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

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

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

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

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

RESULTS

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

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

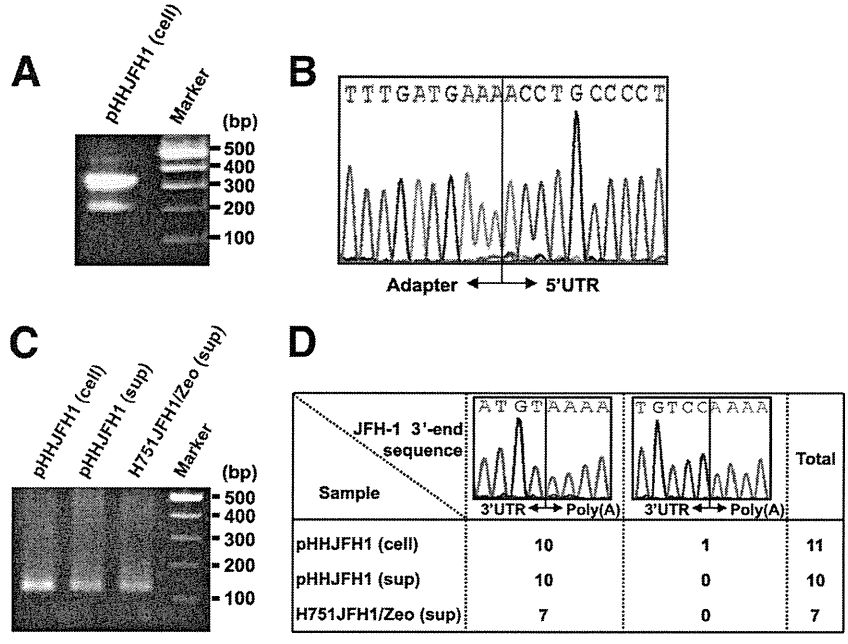

FIG. 1. Determination of the nucleotide sequences at the 5′-and 3′ ends of HCV RNA produced by the Pol I system. (A and B) 5′RACE and sequence analysis. A synthesized RNA adapter was ligated to RNA extracted from cells transfected with pHHJFH1. The positive-strand HCV RNA was reverse transcribed, and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

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

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

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

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

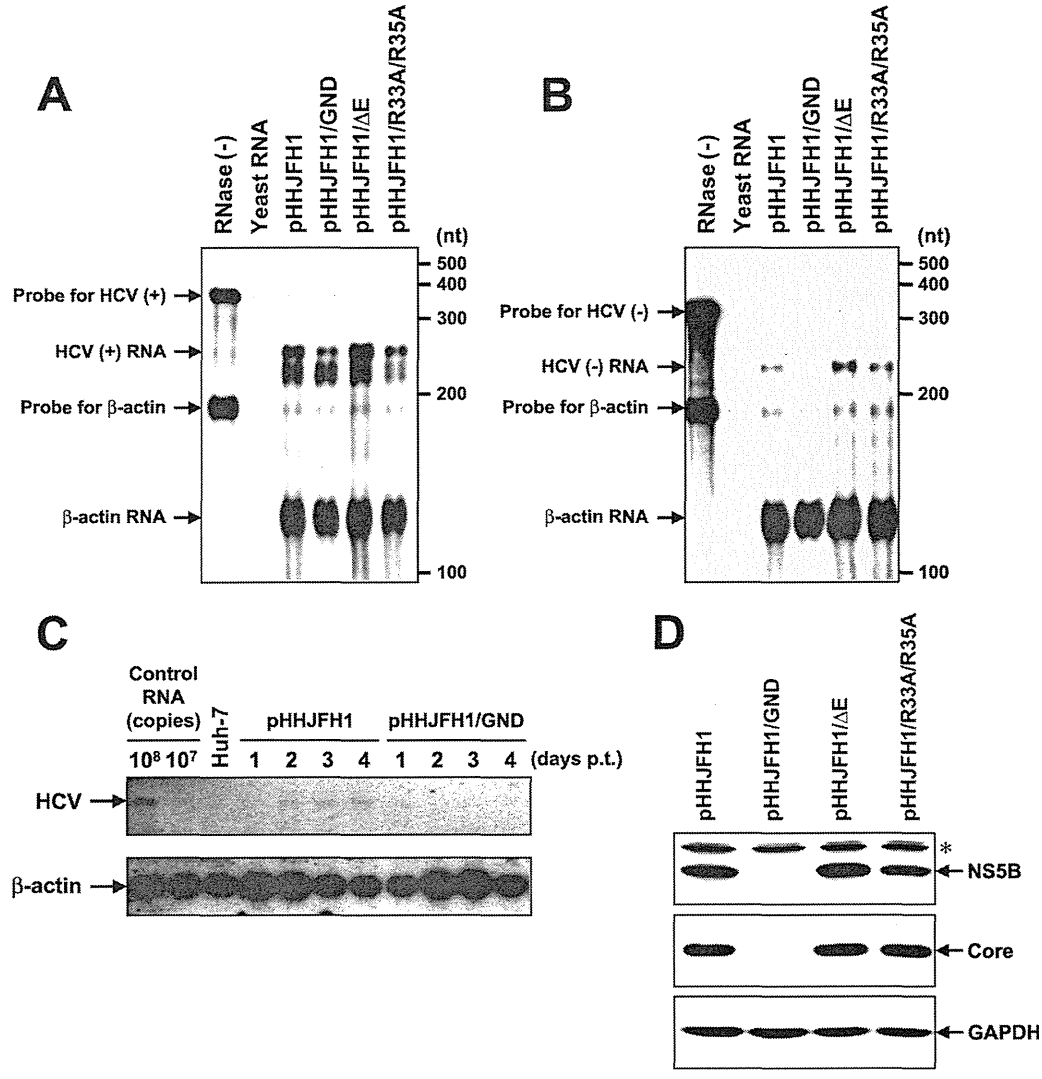

FIG. 2. HCV RNA replication and protein expression in cells transfected with Pol I-driven plasmids. (A and B) Assessment of HCV RNA replication by RPA. Pol I-driven HCV-expression plasmids were transfected into Huh-7 cells. Total RNA was extracted from the cells on day 5 p.t. and positive (A)- and negative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

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

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

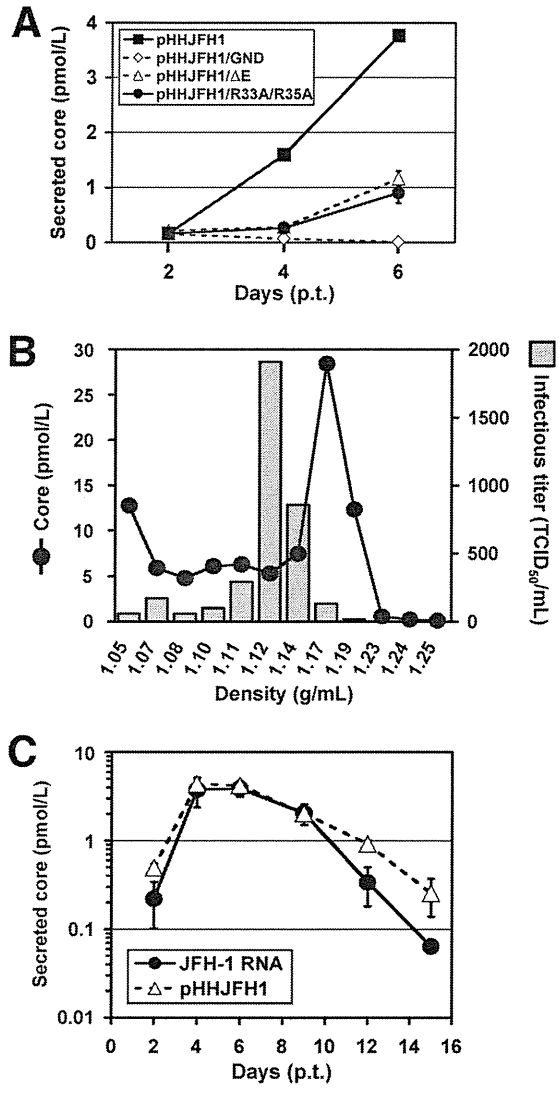

FIG. 3. HCV released from cells transfected with Pol I-driven plasmids. (A) HCV particle secretion from the transfected cells. The culture supernatant of Huh-7 cells transfected with Pol I-driven plasmids containing wild-type or mutated HCV genome were harvested on days 2, 4, and 6 and assayed for HCV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

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

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

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

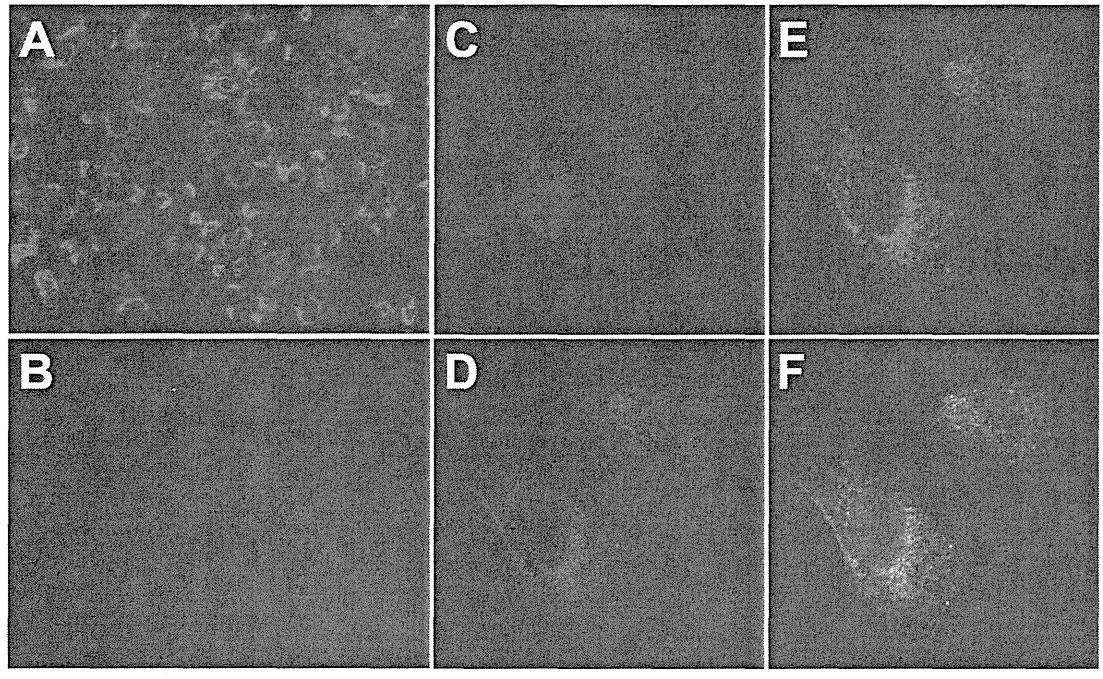

FIG. 4. Indirect immunofluorescence analysis of H751JFH1/Zeo cells. (A and B) H751JFH1/Zeo cells (A) and parental Huh7.5.1 cells (B) were immunostained with an anti-NS5A antibody. (C to F) The subcellular colocalization of *de novo*-synthesized HCV RNA and NS5A in H751JFH1/Zeo cells was analyzed. The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

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

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

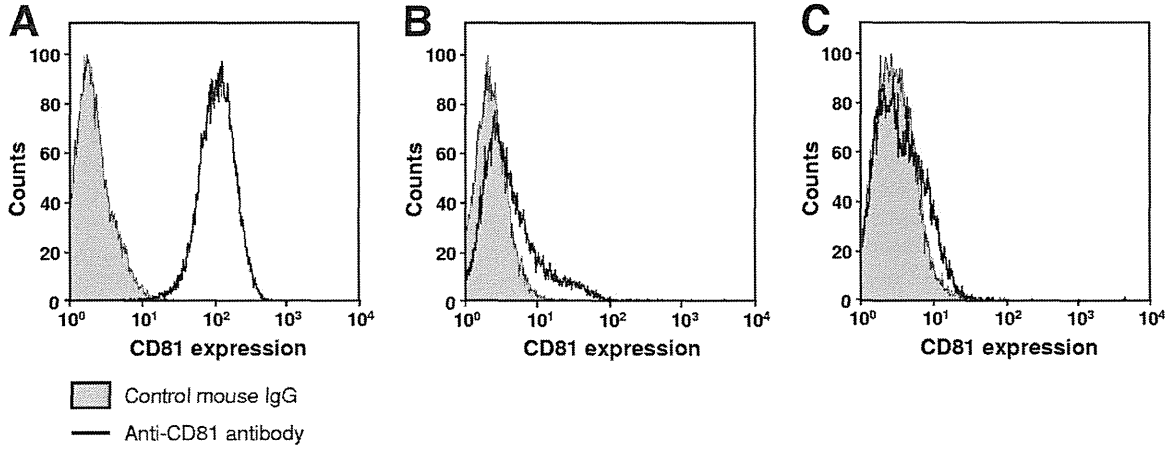

FIG. 5. Loss of CD81 expression in H751JFH1/Zeo cells. The cell surface expression of CD81 on Huh7.5.1 cells (A), H751JFH1/Zeo clone H751-1 (B), and clone H751-50 (C) was analyzed by flow cytometry after being stained with anti-CD81 antibody.

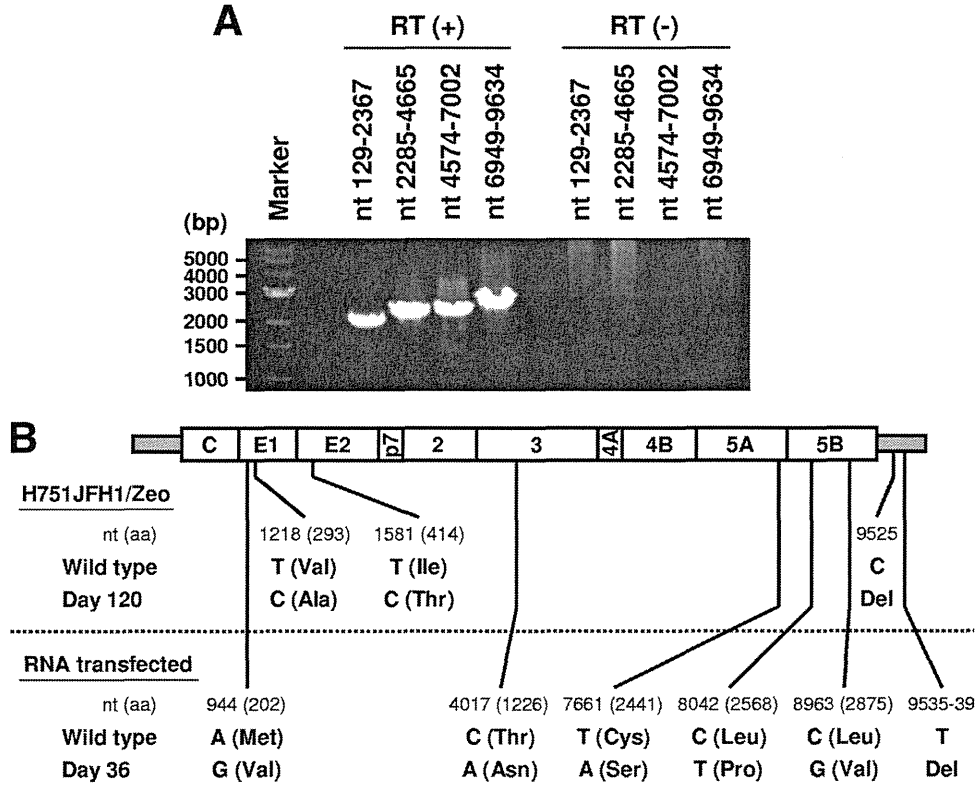

FIG. 6. Genome mutations of HCV secreted from H751JFH1/Zeo cells. (A) RT-PCR of HCV genome extracted from the culture supernatant of H751JFH1/Zeo cells. Viral RNA sequences were reverse transcribed [RT (+)] or not [RT (−)], followed by amplification with primer pairs encompassing the specified HCV genome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

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

DISCUSSION

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

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

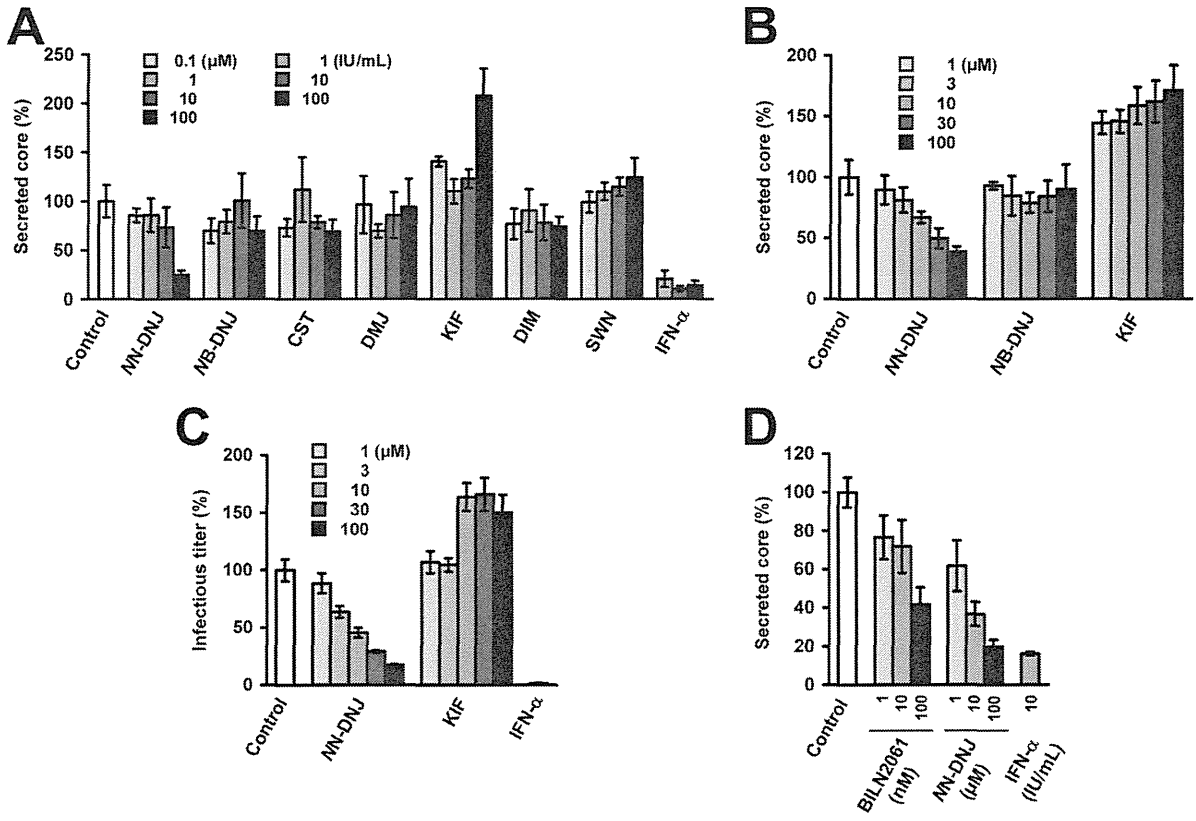

FIG. 7. Effects of glycosylation inhibitors on HCV production from H751JFH1/Zeo cells. (A and B) Effects of glycosylation inhibitors on the secretion of HCV core protein. H751JFH1/Zeo cells were seeded at a density of 1×10^4 cells/well in a 96-well culture plate (A) or 3×10^4 cells/well in a 12-well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

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

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

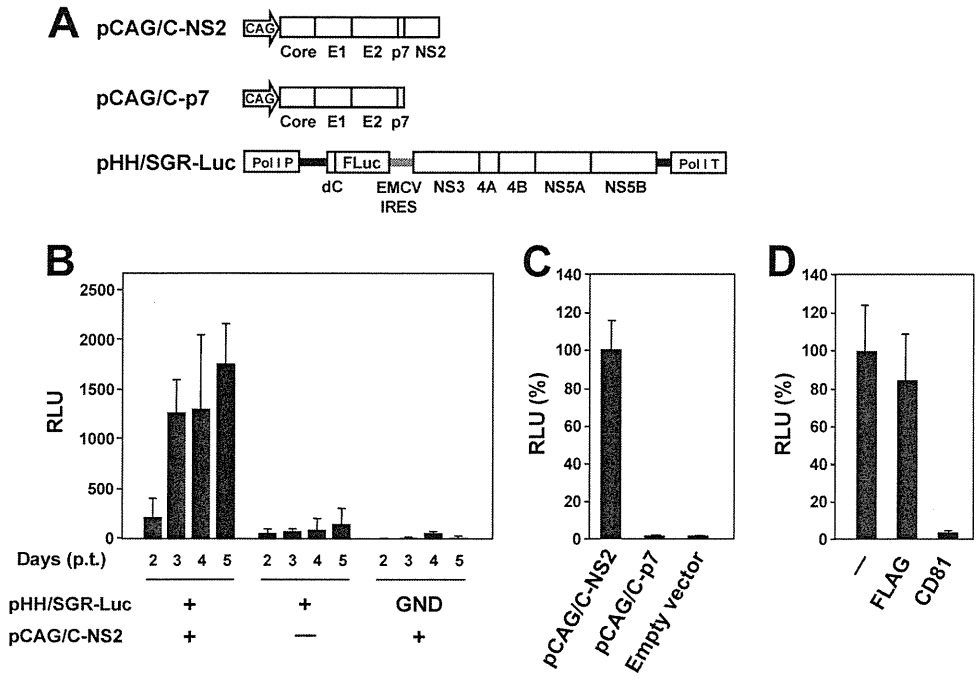

FIG. 8. Establishment of a *trans*-packaging system involving two-plasmid transfection. (A) Schematic representation of the plasmids used for the production of HCV-LP. HCV polyproteins are indicated by the open boxes. Bold lines indicate the HCV UTR. EMCV IRES is denoted by gray bars. The firefly luciferase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

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

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

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

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

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

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

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特集II　C型肝炎ウイルスの感染・増殖メカニズムと臨床応用

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

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

はじめに

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

対象と方法

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

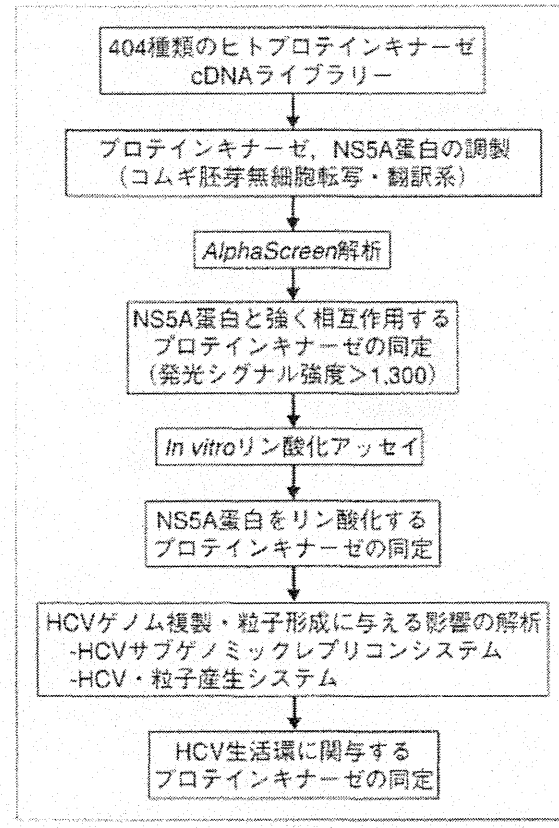

図1　解析方法の概要

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

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を用いて解析した．

1．NS5A蛋白と強く相互作用するプロテインキナーゼの探索

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

2．NS5A蛋白に対するリン酸化能の評価

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

3．HCVゲノム複製，粒子形成に与える影響の解析

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

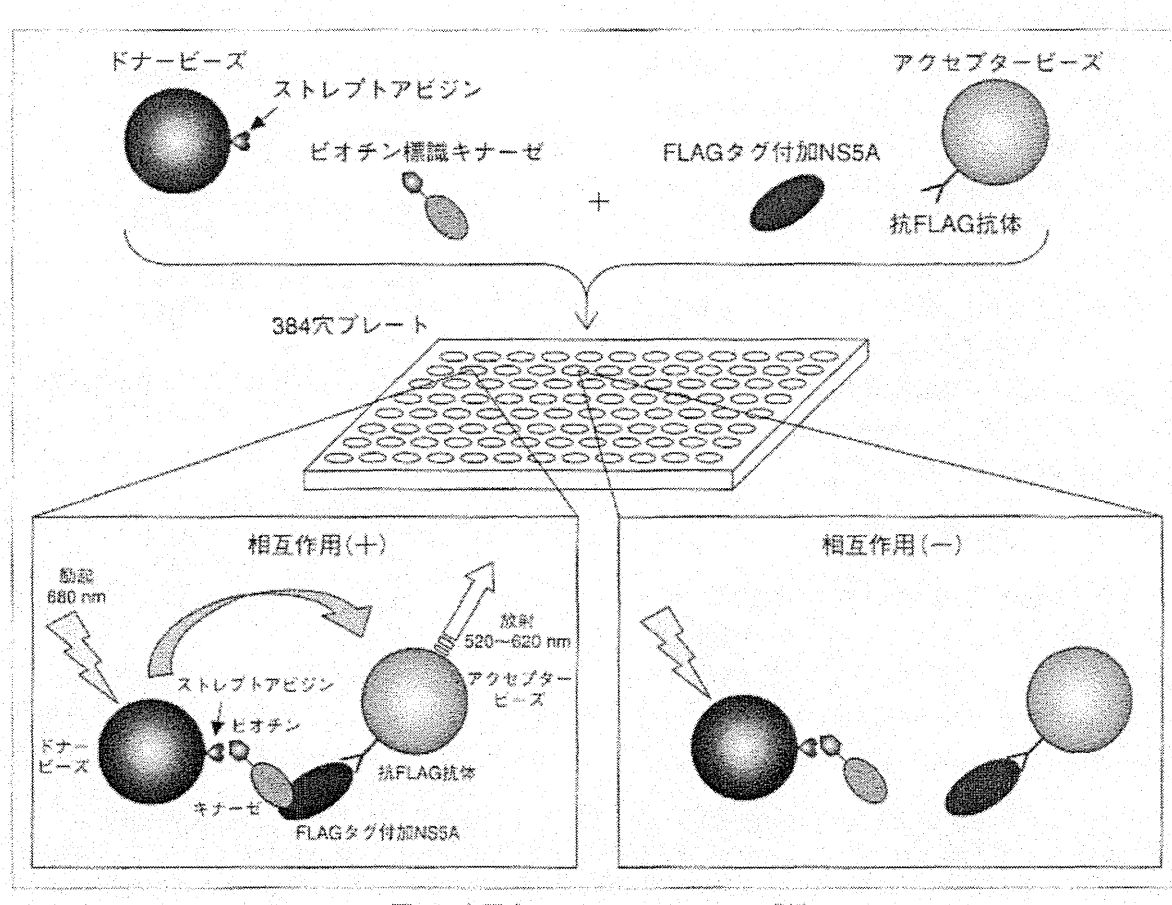

図2　本研究における*AlphaScreen*解析

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

結　果

1. NS5A蛋白と強く相互作用するプロテインキナーゼの探索

*AlphaScreen*法による解析結果を表1に示す．発光シグナル強度1,300以上を強固な蛋白間相互作用と想定した時，このカットオフ値以上のシグナル強度を示したプロテインキナーゼは89種類であった．このうち79種類がNS5A蛋白のリン酸化に重要とされるセリン/スレオニンプロテインキナーゼであり，このグループの中にはNS5A蛋白をリン酸化するとすでに報告されているカゼインキナーゼ(CK)2[19]の触媒サブユニットであるCK2α2も含まれていた．

2. NS5A蛋白に対するリン酸化能の評価

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

3. HCVゲノム複製，粒子形成に与える影響の解析

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

表1　*AlphaScreen*解析結果

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

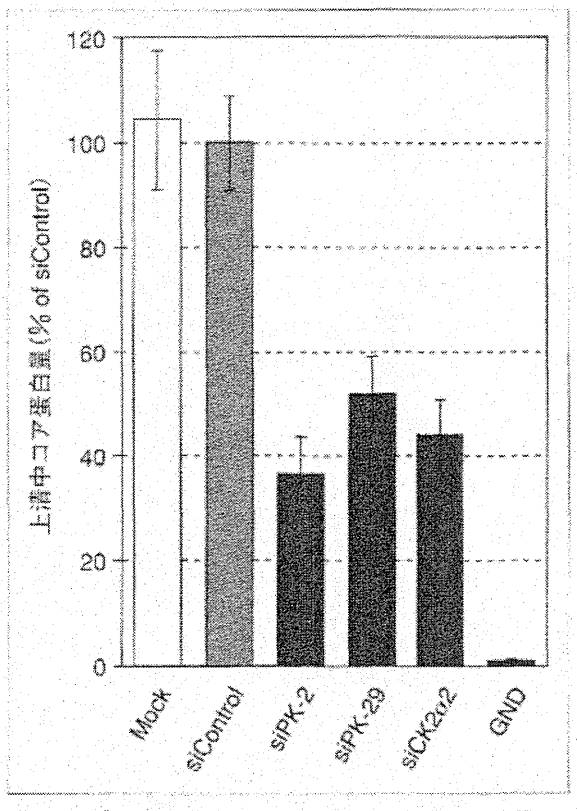

図3　プロテインキナーゼのノックダウンが分泌HCV粒子量に与える影響
siRNA, HCV RNA導入後3日目の上清中コア蛋白量をcontrol siRNA(siControl)導入時の値を100%として算出し，グラフ化した．Mock：全長HCV RNAの導入のみでsiRNAの導入はなし．GND：複製活性のない変異HCV RNAの導入のみでsiRNAの導入はなし．

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

考　察

NS5A蛋白はHCVの複製増殖やインターフェロン感受性，病原性発現などに関与する多機能蛋白である[7]．リン酸化蛋白であり，NS5A蛋白のリン酸化はウイルスゲノム複製や感染性ウイルス粒子の形成に重要な役割を担うことが報告されている[13,14]．したがって，NS5A蛋白のリン酸化を制御するプロテインキナーゼの同定は，HCV生活環をより理解する上で重要であることに加え，新たな創薬ターゲットとしても魅力的である．

阻害剤や酵母由来の精製プロテインキナーゼを用いた解析から，現在までにAKT, p70S6K, MEK, CK1, CK2など数種類のプロテインキナーゼがNS5A蛋白のリン酸化に関与するものとして報告されている[8,9]．しかし，いずれの報告も解析対象のプロテインキナーゼ数は数十～百前後であり，また，HCV生活環への関与についても十分な検討がなされていない．そこで，今回われわれは404種類のヒトプロテインキナーゼを対象とし，NS5A蛋白に対するリン酸化能を有し，かつ，HCV生活環に関与するプロテインキナーゼの同定を試みた．同定された3種類のプロテインキナーゼの中にはCK2の触媒サブユニットであるCK2α2が含まれていたが，CK2はNS5A蛋白のリン酸化とHCV粒子形成に関与することが報告されており[15]，本解析結果の妥当性が高いことを示している．

今回の解析で同定された3種類のプロテインキナーゼは，いずれもsiRNAによるノックダウンで複製活性には影響を与えずにウイルス粒子分泌量を低下させたことから，HCV生活環の後期過程であるウイルス粒子形成(もしくはそれ以降のステップ)に関与し，この過程を正に制御している可能性が示唆された．さらに，PK-2のノックダウンはNS5A蛋白の高リン酸化を著しく抑制しており，このプロテインキナーゼの粒子形成過程への作用はNS5A蛋白の高リン酸化制御を介している可能性が考えられた．最近，台湾の研究チームがサブゲノミックレプリコン細胞を用いて1,210種類のヒトプロテインキナーゼおよびホスファターゼを対象とした網羅的RNAiスクリーニングを行い，HCVゲノム複製にpolo-like kinase 1(Plk1)というプロテインキナーゼが関与することを報告した[10]．Plk1の作用点はHCVの複製過程であるが，その作用はわれわれが同定したPK-2と同様NS5A蛋白の高リン酸化制御を介する．NS5A蛋白の高リン酸化にはその中央領域に存在する複数のセリン残基が関与すると報告されているが[11,12]，同じリン酸化パターンでも責任プロテインキナーゼやNS5A蛋白のリン酸化部位の違いによりHCV生活環における作用点が異なる可能性は十分に考えられる．この相違を明らかにするためには，今後，責任プロテインキナーゼによるリン酸化部位の同定や同定部位のリン酸化がHCV生活環に与える影響につき解析する必要があるであろう．

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

おわりに

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

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特集 第57回日本ウイルス学会シンポジウム特集

5. C型肝炎ウイルスの複製と粒子形成

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

はじめに

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

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

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HCVゲノム複製

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

HCVゲノム複製に関与する宿主因子としては，これまで

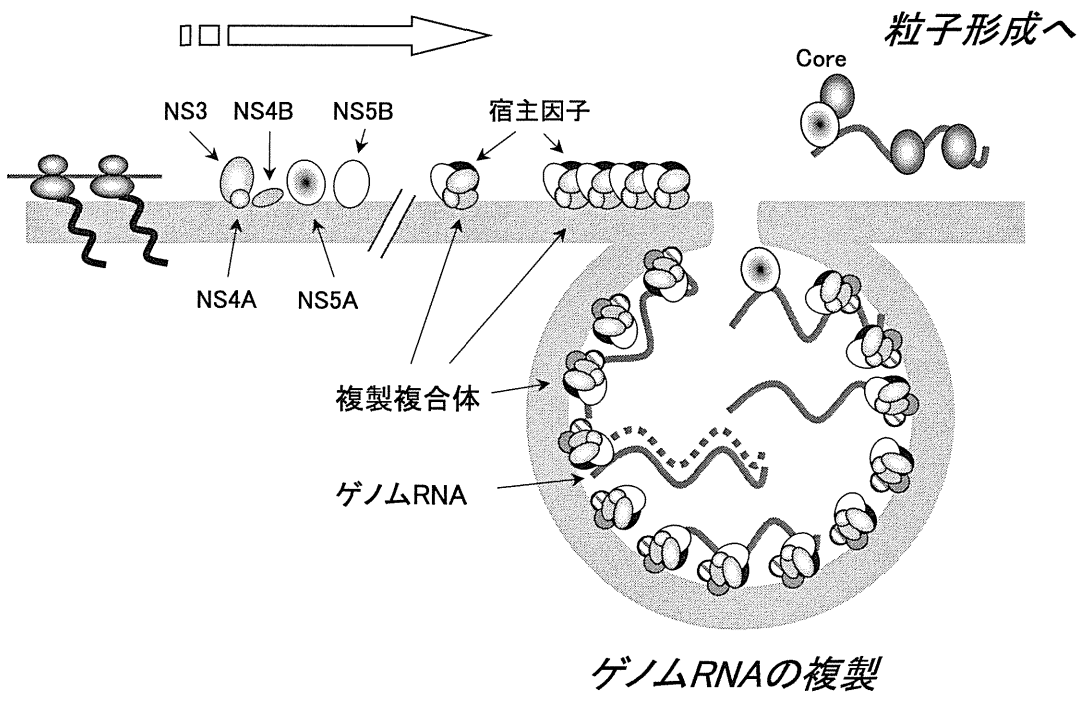

図1 HCV ゲノム複製複合体の形成
翻訳された前駆体蛋白は宿主細胞のシグナルペプチダーゼ，シグナルペプチドペプチダーゼ及び2種類の HCV プロテアーゼによって切断される．NS3，NS4A，NS4B，NS5A，NS5B 蛋白は相互に会合，また宿主蛋白を取り込んだ形で複製複合体を形成する．複製複合体が膜上で高密度化すると，ウイルス蛋白間の相互作用等により膜構造の変化がおこり，小胞構造体が形成される．その際，取り込まれたプラス鎖ゲノム RNA からマイナス鎖（点線で記載）が作られさらにプラス鎖 RNA が合成される．新生されたゲノム RNA と NS5A 蛋白との複合体が Core 蛋白と会合しヌクレオキャプシド形成が進む．

に VAP, FKBP3, Hsp90, hBind-1, FBL2, Cyclophilin B など10数種類の蛋白が同定されている．VAP-A/B 及び SNARE 様蛋白は NS5A, NS5B と結合し，複製複合体形成に働くと考えられる[9, 12]．最近，VAP-B のスプライシングバリアントである VAP-C が，VAP-A/B と NS5B との結合を競合的に阻害することによって HCV 複製を抑制しうることも報告された[21]．また，NS5A と相互作用する FKBP8 や hBind-1 などのコシャペロンが Hsp90 を複製複合体へ運ぶことが HCV 複製に重要であることが示された[30, 31, 36, 37]．Cyclophilin B の関与については，免疫抑制剤シクロスポリンの持つ抗 HCV 活性の作用機序解析を端緒として明らかとなった[41]．FBL2 はゲラニルゲラニル化されて NS5A と結合し複製複合体に取り込まれる[40]．しかしながら，HCV 複製複合体の形成過程，機能などについて未だ十分に解明されているとは言えない．

CKB：新たに見出された HCV 複製関連因子

そこで我々は，比較プロテオーム解析によって，HCV 複製複合体を構成し複製調節に働く新規宿主因子の探索を行った．すなわち，前述のように HCV ゲノムが複製する細胞中の DRM 分画では複製活性が保持されることから，サブゲノムレプリコンを有する Huh-7 細胞及び親株細胞からそれぞれ DRM 分画を調製し，各蛋白レベルを両分画間で比較し，レプリコン細胞の DRM で存在量が顕著に高かった蛋白27種類を同定した[13]．その中には，分子シャペロンなど蛋白ホールディングに関わるもの，代謝，生合成系の酵素，細胞骨格形成蛋白などが含まれていた．これらの蛋白が実際に HCV の複製に関与するかどうかを調べるため，各分子に対する siRNA をレプリコン細胞へ導入し細胞内 HCV RNA レベルの変化を解析した結果，HCV ゲノム複製を正に制御しうる因子として creatine kinase B (CKB)などを同定した．CKB については，遺伝子ノックダウンの他，阻害剤 cyclocreatine の添加，ドミナントネガティブ体の強制発現によっても HCV 複製，感染性ウイルス産生が抑制されることを示した[13]．creatine kinase は，エネルギーを多く必要とする，あるいはエネルギーを急速に必要とする組織での ATP の供給，ATP レベルの維持に重要な酵素であり，高エネルギーリン酸結合を持つクレアチンリン酸から ADP へリン酸基を転移し ATP 生成に働く．ATP のエネルギーを必要とする生化学反応系において，クレアチンリン酸と共に ATP の再生系として利用される．CKB は creatine kinase のアイソフォームの一種であり肝

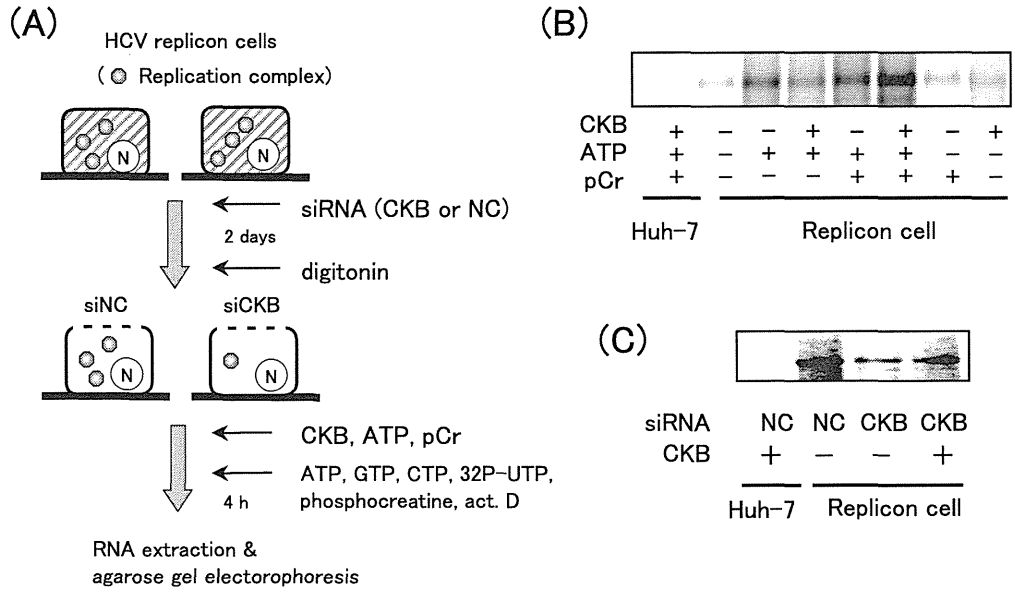

図 2　CKB は HCV replicase 活性を亢進させる [13]

(A) セミインタクトレプリコン細胞を用いた HCV replicase assay. (B) 作製したセミインタクト細胞（Huh7 または Replicon cell）に精製 CKB, ATP, クレアチンリン酸（pCr）を図のように添加し複製活性を調べた. (C) レプリコン細胞に CKB に対する siRNA または陰性コントロール siRNA（NC）を導入した後セミインタクト化した. それぞれに CKB を添加（または未添加）し複製活性を解析した.

臓を含む多様な組織で発現している. 興味深いことに, CKB は HCV の感染や複製によってその発現が亢進する訳ではないが, HCV レプリコン細胞では DRM 分画に enrich されることが示された[13]. すなわち, HCV ゲノムが複製している細胞においては, CKB が特定の細胞内分画に集積する現象が起こりうると考えられたため, 次に, CKB が HCV 蛋白と相互作用し, それによって CKB が DRM へリクルートされるという作業仮説を立て検証を行った. 免疫沈降解析の結果, CKB は, HCV 非構造蛋白のうち NS4A と相互作用すること, その相互作用には CKB の C 末端側と NS4A の中央領域が重要であることが明らかとなった[13]. NS4A は 54 アミノ酸残基からなるポリペプチドで, その中央部を介して NS3 と結合し NS3 セリンプロテアーゼ活性の cofactor として機能している. また, N 末端側は膜貫通領域[7], C 末端側は NS5A の高リン酸化に関わる領域[23] と報告されている. CKB との相互作用に重要な NS4A 領域は NS3 との結合に関わる領域に近接すると考えられたが, 免疫沈降法の結果, CKB は NS3-4A 結合を阻害する訳ではなく, CKB-NS4A-NS3 三者の複合体が形成されうることが示された. また, NS4A との結合部位を欠損した CKB では, DRM 分画局在性が低下し, HCV 複製への関与がキャンセルされた[13].

さらに, 複製複合体への CKB の介入が HCV replicase 活性に重要かどうかを明らかにするため, セミインタクトレプリコン細胞を使った replicase assay を行った. これは Miyanari らによって開発された方法で, レプリコン細胞をジギトニン処理したセミインタクト状態で HCV RNA 複製をモニターし, 複製活性に対するプロテアーゼ, 界面活性剤処理などの影響を解析することができる[28]（**図 2A**）. セミインタクトレプリコン細胞に ATP を加えると replicase 活性の上昇が認められるが, ここへさらに精製 CKB 及びクレアチンリン酸（pCr）を添加すると同活性が顕著に上昇することがわかった（**図 2B**）. また, レプリコン細胞から CKB をノックダウンしておいたセミインタクト細胞では replicase 活性は低下するが, そこへ CKB を添加すると活性の回復が観察された（**図 2C**）. replicase 活性のうち, NS3-4A が関与する ATP 依存的反応は RNA helicase 活性であるが, 実際にこの活性が CKB, pCr 添加によって亢進することを in vitro helicase assay で確認した[13].

以上の成績より, CKB を介した HCV 複製分画への ATP 供給が同ウイルスのゲノム複製調節に寄与していると結論づけた. CKB は NS4A との結合を通じて HCV 複製複合体へリクルートされ, HCV ゲノム複製調節に役割を果たすと考えられた.

HCV 粒子形成機構に関する最近の知見

細胞内中性脂肪に蓄積に用いられる脂肪滴が HCV の感染性粒子形成に重要な役割を果たすことが 2007 年に報告[27]されて以来, HCV 粒子形成の分子機構に関する研究は, 脂肪滴のバイオロジー, リポ蛋白産生などの脂質代謝と関連

づけながら進められている．脂肪滴は小胞体由来膜構造等の小器官と相互作用しながら細胞質内で動的な振舞いを見せる．HCV増殖細胞の中では，脂肪滴膜上にHCVヌクレオキャプシド形成を担うCore蛋白が局在しており，膜構造に随伴したE1，E2蛋白また非構造蛋白も，膜間またはCore蛋白との相互作用によって脂肪滴周辺へ集合する．このようにして感染性HCV粒子の形成は脂肪滴周辺環境で効率よく進行すると考えられる[27,29]．筆者らは，非構造蛋白NS5Aが脂肪滴の周囲で粒子形成の初期過程に関与することを報告した[26]．複製複合体で新生されたウイルスゲノムRNAをNS5Aが捕捉した後，NS5AのC末端領域とCoreとの相互作用を介して，ゲノムRNA-NS5A-Core複合体が作られる．これによりCoreによるRNAパッケージングが開始される，というモデルを提唱した（**図1**）．HCV粒子形成におけるNS5Aの役割は他の研究グループからも報告されている[4,38]．

HCV感染患者の血中ウイルスは多様な密度（約1.05から1.25g/mL）を示すことが知られている．低密度域のHCV粒子は，アポリポ蛋白を含みトリグリセリドに富んだ超低比重リポ蛋白VLDLが会合したかたちで存在するものと推定され，このような低密度粒子は高い感染性を有することがチンパンジー，培養細胞での感染実験で示されている[6,14,24]．また筆者らはHCV粒子表面のコレステロール，スフィンゴ脂質が感染性に重要であることを示した[2]．VLDLの構成因子であるアポリポ蛋白B（apoB）及びアポリポ蛋白E（apoE）がHCV粒子形成に関与すると報告されており[5,8,10,15,18]，HCVエンベロープ蛋白の細胞外への分泌はapoB陽性リポ蛋白のアセンブリーに依存している[16]．apoEについては遺伝子ノックダウンによってHCVの細胞への侵入過程，ゲノム複製は影響をうけないものの感染性HCVの産生は顕著に低下すること[18]，NS5Aと相互作用することなどが見出されている[5]．また，VLDLの産生を低下させるミクロソームトリグリセリド転移蛋白阻害剤によって感染性HCV産生は有意に抑制される[18]．

最近，HCV非構造蛋白についてNS5A以外にNS3，NS2も感染性粒子の形成に関与することが報告されている．NS3のC末端側helicase領域が粒子形成に係わっているとされている[25]．NS2についてもmutagenesis解析から膜貫通領域，C末端領域など粒子形成に重要な領域が見出されている[17,19,20,34]（鈴木ら未発表）．

おわりに

インターフェロンを基軸とした化学療法の進歩により，最近テレビで流れているように「C型肝炎は治る病気になりました」と言っても過言でないのかもしれない．しかしながら，現行の治療法に対する無効例，治療終了後に肝炎が再燃するケースも依然として多い．化学療法の治療効果をより高め，症状，体質などの異なる様々な症例に対応するためには，作用標的の異なる種々の抗HCV剤の創薬化が重要である．現在，HCVプロテアーゼ，ポリメラーゼ，NS5Aをそれぞれ標的とした化合物あるいはシクロスポリン誘導体などによるHCV複製阻害剤の開発が進んでいる．本稿で紹介したように，HCVゲノム複製調節に働く種々の宿主因子が同定され，感染性粒子形成の分子機構も明らかにされつつある．これらは次世代抗HCV薬開発のための新たな標的になるものと期待される．

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