

Short
Communication

Structural requirements of virion-associated cholesterol for infectivity, buoyant density and apolipoprotein association of hepatitis C virus

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Our earlier study has demonstrated that hepatitis C virus (HCV)-associated cholesterol plays a key role in virus infectivity. In this study, the structural requirement of sterols for infectivity, buoyant density and apolipoprotein association of HCV was investigated further. We removed cholesterol from virions with methyl β -cyclodextrin, followed by replenishment with 10 exogenous cholesterol analogues. Among the sterols tested, dihydrocholesterol and coprostanol maintained the buoyant density of HCV and its infectivity, and 7-dehydrocholesterol restored the physical appearance of HCV, but suppressed its infectivity. Other sterol variants with a 3β -hydroxyl group or with an aliphatic side chain did not restore density or infectivity. We also provide evidence that virion-associated cholesterol contributes to the interaction between HCV particles and apolipoprotein E. The molecular basis for the effects of different sterols on HCV infectivity is discussed.

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Hepatitis C virus (HCV) is a major cause of liver diseases, and is an enveloped, plus-strand RNA virus of the genus *Hepacivirus* of the family *Flaviviridae*. The mature HCV virion is considered to consist of a nucleocapsid, an outer envelope composed of the viral E1 and E2 proteins and a lipid membrane. Production and infection of several enveloped viruses, such as human immunodeficiency virus type 1 (HIV-1), hepatitis B virus and varicella-zoster virus (Bremer *et al.*, 2009; Campbell *et al.*, 2001; Graham *et al.*, 2003; Hambleton *et al.*, 2007), are dependent on cholesterol associated with virions. However, except for HIV-1 (Campbell *et al.*, 2002, 2004), there is limited information about the effects of replacing cholesterol with sterol analogues on the virus life cycle. We demonstrated the higher cholesterol content of HCV particles compared with host-cell membranes, and that HCV-associated cholesterol plays a key role in virion maturation and infectivity (Aizaki *et al.*, 2008). Recently, by using mass spectrometry, Merz *et al.* (2011) identified cholesteryl esters, cholesterol,

phosphatidylcholine and sphingomyelin as major lipids of purified HCV particles.

To investigate further the effect of the structural requirement for cholesterol on the infectivity, buoyant density and apolipoprotein association of HCV, depletion of virion-associated cholesterol and substitution of endogenous cholesterol with structural analogues (Fig. 1a) was used in this study. HCVcc (HCV grown in cell culture) of the JFH-1 isolate (Wakita *et al.*, 2005), prepared as described previously (Aizaki *et al.*, 2008), was treated with 1 mM methyl β -cyclodextrin (B-CD), which extracts cholesterol from biological membranes, for 1 h at 37 °C. The cholesterol-depleted virus was then incubated with exogenous cholesterol or cholesterol analogues at various concentrations for 1 h. After removal of B-CD and free sterols by centrifugation at 38 000 r.p.m. (178 000 g) for 2.5 h, the treated particles were used to infect Huh7 cells, kindly provided by Dr Francis V. Chisari (The Scripps Research Institute, La Jolla, CA, USA), and their infectivity was determined by quantifying the viral core protein in cells using an enzyme immunoassay (Ortho-Clinical Diagnostics) at 3 days post-infection (p.i.). Virus infectivity, which fell to <20% after B-CD treatment, was

A supplementary table and figure are available with the online version of this paper.

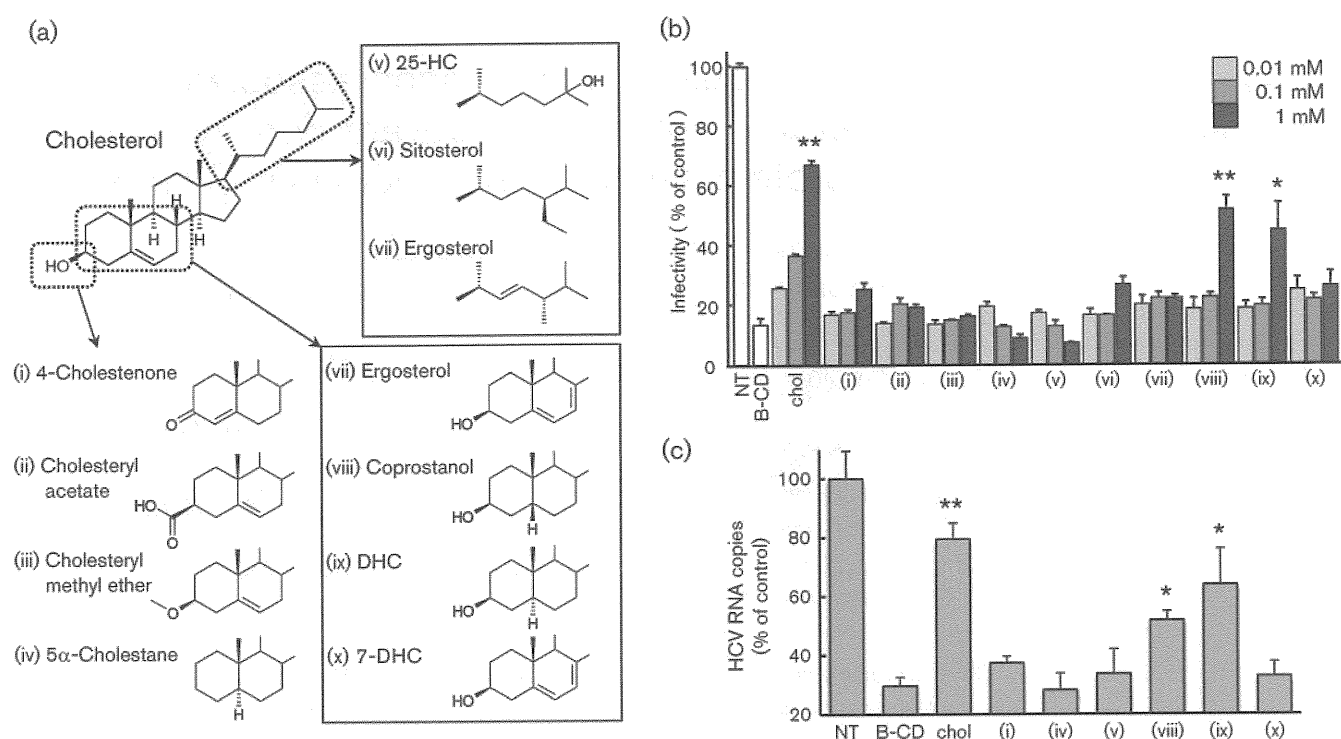


Fig. 1. Role of virion-associated cholesterol analogues in virus infection. (a) Structures of sterols used in this study. Variations in the 3 β -hydroxyl group (lower left), aliphatic side chain (upper right) or ring structure (lower right) of cholesterol are shown. (i–x) Compounds studied in (b) and (c). (b) Effect of replenishment with sterols on HCV infectivity. Intracellular HCV core levels were determined at 72 h p.i. as the indicator of infectivity, which is represented as a percentage of the untreated HCVcc level (NT). (c) Effects of virion-associated sterols on virus internalization. HCV RNA copies in cells after virus internalization were quantified and are shown as percentages of the untreated HCVcc level (NT). (b, c) Means + SD of four samples are shown. * $P < 0.05$; ** $P < 0.01$, compared with B-CD-treated virus (unpaired Student's *t*-test). Data are representative of at least two experiments.

recovered by addition of cholesterol at 0.01–1 mM in a dose-dependent manner (Fig. 1b). Among the cholesterol analogues tested, variants with a 3 β -hydroxyl group (4-cholestenone, cholesteryl acetate, cholesteryl methyl ether and 5 α -cholestane) or variants with an aliphatic side chain [25-hydroxycholesterol (25-HC), sitosterol and ergosterol] exhibited no or little effect on the recovery of infectivity of B-CD-treated HCV (Fig. 1b, lanes i–vii). In contrast, addition of variants in the structure of the sterol rings [coprostanol or dihydrocholesterol (DHC)] at 1 mM restored infectivity to around 50% compared with non-treated virus control (Fig. 1b, lanes viii and ix). Other variants in the ring structure [7-dehydrocholesterol (7-DHC) and ergosterol, which is also a variant with an aliphatic side chain as indicated above] did not show any increase in the infectivity of B-CD-treated virus (Fig. 1b, lanes x and vii).

We demonstrated previously that HCV-associated cholesterol plays an important role in the internalization step of the virus, but not in cell attachment during virus entry (Aizaki *et al.*, 2008). The effect of virion-associated cholesterol analogues on virus attachment to cells and

following internalization was determined. HCVcc, treated with B-CD with or without subsequent replenishment with sterols, was incubated with Huh7-25-CD81 cells, which stably express CD81 (Akazawa *et al.*, 2007), for 1 h at 4 °C. As an internalization assay, the incubation temperature was shifted to 37 °C post-binding procedure and maintained for 2 h. The cells were then treated with 0.25% trypsin for 10 min at 37 °C, by which >90% of HCV bound to the cell surface was removed (data not shown; Aizaki *et al.*, 2008). Internalized HCV was quantified by measuring the viral RNA in cell lysates by real-time RT-PCR (Takeuchi *et al.*, 1999). B-CD treatment or supplementation with sterols of B-CD-treated HCV had little or no effect on virus attachment to the cell surface (data not shown). Regarding virus internalization (Fig. 1c), treatment of HCVcc with 1 mM B-CD resulted in approximately 70% reduction of viral RNA. The reduced level of the internalized HCV recovered markedly to approximately 80% of the untreated HCVcc level by replenishment with 1 mM cholesterol. In agreement with the results shown in Fig. 1(b), addition of coprostanol or DHC to the B-CD-treated virus caused a significant recovery of virus internalization, suggesting that coprostanol and DHC associated with the

virion have the ability to play a role in HCV internalization into cells, in a manner comparable to cholesterol (Fig. 1c, lanes viii and ix). No or only a little recovery of virus internalization was observed by loading with other cholesterol analogues, such as 4-cholestenone, 5 α -cholestane, 25-HC or 7-DHC (Fig. 1c, lanes i, iv, v and x).

To monitor the effect of cholesterol analogues on the physical characteristics of HCV, we next investigated buoyant-density profiles by using sucrose density-gradient centrifugation, in which untreated, B-CD-treated and sterol-replenished HCVcc were concentrated and layered onto continuous 10–60% (w/v) sucrose density gradients, followed by centrifugation at 35 000 r.p.m. (151 000 g) for 14 h. Fractions were collected and analysed for the core protein. Fig. 2 shows that the virus density became higher after treatment with B-CD and that cholesterol-replenished virus shifted the density of B-CD-treated HCV to the non-treated level. Consistent with the result shown in Fig. 1(b), no effect on restoration of the buoyant densities of HCV was observed using variants with modifications in either the 3 β -hydroxyl group (4-cholestenone, cholesteryl acetate and 5 α -cholestane) or the aliphatic side chain (25-HC and sitosterol). In contrast, variants in the sterol ring structure (coprostanol, DHC and 7-DHC) had an ability to recover the density of B-CD-treated virus to that of non-treated virus.

Incorporation efficiency of the sterols into the cholesterol-depleted HCVcc was further determined by gas chromatography with flame ionization detection (see Supplementary Table S1, available in JGV Online). Under the experimental

conditions used, exogenously supplied cholesterol after B-CD treatment was able to restore cholesterol content in HCVcc almost to initial levels. When 4-cholestenone, cholesteryl acetate, 25-HC, DHC or 7-DHC was added to B-CD-treated HCVcc, virion-associated sterol levels were 146, 157, 68, 96 or 73%, respectively, of that of the non-treated control. The proportion of cholesterol analogues to the total sterols incorporated was $\geq 30\%$ when 4-cholestenone, cholesteryl acetate, DHC or 7-DHC was used; however, the proportion in the case of 25-HC was only 3%. It may be that the hydrophilic modification of the aliphatic side chain leads to poor association with HCVcc.

Collectively, exogenous variants with the 3 β -hydroxyl group, such as 4-cholestenone and cholesteryl acetate, can be incorporated into B-CD-treated HCVcc, but resulted in no recovery of virus infectivity, indicating the importance of the 3 β -hydroxyl group of cholesterol associated with the virus envelope in HCV infectivity. In contrast, two variants with modification in their sterol ring structures, coprostanol and DHC, have the ability to substitute for cholesterol. However, 7-DHC, another variant within the sterol ring, is incorporated readily into the depleted virion and restores the virus density, HCV replenished with 7-DHC is not infectious. These facts suggest that reduced forms of the sterol ring (coprostanol and DHC) in virion-associated cholesterol can be permitted for maintaining virus infectivity. However, a molecule with an additional double bond in the ring structure (7-DHC) seems to fail to exhibit infectivity, presumably because the change reduces structural flexibility in the

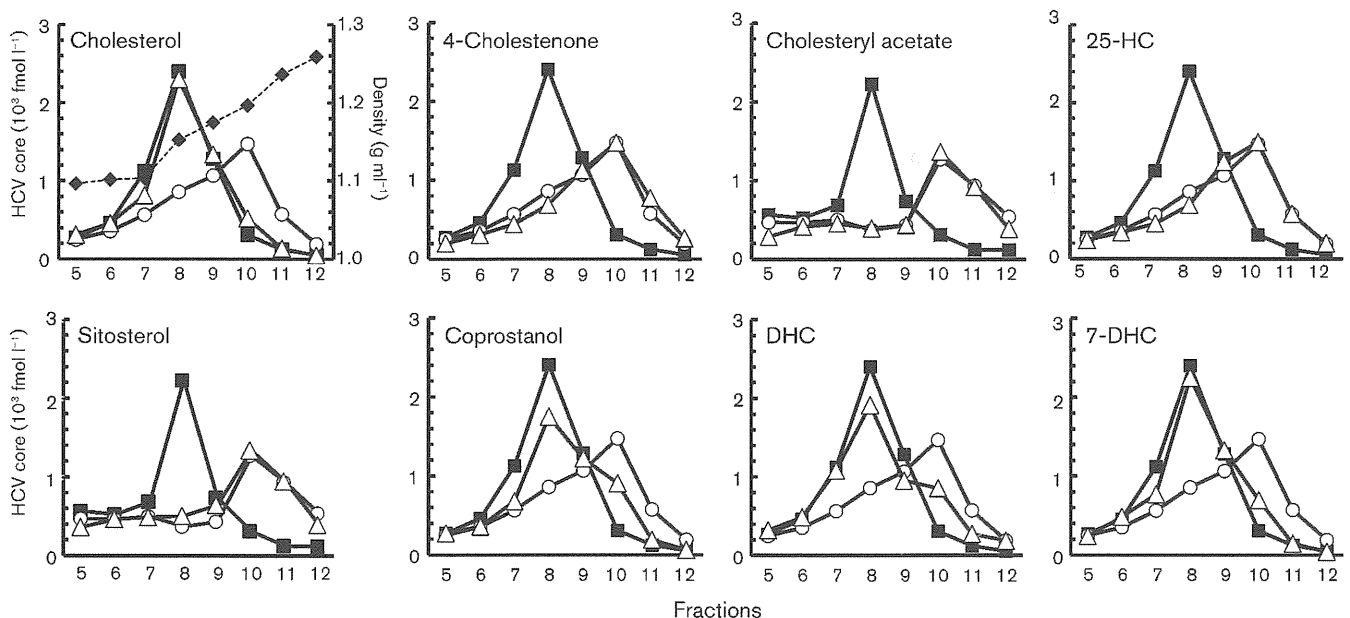


Fig. 2. Sucrose density-gradient profiles of lipid-modified HCV. Core protein concentration in each fraction of untreated HCVcc (■), B-CD-treated HCVcc (○) or HCVcc replenished with sterols (△) was determined. Corresponding densities of fractions are shown as a dashed line (◆).

sterol molecule and consequently in the virion structure. Coprostanol and DHC are *cis* and *trans* isomers, which are often known to have different physical properties. However, based on their molecular models, these two sterols, as well as cholesterol, possibly have similar spatial arrangements of the aliphatic side chain, the hydroxyl group and four-ring region because of their structural flexibility. In contrast, the spatial arrangement of 7-DHC does not seem comparable to that of cholesterol. Campbell *et al.* (2004) reported that replacement of HIV-1-associated cholesterol with raft-inhibiting sterols, including coprostanol, suppresses HIV-1 infectivity, whereas replacement with raft-promoting analogues such as DHC and 7-DHC (Megha *et al.*, 2006; Wang *et al.*, 2004; Xu & London, 2000; Xu *et al.*, 2001) maintains infectivity, demonstrating the importance of the raft-promoting properties of virion-associated cholesterol in HIV-1 infectivity (Campbell *et al.*, 2004). It is therefore likely that HCV-associated cholesterol is involved, at least in part, in virus infectivity via a molecular basis independent of lipid-raft formation.

The density of blood-circulating HCV is heterogeneous, ranging approximately from <1.06 to 1.25 g ml^{-1} , and it is proposed that low-density virus is associated with very-low-density lipoprotein (VLDL) and/or low-density lipoprotein (LDL) (André *et al.*, 2002; Thomssen *et al.*, 1993). It has recently been demonstrated that the pathway for VLDL assembly plays a role in assembly and maturation of infectious HCVcc (Icard *et al.*, 2009). HCVcc with low density, which is presumably associated with VLDL or VLDL-like lipoproteins, was found to possess higher infectivity than that with high density (Lindenbach *et al.*, 2006). This study, as well as our earlier work, indicated that removal of cholesterol from HCVcc by B-CD increased the buoyant density of the virus and reduced its infectivity. Thus, one may hypothesize that the virion-associated cholesterol plays a role in the formation of a complex with lipoproteins or apolipoproteins. To address this, the interaction between apolipoproteins and HCVcc with or without B-CD treatment was investigated by co-immunoprecipitation (Co-IP kit; Thermo Scientific). Virus samples were subjected separately to AminoLink Plus coupling resin, which was conjugated with a monoclonal antibody (mAb) against apolipoprotein E (ApoE) or apolipoprotein B (ApoB), and incubated at 4°C for 4 h. After washing, total RNAs were extracted from the resulting resin beads by using TRIzol reagent (Invitrogen), followed by quantification of HCV RNA as described above (Takeuchi *et al.*, 1999). As indicated in Fig. 3(a), only a fraction of HCVcc was precipitated with an anti-ApoB mAb. In contrast, an anti-ApoE mAb was able to coprecipitate a considerable amount of the virus. It is of interest that B-CD-treated HCVcc hardly reacted with the mAb; however, the cholesterol-replenished virus was found to recover its reactivity, suggesting a role for virion-associated cholesterol in the formation of the HCV-lipoprotein/apolipoprotein complex. The results obtained are consistent with findings indicating that HCVcc can be

captured with anti-ApoE antibodies, but capture with anti-ApoB antibodies is inefficient (Chang *et al.*, 2007; Hishiki *et al.*, 2010; Huang *et al.*, 2007; Jiang & Luo, 2009; Merz *et al.*, 2011; Nielsen *et al.*, 2006; Owen *et al.*, 2009), as well as with a recent model of structures of infectious HCV, in which HCVcc looks like ApoE-positive and primarily ApoB-negative lipoproteins (Bartenschlager *et al.*, 2011). We further tested the ApoE distribution in the density-gradient fractions of HCVcc samples (see Supplementary Fig. S1, available in JGV Online). With or without cholesterol depletion, ApoE was detected at a wide range of concentrations: 1.04 g ml^{-1} (fraction 1) to 1.17 g ml^{-1} (fraction 9). However, its level in the fractions at 1.10 g ml^{-1} (fraction 5) to approximately 1.17 g ml^{-1} was moderately decreased in the case of B-CD-treated virus.

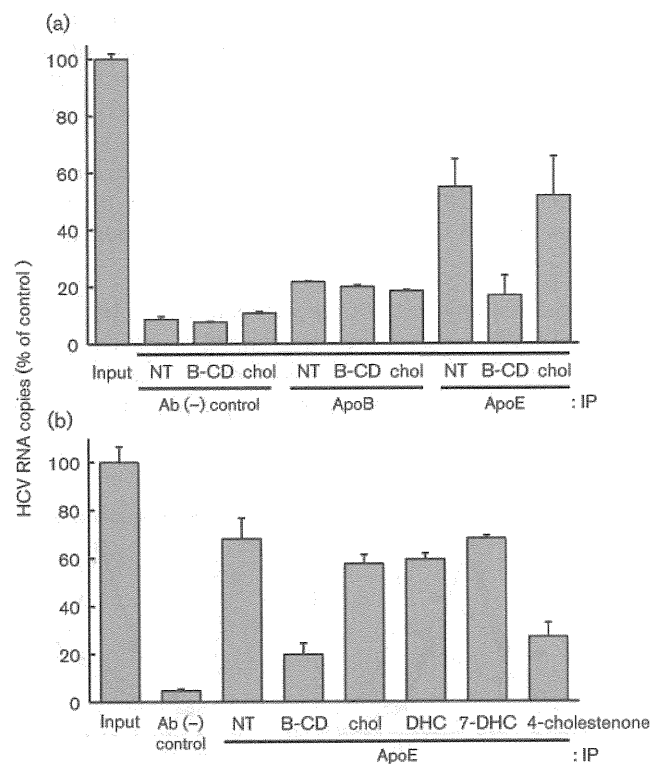


Fig. 3. Effect of virion-associated sterols on HCV-apolipoprotein interaction. (a) HCVcc samples with no treatment (NT), B-CD-treated (B-CD) or replenished with cholesterol (chol) were incubated with an amine-reactive resin coupling either an anti-ApoB mAb (ApoB) or an anti-ApoE mAb (ApoE). Control resin that is composed of the same material as above, but is not activated, was used as a negative control [Ab (-) control]. (b) B-CD-treated HCVcc was incubated with cholesterol (chol), DHC, 7-DHC or 4-cholestenone, followed by immunoprecipitation with the resin coupling with anti-ApoE mAb. (a, b) HCV RNAs in the immunoprecipitates were quantified and are indicated as percentages of the amount of input HCVcc RNA. Means \pm SD of three samples are shown. Data are representative of three experiments.

Whether cholesterol analogues could have a comparable role in HCV association with lipoprotein was examined further (Fig. 3b). Addition of DHC or 7-DHC, but not 4-cholestenone, to B-CD-treated HCVcc resulted in the recovery of coprecipitation of the virus with anti-ApoE. The results are correlated with the effect of sterols on the restoration of the buoyant densities of lipid-modified HCVcc (Fig. 2), suggesting that virion-associated cholesterol variants with modification in the sterol rings, but not in either the 3β -hydroxyl group or the aliphatic side chain, may tolerate the interaction between HCV and ApoE-positive lipoprotein.

Given that 7-DHC restored the association of HCV with ApoE and virion buoyant density, but did not restore infectivity, cholesterol and/or its analogues might affect the ability of virion membranes to fuse with the cell, independent of ApoE association. As cholesterol is an important mediator of membrane fluidity, one may hypothesize that HCV-associated cholesterol is involved in infectivity through modulation of the membrane fluidity. It has been reported that, in patients with Smith–Lemli–Opitz syndrome, a disorder of the cholesterol-synthesis pathway, cholesterol content decreases and 7-DHC increases in the cell membranes, leading to alteration of phospholipid packing in the membrane and abnormal membrane fluidity (Tulenko *et al.*, 2006).

It is now accepted that maturation and release of infectious HCV coincide with the pathway for producing VLDLs, which export cholesterol and triglyceride from hepatocytes. This study revealed roles for the structural basis of virion-associated cholesterol in the infectivity, buoyant density and apolipoprotein association of HCV. Although it was shown that HCV virions in infected patients, so-called lipo-viro particles, exhibited certain biochemical properties such as containing ApoB, ApoC and ApoE (Diaz *et al.*, 2006; Bartenschlager *et al.*, 2011), our studies provide useful information and the basis for future investigations toward a deeper understanding of the biogenesis pathway of infectious HCV particles.

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Identification of hepatitis C virus genotype 2a replicon variants with reduced susceptibility to ribavirin

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ABSTRACT

Ribavirin (RBV), a nucleoside analogue, is used in the treatment of hepatitis C virus (HCV) infection in combination with interferons. However, potential mechanisms of RBV resistance during HCV replication remain poorly understood. Serial passage of cells harboring HCV genotype 2a replicon in the presence of RBV resulted in the reduced susceptibility of the replicon to RBV. Transfection of fresh cells with RNA from RBV-resistant replicon cells demonstrated that the RBV resistance observed is largely replicon-derived. Four major amino acid substitutions: T1134S in NS3, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B region, were identified. Site-directed mutagenesis of these mutations into the replicon indicated that Y2471H plays a role in the reduced susceptibility to RBV and leads to decrease in replication fitness. The results, in addition to analysis of sequence database, suggest that HCV variants with reduced susceptibility to RBV identified are preferential to genotype 2a.

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1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases, such as chronic hepatitis, cirrhosis and hepatocellular carcinoma, affecting approximately 170 million people worldwide (WHO, 2000). HCV belongs to the genus Hepacivirus of the family Flaviviridae, and its genome is a single-stranded, positive-sense RNA of 9.6 kb. HCV displays marked genetic heterogeneity and is currently classified into 6 major genotypes and more than 50 subtypes. HCV genotypes have regional distribution and, of those, genotypes 1 and 2 are detected worldwide (Simmonds et al., 2000). Current standard therapy for chronic hepatitis C consists of the combination of pegylated interferon alpha (IFN- α) in combination with ribavirin (RBV). However, approximately 50% of treated patients infected with genotype 1 do not respond or show only a partial or transient response and treatment is limited by the adverse effects of both agents (Manns et al., 2001; Fried et al., 2002).

HCV replication is associated with a high rate of mutation that gives rise to a mixed and changing population of mutants, known as quasispecies (Martell et al., 1992; Domingo, 1996). The characteristic of HCV may have important implications concerning viral persistence, pathogenicity and resistance to antiviral agents

(Domingo, 1996; Forns et al., 1999; Farci and Purcell, 2000). Most previous studies on the possible relationship between HCV quasispecies and response to chemotherapy have been carried out in HCV genotype 1 patients. In addition, several studies have successfully demonstrated that the HCV subgenomic replicon is derived from genotype 1, which typically contains HCV nonstructural genes placed downstream of the neomycin phosphotransferase gene, in selecting variants resistant to antiviral inhibitors. Two studies have demonstrated the identification of HCV genotype 1 mutants responsible for decreased sensitivity to RBV (Young et al., 2003; Pfeiffer and Kirkegaard, 2005). However, little is known about the generation of genotype 2 isolates resistant to antivirals including RBV, or the molecular mechanisms that confer resistance.

In this study, we report the generation and characterization of HCV genotype 2a replicon variants with reduced susceptibility to RBV. The impacts of major amino acid substitutions observed on RBV susceptibility and viral replication capacity were also examined.

2. Materials and methods

2.1. Compounds

RBV and IFN- α were purchased from MP Biomedicals (Eschwege, Germany) and Dainippon Sumitomo Pharma (Osaka, Japan), respectively.

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Table 1
Primers used for PCR and nucleotide sequencing.

Region	Primer name	Nucleotide sequence	Position ^a	Polarity
NS3–4A–4B region	PCR primers			
	JF1S	GAAAAACACGATGATACCATG	1756–1776	Sense
	JF1AS	AACCCAGTCCCACACGTC	4650–4633	Antisense
	Sequencing primers			
	JF5S	CACTTTCAGTGACAACAGCA	2322–2341	Sense
	JF6S	CGCCACCGACGCCCTCATGA	3003–3022	Sense
NS5A–NS5B region	JF4AS	CTGGTCGACAACGGACTGGT	4109–4090	Antisense
	PCR primers			
	JF2S	TGCTCCGGATCCTGGCTC	4612–4629	Sense
	JF2AS	TACCTAGTGTGTCGCCCTCTA	7786–7806	Antisense
	Sequencing primers			
	JF3S	TGAGTCCATGCTAACAGA	5209–5228	Sense
	JF4S	TCGAGGGGGAGCCTGGAGAT	5870–5889	Sense
	JF3AS	GAGTGTCTAACTGTTTCCAG	7220–7200	Antisense

^a Reference strain: Gene Bank accession no. AB114136.

2.2. Cell culture

The human hepatoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with MEM non-essential amino acids (Invitrogen) 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO₂ incubator. HCV replicon cells JFH-1/4-1 (Miyamoto et al., 2006), which are Huh-7-derived cells carrying a subgenomic replicon of JFH-1 (Kato et al., 2003) were maintained in the Huh-7 medium as above, supplemented with 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan).

2.3. Quantification of HCV RNA

Total RNA was isolated from harvested cells using Trizol (Invitrogen). Copy numbers of the viral RNA were determined by real-time RT-PCR involving single-tube reactions and performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, CA, USA), as described previously (Aizaki et al., 2003; Takeuchi et al., 1999).

2.4. Cell viability assay

Cells were seeded at density of 5×10^4 cells/well in 24-well plates and RBV at various concentrations was added on the next day. Cultures were further incubated for 3 days at 37 °C under a humidified 5% CO₂ atmosphere. Cytotoxicity assay was performed by Cell Titer-GLO™ Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activities were quantified with LUMAT LB 9501 (Berthold Technologies, Bad Wilbad, Germany).

2.5. Isolation and nucleotide sequencing of HCV nonstructural regions from replicon-containing cells

Total cellular RNA was isolated from replicon cells with or without RBV treatment as described above. cDNA synthesis was carried out by using Super Script™ III First-Strand Synthesis System for RT-PCR (Invitrogen) with primer JF1AS for NS3/4B region and JF2AS for NS5A/B region. Two cDNA fragments, corresponding to NS3–NS4B and NS5A–NS5B regions, were amplified by PCR using Takara EX Taq DNA polymerase (Takara BIO, Kyoto, Japan) and specific primers (Table 1; Date et al., 2004). PCR products were subcloned into pGEM-T vector (Promega) and inserts were sequenced using QIA prep^R Spin Mini Prep kit (QIAGEN, Tokyo, Japan). Nucleotide sequences were analyzed with the 3100 Avant Genetic Analyzer (PE Applied Biosystems).

2.6. Plasmid constructions

pSGR-JFH1/luc, a subgenomic replicon construct with luciferase reporter derived from HCV genotype 2a JFH-1 isolate was reported previously (Miyamoto et al., 2006). Mutant replicons carrying T1134S, P1969S, V2405A, and Y2471H were created by PCR-based site-directed mutagenesis and cDNA fragments containing the above mutations were inserted into the corresponding sites of pSGR-JFH/luc. All plasmids were confirmed by sequencing the entire PCR-generated inserts. Each mutant is referred to by the original amino acid (one letter code) followed by the residue positions within the complete open reading frame of full-length JFH-1 and the substituted amino acid (one letter code).

2.7. RNA synthesis and transient replication assay

The transient replication assay method was described previously (Kato et al., 2005). Briefly, purified plasmids of pSGR-JFH1/Luc, -JFH1/Luc-T1134S, -JFH/Luc-P1969S, -JFH/Luc-V2405A and -JFH/Luc-Y2471H were linearized with XbaI and were treated with proteinase K and SDS, followed by phenol–chloroform extraction. RNA was synthesized with Ampliscribe™ T7 Transcription Kits (Epicentre BIO Technologies, Madison, WI, USA). Each transcribed RNA (5 µg) was electroporated into 2.5×10^6 of Huh7 cells pulsed at 290 mV, 975 µFD with Gene pulser II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Transfected cells were resuspended in growth medium without selection antibiotics and were plated in 24-well plates at 6×10^4 cells per well. Cells were harvested at different time points post-transfection and were lysed in Passive Lysis Buffer (Promega). Luciferase activity in cells was determined using the Luciferase Assay System (Promega).

3. Results

3.1. Selection of replicon variants derived from genotype 2a with reduced susceptibility to RBV

It has been reported that RBV inhibits HCV RNA replication in Huh-7 cells bearing the viral subgenomic replicon RNAs with the EC₅₀ (50% effective concentration) values of 15–225 µM (Zhou et al., 2003; Tanaka et al., 2004; Kato et al., 2005; aus dem Siepen et al., 2007). To select for RBV-associated replicon variants, cells bearing a genotype 2a HCV replicon were serially passed in the presence of 200 µM RBV as well as 1 mg/ml G418. After 20-week treatment, variant cells were then tested for RBV resistance. HCV RNA levels were determined after a 72-h incubation with various concentrations of RBV in the absence of G418, and about 5-fold-reduced susceptibility to RBV was observed in the variant replicon

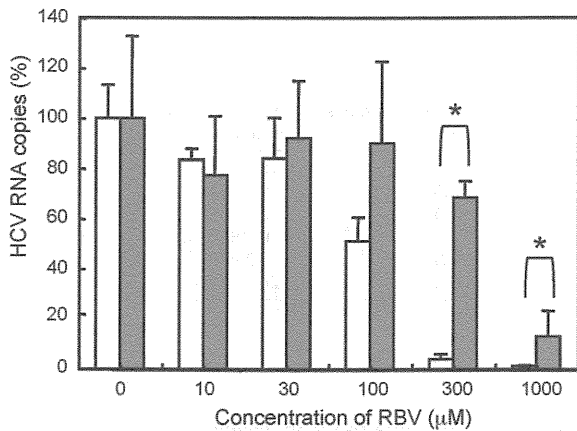


Fig. 1. Inhibitory effect of RBV on HCV RNA levels in genotype 2a replicon cells after long-term treatments with RBV. The replicon cells were serially passaged in 0 or 200 μM RBV for 20 weeks. The cells were then split and incubated with fresh RBV at various concentrations in the absence of G418 for 3 days, followed by the determination of HCV RNA. Clear bars, passage in the absence of RBV; gray bars, passage in the presence of RBV. HCV RNA copies per microgram of total RNA were normalized as percentages of those of untreated (RBV 0 μM). Each data point is presented as the mean of three independent determinations with standard deviation. * $p < 0.05$.

cells; the EC_{50} values for the variant and wild-type replicon cells were 470 and 102 μM , respectively (Fig. 1). Comparable cytotoxic effects of RBV were observed against wild-type and variant replicon cells, with the CC_{50} (50% cytotoxicity concentration) values of 151 and 156 μM , respectively (data not shown).

3.2. Mapping RBV resistance to cell line or replicon RNA

To test whether reduced susceptibility to RBV in the variant cells observed as above was due to the appearance of mutations within the viral RNA or was cell-derived, total RNAs from the variant and wild-type replicon cells were extracted and used for retransfection of naïve Huh7 cells. Retransfected cells resistant to G418 were established after 4 weeks of cultures in the presence of 1 mg/ml G418 and were assessed for HCV RNA replication sensitivity to RBV (Fig. 2A). HCV RNA levels in the cells obtained from the wild-type replicon were inhibited by 56, 89 and 97% with 100, 300 and 1000 μM RBV, respectively. By contrast, the culture retransfected with RNA derived from the variant replicon cells exhibited inhibition levels of 13, 29 and 89% with the corresponding concen-

trations of RBV. EC_{50} values were calculated to be 93 and 449 μM , respectively. We confirmed the presence of replicon mutations, as described below, in the cells retransfected with RNA derived from the variant replicon cells.

In order to explore the possibility for cell-derived resistance, both wild-type and variant replicon cells were cured of viral RNAs by IFN treatment; cells were passaged with media containing 100 IU/mL IFN- α in the absence of G418 for 2 months. To compare RBV sensitivity, cured cells were transiently transfected with the wild-type JFH-1 subgenomic replicon RNA and were treated with various concentrations of RBV for 72 h. Similar anti-HCV effects of RBV were observed in the cured cells derived from wild-type and variant replicons, with the EC_{50} values of 147 and 118 μM , respectively (Fig. 2B). Thus, the results suggest that the RBV resistance observed may arise by mutations in the replicon rather than by changes in the cells.

3.3. HCV mutations in replicon variant with reduced susceptibility to RBV

It has been reported that mutations in RNA virus genomes responsible for RBV resistance are mostly present in the coding region for the viral RNA-dependent RNA polymerase (RdRp). On the other hand, it is known that RBV works as an RNA mutagen to generate rapidly mutating viral RNA and that NS5B RdRp and other nonstructural proteins in HCV are involved in the viral replication complex, playing key roles in genome replication. Therefore, we sequenced the coding regions for NS3 through NS5B proteins of the replicon molecules in order to determine whether mutations associated with RBV resistance were generated. As shown in Table 2, there were numerically more synonymous and non-synonymous mutations in the RBV-resistant variant replicon cells (RBV treatment) when compared with untreated replicative conditions (No-treatment) across most regions examined. Mutation frequencies of NS3, NS4B and NS5A regions of RBV treatment were significantly higher than those of No-treatment. The total number of synonymous mutations in the RBV-resistant variant replicon cells was 3 times higher than that under untreated replicative conditions, and the number of non-synonymous mutations in the RBV-resistant variant replicon cells was 1.5 times higher than that under untreated replicative conditions. The number of both synonymous and non-synonymous mutations (NS3, NS4B, NS5A and NS5B regions) in the RBV-resistant replicon cells was greater than that in the control cells. We also found a large number of transition

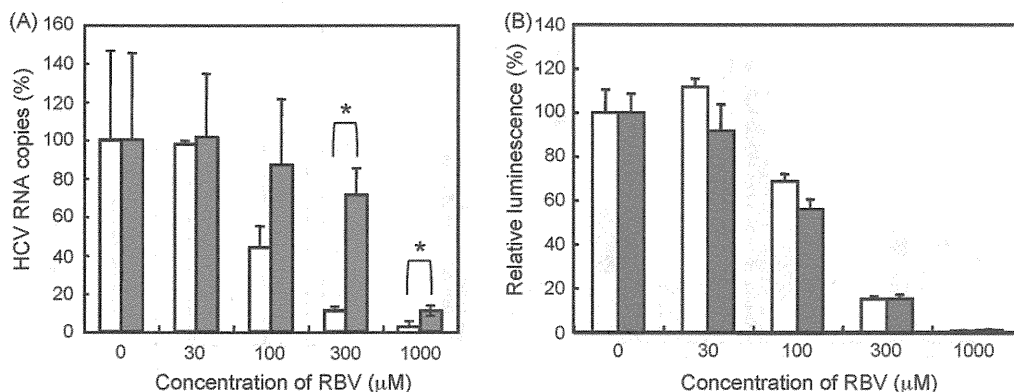


Fig. 2. Testing for replicon-derived resistance (A) or for cell-derived resistance (B). (A) Total RNA from RBV-resistant- or wild-type replicon cells was transfected into naïve Huh7 cells. After selection in 1 mg/ml G418 for 4 weeks, re-established replicon cells, wild-type derived (clear bars) and RBV resistance derived (gray bars), were treated with increasing concentrations of RBV in the absence of G418 for 3 days. HCV RNA copies per microgram total RNA were assessed and the levels from wild-type cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations. * $p < 0.05$. (B) RBV-resistant- or wild-type replicon cells were cured by passage in IFN- α in the absence of G418. Cured cells were transiently transfected with the replicon RNA derived from pSGR-JFH1/luc. Transient replication assay of transfectants derived from wild-type (clear bars) and RBV resistance (gray bars) was performed after treatment with various concentrations of RBV for 72 h. The values for wild-type-derived cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations.

Table 2
Mutation frequencies in HCV NS regions after 20-weeks culture with or without RBV treatment.

Region	nt length	No-treatment			RBV treatment		
		No. of non-synonymous mutations ^a	No. of synonymous mutations ^a	Mutation frequency (10 ⁻³)	No. of non-synonymous mutations ^a	No. of synonymous mutations ^a	Mutation frequency (10 ⁻³)
NS3	1893	1.7 ± 2.1	2.3 ± 1.5	2.1	4.7 ± 2.4	6.5 ± 2.5	5.9 ^b
NS4A	165	1.0 ± 1.0	0.3 ± 0.6	8.1	0.3 ± 0.5	0.5 ± 0.9	4.4
NS4B	780	1.3 ± 1.2	0.3 ± 0.6	2.1	2.3 ± 1.5	2.5 ± 1.2	4.7 ^c
NS5A	1380	4.0 ± 1.2	2.0 ± 1.2	4.3	5.9 ± 1.2	6.2 ± 2.4	12.2 ^c
NS5B	1773	4.5 ± 1.5	2.3 ± 1.5	3.8	4.8 ± 1.8	4.2 ± 1.1	9.0
NS3–NS5B	5991	12.5 ± 2.7	7.3 ± 2.7	–	17.8 ± 4.5	20.1 ± 4.6	–

^a Values are means ± standard deviations.^b $p < 0.05$ relative to No-treatment by the unpaired *t*-test.^c $p < 0.01$ relative to No-treatment by the unpaired *t*-test.

mutations in RBV-resistant cells, particularly G-to-A and C-to-U transitions, as expected from previous studies. Although mutations were distributed throughout nonstructural regions, four major amino acid substitutions; T1134S in the NS3 region, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B, not seen in wild-type cells were observed in most of the subclones among RBV-resistant replicon cells. T1134S, P1969S, V2405A, and Y2471H were present, respectively, in 7 of 11, 6 of 11, 8 of 13, and 7 of 13 PCR subclones sequenced.

3.4. Effects of T1134S, P1969S, V2405A, and Y2471H on RBV susceptibility

To test the possibility that any of the four mutations as identified confer resistance to RBV, we introduced these mutations individually into the JFH-1 subgenomic replicon containing a luciferase reporter gene. Cells transfected with mutant- or wild-type replicon RNA grown in the presence of various concentrations of RBV for 2 or 3 days. As demonstrated in Fig. 3A, the replication levels of all four mutant replicons (SGR-JFH1/Luc-T1134S, -P1969S, -V2405A, and -Y2471H) in the presence of 125 or 500 μ M RBV were higher than those of the wild-type replicon. In particular, the Y2471H mutant significantly reduced susceptibility to RBV; replication levels of SGR-JFH1/Luc-Y2471H were 3–5-fold higher when compared to those of wild-type under the present assay conditions.

The relative replication activity of these mutant replicons was further determined in 3-day replication assay without drug treatment (Fig. 3B). All mutant replicons exhibited reduced efficiency

relative to the wild-type replicon. Levels of the Y2471H-mutated replicon were approximately 30% of those of the wild-type, thus suggesting that replicon mutants with reduced sensitivity to RBV are associated with decreased replication fitness.

4. Discussion

It is generally accepted that, during chemotherapy against viral infection, high rates of viral replication and high frequencies of mutation lead to generation of drug-resistant mutants. Although several potential mechanisms for the inhibition of HCV replication by RBV have been proposed, the molecular mechanisms involved in the generation of RBV-resistant HCV remain poorly understood.

This study found that long-term treatment of HCV JFH-1-derived replicon cells with RBV leads to selection of preferential mutations in NS3 (T1134S), NS4B (P1969S), NS5A (V2405A) and NS5B (Y2471H) genes. Each mutation only required a single nucleotide change, and P1969S, V2405A and Y2471H are transition mutations, which are known to be commonly caused by incorporated RBV. Site-directed mutagenesis of these mutations into the replicon demonstrated that Y2471H plays a role in reduced susceptibility to RBV.

Crystal structure information revealed that HCV RdRp is organized into an arrangement with palm, fingers, and thumb subdomains (Lesburg et al., 1999). Residue 2471 (the 33rd position of NS5B) is present in the N-terminal loop region that bridges the fingers. Although this site is apparently distant from the active site of the polymerase in the palm region, it has been reported

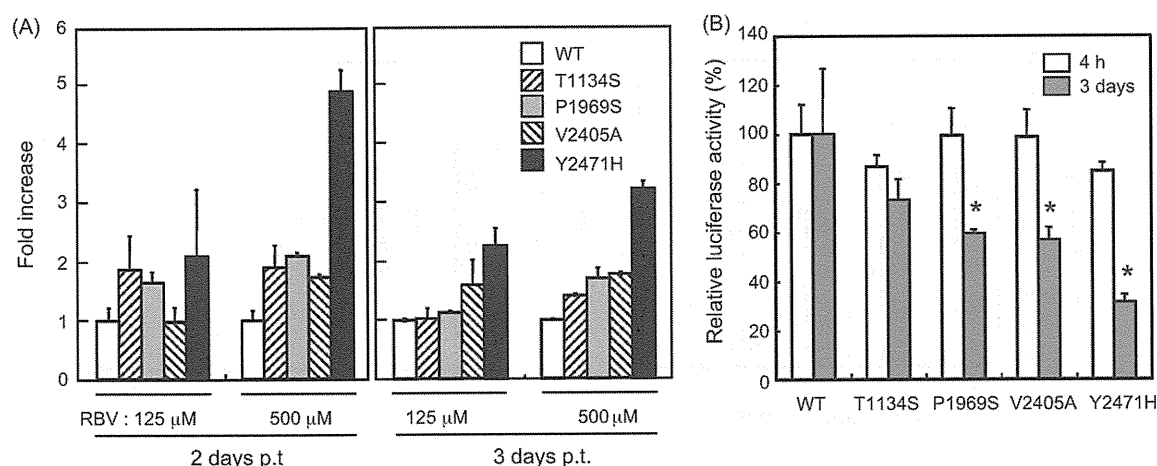


Fig. 3. Impact of major mutations in NS3–NS5B regions on RBV susceptibility (A) and replication capacity (B). Mutated replicons carrying single residue substitutions (T1134S, P1969S, V2405A, and Y2471H) were constructed and used for transient replication assay. Cells were transfected with either wild-type (WT) or with mutant replicon RNA in the absence or presence (125, 500 μ M) of RBV. Luciferase activity was assessed at 4 h, 2 days and 3 days post-transfection (p.t.). (A) Luciferase activities of WT were set at 1, and the fold increases in the activities of mutants were plotted. (B) Luciferase activities in the absence of RBV at 4 h and 3 days post-transfection were shown. The activities of mutants were normalized as percentages of the WT activities. Data from triplicate samples were averaged and indicated with standard deviations. * $p < 0.05$ against WT.

that small molecules, such as benzimidazole compounds, are able to specifically bind the fingers-thumb interface and inhibit polymerase activity (Herlihy et al., 2008), thus suggesting that amino acid substitutions in the loop region may affect RNA polymerization. The involvement of tyrosine residue at position 415 of HCV NS5B in RBV resistance has been previously described for patients with genotype 1a infection and for the genotype 1b replicon (Young et al., 2003). Although the mechanism for resistance remains elusive, it has been hypothesized that RBV interacts with RdRp around this residue, which is located in the thumb subdomain, thus affecting RNA polymerization (Young et al., 2003).

Based on analysis of available sequences from Genbank, tyrosine at the 33rd residue of NS5B is conserved in all isolates of genotype 2a, but not in other genotypes. In genotype 1a and 1b isolates, 96% contain histidine and only a small population contains tyrosine or asparagine at the site. All the isolates of genotypes 3, 4, 5 and 6 contain histidine, whereas phenylalanine is conserved for genotype 2b. It should be noted that V2405 and P1969 are also completely conserved for genotype 2a but not for other genotypes. Therefore, it is likely that the identified HCV variants with reduced susceptibility to RBV are genotype-specific. It will be of interest to determine whether HCV genotype 2a is intrinsically more sensitive to RBV when compared with other genotypes.

At present, at least 4 mechanisms of action of RBV are proposed (Lau et al., 2002). They include (1) direct inhibition of the HCV replication machinery, (2) as an RNA mutagen that drives a rapidly mutating RNA virus over the threshold to “error catastrophe”, (3) inhibition of the host enzyme inosine monophosphate dehydrogenase (IMPDH), and (4) enhancement of host T-cell-mediated immunity against viral infection. In addition to the direct inhibition, it is also possible that other mechanisms such as error-prone and IMPDH-inhibition are involved in HCV escape from RBV treatment. Further investigation of the interaction of HCV variants with the viral and cellular factors involved in viral resistance may improve understanding of the mechanism(s) of RBV resistance.

In conclusion, RBV encountered resistance from the HCV genotype 2a replicon largely mediated by mutations in the N-terminal region of NS5B. Although whether these mutagenic effects are also demonstrable in IFN-RBV combination therapy will require further studies, the mutations identified in this study represent the first drug-resistant variants belonging to HCV genotype 2a. The drug resistance patterns found in this study may be of benefit in prediction in vivo resistance profiles and the development of next-generation nucleoside analogues as anti-HCV drugs.

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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 AseI, 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% CO₂ incubator. *N*-Nonyl-deoxyjirimycin (NN-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-deoxyjirimycin (NB-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, Germany) 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 -³²P]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 (TCID₅₀) assay, as previously described (23, 26). Briefly, naive Huh7.5.1 cells were seeded at a density of 10⁴ 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 × 10⁴ 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	GTACCCCATGAGGTCCGCAAAG
	45-nt RNA adapter	GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA
	5'RACEouter-S	GCTGATGGCGATGAATGAACACTG
	5'RACEouter-R	GACCGCTCCGAAGTTTTCCCTTG
	5'RACEinner-S	GAACACTGCGTTTGCTGGCTTTGATG
	5'RACEinner-R	CGCCCTATCAGGCAGTACCACAAG
3'RACE	CAC-T35	CACTTT
	3X-10S	ATCTTAGCCCTAGTCACGGC
nt 129-2367	44S (1st PCR)	CTGTGAGGAACACTGTCTT
	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	CGGGCTCACGGACCTTTCAC

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 μ l of supernatant from the transfected cells. The cells were incubated for 72 h, followed by lysis with 100 μ l 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 μ g 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/ Δ E 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).

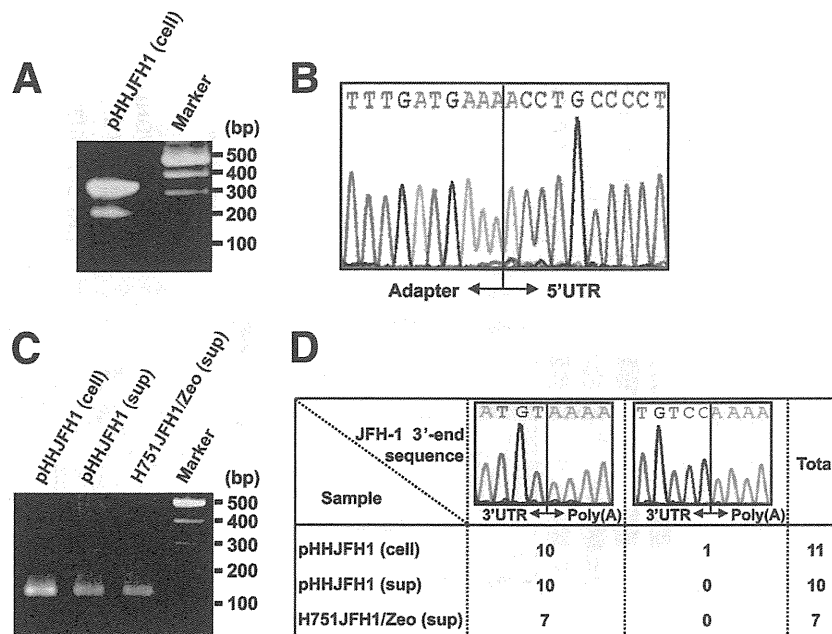


FIG. 1. Determination of the nucleotide sequences at the 5'- and 3'- ends of HCV RNA produced by the Pol I system. (A and B) 5'RACE and sequence analysis. A synthesized RNA adapter was ligated to RNA extracted from cells transfected with pHHJFH1. The positive-strand HCV RNA was reverse transcribed, and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

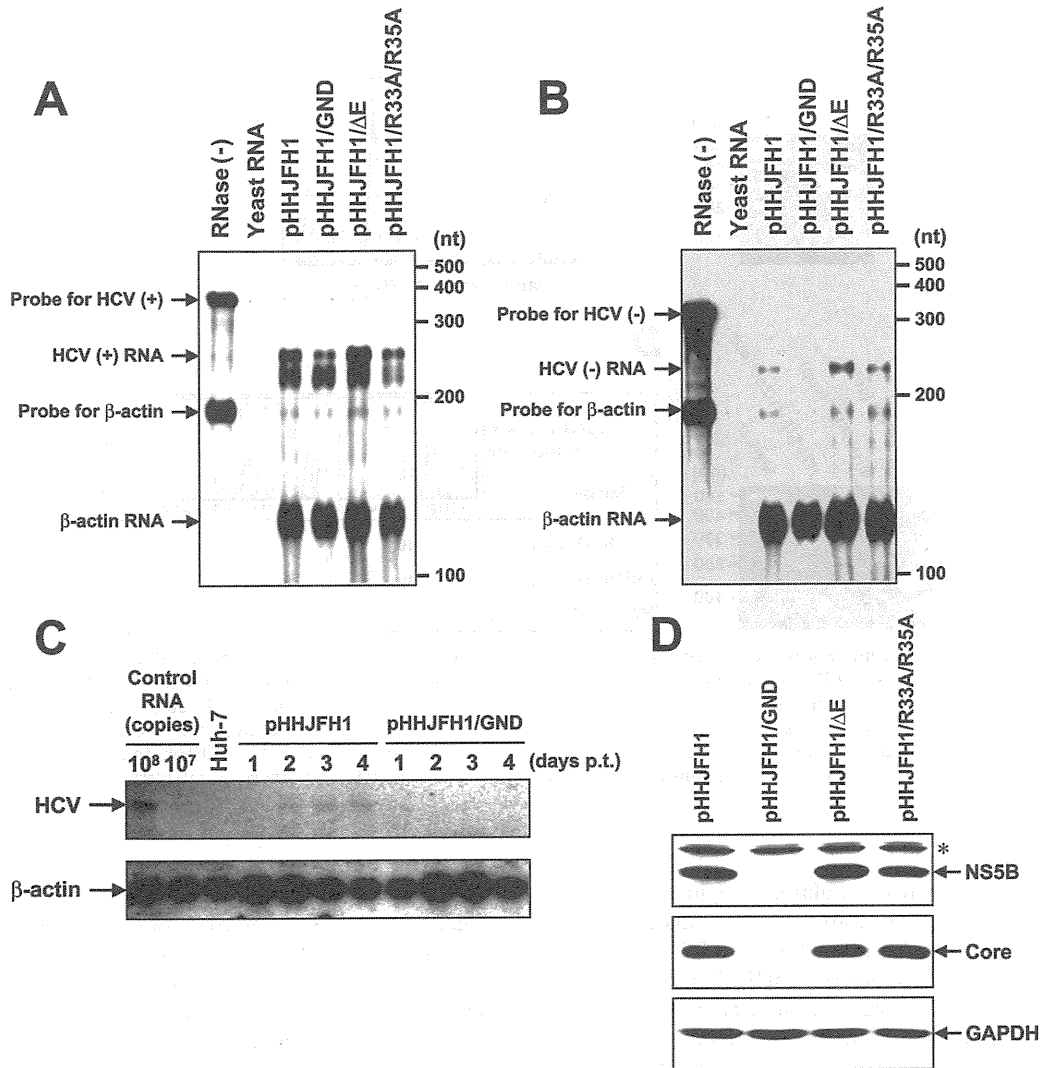


FIG. 2. HCV RNA replication and protein expression in cells transfected with Pol I-driven plasmids. (A and B) Assessment of HCV RNA replication by RPA. Pol I-driven HCV-expression plasmids were transfected into Huh-7 cells. Total RNA was extracted from the cells on day 5 p.t. and positive (A)- and negative (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 $\sim 10^3$ TCID₅₀/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

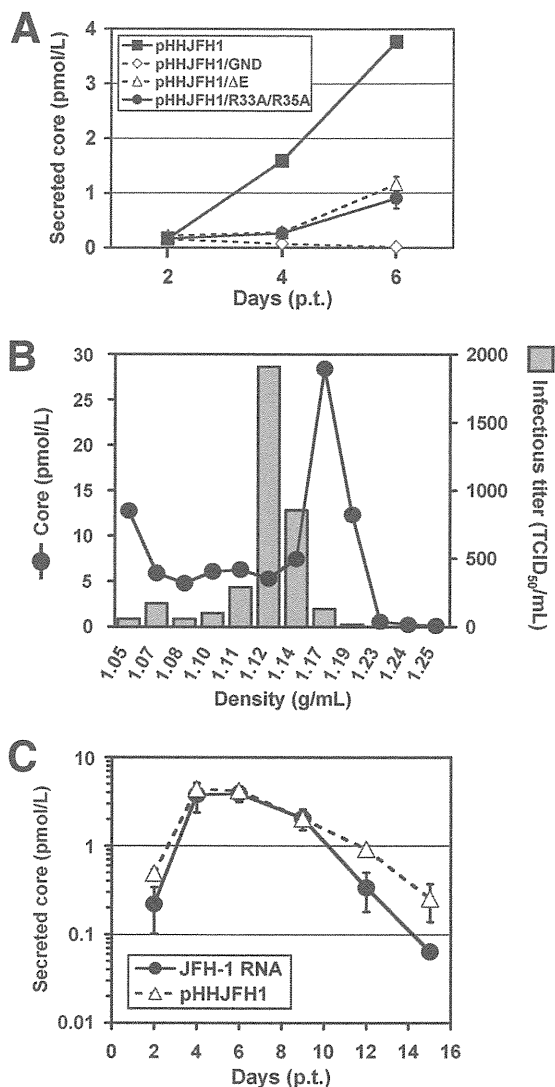


FIG. 3. HCV released from cells transfected with Pol I-driven plasmids. (A) HCV particle secretion from the transfected cells. The culture supernatant of Huh-7 cells transfected with Pol I-driven plasmids containing wild-type or mutated HCV genome were harvested on days 2, 4, and 6 and assayed for HCV 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- μ m-pore-size filter, and concentrated \sim 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 μ g 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 NN-DNJ, which is an inhibitor of ER α -glucosidases, resulted in a dose-dependent reduction in secreted core protein. NN-DNJ was observed to have an IC_{50} (i.e., the concentration inhibiting 50% of core protein secretion) of ~ 20 μ M. 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 NN-DNJ and KIF on the production of infectious HCV (Fig. 7C). As expected, NN-DNJ reduced the production of infectious virus in a dose-dependent manner, while production increased in the presence of KIF at 10 to 100 μ M. Since NN-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 NN-DNJ was reproducibly observed using the cell line after 1 year of culturing

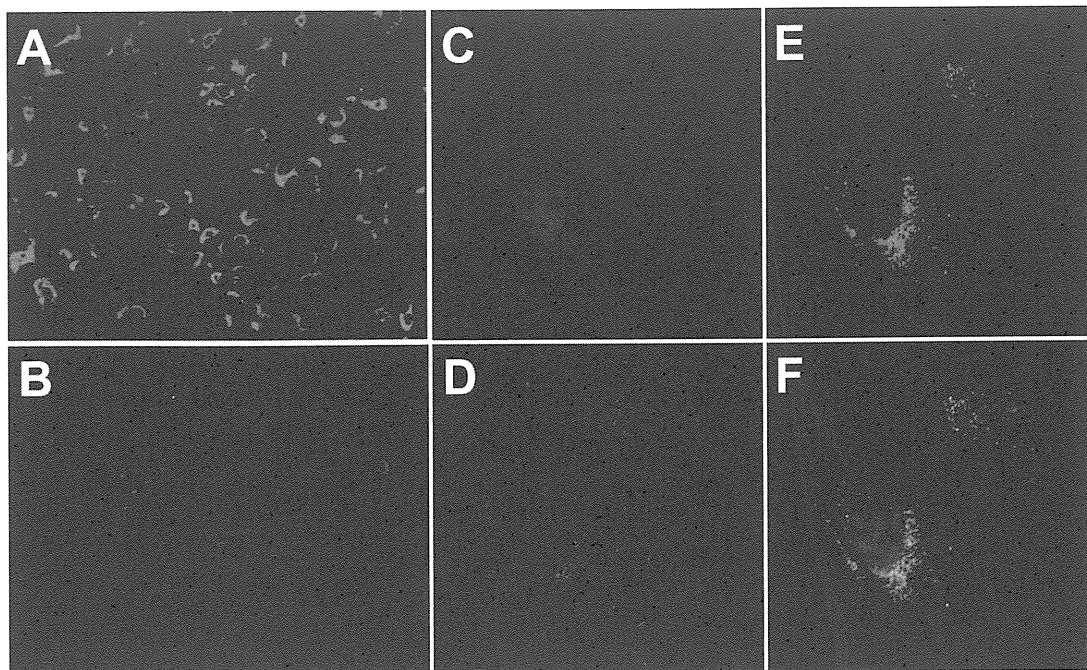


FIG. 4. Indirect immunofluorescence analysis of H751JFH1/Zeo cells. (A and B) H751JFH1/Zeo cells (A) and parental Huh7.5.1 cells (B) were immunostained with an anti-NS5A antibody. (C to F) The subcellular colocalization of *de novo*-synthesized HCV RNA and NS5A in H751JFH1/Zeo cells was analyzed. The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

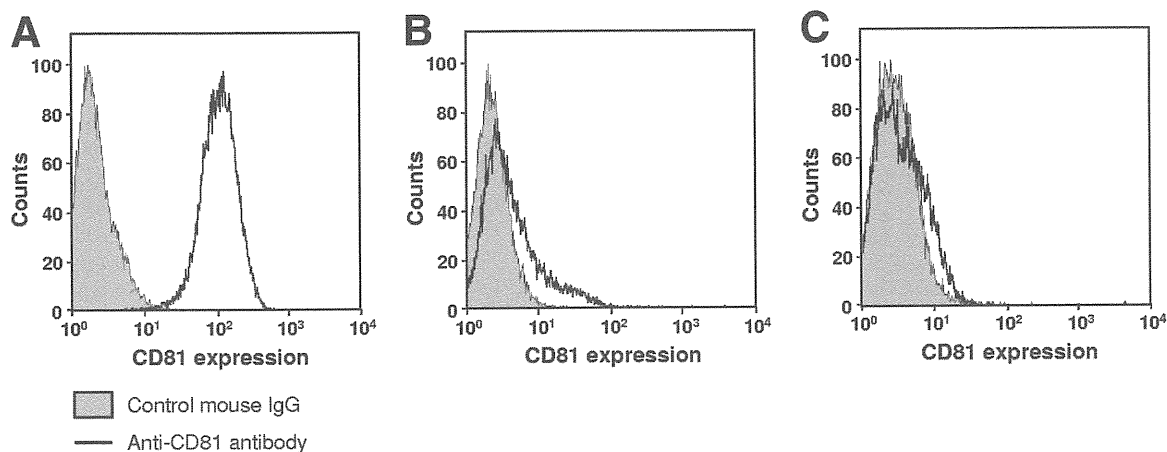


FIG. 5. Loss of CD81 expression in H751JFH1/Zeo cells. The cell surface expression of CD81 on Huh7.5.1 cells (A), H751JFH1/Zeo clone H751-1 (B), and clone H751-50 (C) was analyzed by flow cytometry after being stained with anti-CD81 antibody.

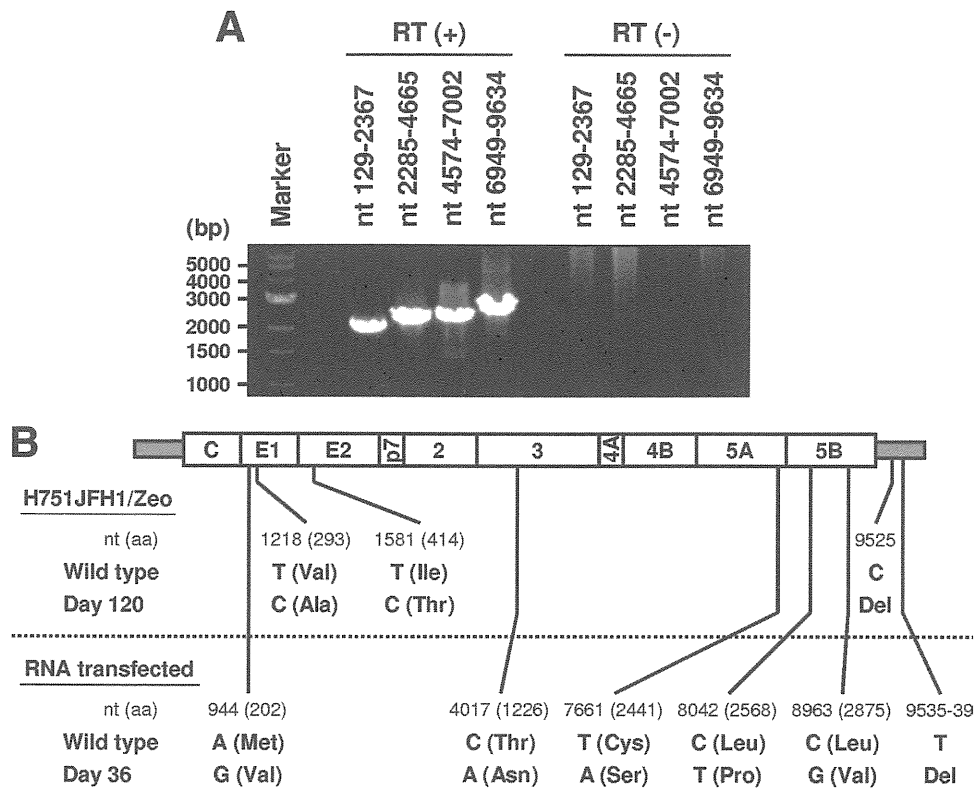


FIG. 6. Genome mutations of HCV secreted from H751JFH1/Zeo cells. (A) RT-PCR of HCV genome extracted from the culture supernatant of H751JFH1/Zeo cells. Viral RNA sequences were reverse transcribed [RT (+)] or not [RT (-)], followed by amplification with primer pairs encompassing the specified HCV genome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

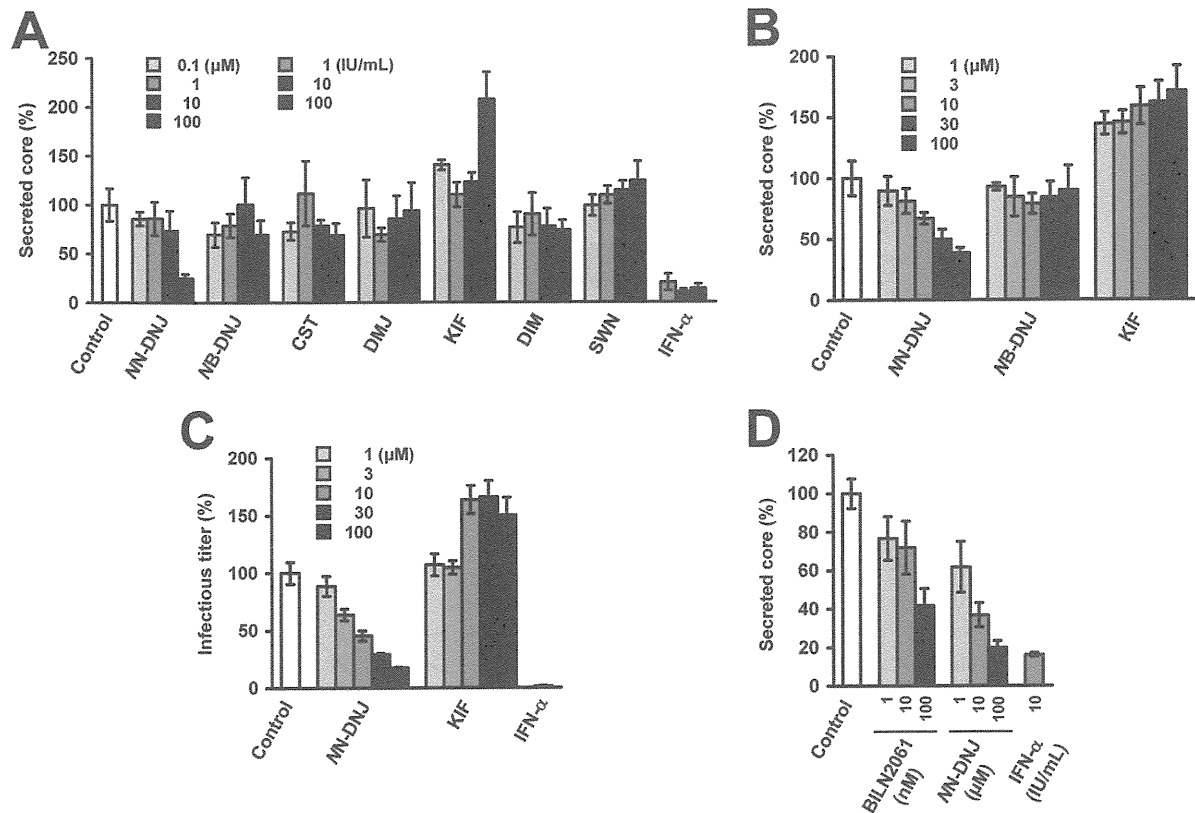


FIG. 7. Effects of glycosylation inhibitors on HCV production from H751JFH1/Zeo cells. (A and B) Effects of glycosylation inhibitors on the secretion of HCV core protein. H751JFH1/Zeo cells were seeded at a density of 1×10^4 cells/well in a 96-well culture plate (A) or 3×10^4 cells/well in a 12-well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

rect 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