*gray bar*), and the recipients carrying the minor-allele homozygote who received a transplant from donors carrying the same minor allele (*black bar*). Statistical analysis was performed using Fisher's exact probability test. SNP, single nucleotide polymorphism.

in the IL28B gene of both the recipients and the donors (Figure 1*D–F*). These results indicate that IL28B genetic variations of not only the recipients but also the donors are significantly associated with the response to PEG-IFN/RBV therapy for recurrent hepatitis C after OLT.

IL28 Messenger RNA Expression and IL28B Genetic Variation

To analyze the correlation between IL28B genetic variation and IL28 messenger (mRNA) expression, the expression level of IL28 mRNA was compared between major allele homozygous and minor allele–positive recipients and/or donors. The amounts of IL28 mRNA in the livers were comparable with those in peripheral blood mononuclear cells (P = NS; Supplementary Figure 1). The levels of IL28 mRNA in the resected livers were significantly higher in the patients carrying the major allele homozygote than those carrying the minor allele heterozygote or homozygote (8.88% ± 6.09% vs 4.45% ± 3.31%; P = .010, Figure 2). In addition, the expression of IL28 mRNA in the liver grafts was also higher in the donors carrying the major allele homozygote than those carrying the minor allele heterozygote or homozygote (6.82% ± 6.51% vs 2.61% ± 1.75%; P = .009, Figure 2). In the transplanted liver before antiviral therapy, a high level of IL28 mRNA expression was observed only in the recipients who were homozygous for the major allele and who were transplanted with a liver from a recipient with the same allele, whereas other combinations exhibited a lower level of IL28 mRNA expression in the transplanted livers (Figure 2). These results suggest that IL28B genetic variation in both recipients and donors is closely associated with the expression of IL28 mRNA in the transplanted livers and might be involved in determining IFN sensitivity after OLT. To confirm the potential role of IL28 mRNA expression in IFN sensitivity, the expression of IL28 mRNA in the transplanted liver was significantly higher in the SVR patients than that in the NR patients (8.03% ± 6.22% vs 2.34% ± 1.82%; P = .046; Figure 3).

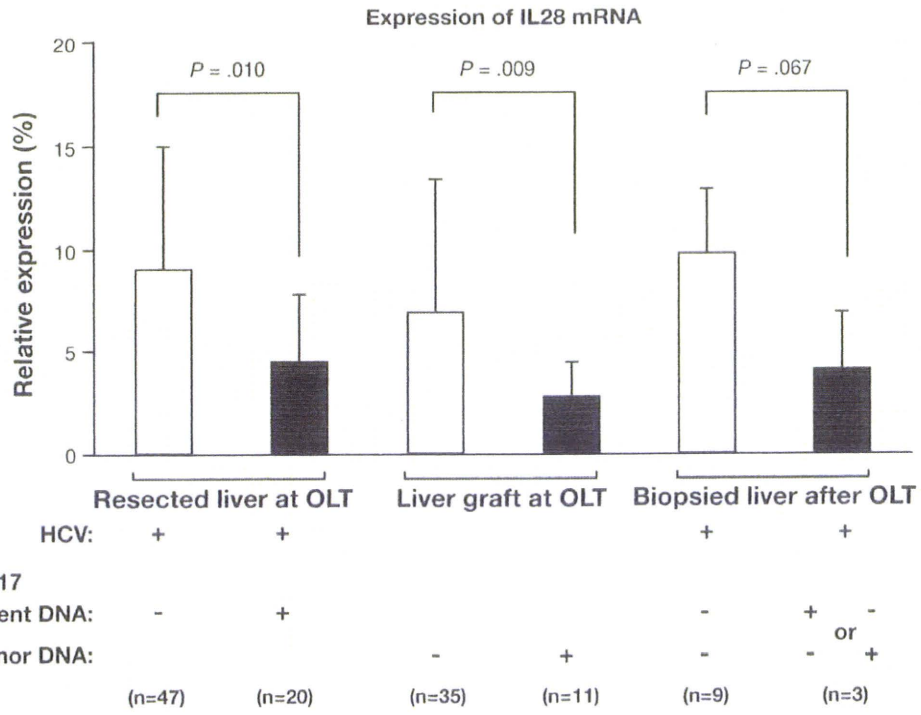

Figure 2. The expression of IL28 messenger RNA (*mRNA*) in the resected livers (n = 67), the liver grafts (n = 46), and the transplanted livers (n = 12). *White* and *black bars* indicate the major allele homozygote and minor allele heterozygote or homozygote, respectively. In the transplanted liver, the *white bar* indicates the major allele homozygote in both recipients and donors. The *black bar* indicates cases carrying the major homozygous allele (either the recipients or the donors). The statistical analysis was performed using Student's *t* test. HCV, hepatitis C virus; OLT, orthotopic liver transplantation.

Association Between Genetic Variations in IL28B and Mutations in HCV-RNA

Recently, we demonstrated the significant impact of HCV-RNA mutations in the Core and NS5A regions on IFN sensitivity after OLT.[14] Therefore, we next examined the association between genetic variations of IL28B in resected liver tissue and mutations in HCV-RNA on the IFN sensitivity after OLT. The mutation rates in amino acid residues 70 and 91 in the Core region were 48% (25 of 52) and 35% (18 of 52), respectively, and in the non-DW, carrying mutation in either positions, ratio was 44% (23 of 52). The number of mutations in the ISDR and IRRDR of NS5A were 1.56 ± 1.83 (range, 0−7) and 5.00 ± 2.74 (range, 1−13), respectively. The minor allele positive ratio at rs8099917 in the non-DW group was significantly higher than that in the DW group (41% vs 13%; P = .025; Figure 4*A*). On the other hand, minor allele−positive ratios in the ISDR < 2 and IRRDR < 6 groups were comparable with those in the ISDR ≥ 2 and IRRDR ≥ 6 groups (31% vs 25% and 33% vs 19%, respectively; P = NS, Figure 4*B* and *C*). These results suggest that viral mutations in the Core but not in the NS5A region are associated with IL28B genetic variation.

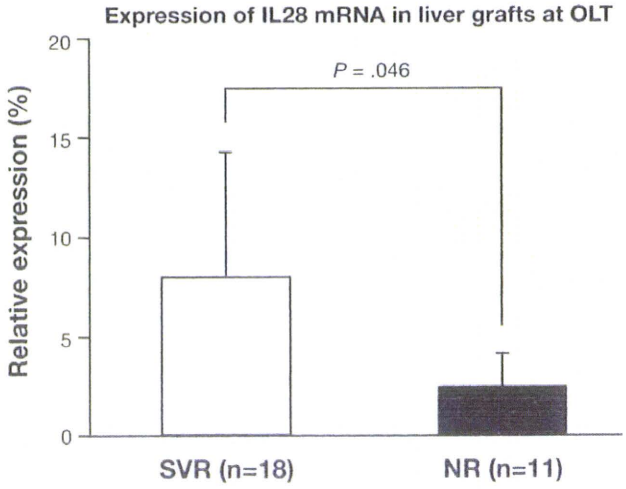

Figure 3. Expression of IL28 messenger RNA in the transplanted livers in recipients with sustained viral response (*SVR*) (*white*, n = 18) and nonresponse (*NR*) (*black*, n = 11). The statistical analysis was performed using Student's *t* test. OLT, orthotopic liver transplantation.

Combined Genetic Analysis of the IL28B Gene and HCV-RNA in the Prediction of IFN Sensitivity After OLT

Although the sensitivity and specificity of using IL28B genetic variations to predict the achievement of SVR were 62% to 87% in the chronic hepatitis C patients, the specificity of the genetic variation for predicting SVR was lower in recipients after OLT (Table 2).[16,18,19] Therefore, we assessed the impact of HCV-RNA mutations on

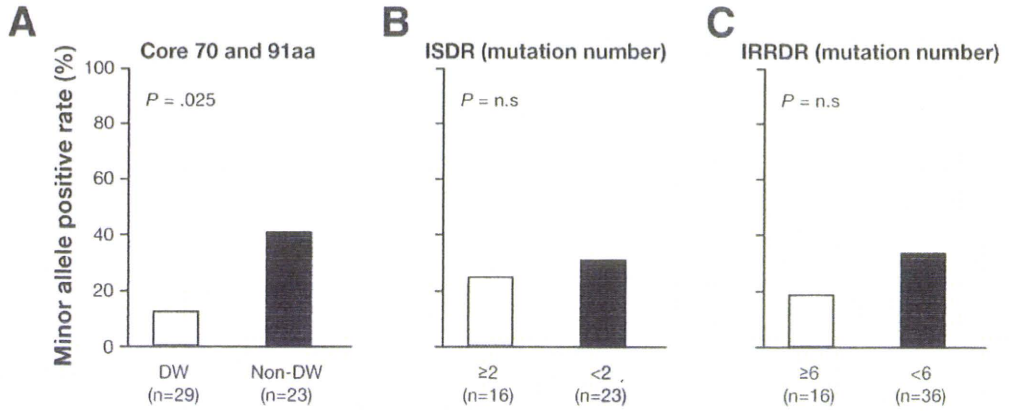

Figure 4. Association of genetic variations of IL28B in rs8099917 and mutations of hepatitis C virus (HCV)-RNA in the resected HCV-infected liver. (*A*) Comparison of the minor allele-positive rate in rs8099917 between a double-wild (*DW*) group with no mutations in amino acids 70 and 91 in the Core region (*white bar*) and a non-DW bearing 1 or more such mutations (*black bar*). (*B*) Comparison between the number of mutations of the interferon sensitivity-determining region (*ISDR*) ≥2 group (*white bar*) and <2 group (*black bar*). (*C*) Comparison between the number of mutations in the interferon-ribavirin resistance-determining region (*IRRDR*) ≥6 group (*white bar*) and <6 group (*black bar*).

the achievement of SVR in the recipients carrying the major allele homozygote and in those receiving transplantation from donors carrying the major allele homozygote. In our previous report, IFN sensitivity was scored according to the viral factors that participate in the IFN sensitivity.[14] Briefly, the patients were divided into 4 groups based on the number of positive factors, including the DW in the Core region, ISDR ≥ 2 and IRRDR ≥ 6 in the NS5A region, and this positive number was used as a prediction score. In the recipients carrying the major allele homozygote, the percentage of patients achieving SVR based on a prediction score of 0 (n = 8) was 13%, and those achieving SVR based on a score of 1 (n = 14), 2 (n = 7), or 3 (n = 4) were 43%, 86%, and 100%, respectively (P = .006; Figure 5*A*). In the donors carrying the major allele homozygote, the percentage of those achieving SVR based on a score of 0 (n = 9) was 11%, and those achieving SVR based on a score of 1 (n = 6), 2 (n = 4), or 3 (n = 2) were 33%, 75%, and 100%, respectively (P = .037; Figure 5*B*). These results suggest that combined genetic analysis of IL28B in both recipients and donors and of the Core and NS5A regions in HCV-RNA has the potential to predict SVR to PEG-IFN/RBV therapy after OLT.

Table 2. Comparison of Sensitivity and Specificity for SVR Between the Current Analysis (After Liver Transplantation) and Previous Analysis (Before Liver Transplantation)

	Sensitivity (%)	Specificity (%)
Current analysis (after Liver Transplantation)		
rs8099917 (Recipient DNA)	91	45
rs8099917 (Donor DNA)	91	45
Core aa70, 91 (Double Wild)	57	74
ISDR mutation number ≥2	64	71
IRRDR mutation number ≥6	75	78
Combined analysis (rs8099917 and HCV-RNA)	83	82
Previous analysis (before Liver Transplantation)		
rs8099917 (Tanaka et al 2009)	81	87
rs12979860 (McCarthy et al 2010)	63	76
rs12979860 (Montes-Cano et al 2010)	67	62

HCV, hepatitis C virus; IRRDR; interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; SVR, sustained viral response.

Discussion

In this study, we demonstrated that genetic variations in IL28B of both recipients and donors were significantly associated with IFN sensitivity, including SVR and ETR after OLT. These genetic variations were significantly associated with IL28 mRNA expression in both the resected liver derived from the recipients and in the donated liver. In addition, the current study revealed that HCV-RNA mutations in the Core but not in the NS5A region were significantly associated with IL28B genetic variations. Furthermore, the combined genetic analysis of IL28B and HCV-RNA was useful to predict the response to PEG-IFN/RBV therapy in patients with recurrent HCV infection after OLT.

The predictive factors for IFN sensitivity have been investigated extensively and several viral and host factors have been identified. Among the viral factors identified, the viral genotype is the most important and well-established predictive factor determining IFN sensitivity.[23] The SVR rate in patients with genotype 1 has been reported to be low (40%−50%), while that in patients with genotypes 2 and 3 has been reported to be high (70%−80%).[24] In addition, many reports have shown that mutations in the Core and NS5A regions are useful for predicting the response to IFN therapy.[11–13,24–26]

Several host factors have also been reported to be associated with the efficacy of IFN-centered antiviral therapy. The increased expression of ISGs at baseline