antibiotic-free DMEM overnight, and each plate was transfected with 10 nM siRNAs by X-tremeGENE (Roche Diagnostics) according to the manufacturer's protocol. Forty-eight hours post-transfection, the total RNA and protein extracts were prepared and subjected to real-time RT-PCR and immunoblot analyses, respectively.

Quantitation of HCV RNA and core protein

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time RT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, CA) as described previously (Aizaki et al., 2004; Murakami et al., 2006). HCV core protein levels in the cells and in the supernatant were quantified using an HCV core enzyme-linked immunosorbent assay (Ortho-Clinical Diagnostics, Tokyo, Japan).

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

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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-

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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*-butyldeoxynojirimycin (*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).

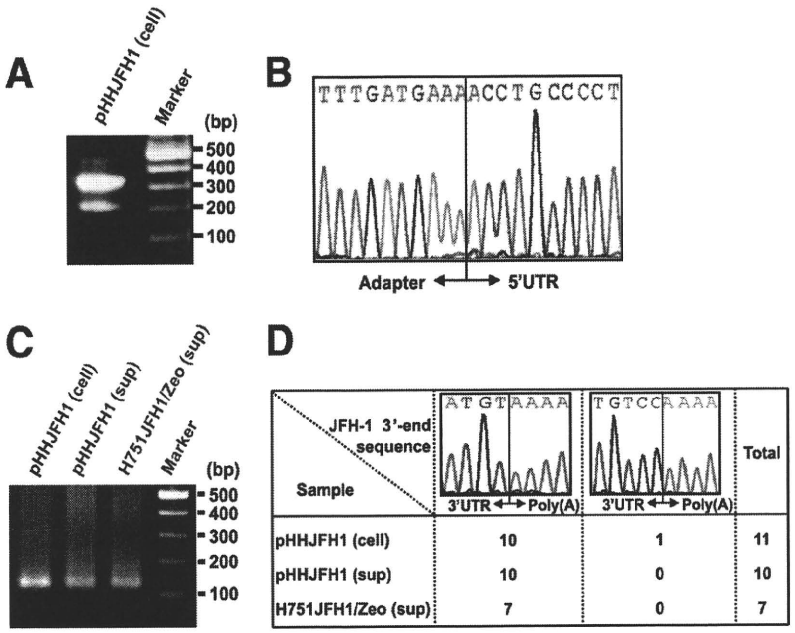

FIG. 1. Determination of the nucleotide sequences at the 5′-and 3′ ends of HCV RNA produced by the Pol I system. (A and B) 5′RACE and sequence analysis. A synthesized RNA adapter was ligated to RNA extracted from cells transfected with pHHJFH1. The positive-strand HCV RNA was reverse transcribed, and 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/

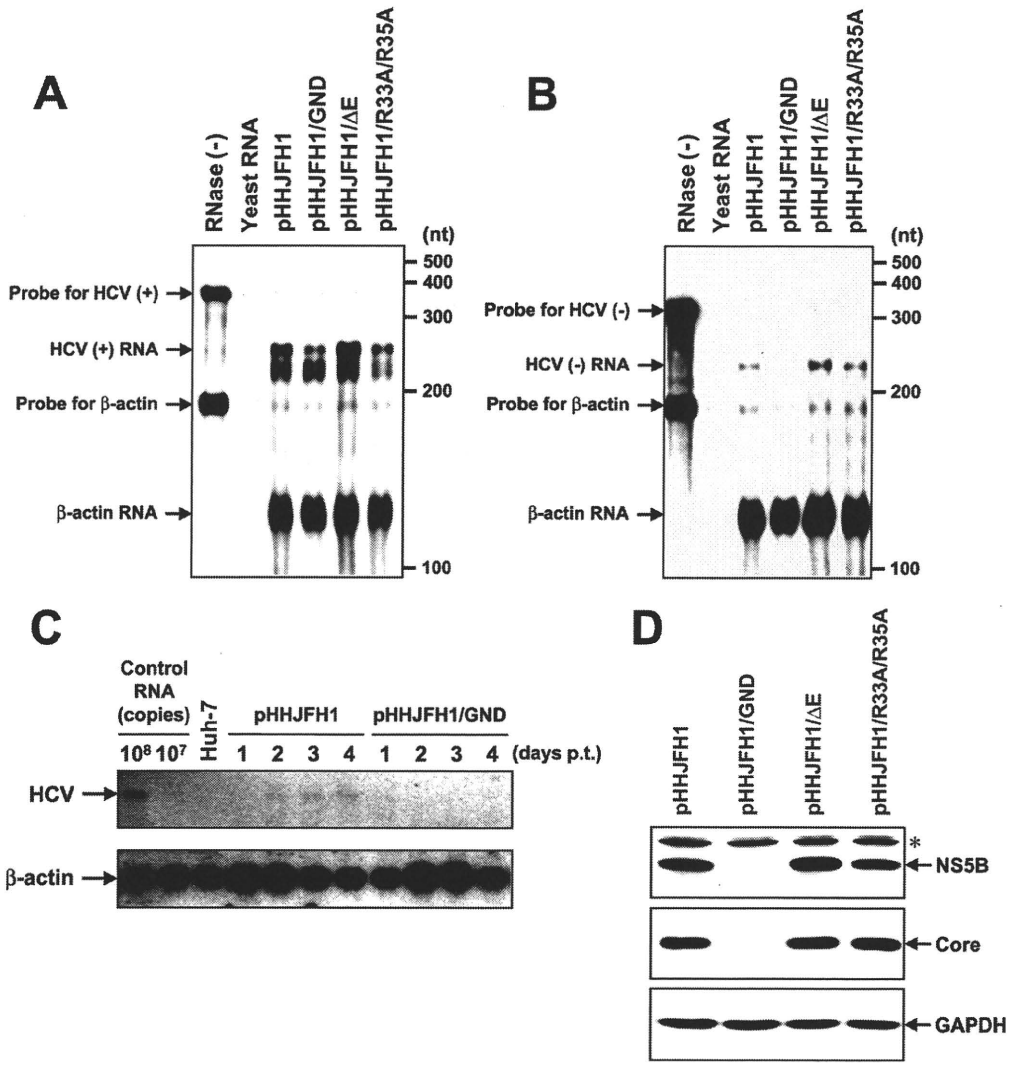

FIG. 2. HCV RNA replication and protein expression in cells transfected with Pol I-driven plasmids. (A and B) Assessment of HCV RNA replication by RPA. Pol I-driven HCV-expression plasmids were transfected into Huh-7 cells. Total RNA was extracted from the cells on day 5 p.t. and positive (A)- and negative (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

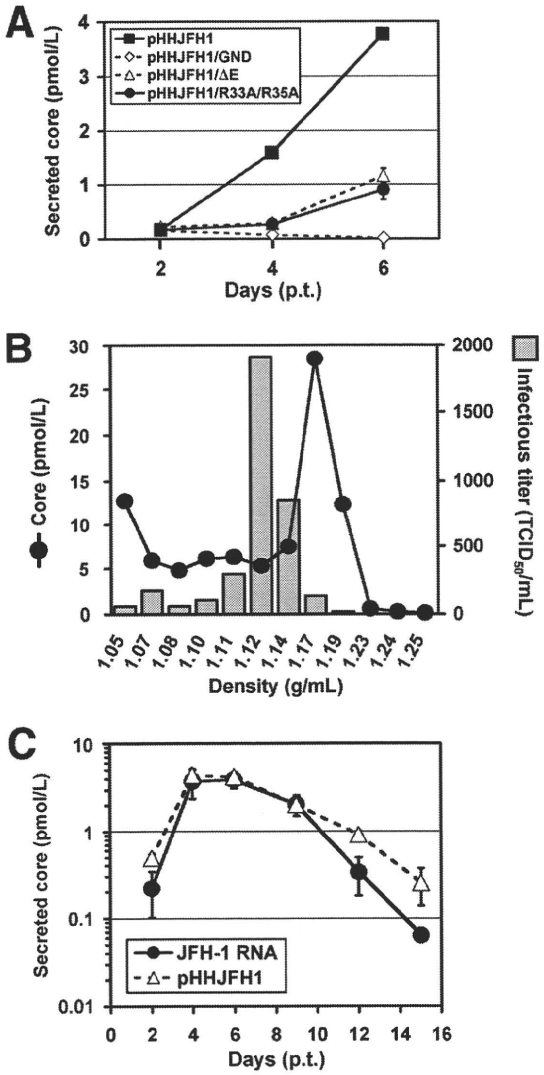

FIG. 3. HCV released from cells transfected with Pol I-driven plasmids. (A) HCV particle secretion from the transfected cells. The culture supernatant of Huh-7 cells transfected with Pol I-driven plasmids containing wild-type or mutated HCV genome were harvested on days 2, 4, and 6 and assayed for HCV 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

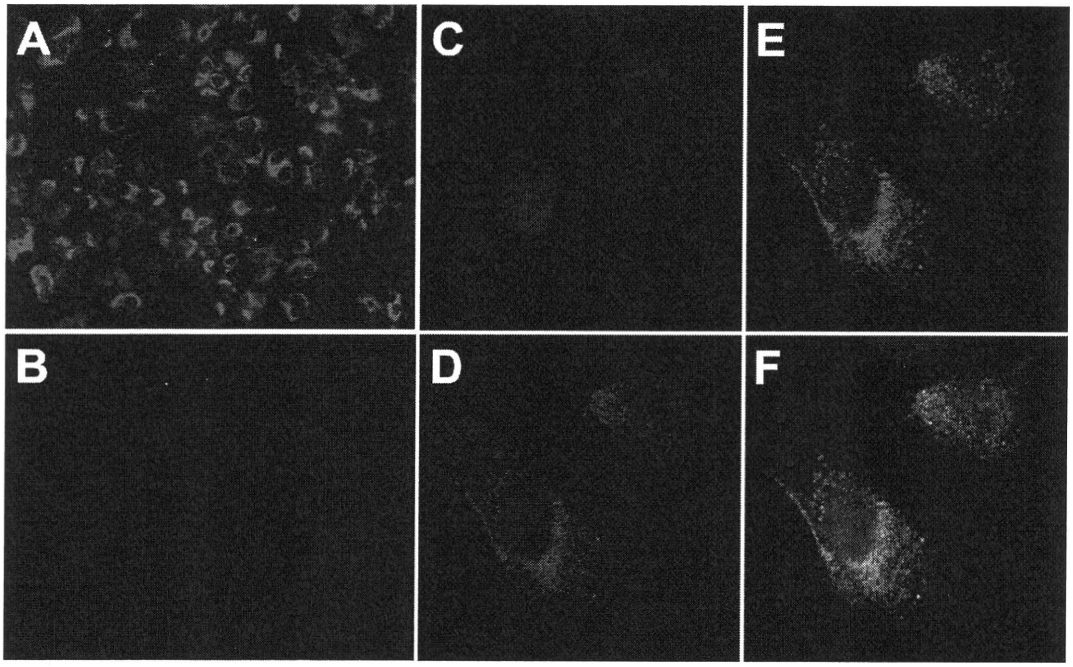

FIG. 4. Indirect immunofluorescence analysis of H751JFH1/Zeo cells. (A and B) H751JFH1/Zeo cells (A) and parental Huh7.5.1 cells (B) were immunostained with an anti-NS5A antibody. (C to F) The subcellular colocalization of *de novo*-synthesized HCV RNA and NS5A in H751JFH1/Zeo cells was analyzed. The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

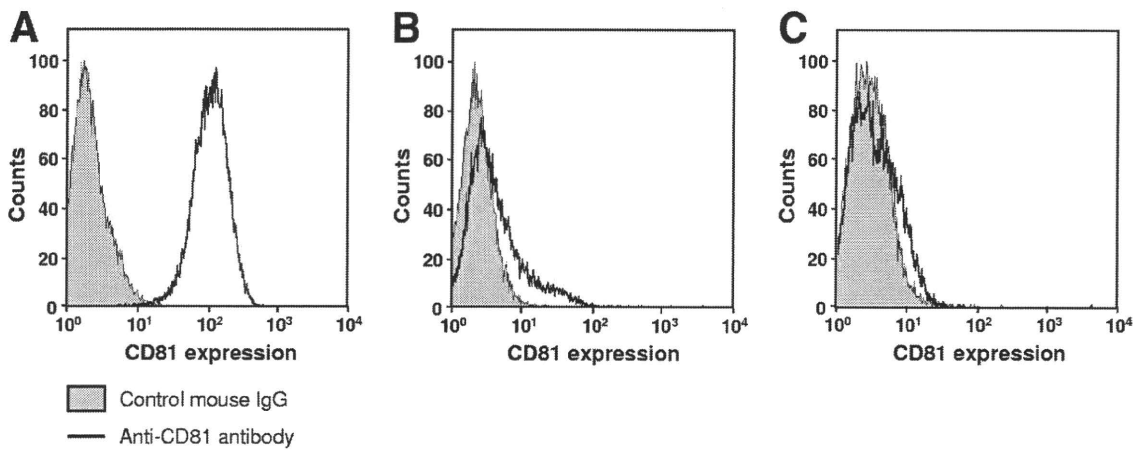

FIG. 5. Loss of CD81 expression in H751JFH1/Zeo cells. The cell surface expression of CD81 on Huh7.5.1 cells (A), H751JFH1/Zeo clone H751-1 (B), and clone H751-50 (C) was analyzed by flow cytometry after being stained with anti-CD81 antibody.

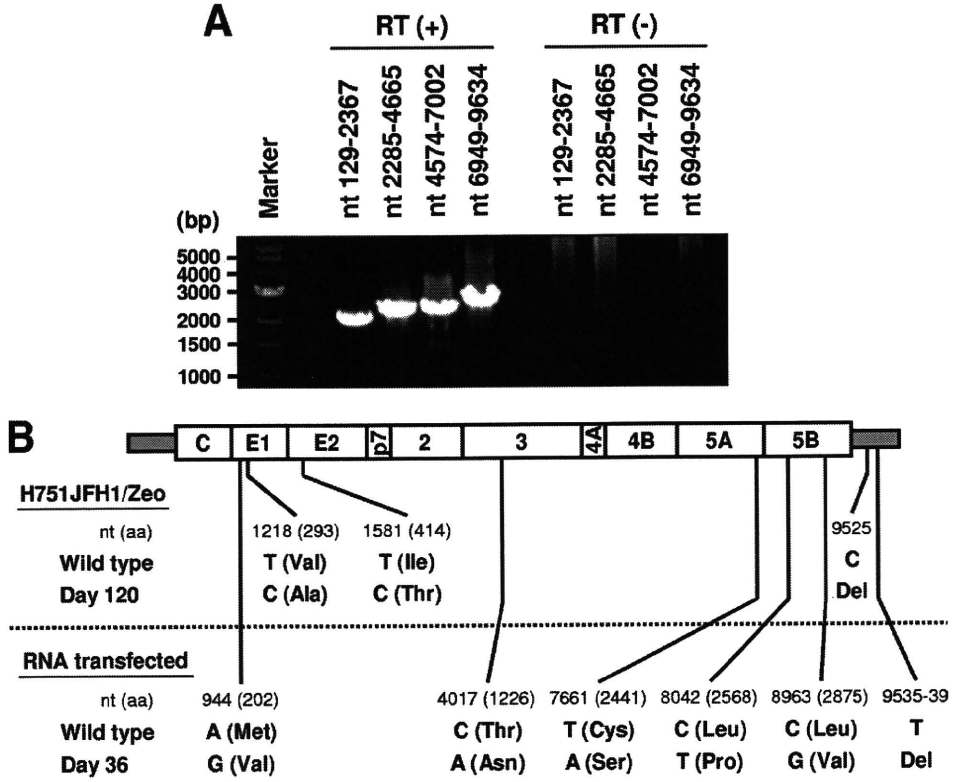

FIG. 6. Genome mutations of HCV secreted from H751JFH1/Zeo cells. (A) RT-PCR of HCV genome extracted from the culture supernatant of H751JFH1/Zeo cells. Viral RNA sequences were reverse transcribed [RT (+)] or not [RT (−)], followed by amplification with primer pairs encompassing the specified HCV genome 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

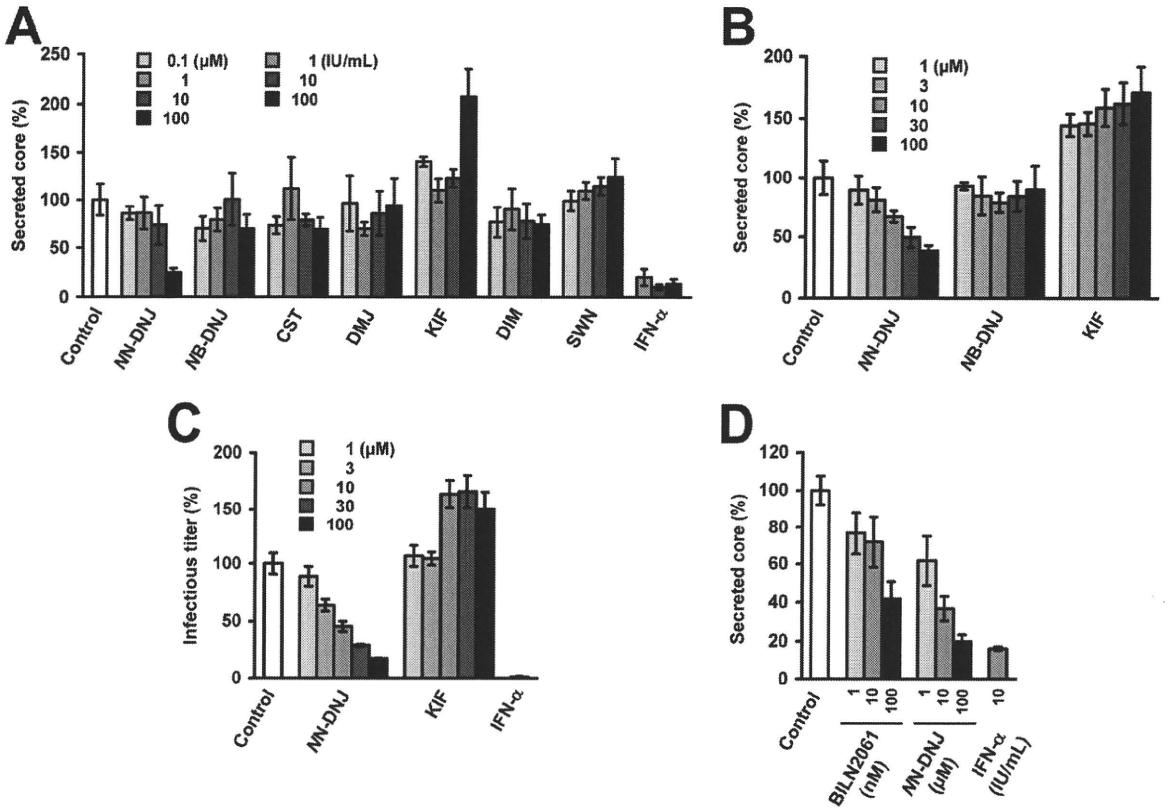

FIG. 7. Effects of glycosylation inhibitors on HCV production from H751JFH1/Zeo cells. (A and B) Effects of glycosylation inhibitors on the secretion of HCV core protein. H751JFH1/Zeo cells were seeded at a density of 1×10^4 cells/well in a 96-well culture plate (A) or 3×10^4 cells/well in a 12-well 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

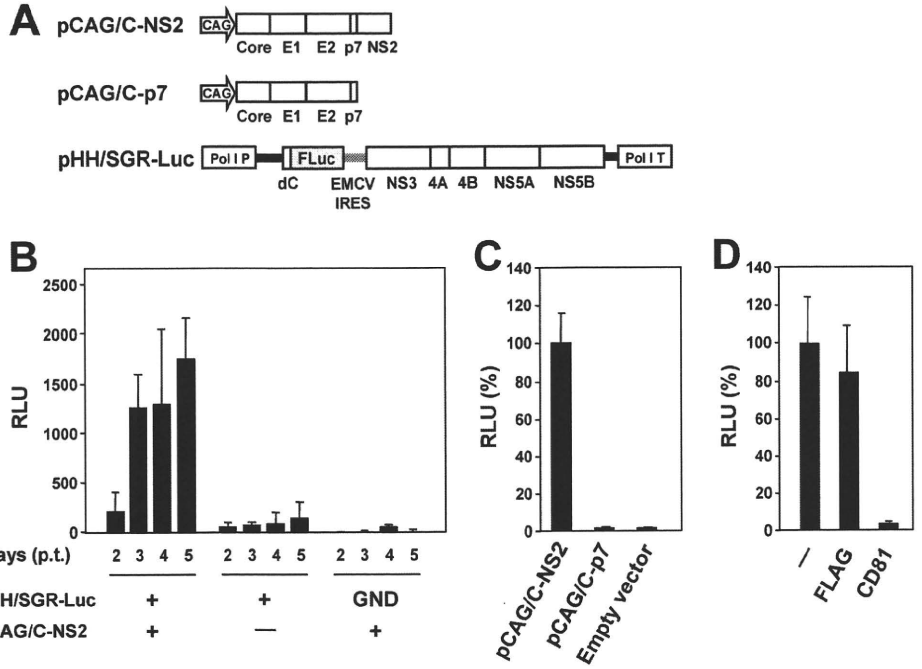

FIG. 8. Establishment of a *trans*-packaging system involving two-plasmid transfection. (A) Schematic representation of the plasmids used for the production of HCV-LP. HCV polyproteins are indicated by the open boxes. Bold lines indicate the HCV UTR. EMCV IRES is denoted by gray bars. The firefly luciferase 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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特集II　C型肝炎ウイルスの感染・増殖メカニズムと臨床応用

HCV NS5A蛋白のリン酸化に関与する新規セリン/スレオニンプロテインキナーゼの探索*

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Key Words：hepatitis C virus(HCV), NS5A, phosphorylation, protein kinase, *AlphaScreen*

はじめに

C型肝炎ウイルス(HCV)の非構造蛋白であるNS5A蛋白はリン酸化蛋白で，主にセリン，スレオニン残基のリン酸化状態により高リン酸化型および低リン酸化型と呼ばれる分子量の異なる2種類の蛋白として存在する[1]．NS5A蛋白のリン酸化はウイルスゲノム複製だけでなく，感染性ウイルス粒子の形成にも重要な役割を担うことが報告[2)~4)]されているが，そのリン酸化部位やリン酸化に関与するプロテインキナーゼの同定等，リン酸化機構の詳細は明らかになっていない．今回われわれは網羅的手法を用いてNS5A蛋白のリン酸化に関与する新規プロテインキナーゼの同定を試みたので報告する．

対象と方法

解析方法の概要を図1に示す．NS5A蛋白のリン酸化に関与するプロテインキナーゼを同定するために，まず，NS5A蛋白と強く相互作用するプロテインキナーゼの探索を行った．次に，強い相互作用が認められたプロテインキナーゼに関して，NS5A蛋白に対するリン酸化能を調べた．最後に，同定されたプロテインキナーゼがHCVゲノム複製，粒子形成に与える影響を培養細胞

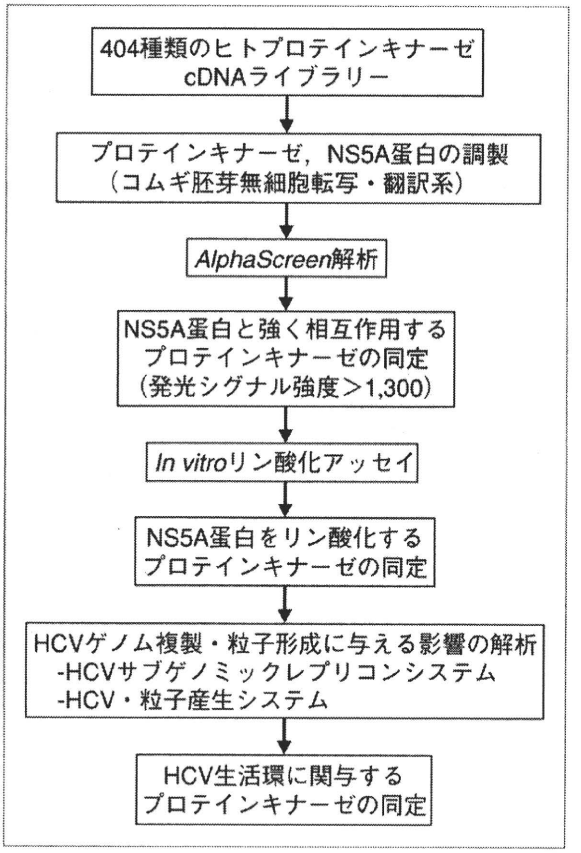

図1　解析方法の概要

* Identification of novel serine/threonine protein kinases responsible for HCV NS5A phosphorylation.

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を用いて解析した．

1．NS5A蛋白と強く相互作用するプロテインキナーゼの探索

NS5A蛋白をコムギ胚芽無細胞転写・翻訳系で合成した．また，404種類のヒトプロテインキナーゼを包括するcDNAライブラリーから同様の方法でプロテインキナーゼを取得した．NS5A蛋白とプロテインキナーゼの相互作用はハイスループットな定量解析が可能である*AlphaScreen*法を用いて解析した．本研究における*AlphaScreen*解析の概要を図2に示す．

2．NS5A蛋白に対するリン酸化能の評価

NS5A蛋白に対するリン酸化能の評価は，精製プロテインキナーゼを[γ-^{32}P]ATP存在化において精製NS5A蛋白と混和し，SDS-PAGEで展開後，オートラジオグラフィーを用いてリン酸化NS5A蛋白のバンドを検出することにより行った(*in vitro* リン酸化アッセイ)．

3．HCVゲノム複製，粒子形成に与える影響の解析

HCVゲノム複製能はサブゲノミックレプリコンRNAを用いて，また，粒子形成能は全長HCV RNAもしくは感染性ウイルス粒子を用いて解析した．細胞にはヒト肝癌由来細胞株(HuH-7)およびその派生株を使用した．HCV RNA導入細胞，HCV感染細胞におけるプロテインキナーゼの発現をsiRNAによりノックダウンし，HCVゲノム複製能，粒子形成能に与える影響を解析した．また，NS5A蛋白のリン酸化状態をウエスタンブロッティング法により解析した．培養上清中の感染性ウイルス粒子量の測定は，培養上清を非感染細胞に処理後，感染巣(フォーカス)をカウントし，1 m*l*あたりのフォーカス形成単位(FFU)を算出することにより行った．

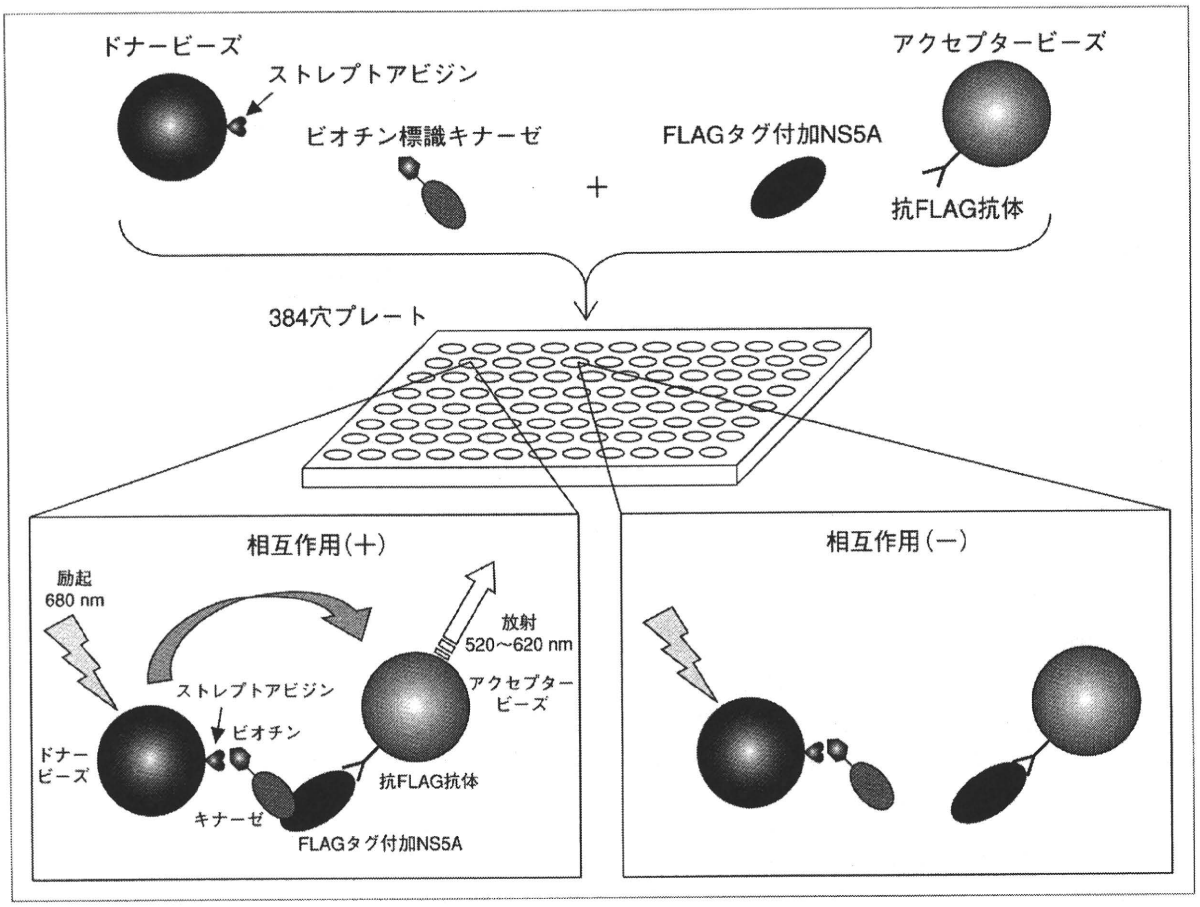

図2　本研究における*AlphaScreen*解析

ビオチン標識プロテインキナーゼとFLAGタグ付加NS5A蛋白をドナービーズ，アクセプタービーズとともに同一ウェルに分注し，ウェル内でドナービーズに結合したプロテインキナーゼがアクセプタービーズに結合したNS5A蛋白と生物学的に相互作用し，2つのビーズが近接した状態の時にのみ発光シグナルが検出される．

結　果

1. NS5A蛋白と強く相互作用するプロテインキナーゼの探索

*AlphaScreen*法による解析結果を表1に示す．発光シグナル強度1,300以上を強固な蛋白間相互作用と想定した時，このカットオフ値以上のシグナル強度を示したプロテインキナーゼは89種類であった．このうち79種類がNS5A蛋白のリン酸化に重要とされるセリン/スレオニンプロテインキナーゼであり，このグループの中にはNS5A蛋白をリン酸化するとすでに報告されているカゼインキナーゼ(CK)2[4)5)]の触媒サブユニットであるCK2α2も含まれていた．

表1　*AlphaScreen*解析結果

発光シグナル強度	プロテインキナーゼ数
5,000～	7
4,500～5,000	1
4,000～4,500	5
3,500～4,000	4
3,000～3,500	7
2,500～3,000	10
2,000～2,500	11
1,500～2,000	23
1,000～1,500	62
500～1,000	143
～ 500	131

2. NS5A蛋白に対するリン酸化能の評価

*AlphaScreen*解析でスクリーニングされた79種類のセリン/スレオニンプロテインキナーゼに対して*in vitro*リン酸化アッセイを行ったところ，9種類にNS5A蛋白に対する強いリン酸化活性が認められた．

3. HCVゲノム複製，粒子形成に与える影響の解析

NS5A蛋白をリン酸化する9種類のプロテインキナーゼがHCV生活環に役割を有するか否かを調べるために，各プロテインキナーゼの細胞内発現をノックダウンした状態でHCVを感染させ，感染後のウイルス粒子産生量を解析した．ヒト肝癌由来細胞株において発現が認められなかったり，ノックダウンにより著しい細胞障害を示したプロテインキナーゼはこの解析から除外し，7種類のプロテインキナーゼを対象とした．CK2α2ノックダウン細胞から分泌される感染性ウイルス粒子量(ウイルス感染力価)は，mock処理細胞もしくはコントロールsiRNA導入細胞の約1/2に抑制された．この結果はCK2が感染性HCV粒子の産生を制御するという過去の報告内容[4)]を支持するものであった．さらに，ノックダウンによりCK2α2以上に感染性ウイルス粒子分泌量を低下させる新規プロテインキナーゼを2種類見出した(PK-2およびPK-29)．次に，これら2種類のプロテインキナーゼがHCV生活環の中のどのステップにかかわっているのかをより詳細に調べるために，プロテインキナーゼノックダウン細胞におけるRNA複製能をサブゲノミックレプリコンシステムを用いて，また，HCV粒子形成能をウイルス感染が成立しないHuH-7細胞[6)]を用いたHCV産生システムで解析した．HCV RNA複製はレポーターとしてレプリコンに挿入されたルシフェラーゼ遺伝子の発現を指標にして定量的に評価した．2種類の新規プロテインキナーゼおよびCK2α2ノックダウン細胞におけるRNA複製能はmock処理細胞もしくはコントロールsiRNA導入細胞の複製能と同程度であり，これらのプロテインキナーゼの作用点はゲノム複製のステップではないことが示唆された．図3にHCV粒子形成能の結果を示す．全長HCV RNAをプロテインキナーゼsiRNAとともにエレクトロポレーション法で細胞に導入し，導入後3日目の上清中コア蛋白量を測定した．2種類の新規プロテインキナーゼおよびCK2α2ノックダウン細胞から分泌されるコア蛋白量はmock処理細胞もしくはコントロールsiRNA導入細胞における分泌コア蛋白量の1/3～1/2に減少し，ウイルス粒子形成過程がこれらのプロテインキナーゼの作用点である可能性が示唆された．最後に，2種類の新規プロテインキナーゼが培養細胞内においてもNS5A蛋白のリン酸化に関与するか否かを調べるために，プロテインキナーゼノックダウン細胞にHCVを感染させ，NS5A蛋白のリン酸化状態を解析した．PK-2ノックダウン細胞では，コントロールsiRNA導入細胞と比べて，高リン酸化型NS5A蛋白の発現低下および高リン酸化型NS5A蛋白/低リン酸化型NS5A蛋白比の減少を認めた．一方，PK-29ノックダウン細胞におけるNS5A蛋白のバンドパ

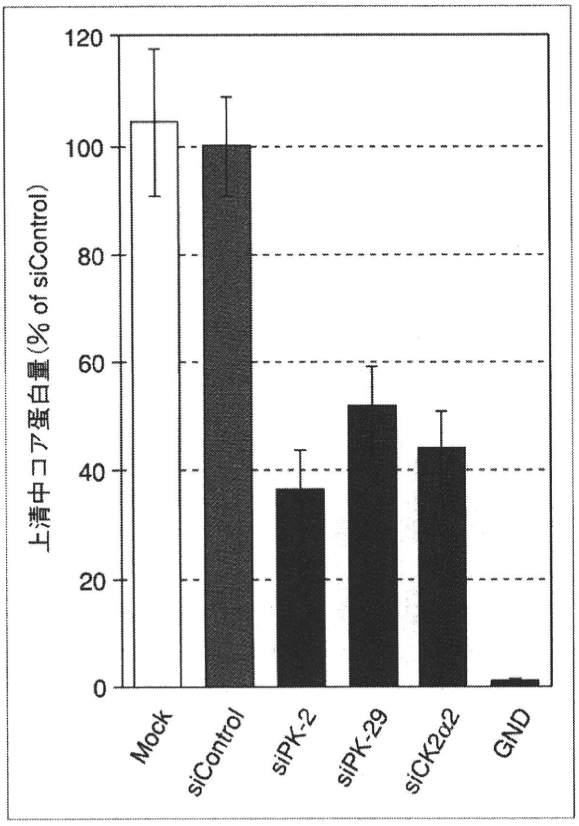

図3　プロテインキナーゼのノックダウンが分泌HCV粒子量に与える影響

siRNA, HCV RNA導入後3日目の上清中コア蛋白量をcontrol siRNA(siControl)導入時の値を100%として算出し，グラフ化した．Mock：全長HCV RNAの導入のみでsiRNAの導入はなし．GND：複製活性のない変異HCV RNAの導入のみでsiRNAの導入はなし．

ターンはコントロールsiRNA導入細胞のNS5A像と同様のパターンを呈していた．

考　察

NS5A蛋白はHCVの複製増殖やインターフェロン感受性，病原性発現などに関与する多機能蛋白である[7]．リン酸化蛋白であり，NS5A蛋白のリン酸化はウイルスゲノム複製や感染性ウイルス粒子の形成に重要な役割を担うことが報告されている[1)3)4)]．したがって，NS5A蛋白のリン酸化を制御するプロテインキナーゼの同定は，HCV生活環をより理解する上で重要であることに加え，新たな創薬ターゲットとしても魅力的である．

阻害剤や酵母由来の精製プロテインキナーゼを用いた解析から，現在までにAKT, p70S6K, MEK, CK1, CK2など数種類のプロテインキナーゼがNS5A蛋白のリン酸化に関与するものとして報告されている[8)9)]．しかし，いずれの報告も解析対象のプロテインキナーゼ数は数十～百前後であり，また，HCV生活環への関与についても十分な検討がなされていない．そこで，今回われわれは404種類のヒトプロテインキナーゼを対象とし，NS5A蛋白に対するリン酸化能を有し，かつ，HCV生活環に関与するプロテインキナーゼの同定を試みた．同定された3種類のプロテインキナーゼの中にはCK2の触媒サブユニットであるCK2α2が含まれていたが，CK2はNS5A蛋白のリン酸化とHCV粒子形成に関与することが報告されており[4)5)]，本解析結果の妥当性が高いことを示している．

今回の解析で同定された3種類のプロテインキナーゼは，いずれもsiRNAによるノックダウンで複製活性には影響を与えずにウイルス粒子分泌量を低下させたことから，HCV生活環の後期過程であるウイルス粒子形成(もしくはそれ以降のステップ)に関与し，この過程を正に制御している可能性が示唆された．さらに，PK-2のノックダウンはNS5A蛋白の高リン酸化を著しく抑制しており，このプロテインキナーゼの粒子形成過程への作用はNS5A蛋白の高リン酸化制御を介している可能性が考えられた．最近，台湾の研究チームがサブゲノミックレプリコン細胞を用いて1,210種類のヒトプロテインキナーゼおよびホスファターゼを対象とした網羅的RNAiスクリーニングを行い，HCVゲノム複製にpolo-like kinase 1(Plk1)というプロテインキナーゼが関与することを報告した[10)]．Plk1の作用点はHCVの複製過程であるが，その作用はわれわれが同定したPK-2と同様NS5A蛋白の高リン酸化制御を介する．NS5A蛋白の高リン酸化にはその中央領域に存在する複数のセリン残基が関与すると報告されているが[11)～14)]，同じリン酸化パターンでも責任プロテインキナーゼやNS5A蛋白のリン酸化部位の違いによりHCV生活環における作用点が異なる可能性は十分に考えられる．この相違を明らかにするためには，今後，責任プロテインキナーゼによるリン酸化部位の同定や同定部位のリン酸化がHCV生活環に与える影響につき解析する必要があるであろう．

NS5A蛋白がHCV粒子形成に関与する過程においてキャプシド蛋白であるコア蛋白との相互作用[2)3)]や粒子形成の場である脂肪滴周辺膜への局在[15)]は必須であり，いずれにもNS5A蛋白のリン酸化が重要であると考えられている[3)4)]．同定されたプロテインキナーゼがNS5A蛋白のリン酸化制御を介して粒子形成過程のどのステップに関与しているのかを現在解析中である．また，HCV侵入過程に関与するプロテインキナーゼの報告[16)]もあることから，同定プロテインキナーゼがこの侵入過程にも役割を有するかどうか解析予定である．

おわりに

NS5A蛋白と相互作用し，NS5A蛋白をリン酸化する新規セリン/スレオニンプロテインキナーゼを網羅的手法により同定した．さらに，この中から感染性HCV産生を制御する2種類の新規プロテインキナーゼを取得した．本研究は，HCVゲノム複製・粒子形成機構の解明や新たな創薬ターゲットの同定に道を拓く可能性がある．

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5. C型肝炎ウイルスの複製と粒子形成

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輸血による新たな感染は激減したものの，C型肝炎ウイルス（HCV）キャリアは我が国だけで約200万人とされ，キャリアからの発症予防，慢性肝炎からの肝硬変化，発がん阻止は，高齢化社会を迎え非常に重要な課題である．HCV生活環の各過程の調節機構を分子レベルで明らかにすることにより，治療薬開発のための新たな分子標的が見出される．HCVゲノム複製機構については，近年，複製複合体の性状解析が進み，複製調節に関与する様々な宿主因子が同定されている．筆者らは，ATP産生に重要なcreatine kinase B（CKB）がHCV複製に関与することを見出した．CKBはNS4Aと相互作用して複製の場へリクルートされエネルギー供給に寄与する可能性を示した．この他，粒子形成過程と宿主脂質代謝系との関連など最近のトピックスを紹介する．

はじめに

肝炎，肝硬変，肝がんの主要な原因因子であるC型肝炎ウイルス（HCV）は，約9.6 kbの一本鎖のプラス鎖RNAをゲノムとし，フラビウイルス科（*Flaviviridae*）のヘパシウイルス属（*Hepacivirus*）に分類されている．約3010アミノ酸からなる前駆体蛋白質が，小胞体に存在するシグナルペプチダーゼとシグナルペプチドペプチダーゼ，及びウイルス自身がコードする2種類のプロテアーゼによってプロセシングをうけ，ウイルス粒子を形成する構造蛋白質（Core，E1，E2）とウイルス粒子に含まれない非構造蛋白質（NS2，NS3，NS4A，NS4B，NS5A，NS5B）が作られる．E2のC末端側にはp7と呼ばれる小分子が存在するが，ウイルス粒子に含まれるかは不明である[35]．

1999年，培養細胞でHCVサブゲノムRNAが自律複製するレプリコンシステムが開発され，ゲノム複製機構の研究は進展を見せ，さらに，2005年，劇症肝炎患者から単離されたJFH-1株のゲノムRNAから感染性粒子が効率よく産生されることが見出され，HCVの生活環全般に関する分子生物学的研究が可能となった．

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HCVゲノム複製

この10年の間にレプリコンシステムを使った解析からHCVのゲノム複製機構について多くの知見が得られた．HCVレプリコンRNAが複製する細胞の電顕観察から，Membranous webと呼ばれる小胞様構造がHCVゲノム複製の場と推定されている[11]．一方，生化学的解析から，NP-40やTriton X-100などの非イオン性界面活性剤処理で不溶性となる膜分画（DRM分画）にHCV複製活性が保持されることが示され[1,33]，コレステロール合成阻害剤やスフィンゴ脂質合成阻害剤を用いた解析などから，HCVのゲノム複製には脂質ラフト様膜構造が関与することを示唆する知見が蓄積されている[1,32,39]．一般には，Membranous webは小胞体由来と考えられ，脂質ラフトは小胞体に存在しないとされることから，HCV複製の足場となる膜構造の性状を明らかにするためには，更に詳細な解析が必要である．いずれにしても，DRM分画にはウイルスゲノムRNAが鋳型となってマイナス鎖が作られ，さらにそれからプラス鎖RNAが合成される活性が存在する．そしてこの膜分画には，NS3～NS5Bの5種類のHCV非構造蛋白及び宿主細胞由来因子からなる複製複合体が存在することが示されている[3,22,28]（**図1**）．

HCVゲノム複製に関与する宿主因子としては，これまで