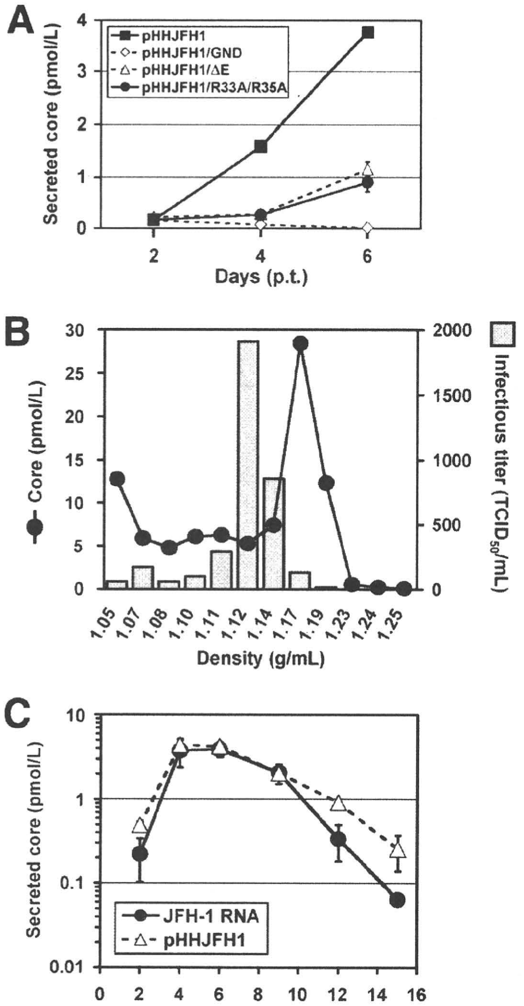

FIG. 3. HCV released from cells transfected with Pol I-driven plasmids. (A) HCV particle secretion from the transfected cells. The culture supernatant of Huh-7 cells transfected with Pol I-driven plasmids containing wild-type or mutated HCV genome were harvested on days 2, 4, and 6 and assayed for HCV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

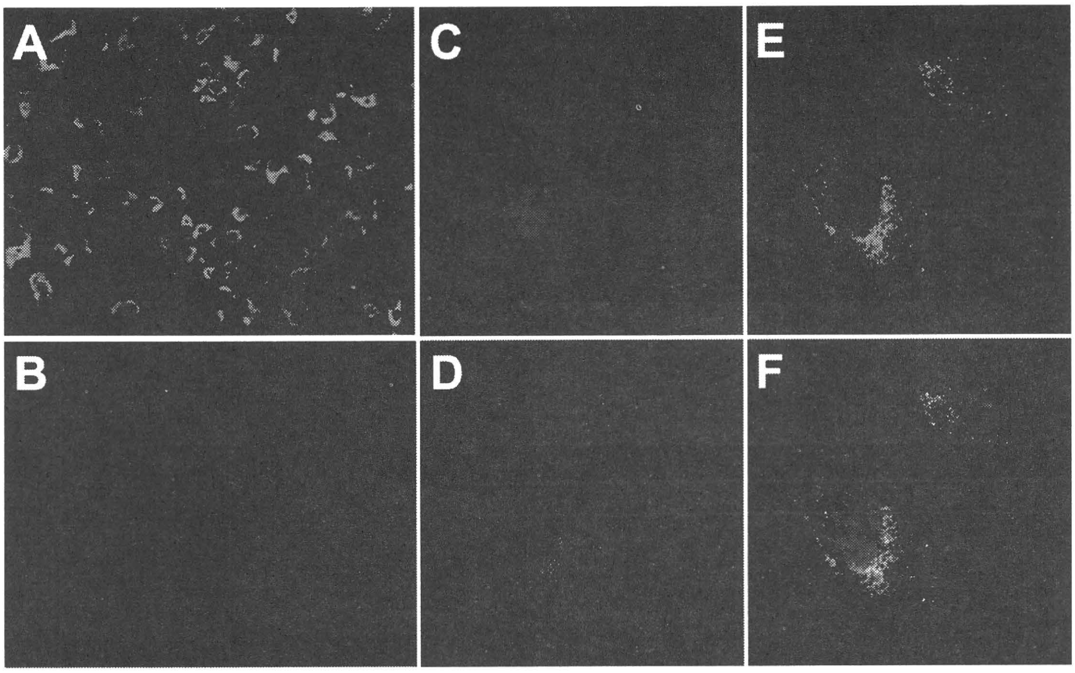

FIG. 4. Indirect immunofluorescence analysis of H751JFH1/Zeo cells. (A and B) H751JFH1/Zeo cells (A) and parental Huh7.5.1 cells (B) were immunostained with an anti-NS5A antibody. (C to F) The subcellular colocalization of *de novo*-synthesized HCV RNA and NS5A in H751JFH1/Zeo cells was analyzed. The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

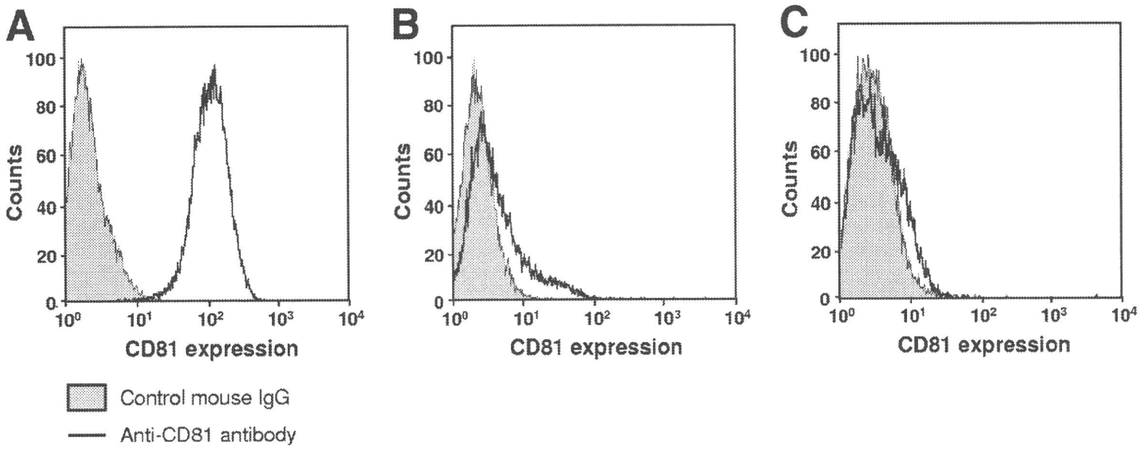

FIG. 5. Loss of CD81 expression in H751JFH1/Zeo cells. The cell surface expression of CD81 on Huh7.5.1 cells (A), H751JFH1/Zeo clone H751-1 (B), and clone H751-50 (C) was analyzed by flow cytometry after being stained with anti-CD81 antibody.

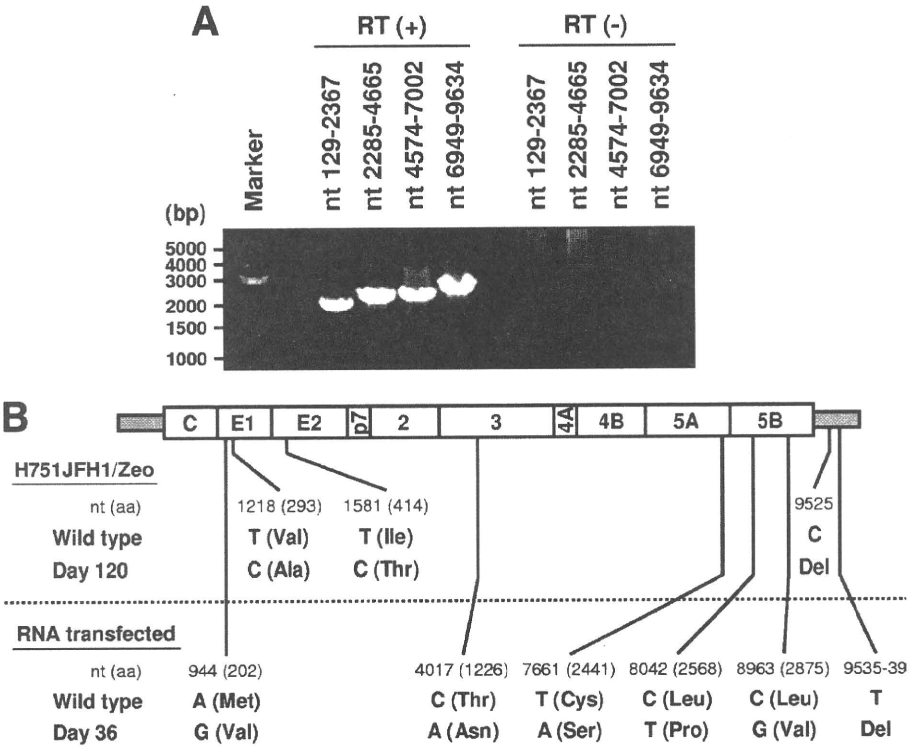

FIG. 6. Genome mutations of HCV secreted from H751JFH1/Zeo cells. (A) RT-PCR of HCV genome extracted from the culture supernatant of H751JFH1/Zeo cells. Viral RNA sequences were reverse transcribed [RT (+)] or not [RT (−)], followed by amplification with primer pairs encompassing the specified HCV genome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

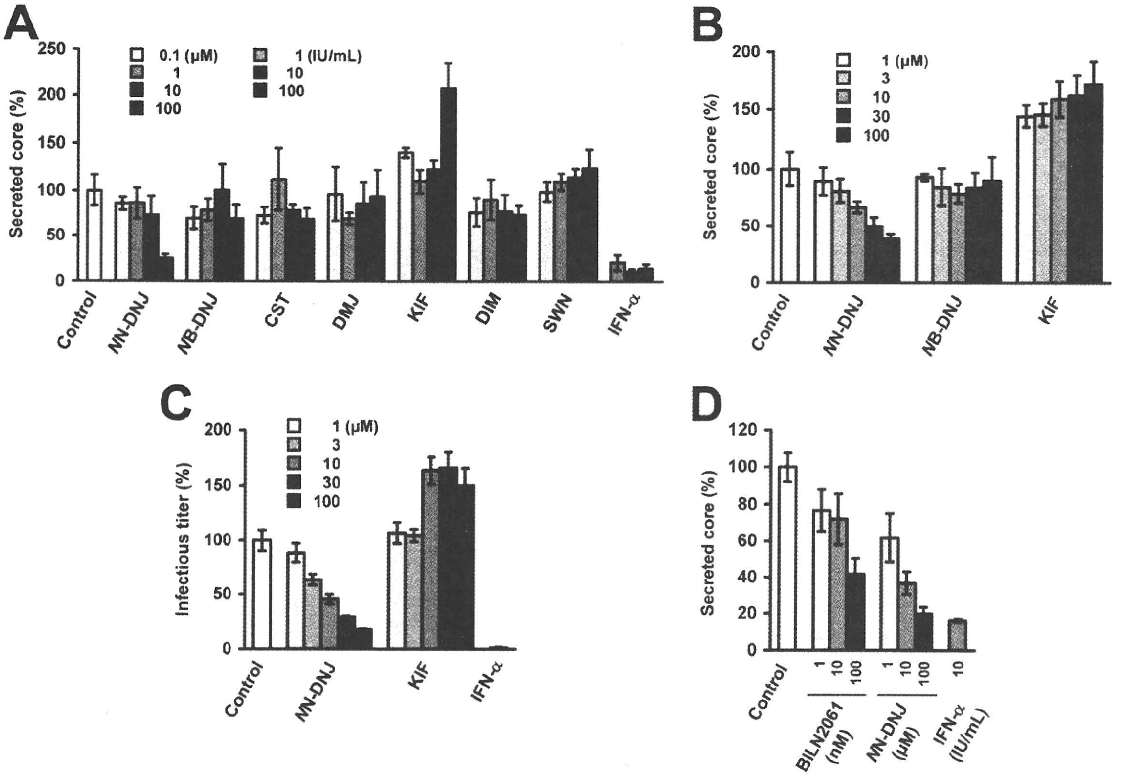

FIG. 7. Effects of glycosylation inhibitors on HCV production from H751JFH1/Zeo cells. (A and B) Effects of glycosylation inhibitors on the secretion of HCV core protein. H751JFH1/Zeo cells were seeded at a density of 1 × 10^4 cells/well in a 96-well culture plate (A) or 3 × 10^4 cells/well in a 12-well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

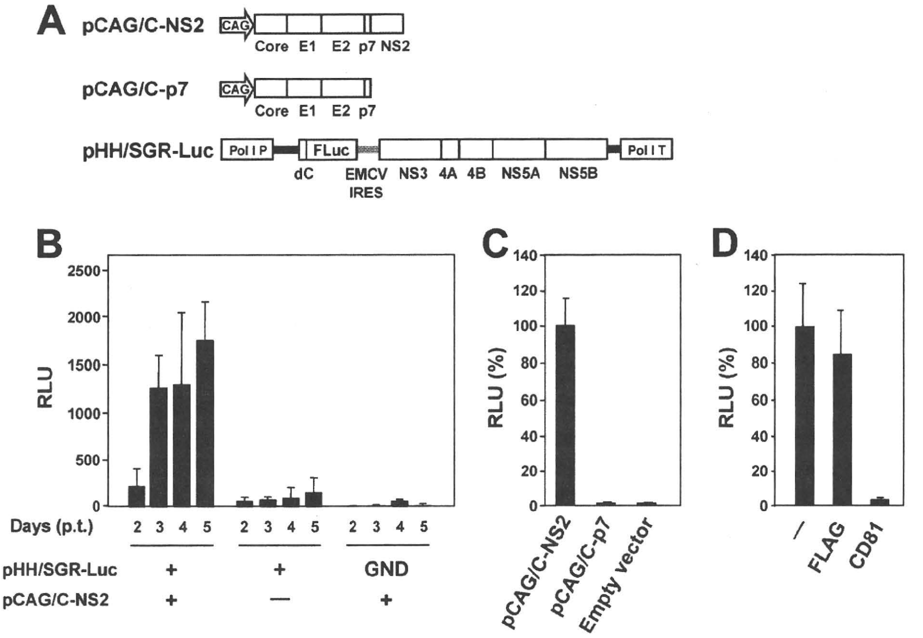

FIG. 8. Establishment of a *trans*-packaging system involving two-plasmid transfection. (A) Schematic representation of the plasmids used for the production of HCV-LP. HCV polyproteins are indicated by the open boxes. Bold lines indicate the HCV UTR. EMCV IRES is denoted by gray bars. The firefly luciferase 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-mannosidase 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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Involvement of PA28γ in the Propagation of Hepatitis C Virus

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We have reported previously that the proteasome activator PA28γ participates not only in degradation of hepatitis C virus (HCV) core protein in the nucleus but also in the pathogenesis in transgenic mice expressing HCV core protein. However, the biological significance of PA28γ in the propagation of HCV has not been clarified. PA28γ is an activator of proteasome responsible for ubiquitin-independent degradation of substrates in the nucleus. In the present study, knockdown of PA28γ in cells preinfection or postinfection with the JFH-1 strain of HCV impaired viral particle production but exhibited no effect on viral RNA replication. The particle production of HCV in PA28γ knockdown cells was restored by the expression of an small interfering RNA (siRNA)-resistant PA28γ. Although viral proteins were detected in the cytoplasm of cells infected with HCV, suppression of PA28γ expression induced accumulation of HCV core protein in the nucleus. HCV core protein was also degraded in the cytoplasm after ubiquitination by an E3 ubiquitin ligase, E6AP. Knockdown of PA28γ enhanced ubiquitination of core protein and impaired virus production, whereas that of E6AP reduced ubiquitination of core protein and enhanced virus production. Furthermore, virus production in the PA28γ knockdown cells was restored through knockdown of E6AP or expression of the siRNA-resistant wild-type but not mutant PA28γ incapable of activating proteasome activity. *Conclusion:* Our results suggest that PA28γ participates not only in the pathogenesis but also in the propagation of HCV by regulating the degradation of the core protein in both a ubiquitin-dependent and ubiquitin-independent manner. (HEPATOLOGY 2010;52:411-420)

Over 170 million individuals worldwide are infected with hepatitis C virus (HCV), which is a major etiological agent of liver diseases, including hepatic steatosis, cirrhosis, and hepatocellular carcinoma (HCC).[1] HCV is classified into the genus Hepacivirus of the *Flaviviridae* family and has a positive, single-strand RNA genome that encodes a single polyprotein consisting of about 3,000 amino acids.[2] The N-terminal one-third of the polyprotein is occupied by the structural proteins, and the remaining portion consists of nonstructural proteins involved in viral replication and assembly. Host and viral proteases cleave the appropriate sites of the polyprotein, resulting in generation of at least 10 viral proteins. The capsid (core), E1 and E2 proteins, and p7 are cleaved off by signal peptidase from the polyprotein. Furthermore, the C-terminal signal sequence of the core protein is processed by signal peptide peptidase.[3] Our recent data indicate that signal peptide peptidase cleaves the polyprotein between Phe^{177} and Leu^{178} in the signal sequence, and this processing is required for HCV propagation.[4] The mature core proteins make nucleocapsid with viral RNA, and HCV particles bud into the lumen of the endoplasmic reticulum bearing E1 and E2 glycoproteins on the host lipid components, and are released from the host cells.

Several reports suggest that HCV core protein plays an important role in the development of various outcomes of liver failure, including steatosis and HCC.[5,6]

Abbreviations: HA, hemagglutinin; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; JEV, Japanese encephalitis virus; moi, multiplicity of infection; shRNA, short hairpin RNA; siRNA, small interfering RNA.

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We have reported previously that HCV core protein specifically interacts with a proteasome activator PA28γ/REGγ in the nucleus and is digested by a PA28γ-dependent proteasome activity.[7] *In vivo* experiments in a mouse model suggest that PA28γ plays a critical role in the pathogenesis induced by HCV core protein.[8,9] PA28γ forms a homoheptamer in the nucleus and enhances the proteasome-mediated cleavage after basic amino acid residues, whereas PA28α and PA28β exhibit 41% and 34% homology to PA28γ, respectively, and form a heteroheptamer in the cytoplasm to activate cleavage after hydrophobic, acidic, or basic amino acid residues.[10] Recently, several groups reported that PA28γ interacts with steroid receptor coactivator-3 and cell cycle suppressors such as $p21^{WAF1/CIP1}$, $p16^{INK4A}$, and $p19^{ARF}$, and enhances the degradation of these proteins in a ubiquitin- and adenosine triphosphate–independent manner.[11-13] Furthermore, other mechanisms of ubiquitin-independent degradation have been considered for cell cycle regulation, summarized in the review of Jariel -Encontre et al.[14] However, the precise physiological functions of PA28γ are largely unknown *in vivo*, because PA28γ-knockout mice exhibit only mild growth retardation and live approximately as long as their control littermates.[15,16]

HCV core protein is degraded in a PA28γ-dependent and ubiquitin-independent manner in the nucleus,[7,17] while E6AP is also involved in the degradation of the core protein in a ubiquitin-dependent manner.[17,18] E6AP is a member of E3 ligases, which catalyze ubiquitin ligation of host and foreign proteins. Knockdown of E6AP suppressed degradation of HCV core protein and enhanced the release of infectious particles, suggesting that E6AP negatively regulates HCV propagation.[18] However, the role of PA28γ in the propagation of HCV has not yet been characterized. In this study, we examined the biological significance of PA28γ in the propagation of HCV.

Materials and Methods

Transfection, Immunoblotting, and RNA Interference. Plasmid DNA was transfected into Huh7OK1 cells by way of liposome-mediated transfection using Lipofectamine LTX with Plus reagent (Invitrogen, Carlsbad, CA). Expression of HCV core protein was determined by way of enzyme-linked immunosorbent assay as described.[19] Immunoblotting was performed as described.[8] The small interfering RNAs (siRNAs) targeted to the PA28γ gene were purchased from Ambion (Austin, TX) and were introduced into the cell lines using Lipofectamine RNAiMax (Invitrogen). siRNAs with the Ambion siRNA ID numbers 138669 and 138670 were designated as siPA28γ1 and siPA28γ2, respectively. Antibodies and plasmids are described in the Supporting Information.

Cell Lines and Virus Infection. All cell lines were cultured at 37°C under the conditions of humidified atmosphere and 5% CO_2. The human hepatoma cell line Huh7OK1 and derivative cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with nonessential amino acids, sodium pyruvate, and 10% fetal bovine serum. The Huh7-derived cell line harboring a subgenomic or a full-length HCV replicon RNA[20] was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, and 1 mg/mL G418 (Nakarai Tesque, Kyoto, Japan). Huh7OK1 cells were transfected with pSilencer-shPA28γ4 or a control plasmid, pSilencer 2.1 U6 hygro negative control (Ambion), and drug-resistant clones were selected by treatment with hygromycin (Wako, Tokyo, Japan) at a final concentration of 100 μg/mL. Huh7OK1 cells transfected with the control plasmid were selected with puromycin and designated as shCntrl, whereas those transfected with pSilencer-shPA28γ4 were established by limited dilution,[8] and two of the resulting cell lines were designated as KD5 and KD7. Plasmids encoding wild-type or mutant PA28γ complementary DNAs resistant to siRNA against PA28γ were prepared by using the silent mutations as reported.[8] These plasmids were transfected into Huh7OK1 cells and cultivated in medium containing 0.1 μg/mL of puromycin for 2 days. The surviving cells were used for virus infection. The shCtrl and KD5 cells were transformed with pSilencer shE6AP or pSilencer 3.1 H1 puro negative control (Ambion) and treated with 0.1 μg/mL of puromycin for 2 days. The surviving cells were infected with JFH-1 virus at a multiplicity of infection (moi) of 0.05. The viral RNA derived from the plasmid pJFH1 was transcribed and introduced into Huh7OK1 cells according to the method of Wakita et al.[21] The infectivity of JFH1 strain was determined using a focus-forming assay[21] and is expressed in focus-forming units. The Huh7 cell line harboring subgenomic replicon RNA of the Con1 or JFH1 strain was prepared according to the method of Pietschmann et al.[22] The infectivity of the Japanese encephalitis virus (JEV) was determined by an immunostaining focus assay as described[23] and is expressed in focus-forming units. Colony formation and replication assays, quantitative reverse-transcription polymerase chain reaction, and estimation of cell growth was performed as described in the Supporting Information.

Immunofluorescent Staining. Huh7OK1-derived cells were seeded at 0.5 × 10^4 cells/well in an eight-well chamber slide, infected with JFH-1 virus at an moi of 0.3 after incubation at 37°C for 24 hours, stained with Bodipy 558/568 C_{12} according to the method of Targett-Adams et al.[24] at 4 days postinfection, and then fixed at 4°C for 30 mintues with 4% paraformaldehyde in phosphate-buffered saline. After treatment of cells with 1 μg/mL of RNase A, nuclei were stained with 50 μM Heachest 33258. The fixed cells were permeabilized with 20 mM Tris-HCl containing 1% Nonidet P-40 and 135 mM NaCl at room temperature for 5 minutes, reacted with rabbit anti-core or anti-NS5A antibody followed by Alexa Fluor 488-goat antibody to rabbit immunoglobulin G, washed three times with phosphate-buffered saline, and observed with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). The percentage of the area occupied by the core protein in nucleus and cytoplasm was calculated using Image-Pro software (Media Cybernetics). The percentage of the nuclear core protein to the total core protein was examined randomly in 10 fields of every three wells. The percentage of the nuclear NS5A to total NS5A was estimated by the same method as the ratio of the core protein.

Results

Transient Knockdown of PA28γ Prior to or After Infection With HCV Reduces Particle Production. We reported previously that Huh7OK1 cells are as permissive to JFH-1 virus infection as Huh7.5.1 cells.[25] The Huh-7OK1 cell line retained the ability to produce type I IFNs through the RIG-I–dependent signaling pathway upon infection with RNA viruses and exhibited a cell surface expression level of human CD81 comparable to that of the parental cell line. However, the mechanism through which the Huh7 OK1 cell line exhibits highly permissive to JFH-1 virus infection has not been clarified yet. Two siRNAs were used to knock down PA28γ, but only one, siPA28γ1, was used because the other had off-target effects (Supporting Fig. 1). To examine the effect of PA28γ on the propagation of HCV, siPA28γ1 was introduced into Huh7OK1 cells 24 hours before infection. The levels of viral RNA, core protein, and infectious viral titer were determined at 48 and 96 hours postinfection. Viral RNA in the culture supernatant and cells was clearly reduced by the knockdown of PA28γ at 48 and 96 hours postinfection, respectively (Fig. 1A), whereas a significant reduction of core protein expression was detected at 96 hours but not at 48

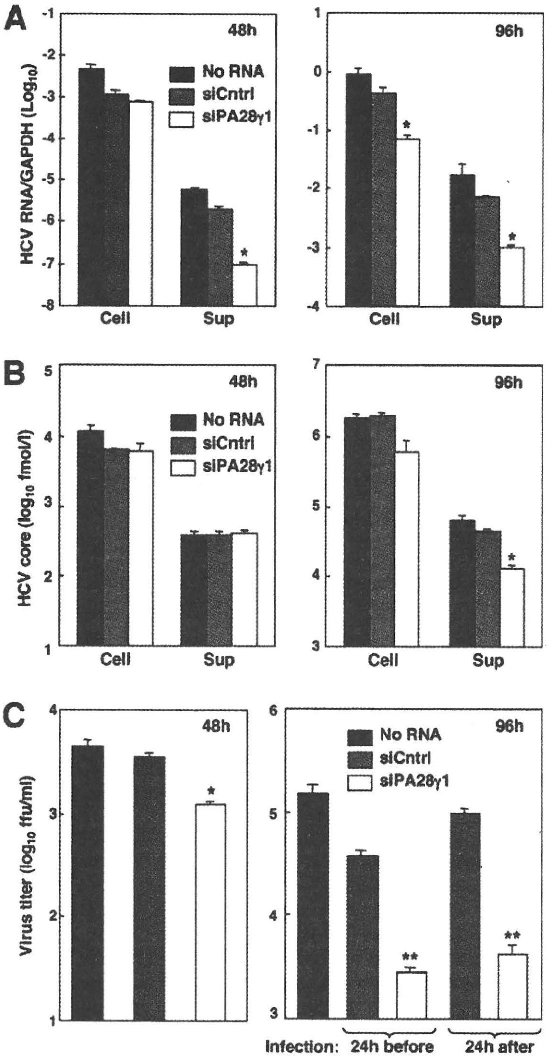

Fig. 1. Transient knockdown of PA28γ before or after infection with HCV reduces particle production. (A) Huh7OK1 cells transfected with a control siRNA (siCntrl) or PA28γ siRNA1 were infected with JFH-1 virus at 24 hours posttransfection and then harvested at 48 hours (left panel) and 96 hours postinfection (right panel). The quantity of HCV RNA in cells and supernatants was determined by way of quantitative reverse-transcription polymerase chain reaction. (B) The expression of HCV core protein in cells and supernatants at 48 hours (left panel) and 96 hours (right panel) postinfection was determined by ELISA. (C) Huh7OK1 cells that were transfected with siCntrl or PA28γ siRNA1 were infected with JFH-1 virus at 24 hours posttransfection. The infectivity of the virus in the culture supernatant was determined by a focus-forming assay at 48 hours postinfection (left panel). Those transfected with the siRNAs at 24 hours before and after infection with JFH-1 virus were determined similarly at 96 hours postinfection (right panel). $*P < 0.05$, $**P < 0.01$ versus control siRNA-transfected cells. Data are representative of three independent experiments.

hours postinfection (Fig. 1B). Infectious viral titer in the culture supernatant was significantly reduced at 48 and 96 hours postinfection by the PA28γ knockdown (Fig. 1C), consistent with the suppression of the viral RNA in the supernatant. Furthermore, a comparable suppression of the production of infectious particles in the supernatant was also achieved by introducing siPA28γ1 into cells even at 24 hours postinfection (Fig. 1C, right panel). These results suggest that PA28γ participates in the regulation of HCV propagation in postentry steps.

Stable Knockdown of PA28γ Impairs Viral Propagation. To establish the PA28γ knockdown cell lines, Huh7OK1 cells were transfected with a plasmid encoding a short hairpin RNA (shRNA) targeted to PA28γ and selected with hygromycin, resulting in two clones—KD5 and KD7—that exhibited a clear reduction of PA28γ expression (Fig. 2A). Although the suppression of PA28γ expression in KD7 cells was slightly more efficient than that in KD5 cells, the growth of KD7 cells was impaired (Fig. 2B). Viral production in the culture supernatants in cells infected with the JFH-1 virus was significantly impaired in PA28γ knockdown KD5 cells compared with control cells (Fig. 2C). The viral RNA and core protein in the supernatant were also reduced in KD5 cells (Fig. 2D). Expression of siRNA-resistant PA28γ in PA28γ knockdown KD5 and KD7 cells recovered virus production in the supernatant to a level similar to that in the control cells transfected with an empty vector, and overexpression of siRNA-resistant PA28γ in control cells slightly enhanced virus production (Fig. 2E). Our previous data suggest that capsid protein of JEV does not bind to PA28γ.[7] To examine whether PA28γ regulates JEV propagation, KD5 and shCntrl cells were infected with JEV at an moi of 0.5. The infectivity of JEV in KD5 cells was similar to that in shCntrl cells (Fig. 2F), suggesting that PA28γ does not participate in the virus production pathway of JEV. These results further support the notion that PA28γ participates in HCV propagation.

Knockdown of PA28γ Exhibits No Effect on Viral RNA Replication. Although knockdown of PA28γ resulted in the suppression of viral particle and RNA production in the culture supernatant at 48 hours postinfection with JHF-1 virus, viral RNA in the cells was not reduced (Fig. 1), suggesting that PA28γ does not participate in viral replication. To gain more insight on this point, we examined the effect of PA28γ knockdown on RNA replication in replicon cells. Transient knockdown of PA28γ through introduction of siPA28γ into the subgenomic HCV replicon cells derived from the Con1 or JFH-1 strain induced no significant reduction of HCV RNA (Fig. 3A). Furthermore, luciferase activities in the stable PA28γ

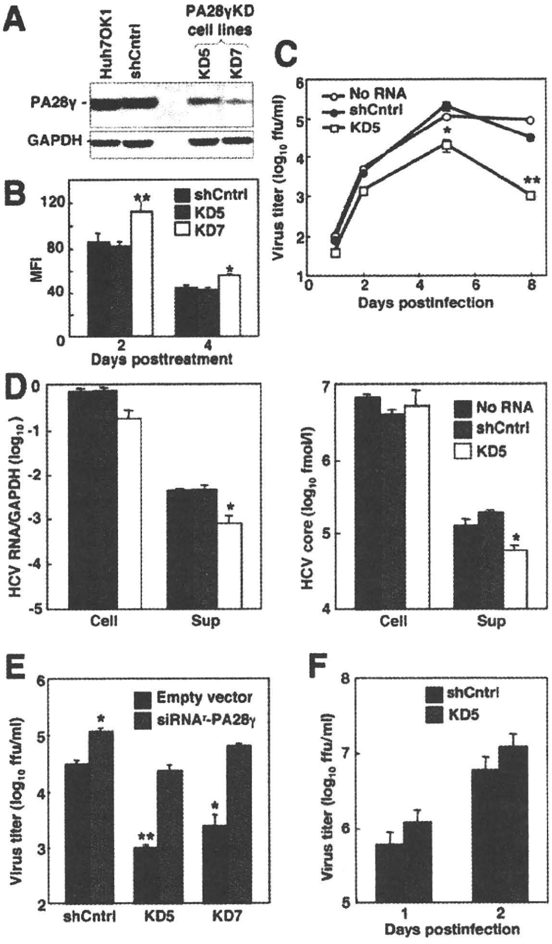

Fig. 2. Establishment of PA28γ knockdown cell lines and propagation of HCV. (A) Huh7OK1 cells were transfected with pSilencer shPA28γ or control plasmid and selected by hygromycin at 48 hours posttransfection. Two PA28γ knockdown cell lines (KD5 and KD7) and one control cell line (shCntrl) were established, and PA28γ knockdown was confirmed by way of immunoblotting. (B) Growth of the cell lines was determined by staining with carboxyfluorescein succinimidyl ester. (C,D) KD5 and shCntrl cell lines were infected with the JFH-1virus at an moi of 0.05. The infectious virus titers in the culture supernatants (C) was determined by way of focus-forming assay. The virus RNA (D, left panel) and the core protein (D, right panel) in both cell and the supernatant were determined at 5 days postinfection by way of ELISA and quantitative reverse-transcription polymerase chain reaction, respectively. (E) The plasmid encoding an siRNA-resistant PA28γ or empty vector was transfected into the cell lines, seeded at 5 × 10^4 cells into a six-well plate after cultivation in the presence of puromycin for 2 days, and infected with JFH-1 virus at an moi of 0.05. The viral titers were determined at 5 days postinfection. *$P < 0.05$, **$P < 0.01$ versus shCntrl cells transfected with an empty vector. (F). KD5 and shCntrl cell lines were infected with the JEV virus at an moi of 0.5. The infectivity of JEV in the supernatant was determined at 1 and 2 days postinfection. Data are representative of three independent experiments.

knockdown cell line KD5 and the control cell line transfected with the subgenomic replicon RNA (WT) were gradually increased until 4 days posttransfection, whereas luciferase activities in the same two cell lines transfected with the polymerase-dead replicon RNA (GND) were decreased in a time-dependent manner (Fig. 3B). Next, to explore the effect of PA28γ knockdown on the viral replication over a longer period, replicon RNA encoding the neomycin-resistance gene was transfected into the cell lines for a colony formation assay. The numbers of colonies in the KD5 cell line after 4 weeks of selection with G418 were similar to those in the control cell line (Fig. 3C). To further clarify the roles of PA28γ on the postreplication steps, *in vitro* transcribed full-length viral RNA was transfected into Huh7OK1 cells, and siPA28γ1 was then introduced into the cells at 24 hours posttransfection of viral RNA. Intracellular core protein was increased in a time-dependent manner, but no significant difference was observed between cells transfected with control siRNA and those transfected with siPA28γ1 (Fig. 3D, left panel). However, infectious virus titers in the supernatant were significantly decreased by the transient and stable knockdown of PA28γ compared with control cells (Fig. 3D, middle and right panels). Furthermore, PA28γ did not contribute to the virus production of JEV (Fig. 2F), suggesting that the general sorting pathway of the flavivirus is functional under

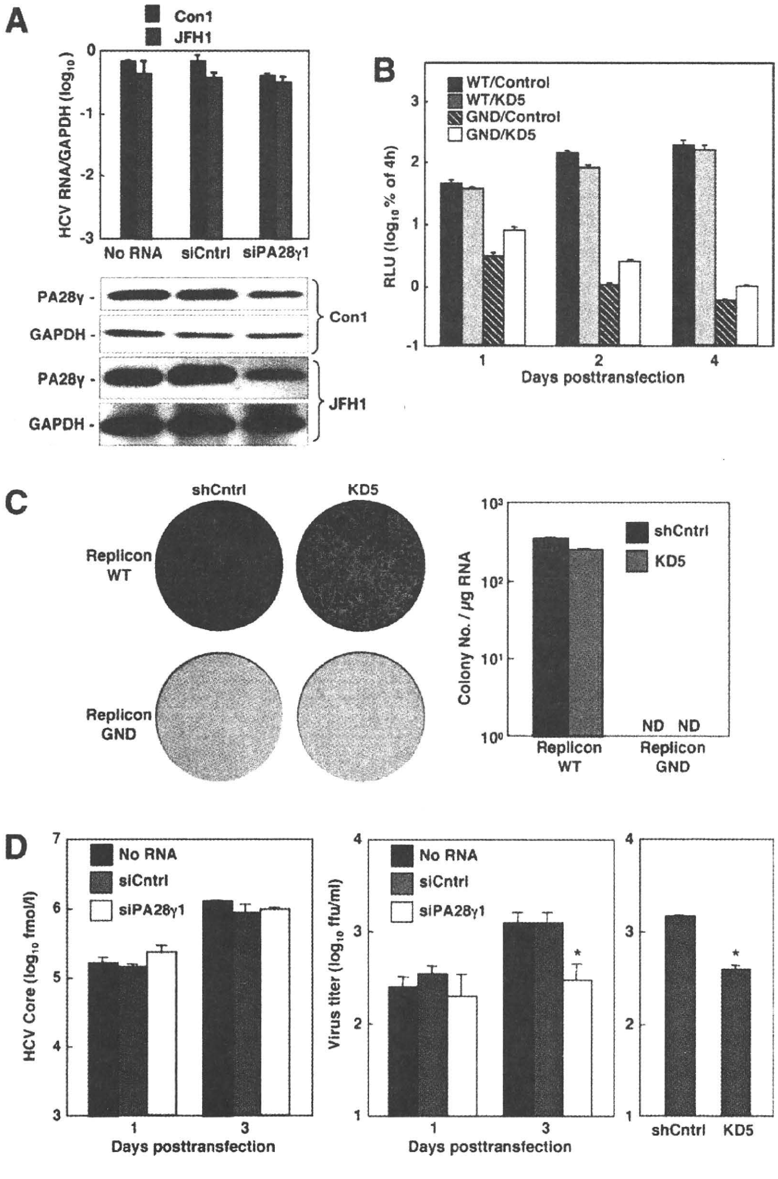

Fig. 3. Effect of PA28γ knockdown on HCV RNA replication. (A) The siCntrl or siPA28γ1 (10 nM) was transfected into the subgenomic HCV replicon cells derived from Con1 and JFH-1 strains. The transfected cells were harvested at 72 hours posttransfection. The replicon RNA was determined by quantitative reverse-transcription polymerase chain reaction at 72 hours posttransfection (upper). PA28γ or glyceraldehyde 3-phosphate dehydrogenase was detected by way of immunoblotting. Cell lysates were subjected to western blotting using antibodies to PA28γ and glyceraldehyde 3-phosphate dehydrogenase (lower). (B) The HCV replicon RNA encoding luciferase gene (WT) or the HCV replicon RNA that has a replication-deficient mutation (GND) was transfected into the shCntrl (Control) and KD5 cell lines. Relative luciferase activity was determined using the activity at 4 hours post-electroporation as a transfection efficiency. (C) Colony formation assay. Replicon RNA encoding the neomycin-resistance gene was transfected into the shCntrl and KD5 cell lines, and the remaining colonies were fixed with 4% paraformaldehyde at 4 weeks posttransfection and then stained with crystal violet. The number of colonies was counted (right). (D) Huh7OK1 cells transfected with 10 μg of *in vitro*-transcribed full-length JFH-1 viral RNA were further transfected with siCntrl or siPA28γ1 at 24 hours posttransfection of viral RNA. The level of HCV core protein in the cells was determined by way of ELISA at 1 and 3 days posttransfection (left). Infectious virus titers in the culture supernatants at 1 and 3 days posttransfection were determined by way of focus-forming assay (middle). Infectious viral titers in the shCntrl or KD5 cells transfected with 10 μg of the infectious viral RNA were determined at 5 days posttransfection (right). $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control cells or cells transfected with siCntrl. Data are representative of three independent experiments.

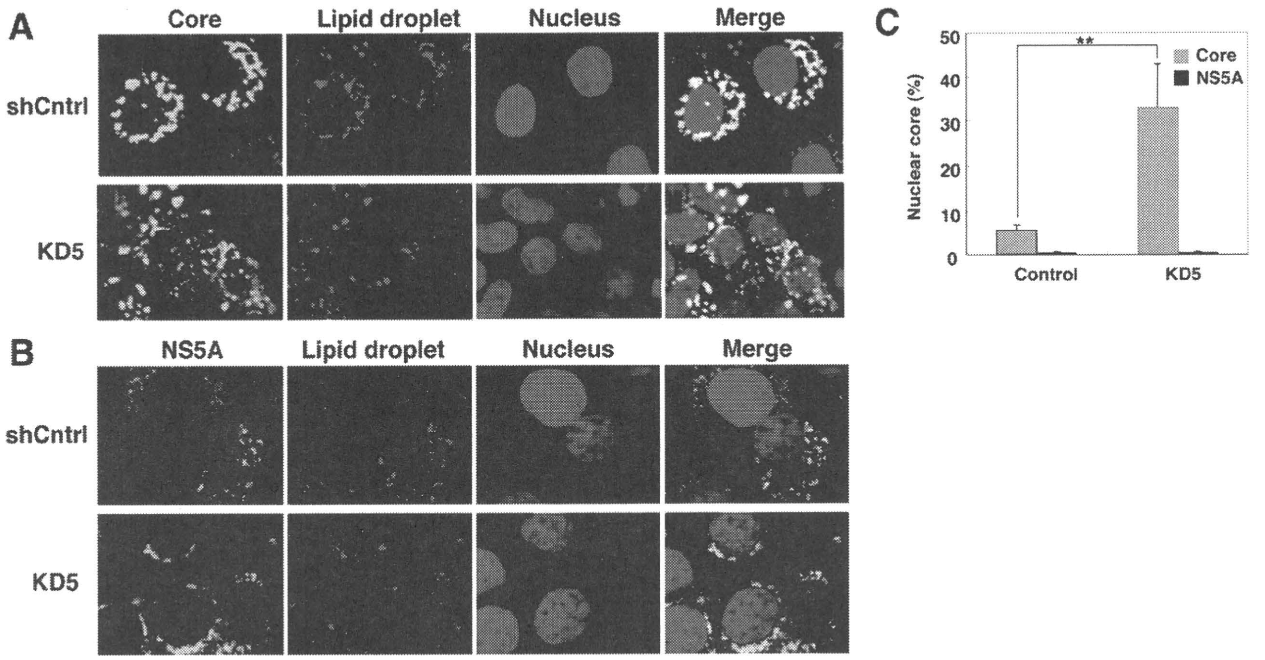

Fig. 4. Effect of PA28γ knockdown on the localization of HCV core protein and lipid droplets. The shCntrl and KD5 cell lines infected with JFH-1 virus were fixed with methanol or paraformaldehyde for 5 minutes at 4 days postinfection. HCV core (A) and NS5A (B) proteins were stained with rabbit antibodies raised against the proteins and Alexa Flour 488-conjugated goat anti-rabbit immunoglobulin G antibody. Lipid droplets were stained with Bodipy 558/568 C12. Nuclei were stained with 50 μM Heachest 33258 after treatment with 1 μg/mL of RNase A. Data are representative of three independent experiments. (C) The percentage of the area occupied by the core protein in nucleus and cytoplasm was calculated using the method described in Materials and Methods. The percentage of the nuclear NS5A to total NS5A was estimated by the same way as the ratio of the core protein. $**P < 0.01$ versus control siRNA-transfected cells.

the PA28γ knockdown condition. These results suggest that PA28γ specifically regulates the postreplication steps in the life cycle of HCV.

Core Protein Is Partially Accumulated in the Nucleus of PA28γ Knockdown Cells. We reported previously that some fraction of HCV core protein migrates into the nucleus and is then degraded by a PA28γ-dependent proteasome pathway.[7] Furthermore, we have demonstrated that HCV core protein is clearly accumulated in the nucleus of the liver cells of PA28γ-knockout mice.[8] However, the role of PA28γ on the intracellular localization of HCV core protein in the infected HCV cells has not been characterized. HCV core protein was chiefly detected in cytoplasm of the control cell line infected with the JFH-1 virus, where it appeared around lipid droplets after staining with Bodipy 558/568 C12 (Fig. 4A, upper panels). In contrast, the core protein was detected not only in the cytoplasm around the surface of lipid droplets, but also in the nucleus in the KD5 cell line (Fig. 4A, lower panels). The NS5A protein was detected in the cytoplasm but not in the nucleus in both the shCntrl and KD5 cell lines (Fig. 4B). The percentage occupied by nuclear core protein to total core protein was increased by about six time levels in the KD5, while the ratio of nuclear NS5A to total NS5A exhibited no difference (Fig. 4C). These results suggest that PA28γ participates in the degradation of HCV core protein in the nucleus.

PA28γ Positively Regulates HCV Propagation by Inhibiting Ubiquitin-Dependent Degradation of Core Protein in Cytoplasm. We reported previously that HCV core protein is degraded by at least two distinct pathways: a ubiquitin-dependent proteasome pathway and a ubiquitin-independent proteasome pathway.[17] The ubiquitin E3 ligase, E6AP, can catalyze ubiquitin ligation of the core protein for ubiquitin-dependent degradation in the cytoplasm,[18] whereas PA28γ participates in the degradation of the core protein through a ubiquitin-independent pathway in the nucleus.[17] We have also demonstrated that PA28γ knockdown leads to enhanced ubiquitination of HCV core protein.[8] However, the interplay between these two pathways in cells infected with HCV has not been determined. To address this point, we examined the effects of knockdown of E6AP or PA28γ on the virus propagation and the ubiquitination of the core protein. JFH-1 virus was inoculated into E6AP- and/or PA28γ knockdown cell lines (Fig. 5A). Transfection of the plasmid encoding shRNA to E6AP into the control cells (shCntrl) increased virus production (Fig. 5A [C-E]) in comparison with that of the

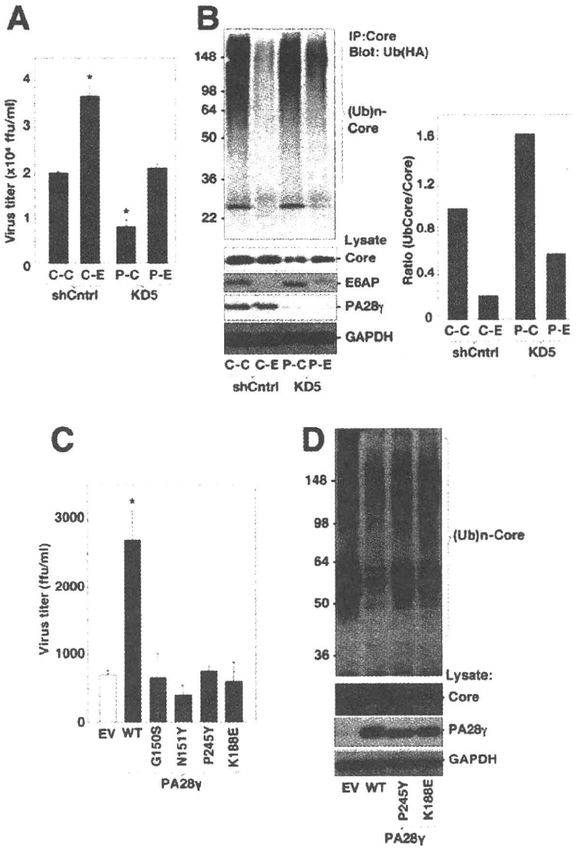

Fig. 5. PA28γ knockdown enhances E6AP-dependent ubiquitination of core protein and reduces virus titer. (A) shCntrl and KD5 cells transfected with plasmids encoding the negative control (C-C and P-C) or E6AP (C-E and P-E) shRNA were treated with puromycin for 2 days. The remaining cells seeded at 2.5×10^4 cells in a 24-well plate were infected with the JFH-1 virus at an moi of 0.05, and infectious virus titers in the supernatants were determined at 72 hours postinfection by way of focus-forming assay. (B) The cells transfected and infected as in (A) were further transfected with a plasmid encoding HA-tagged ubiquitin at 48 hours postinfection. The cells were treated with 10 μM MG132 for 5 hours at 72 hours postinfection and subjected to immunoprecipitation with anti-core monoclonal antibody and immunoblotting with anti-HA antibody. The ratio of ubiquitination of HCV core protein was assessed by the densitometries of the ubiquitinated and unubiquitinated core proteins. (C) KD5 cells transfected with plasmids encoding wild-type or mutant PA28γ were infected with the JFH-1 virus at an moi of 0.05 at 24 hours posttransfection, and the infectious titers in the supernatant were determined at 72 hours postinfection by way of focus-forming assay. (D) KD5 cells transfected with plasmids encoding HCV core protein and HA-tagged ubiquitin, together with wild-type or mutant PA28γ, were treated with 10 μM MG132 for 5 hours at 24 hours posttransfection and subjected to immunoprecipitation with anti-core monoclonal antibody and immunoblotting with anti-HA antibody. EV, empty vector; WT, plasmid encoding wild-type PA28γ. $^*P < 0.05$ versus shCntrl or KD5 cells transfected with the negative control or empty vector. Data are representative of three independent experiments.

control cells transfected with the plasmid encoding control shRNA (Fig. 5A [C-C]). Furthermore, the impaired virus production in the PA28γ knockdown cells (KD5) was restored by the transfection of the plasmid encoding shRNA to E6AP (Fig. 5A [P-E]). Cells expressing hemagglutinin (HA)-tagged ubiquitin infected with the JFH-1 virus were immunoprecipitated by the anti-core antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-HA antibody (Fig. 5B). E6AP knockdown decreased the ratio of ubiquitination of HCV core protein, in contrast to the increase of that by PA28γ knockdown (Fig. 5B, lanes C-E and P-C). Furthermore, E6AP knockdown in the PA28γ knockdown cells restored the ubiquitination of the core protein to a certain extent (Fig. 5B, lane P-E). It was shown that Pro^{245} of PA28γ is critical for binding to the 20S proteasome, and that Gly^{150} and Asn^{151} of PA28γ are important for activation of the proteasome.[26] To further examine the functional significance of PA28γ on HCV propagation, expression plasmids encoding siRNA-resistant PA28γ mutants in which Gly^{150}, Asn^{151}, and Pro^{245} were replaced with Ser (G150S), Tyr (N151Y), and Tyr (P245Y), respectively, were transfected into KD5 cells and inoculated with JFH-1 virus at 24 hours posttransfection. The infectious virus titers in the culture supernatant were determined at 3 days postinfection (Fig. 5C). KD5 cells transfected with the plasmid encoding wild-type PA28γ exhibited a partial recovery of virus production, although those transfected with the plasmid encoding PA28γ G150S, N151Y, or P245Y or with an empty vector exhibited no effect on virus production. Replacing Lys^{188} with Glu in PA28γ (PA28γ K188E) confers the capability of proteasome-mediated cleavage after hydrophobic, acidic, and basic residues such as those exhibited by PA28α.[27] Expression of siRNA-resistant PA28γ K188E in KD5 cells could not restore virus production (Fig. 5D). The ubiquitination of HCV core protein was inhibited by expression of the wild-type PA28γ but not expression of the PA28γ mutants (P245Y or K188E) in KD5 cells (Fig. 5D). Collectively, these results suggest that PA28γ positively regulates HCV propagation by inhibiting degradation of HCV core protein by an E6AP/ubiquitin-dependent proteasome.

Discussion

To explore the role of PA28γ on the life cycle of HCV, we examined the effects of knockdown of PA28γ in Huh7OK1 cells infected with the JFH-1 virus. Knockdown of PA28γ in Huh7OK1 cells before or after infection with the JFH-1 virus impaired production of infectious particles but did not impair viral RNA replication. However, PA28γ knockdown did not affect the production of JEV, of which the capsid protein does not interact with PA28γ, suggesting that PA28γ knockdown does not affect the general sorting pathway of flavivirus. These results suggest that PA28γ is specifically involved in the postreplication steps of HCV life cycle. Our previous report indicated that HCV core protein was accumulated in the nucleus of the hepatocytes of HCV core transgenic/PA28γ knockout mice.[8] PA28γ is located mainly in the nucleus, although a small portion is also located in the cytoplasm[7,28] and can up-regulate trypsin-like proteasome activity, which cleaves after basic amino acid residues.[27] Previous studies have shown that some fraction of HCV core protein is translocated into the nucleus and quickly degraded in the PA28γ-dependent proteasome pathway.[7,8,29] Miyanari et al.[30] demonstrated that the core protein is localized on the surface of lipid droplets and is surrounded by nonstructural proteins, suggesting that HCV particles are assembled near the surface of the lipid droplets. In the present experiments, although HCV core protein was detected on the surface of the lipid droplets in both control and PA28γ knockdown cell lines, it was partially localized in the nucleus in PA28γ knockdown cells but not control cells. Furthermore, localization of HCV core protein on the surface of lipid droplets was impaired in PA28γ knockdown cells (Fig. 4). These results suggest that HCV core protein is partially translocated into the nucleus and degraded in the PA28γ-dependent proteasome pathway in HCV-infected cells and that PA28γ does not directly participate in the particle formation of HCV.

HCV core protein is degraded by at least two proteasome pathways: a ubiquitin-dependent pathway and a ubiquitin-independent and PA28γ-dependent pathway.[17] The E3 ligase E6AP catalyzes ubiquitin ligation to HCV core protein, resulting in enhanced degradation of the core protein in the cytoplasm.[18] Knockdown of E6AP up-regulated virus production in cells infected with the JFH-1 virus,[18] suggesting that E6AP/ubiquitin-dependent degradation of the core protein contributes to an antiviral response. In contrast, knockdown of PA28γ induced up-regulation of the ubiquitination of HCV core protein and down-regulation of the viral production, suggesting that PA28γ-dependent proteasome activity contributes to the proviral response by suppressing E6AP-dependent degradation of the core protein, thereby enhancing viral particle formation. The wild-type PA28γ enhances the trypsin-like activity of proteasome that cleaves peptide bonds after basic residues of the substrates, whereas the PA28γ K188E mutant enhances the proteasome activity that cleaves peptide bonds after hydrophobic, acidic, and basic residues in the manner of PA28α.[27] Therefore, the sizes of fragments produced by the PA28γ-dependent proteasome should be different from those produced by the PA28α/β- or ubiquitination-mediated proteasome. It might be feasible to speculate that the peptide fragments of HCV core protein generated by the PA28γ-dependent proteasome or PA28γ *per se* may be directly or indirectly involved in the suppression of the E6AP-dependent ubiquitination of the core protein. Further studies will be needed to clarify the relationship between E6AP and PA28γ in the degradation and ubiquitination of HCV core protein. Figure 6 shows a schematic diagram of our hypothesis of the regulation of HCV propagation by PA28γ.

HCV core protein was found in not only nuclei but also cytoplasm of the infected KD5 cells (Fig. 4). The down-regulation of virus production should potently reduce a total amount of the core protein in KD5 cells before a clear accumulation of the core protein in nuclei. Furthermore, a small amount of PA28γ was found in the PA28γ knockdown cells, suggesting that E6AP-dependent degradation of HCV core protein is not potently suppressed in the PA28γ knockdown cells. If HCV core protein is constitutively expressed under the PA28γ knockout cells regardless of an amount of infected virus, a clear accumulation of the core protein in nuclei should be found without cytoplasmic expression of the core protein under the PA28γ knockout condition. We reported previously that HCC and liver steatosis in mouse are induced by the HCV core protein in the presence, but not the absence, of PA28γ.[8] Although HCV core protein is predominantly detected in the cytoplasm of the liver cells of PA28$\gamma^{+/+}$ mice,[8,31] HCV core protein was clearly accumulated in the nuclei, but clearly reduced in cytoplasm, of liver cells of PA28$\gamma^{-/-}$ mouse.[8] In addition, ubiquitination of HCV core protein was increased by PA28γ knockdown in the 293T cell line.[8] These results and the data in Fig. 5 suggest that the suppression of PA28γ function enhances the E6AP-dependent degradation of HCV core protein. Hence, the reason there is no difference between PA28$\gamma^{+/+}$ and PA28$\gamma^{-/-}$ mice with respect to the amount of core protein may be due to the competitive regulation of the core protein by E6AP- and PA28γ-dependent degradation mechanisms. E6AP-dependent degradation of HCV core protein in cytoplasm may be enhanced *in vivo* under the PA28γ knockout condition.

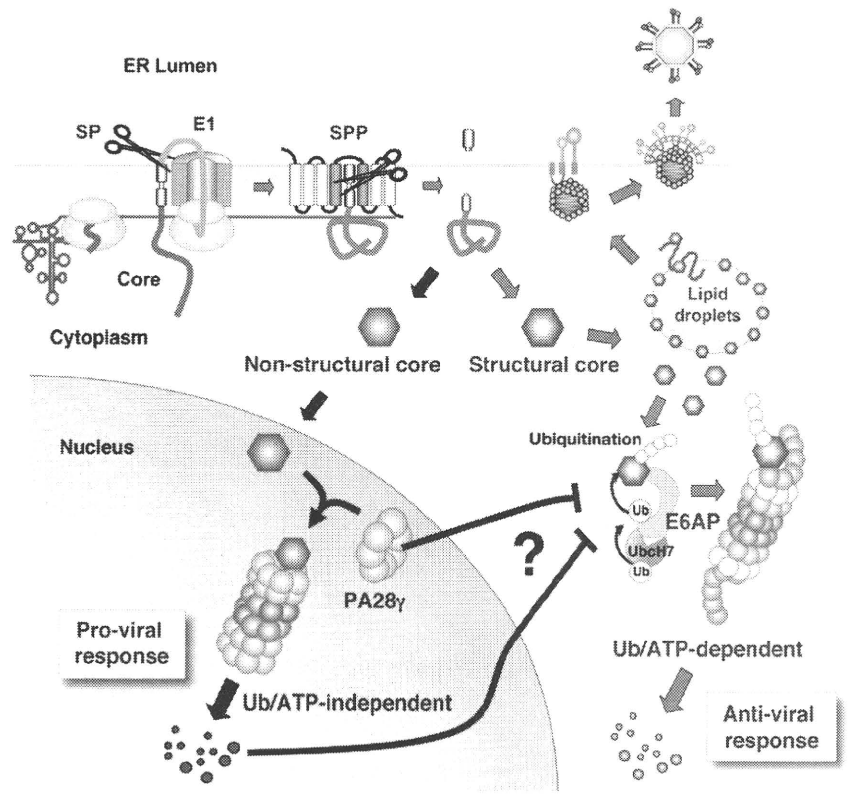

Fig. 6. Schematic diagram of the potential roles of PA28γ in HCV propagation. HCV core protein is cleaved off from the precursor polyprotein by signal peptidase (SP) and the signal sequence is further processed by signal peptide peptidase (SPP). The mature core protein mainly localizes on the lipid droplets close to the endoplasmic reticulum to form a nucleocapsid with the viral RNA genome and is incorporated into virus particles as a structural protein. In addition to the structural protein of HCV, the core protein has characteristics of a nonstructural protein. HCV core protein is degraded through ubiquitin-dependent and ubiquitin-independent proteasome pathways. E6AP catalyzes ubiquitin ligation to HCV core protein and promotes degradation in the cytoplasm, which contributes to the antiviral response. In contrast, the core protein partially migrates into the nucleus and is degraded through a ubiquitin-independent and PA28γ-dependent proteasome pathway, and the core protein fragments generated by the PA28γ pathway or PA28γ *per se* were suggested to participate in the suppression of E6AP-dependent ubiquitination of HCV core protein, which contributes to the proviral response.

In conclusion, in this study we demonstrated that the proteasome activator PA28γ positively regulates particle production of HCV by inhibiting E6AP-dependent ubiquitination of the core protein, in addition to our previous observation that PA28γ plays a crucial role in the development of liver pathology induced by HCV core protein.[8] PA28γ knockout mice exhibit only mild growth retardation.[15,16] Therefore, PA28γ may be a novel and promising antiviral target not only for elimination of HCV from hepatitis C patients but also for intervention in the progression of liver diseases induced by chronic HCV infection.

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