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).

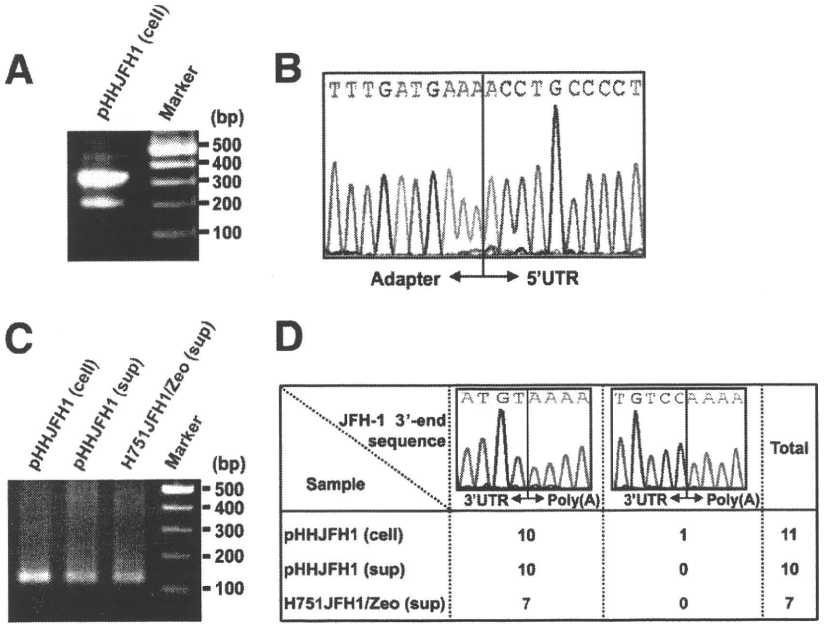

FIG. 1. Determination of the nucleotide sequences at the 5′-and 3′ ends of HCV RNA produced by the Pol I system. (A and B) 5′RACE and sequence analysis. A synthesized RNA adapter was ligated to RNA extracted from cells transfected with pHHJFH1. The positive-strand HCV RNA was reverse transcribed, and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

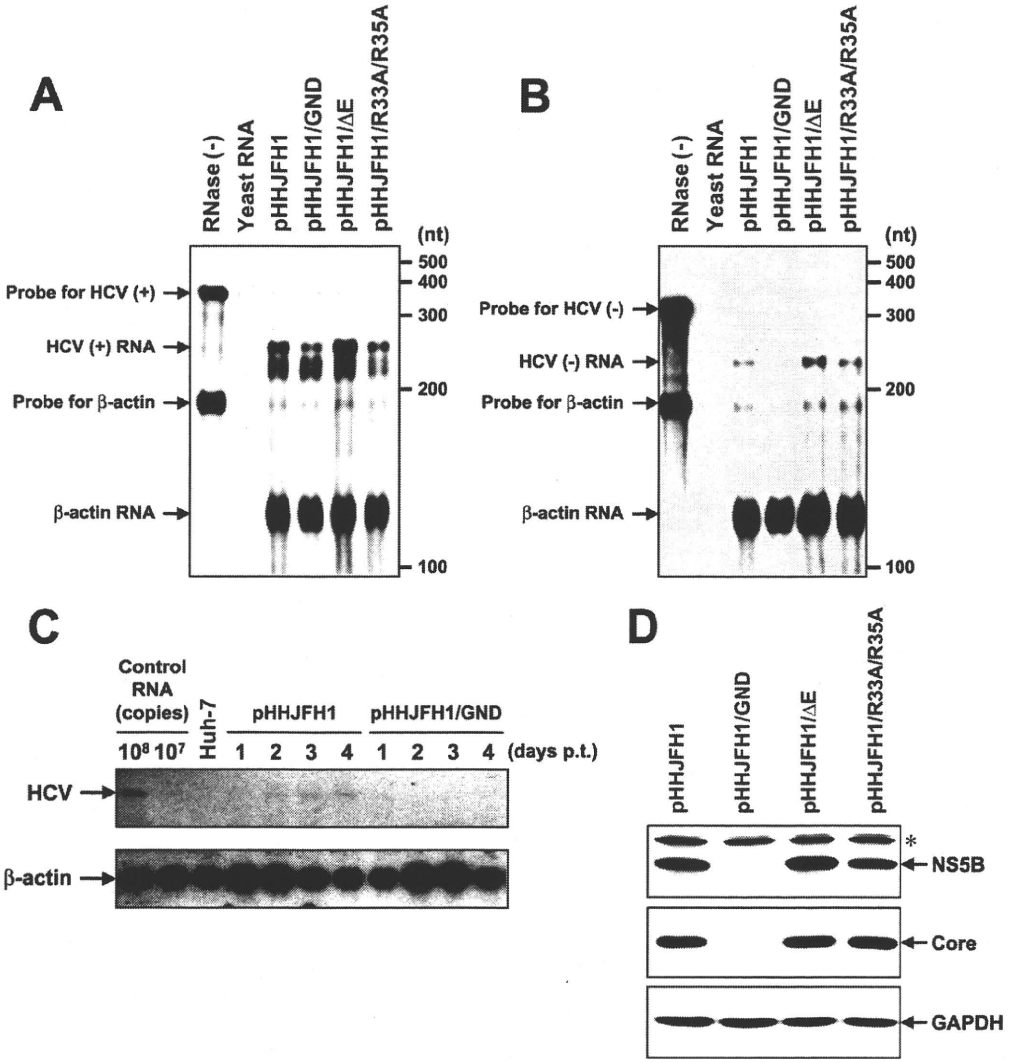

FIG. 2. HCV RNA replication and protein expression in cells transfected with Pol I-driven plasmids. (A and B) Assessment of HCV RNA replication by RPA. Pol I-driven HCV-expression plasmids were transfected into Huh-7 cells. Total RNA was extracted from the cells on day 5 p.t. and positive (A)- and negative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

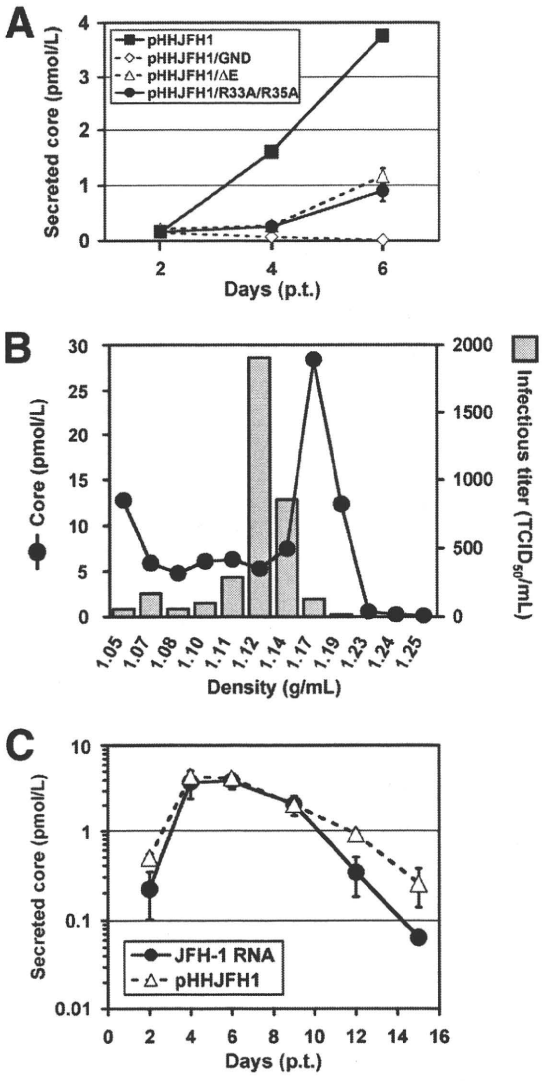

FIG. 3. HCV released from cells transfected with Pol I-driven plasmids. (A) HCV particle secretion from the transfected cells. The culture supernatant of Huh-7 cells transfected with Pol I-driven plasmids containing wild-type or mutated HCV genome were harvested on days 2, 4, and 6 and assayed for HCV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

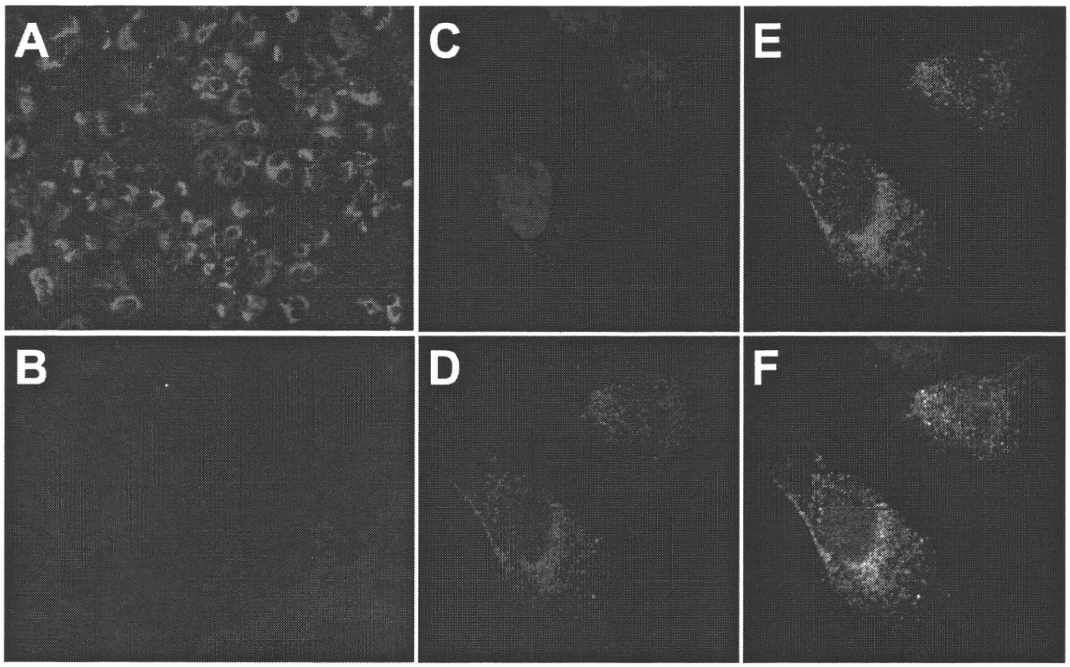

FIG. 4. Indirect immunofluorescence analysis of H751JFH1/Zeo cells. (A and B) H751JFH1/Zeo cells (A) and parental Huh7.5.1 cells (B) were immunostained with an anti-NS5A antibody. (C to F) The subcellular colocalization of *de novo*-synthesized HCV RNA and NS5A in H751JFH1/Zeo cells was analyzed. The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

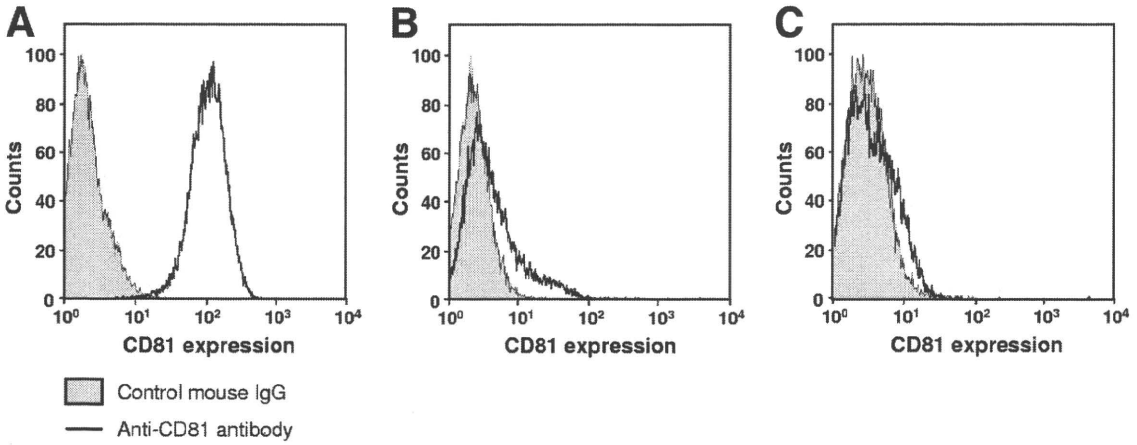

FIG. 5. Loss of CD81 expression in H751JFH1/Zeo cells. The cell surface expression of CD81 on Huh7.5.1 cells (A), H751JFH1/Zeo clone H751-1 (B), and clone H751-50 (C) was analyzed by flow cytometry after being stained with anti-CD81 antibody.

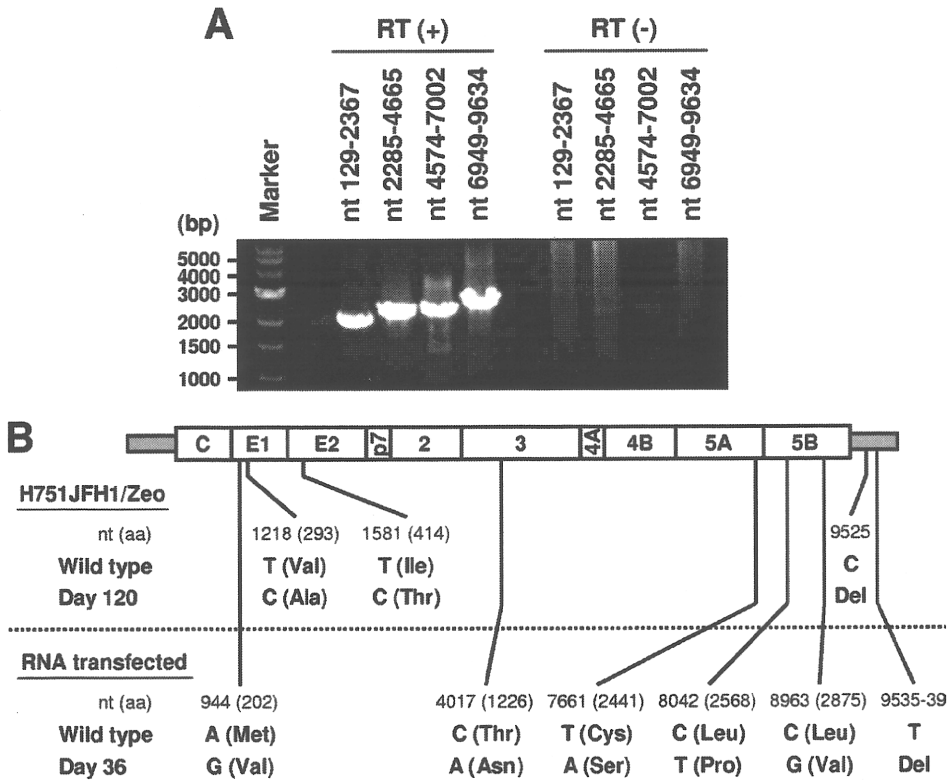

FIG. 6. Genome mutations of HCV secreted from H751JFH1/Zeo cells. (A) RT-PCR of HCV genome extracted from the culture supernatant of H751JFH1/Zeo cells. Viral RNA sequences were reverse transcribed [RT (+)] or not [RT (−)], followed by amplification with primer pairs encompassing the specified HCV genome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

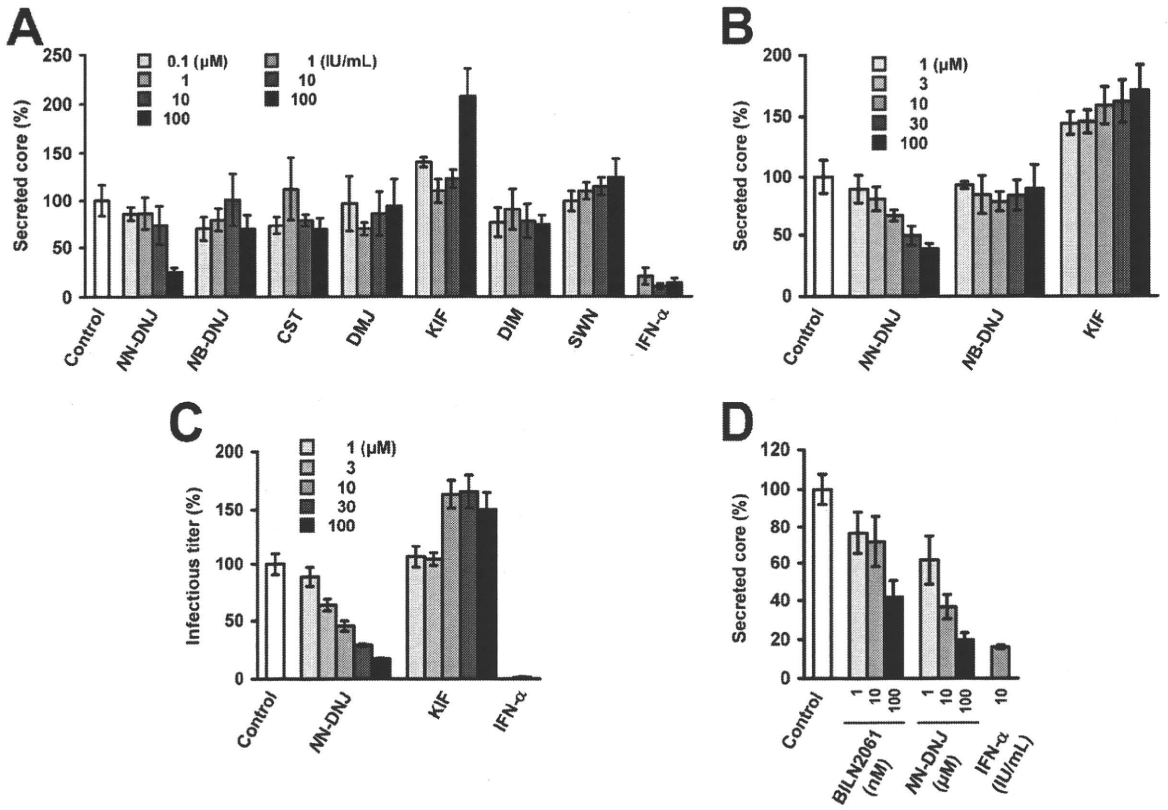

FIG. 7. Effects of glycosylation inhibitors on HCV production from H751JFH1/Zeo cells. (A and B) Effects of glycosylation inhibitors on the secretion of HCV core protein. H751JFH1/Zeo cells were seeded at a density of 1×10^4 cells/well in a 96-well culture plate (A) or 3×10^4 cells/well in a 12-well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

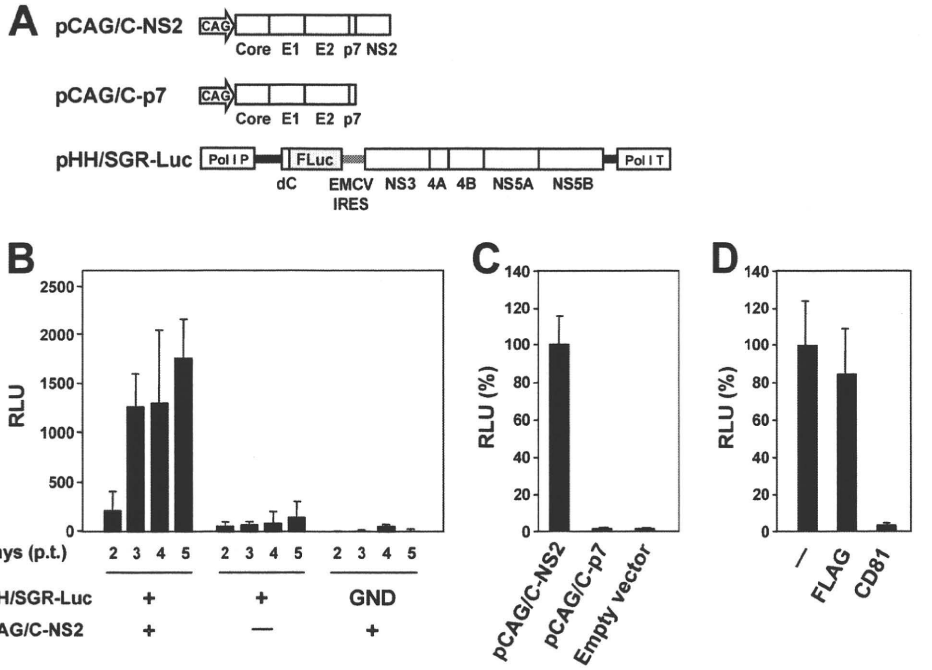

FIG. 8. Establishment of a *trans*-packaging system involving two-plasmid transfection. (A) Schematic representation of the plasmids used for the production of HCV-LP. HCV polyproteins are indicated by the open boxes. Bold lines indicate the HCV UTR. EMCV IRES is denoted by gray bars. The firefly luciferase 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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Identification of hepatitis C virus genotype 2a replicon variants with reduced susceptibility to ribavirin

Su Su Hmwe[a,b], Hideki Aizaki[a], Tomoko Date[a], Kyoko Murakami[a], Koji Ishii[a], Tatsuo Miyamura[a], Kazuhiko Koike[b], Takaji Wakita[a], Tetsuro Suzuki[a,*]

[a] Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[b] Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

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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.

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.

* Corresponding author. Tel.: +81 3 5285 1111; fax: +81 3 5285 1161.
E-mail address: tesuzuki@nih.go.jp (T. Suzuki).

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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
	JF4AS	CTGGTCGACAACGGACTGGT	4109–4090	Antisense
NS5A–NS5B region	PCR primers			
	JF2S	TGCTCCGGATCCTGGCTC	4612–4629	Sense
	JF2AS	TACCTAGTGTGTGCCGCTCTA	7786–7806	Antisense
	Sequencing primers			
	JF3S	TGAGGTCCATGCTAACAGA	5209–5228	Sense
	JF4S	TCGAGGGGGAGCCTGGAGAT	5870–5889	Sense
	JF3AS	GAGTGTCTAACTGTTTCCCAG	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% CO_2 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% CO_2 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 ScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen) with primer JF1AS for NS34AB region and JF2AS for NS5AB 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, 975uFD 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 EC_{50} (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

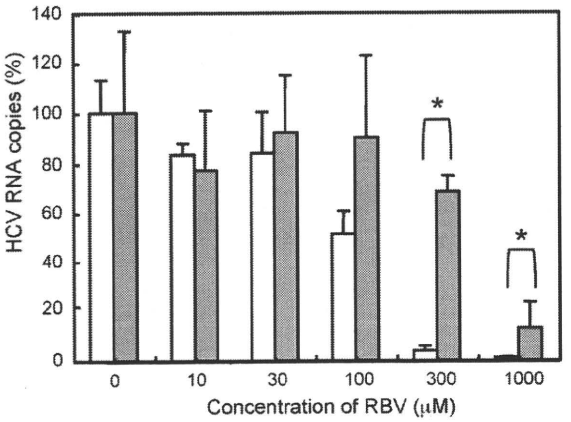

Fig. 1. Inhibitory effect of RBV on HCV RNA levels in genotype 2a replicon cells after long-term treatments with RBV. The replicon cells were serially passaged in 0 or 200 μM RBV for 20 weeks. The cells were then split and incubated with fresh RBV at various concentrations in the absence of G418 for 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 concentrations 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

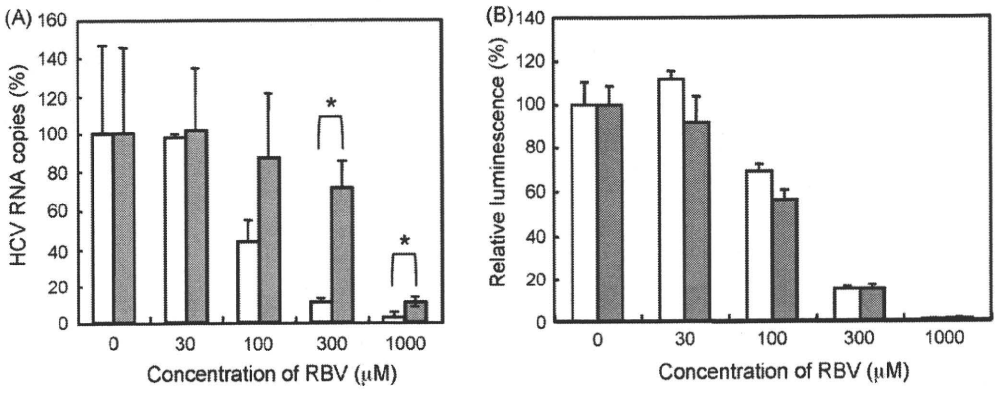

Fig. 2. Testing for replicon-derived resistance (A) or for cell-derived resistance (B). (A) Total RNA from RBV-resistant- or wild-type replicon cells was transfected into naïve Huh7 cells. After selection in 1 mg/ml G418 for 4 weeks, re-established replicon cells, wild-type derived (clear bars) and RBV 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 μM 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

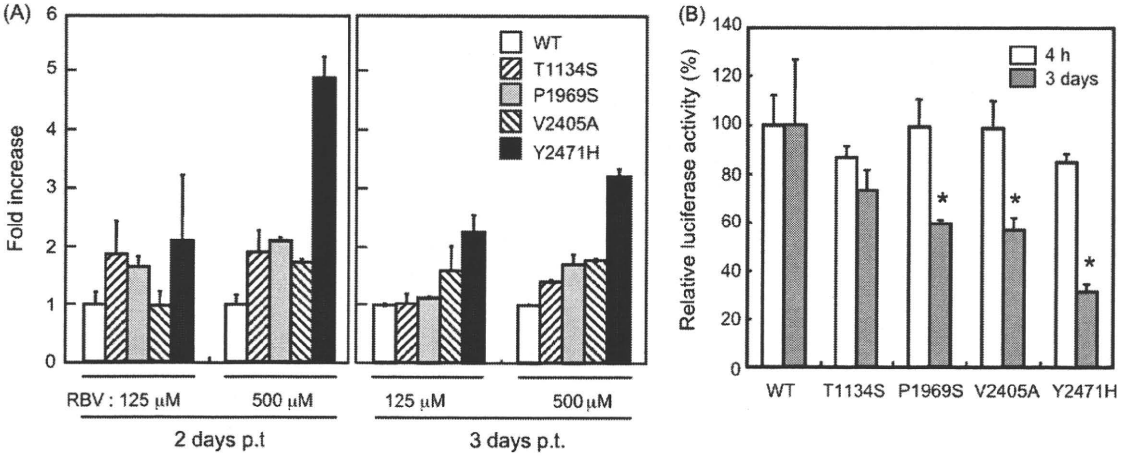

Fig. 3. Impact of major mutations in NS3–NS5B regions on RBV susceptibility (A) and replication capacity (B). Mutated replicons carrying single residue substitutions (T1134S, P1969S, V2405A, and Y2471H) were constructed and used for transient replication assay. Cells were transfected with either wild-type (WT) or with mutant replicon RNA in the absence or presence (125, 500 μM) 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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Biological properties of purified recombinant HCV particles with an epitope-tagged envelope

Hitoshi Takahashi [a,b], Daisuke Akazawa [a,b], Takanobu Kato [a], Tomoko Date [a], Masayuki Shirakura [a,b], Noriko Nakamura [b], Hidenori Mochizuki [b], Keiko Tanaka-Kaneko [c], Tetsutaro Sata [c], Yasuhito Tanaka [d], Masashi Mizokami [e], Tetsuro Suzuki [a], Takaji Wakita [a,*]

[a] Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan
[b] Toray Industries, Inc., Kanagawa, Japan
[c] Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan
[d] Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medicine, Nagoya, Japan
[e] Research Center for Hepatitis & Immunology, Kohnodai Hospital, International Medical Center of Japan, Chiba, Japan

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ABSTRACT

To establish a simple system for purification of recombinant infectious hepatitis C virus (HCV) particles, we designed a chimeric J6/JFH-1 virus with a FLAG (FL)-epitope-tagged sequence at the N-terminal region of the E2 hypervariable region-1 (HVR1) gene (J6/JFH-1/1FL). We found that introduction of an adaptive mutation at the potential *N*-glycosylation site (E2N151K) leads to efficient production of the chimeric virus. This finding suggests the involvement of glycosylation at Asn within the envelope protein(s) in HCV morphogenesis.

To further analyze the biological properties of the purified recombinant HCV particles, we developed a strategy for large-scale production and purification of recombinant J6/JFH-1/1FL/E2N151K. Infectious particles were purified from the culture medium of J6/JFH-1/1FL/E2N151K-infected Huh-7 cells using anti-FLAG affinity chromatography in combination with ultrafiltration. Electron microscopy of the purified particles using negative staining showed spherical particle structures with a diameter of 40–60 nm and spike-like projections. Purified HCV particle-immunization induced both an anti-E2 and an anti-FLAG antibody response in immunized mice. This strategy may contribute to future detailed analysis of HCV particle structure and to HCV vaccine development.

1. Introduction

The hepatitis C virus (HCV) causes chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [1]. HCV is a positive strand RNA virus belonging to the *Hepacivirus* genus in the Flaviviridae family. The HCV genome consists of about 9600 nucleotides and contains three regions: a 5′ non-coding region of 341 nucleotides containing the sequence for the IRES structure, a coding region of about 9000 nucleotides, which encodes about 10 viral proteins, and a 3′ non-coding region of about 200 nucleotides depending on the size of the poly-uridylate track within this region [2,3].

The main therapy for HCV is treatment with pegylated-interferon and rivabirin. However, these agents show little effect in patients that have a high titer of HCV RNA, genotype 1. Thus, it is necessary to develop new, more effective therapies and preventive treatments to counteract HCV infection. As yet, no preventive vaccine is available for HCV. A recombinant HCV vaccine based on the viral envelope protein E1/E2 has been reported that generated neutralizing antibodies (nAb) in animals [4]. These nAbs were capable of limiting HCV pseudoparticles (HCVpp) and HCV cell culture (HCVcc) infection.

Recently, a genotype 2a strain of HCV named JFH-1 was discovered. This strain can efficiently replicate in the Huh-7 cell line [5], and an *in vitro* culture system of infectious HCV has also been successfully developed using the JFH-1 genome [6–8]. The JFH-1 viral production system is expected to become a powerful tool for HCV vaccine development. In this study, we developed a simple strategy for purification of recombinant HCV particles from the media of infected Huh-7 cells for structural analysis and for vaccine development using the JFH-1 genome.

2. Materials and methods

2.1. Plasmids

Plasmid pJ6/JFH-1 was generated from pJFH-1 by replacement of the 5′ untranslated region with the p7 region of J6 [9]. The

* Corresponding author. Address: Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan. Fax: +81 3 5285 1161.
E-mail address: wakita@nih.go.jp (T. Wakita).

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plasmids pJ6/JFH-1/1FL and pJ6/JFH-1/3FL were constructed by introduction of a single (DYKDDDDKGGG) or triple (DYKDHDG-DYKDHDIDYKDDDDKGGG) FLAG-tag sequence, respectively, into pJ6/JFH-1, which replaced part of the E2 HVR1 (amino acids 394–400) region. These two plasmids were then modified by introduction of a Lys residue to replace the Asn at amino acid position 151 of the E2 sequence, creating pJ6/JFH-1/1FL/E2N151K and pJ6/JFH-1/3FL/E2N151K, respectively.

The J6E2 gene (codons 1490–2500) was generated by PCR amplification from pJ6CF. The sense and antisense primers used were: 5′-CACAAGCTTCGCACCCATACTGTTGGGG-3′ and 5′-ACAGGATCCCAT-CGGACGATGTATTTTGTG-3′, respectively. For cloning purposes, HindIII or BamHI sites (underlined) were added to the primers. The amplified DNA was digested and inserted into p3XFLAG-CMV-13 (SIGMA, Saint Louis, MO).

The plasmid CDM-J6E2Fc encodes the J6E2 sequence downstream of the preprotrypsin leader sequence. pCDM-J6E2Fc was digested with SacI and BamHI, and the DNA fragment containing the preprotrypsin leader and J6E2 sequence was inserted into pCD4Rg (a kind gift from Dr. Brian Seed, Harvard Medical School) from which the SacI–BamHI fragment containing the CD4 gene was removed. This ligation resulted in the creation of a plasmid encoding a fusion gene of E2 and human IgG1-Fc.

2.2. Cell culture

The human hepatoma cell line, Huh-7, was maintained in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 incubator.

2.3. In vitro synthesis of HCV RNA and RNA transfection of Huh cells

HCV RNA was synthesized from the plasmids described above *in vitro* using a MEGAscript T7 kit (Ambion, Austin, TX). Synthesized HCV RNA was then electroporated into cells as previously described [10]. The transfected cells were transferred onto 100-mm culture dishes containing culture medium.

2.4. Quantification of HCV core protein and RNA

The HCV core protein in cell culture supernatants or in purified HCV samples was quantified by enzyme immunoassay using a HCV core ELISA kit (Ortho Clinical Diagnostics). HCV RNA in purified HCV samples was quantified by RTD-PCR as previously described [11].

2.5. Deglycosylation with PNGase F

For deglycosylation reactions, the Enzymatic In-Solution N-Deglycosylation kit (Sigma) was used according to the manufacturer's instructions. Briefly, lysates of passaged cells were incubated for 10 min at 100 °C in denaturation buffer and then in the presence of PNGase F enzyme for 1 h at 37 °C. These samples were analyzed by Western blotting as described below using anti-FLAG (SIGMA) and anti-GAPDH (CHEMICON, Temecula, CA) antibodies.

2.6. Sequence analysis

The cDNAs of the HCV genome were synthesized from total RNA isolated from HCV RNA-transfected cells [5]. These cDNA were subsequently amplified using DNA polymerase (*TaKaRa LA Taq*, Takara, Shiga, Japan). The sequence of the amplified DNA was determined by the 3130 Genetic Analyzer (Applied Biosystems, Foster city, CA).

2.7. Purification of recombinant HCV particles

Culture supernatants from Huh-7 cells transfected with FLAG-tagged HCV RNA were harvested. The medium was concentrated by ultrafiltration using the pellicon-2 300 system (Millipore, Bedford, MA) and was subjected to affinity chromatography using an Anti-FLAG M2 affinity gel (Sigma). Virus particles were eluted using the 3×FLAG Peptide (Sigma) and were concentrated by ultracentrifugation for 2 h at 50,000 rpm at 4 °C.

2.8. Determination of the viral infectious titer

The infectious titer was determined by the method as previously described and was expressed as the number of focus-forming units per milliliter (FFU/mL) [6].

2.9. Western blotting

The purified HCV sample was lysed using a buffer containing 0.1 M Tris–HCl (pH 6.8), 4% SDS, 1.2% 2-mercaptoethanol, 20% glycerol, and Bromophenol blue. SDS–PAGE and immunoblotting were performed as previously described [6]. Antibodies used for immunoblotting were: anti-HCV core (clone 2H9) [6], anti-E1 (B7567) [6], and anti-E2 (clone 8D10-3, unpublished).

2.10. Electron microscopy

Concentrated, purified HCV particles were allowed to settle on carbon-coated copper grids and were stained with 4% uranylacetate. The grids were examined in a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan) and were photographed at an instrumental magnification of 50,000×.

2.11. Sucrose density gradient analysis

The purified HCV sample containing 266 fmol of the HCV core was layered on a stepwise sucrose gradient (10–60%, wt/vol) and was centrifuged for 16 h in an SW41 rotor (Beckman Coulter, Fullerton, CA) at 35,000 rpm at 4 °C. After centrifugation, 12 fractions were harvested from the bottoms of the tubes. For each fraction, the core protein concentration was determined using an immunoassay. The HCV RNA titer was determined using RTD-PCR. The infectious titer was determined using an immunofluorescence assay as described above.

2.12. HCV particle-immunization

The purified HCV particles described above were inactivated by UV-irradiation, and 2 pmol of the HCV core protein of the particles were intraperitoneally injected into 4 week old BALB/c female mice ($n = 3$). Immunization was repeated four times at 2-week intervals (0, 2, 4 and 6 weeks). The Sigma Adjuvant System (Sigma), composed of monophosphoryl lipid A and trehalose dicorynomycolate, was used as an adjuvant. Saline alone was injected into control mice. Sera were collected at 1, 3, 5 and 7 weeks after immunization.

2.13. EIA for measurement of anti-E2 and anti-FLAG antibody responses

Recombinant J6E2/Fc or the FLAG peptide antigen (Sigma) was bound to microtiter plates (Nunc, Rochester, NY, USA) overnight at 4 °C, at a concentration of 50 ng per well. Recombinant J6E2/Fc was produced from COS-1 cells transfected with the CDM-J6E2Fc plasmid, which encodes the J6CF-E2 region (aa 384–720) fused with the Fc region of human IgG. The plates were blocked with Blocking One solution (Nacalai Tesque, Kyoto, Japan) and were washed with PBS containing 0.05% Tween 20 (washing buffer). Serum samples were diluted in washing buffer and were transferred to the blocked, antigen coated plates. After a 1.5-h incubation,

the plates were washed and bound antibody was detected using an HRP-conjugated anti-mouse antibody (GE healthcare, Buckinghamshire, England) and 3,3′,5,5′-tetramethylbenzidine (TMBZ) as a substrate (Sumitomo Bakelite, Tokyo, Japan).

3. Results

3.1. Production of recombinant HCV with an epitope-tagged envelope

To facilitate purification of recombinant HCV particles secreted into the culture medium of transfected cells, we constructed recombinant HCV with a FLAG-epitope-tagged envelope, which could then be purified by affinity chromatography using an anti-FLAG-agarose column. The FLAG-tagged HCV genome J6/JFH-1/3FL with the J6CF structural region was constructed by introducing a triple FLAG-tag sequence into the HVR1 of E2 (Fig. 1A). This region was selected for epitope-tag insertion because we predicted that this region would lie on the outside of the virus particles and would be tolerant to amino acid changes. Recombinant HCV particles were produced following transfection of Huh-7 cells with viral RNA, and were secreted into the culture medium.

RNA-transfected cells were passaged every 4 or 5 days. The level of the HCV core protein in the culture supernatant was measured over a period of 70 days (Fig. 1B). In contrast to the gradually increasing level of the core protein in J6/JFH-1 cells over time, the level of the core protein in the supernatants of the J6/JFH-1/3FL RNA-transfected cells decreased over the first 3 weeks post-transfection. Subsequently, the level began to increase and this level became equal to that of the wild-type J6/JFH-1 RNA-transfected cells 35 days post-transfection. This result suggested that after the first 35 days of culture, some mutations were introduced into the HCV genome that conferred efficient virus production during genome replication and/or that the transfected cells were altered in some way that was more favorable for viral production.

3.2. An N151K mutation facilitates the production of FLAG-tagged HCV

To determine if any adaptive mutations had arisen in the viral genome, we sequenced the full length of the HCV genome on days 8 and 35 post-J6/JFH-1/3FL RNA transfection. On day 8 post-transfection, no non-synonymous mutations were detected. However, on day 35, we found a single amino acid mutation at a potential

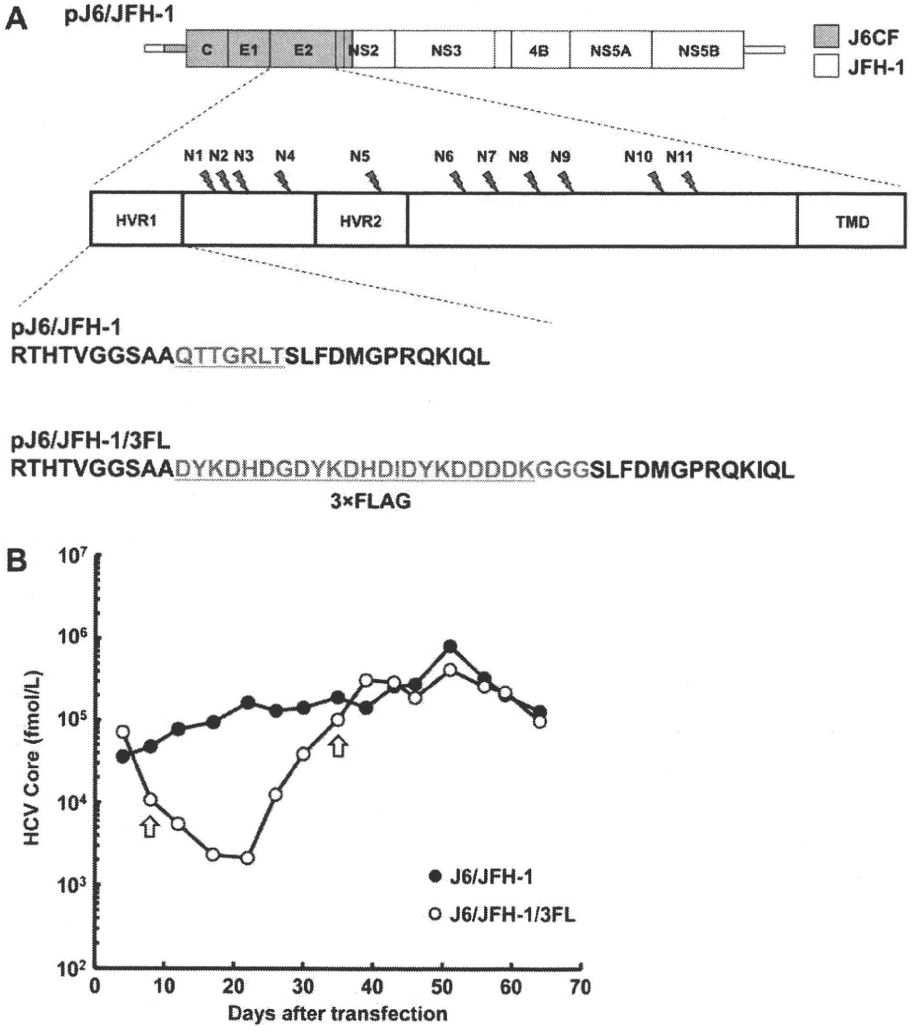

Fig. 1. Time course of HCV core protein secretion in recombinant HCV RNA-transfected cells. (A) Organization of the recombinant HCV construct pJ6/JFH-1/3FL. Open reading frames (thick boxes) are flanked by 5′- and 3′-UTRs (thin boxes). Gray, J6CF; white, JFH-1; HVR, hyper variable region; TMD, transmembrane domain. *N*-Glycosylation sites are indicated by pointers and are numbered N1–N11. The region of pJ6/JFH-1 that is replaced by the 3×FLAG sequence to generate pJ6/JFH-1/3FL is indicated at bottom. (B) HCV core protein secretion into the culture medium after HCV RNA transfection of Huh-7 cells. The HCV core protein was analyzed using an ELISA. Arrows indicate the times at which the J6/JFH-1/3FL HCV genome transfected into HCV RNA-transfected cells was sequenced.

N-glycosylation site of the E2 protein (Fig. 2A) in which asparagine at amino acid position 151 in the E2 protein was changed to lysine (E2N151K). Interestingly, this mutation was identical to that described by Delgrange et al. [12] as a mutation that was important for efficient production of HCV JFH-1. We performed Western blot analysis of cell lysates of transfected cells of different passages, using the anti-FLAG antibody as a probe for E2, to confirm that the N151K mutation abolishes one specific *N*-glycosylation. Indeed, the size of the FLAG-E2 protein was smaller on days 30 and 43 compared to that on day 4 (Fig. 2B). In contrast, the size of FLAG-E2 proteins that were deglycosylated using PNGase F was similar for all of the tested samples (Fig. 2B). This result suggested that the E2N151K mutation abolished *N*-glycosylation at this residue.

To investigate if the E2N151K mutation enhances production of FLAG-tagged HCV, we introduced the E2N151K mutation into the J6/JFH-1/3FL genome (J6/JFH-1/3FL/E2N151K). J6/JFH-1/3FL/E2N151K RNA-transfected cells were then passaged every 4 or 5 days and the level of the HCV core protein in the culture supernatant was measured over a period of 16 days (Fig. 2C). The result clearly showed that the E2N151K mutation contributes to efficient production of FLAG-tagged HCV particles.

We further analyzed the effect of the E2N151K mutation on specific viral infectivity (Table 1). The culture supernatant on day 3 post-transfection of recombinant viral RNA was therefore concentrated by ultrafiltration and tested in an infectious assay. The recombinant virus with the E2N151K mutation exhibited higher specific infectivity than the virus without this mutation. These data suggest that efficient production of infectious particles is impaired by the introduction of a FLAG-tag into the E2 protein but that this deficiency could be compensated for by the introduction of the E2N151K mutation which modifies an *N*-glycosylation site.

3.3. Purification of FLAG-tagged HCV

To purify FLAG-tagged HCV particles, we used a viral construct with a single FLAG-tag, J6/JFH-1/1FL/E2N151K (Fig. 1A), which as efficient in virus production as J6/JFH-1/3FL/E2N151K (data not shown). A total of 10 L of the culture supernatant of Huh-7 cells infected with J6/JFH-1/1FL/E2N151K was collected. This culture medium was concentrated to 300 mL by ultrafiltration and was then subjected to affinity chromatography using an anti-FLAG-agarose column. Bound virus particles were eluted using 10 mL of a

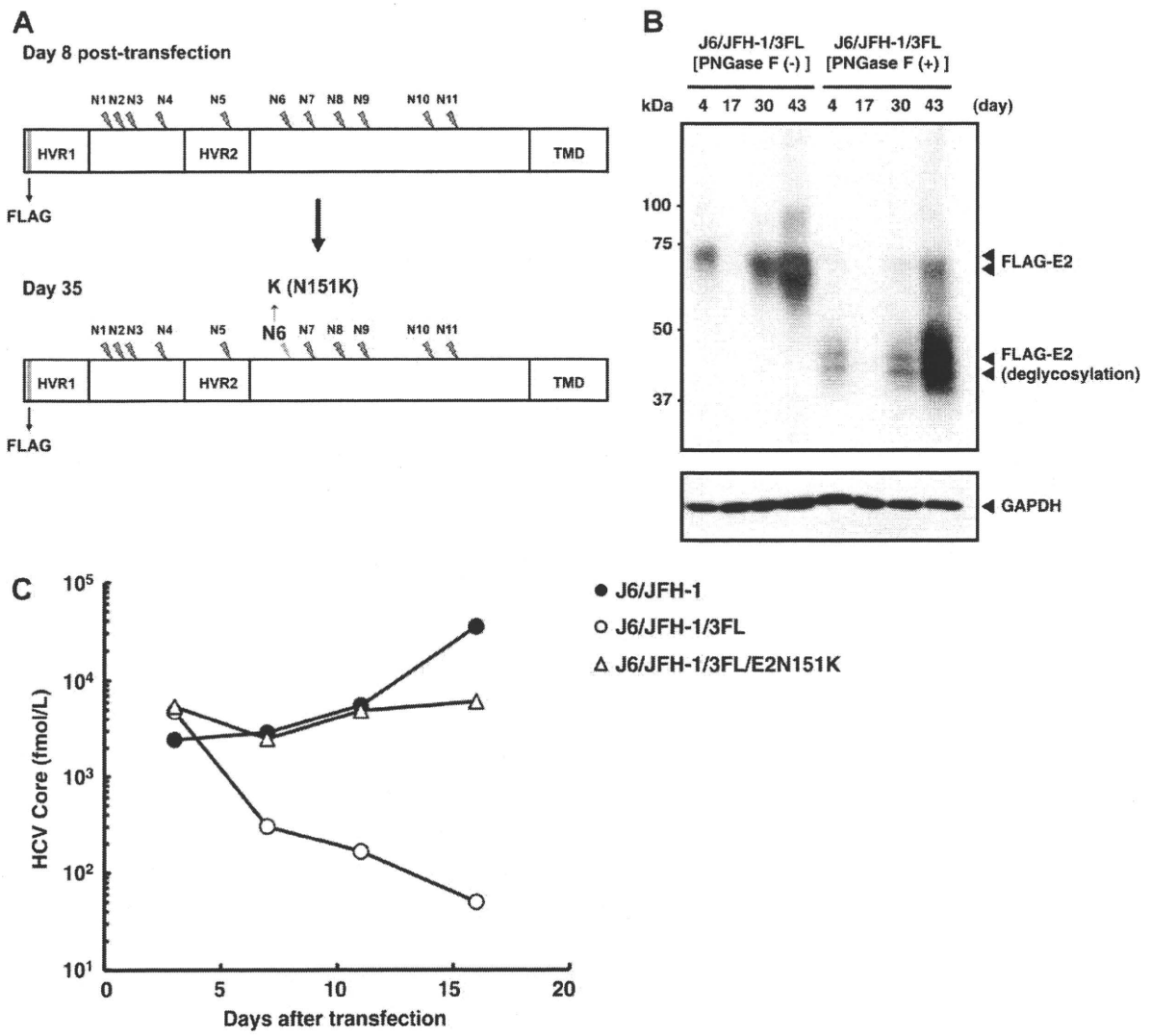

Fig. 2. Characterization of the recombinant HCV genome with an introduced N151K mutation. (A) Schematic diagram of the sequence of the E2 in the J6/JFH-1/3FL HCV RNA-transfected cells on day 8 and day 35 post-transfection. N151K replaces an Asn residue with a Lys residue at the N6 glycosylation site of E2. (B) Western blot analysis of the FLAG-E2 protein in lysates of cells transfected with J6/JFH-1/3FL RNA. Arrowheads indicate intact, and deglycosylated (PNGase F (+)), FLAG-E2 protein (upper panel) and control GAPDH protein (lower panel). (C) HCV core protein secretion into the culture medium following transfection of Huh-7 cells with HCV RNA with or without an introduced N151K mutation.

Table 1
Infectivity of recombinant viruses with or without N151K mutation.

Recombinant virus	Infectious titer ($\times 10^2$ FFU/mL)	HCV core protein ($\times 10^2$ fmol/mL)	Specific infectivity (FFU/HCV core)
J6/JFH-1/3FL	<1.7	1.6	<1.1
J6/JFH-1/3FL/E2N151K	8.3	2.0	4.2

FLAG peptide solution. Finally, the purified HCV particles were concentrated by ultracentrifugation.

The HCV yield and the amount of total protein after each purification step are summarized in Table 2. This purification process resulted in a 5000-fold concentration of the culture supernatant. The recovery of the HCV core protein in the final purified virus preparation was approximately 5%, and the virus purity was increased about 9000-fold compared to its purity in the initial culture medium. Specific infectivity was increased about 4-fold after the final step.

HCV structural proteins in the purified virus sample were examined by Western blotting (Fig. 3A). Core, E1 and E2 proteins were all detected in the purified virus preparation. Interestingly, incorporation of the E2 protein into the purified virus appeared to increase compared to incorporation of the core and E1 proteins. However, this higher apparent incorporation of FLAG-E2, may reflect the presence of free, non-virus incorporated FLAG-E2 proteins that co-purified with the FLAG-tagged virus. We further analyzed the virus particles in the purified preparation by electron microscopy (Fig. 3B–D). Substantial debris was found in the culture

Table 2
HCV yield and properties of purified recombinant HCV after each purification step.

Purification step	Volume (mL)	HCV core protein ($\times 10^2$ fmol/mL)	HCV RNA ($\times 10^7$ copies/mL)	Total protein (μg/mL)	Recovery[a] (%)	Purity[b]	Infectivity ($\times 10^2$ FFU/mL)	Specific infectivity (FFU/HCV core)
Culture supernatant	10,000	1.4	3.5	877	100	1	25	18
Concentrate (after Ultrafiltration)	300	45	57	19,597	96	0.73	743	17
Affinity purification (after Elution)	10	98	324	171	7	469	4240	43
Concentrate (after Ultracentrifugation)	0.2	1440	3220	84	5	9546	94,600	66

[a] Recovery of HCV core protein.
[b] The degree of virus purity was calculated by HCV RNA contents per μg total proteins.

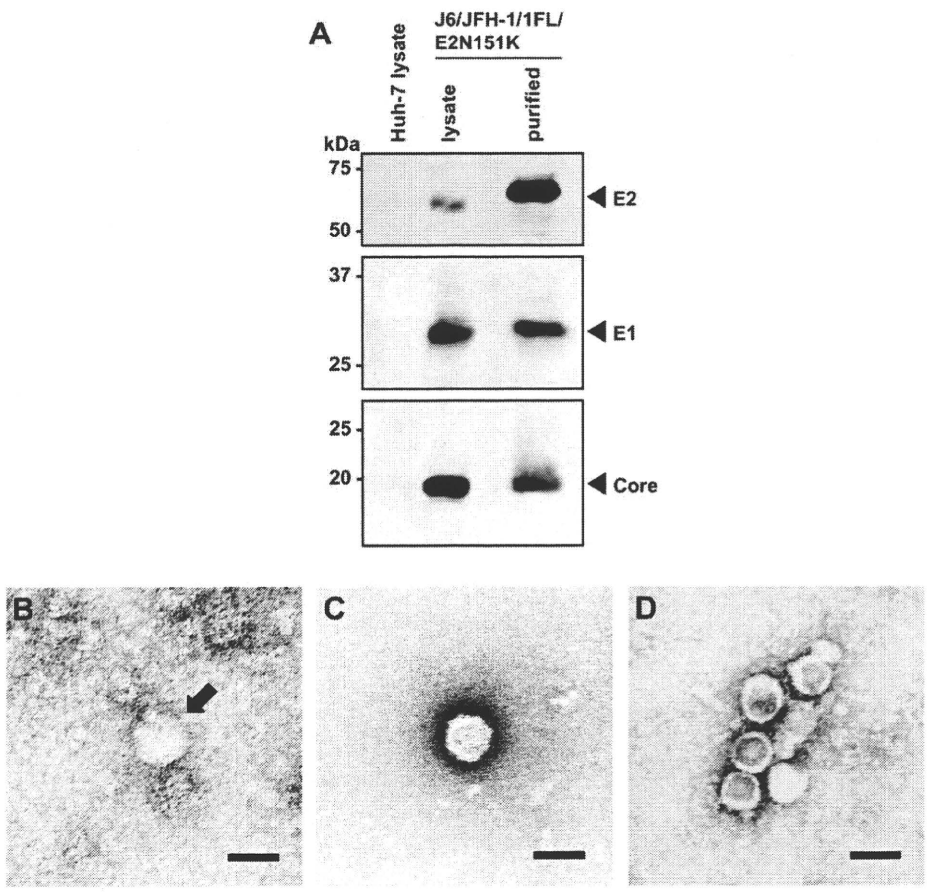

Fig. 3. Analysis of purified HCV particles. (A) Western blot analysis of viral proteins in lysates of, and in HCV particles purified from, whole-cell extracts of Huh-7 cells transfected with J6/JFH-1/1FL N151K RNA. Lysates of non-transfected cells were also analyzed. The arrowheads indicate the positions of the HCV core, E1 and E2 proteins. Marker proteins are shown at left. (B–D) Electron micrographs using negative staining of: (B) An HCV particle from culture media (indicated by an arrow.), (C) A purified HCV particle and (D) Purified HCV particles aggregated by an anti-FLAG antibody. Scale bar, 50 nm.