

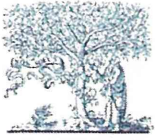
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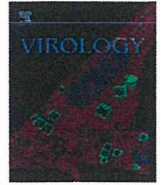
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Trans-complemented hepatitis C virus particles as a versatile tool for study of virus assembly and infection

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ABSTRACT

In this study, we compared the entry processes of *trans*-complemented hepatitis C virus particles (HCVtcp), cell culture-produced HCV (HCVcc) and HCV pseudoparticles (HCVpp). Anti-CD81 antibody reduced the entry of HCVtcp and HCVcc to almost background levels, and that of HCVpp by approximately 50%. Apolipoprotein E-dependent infection was observed with HCVtcp and HCVcc, but not with HCVpp, suggesting that the HCVtcp system is more relevant as a model of HCV infection than HCVpp. We improved the productivity of HCVtcp by introducing adapted mutations and by deleting sequences not required for replication from the subgenomic replicon construct. Furthermore, blind passage of the HCVtcp in packaging cells resulted in a novel mutation in the NS3 region, N1586D, which contributed to assembly of infectious virus. These results demonstrate that our plasmid-based system for efficient production of HCVtcp is beneficial for studying HCV life cycles, particularly in viral assembly and infection.

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Introduction

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic liver diseases (Hoofnagle, 2002). HCV is an enveloped virus of the family *Flaviviridae*, and its genome is a positive-strand RNA consisting of the 5'-untranslated region (UTR), an open reading frame encoding viral proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) and the 3'-UTR (Suzuki et al., 2007).

Host–virus interactions are required during the initial steps of viral infection. It was previously reported that CD81 (Bartosch et al., 2003a, b; McKeating et al., 2004; Pileri et al., 1998), scavenger receptor class B type I (Bartosch et al., 2003a, b; Scarselli et al., 2002), claudin-1 (Evans et al., 2007; Liu et al., 2009) and occludin (Benedicto et al., 2009; Evans et al., 2007; Liu et al., 2009; Ploss et al., 2009) are critical molecules for HCV entry into cells. CD81 interacts with HCV E2 via a second extracellular loop (Bartosch et al., 2003a, b; Hsu et al., 2003) and its role in the internalization process was confirmed (Cormier et al., 2004; Flint et al., 2006). It has also been shown that infectious

HCV particles produced in cell cultures (HCVcc) exist as apolipoprotein E (ApoE)-enriched lipoprotein particles (Chang et al., 2007) and that ApoE is important for HCV infectivity (Owen et al., 2009).

Investigation of HCV had been hampered by difficulties in amplifying the virus *in vitro* before development of robust cell culture systems based on JFH-1 isolates (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Retrovirus-based HCV pseudoparticles (HCVpp), in which cell entry is dependent on HCV glycoproteins, have been used to study virus entry (Bartosch et al., 2003a; Hsu et al., 2003). Vesicular stomatitis virus (VSV)-based pseudotypic viruses bearing HCV E1 and E2 and replication-competent recombinant VSV encoding HCV envelopes have also been available as surrogate models for studies of HCV infection (Mazumdar et al., 2011; Tani et al., 2007).

It was recently shown that HCV subgenomic replicons can be packaged when structural proteins are supplied in *trans* (Adair et al., 2009; Ishii et al., 2008; Masaki et al., 2010; Steinmann et al., 2008). These *trans*-complemented HCV particles (HCVtcp) are infectious, but support only single-round infection and are unable to spread. Establishment of flexible systems to efficiently produce HCVtcp should contribute to studying HCV assembly, in particular encapsidation of the viral genome, and entry to cells with less stringent biosafety and biosecurity measures. Although single-round infection can be achieved by using the HCVcc system with receptor knock-out

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cells, the single-round HCVcc system is not suitable for studying virus entry. We previously described plasmid-based production of HCVcc and HCVtcp (Masaki et al., 2010). Here, we demonstrated that HCVtcp production can be enhanced by introducing the previously reported cell-culture adaptive mutations and by deleting sequences not essential for replication in the subgenomic replicon construct. By providing genotype 1b-derived core-to-p7 in addition to intragenotypic viral proteins, chimeric HCVtcp were generated. Furthermore, blind passage of HCVtcp in the packaging cells resulted in the identification of a novel cell culture-adaptive mutation in NS3 that enables us to establish the efficient production of HCVtcp with structural proteins from various strains. Taken together, our system for producing single-cycle infectious HCV particles should be useful in the study of entry and assembly steps of the HCV life cycles. This technology may also have potential to be the basis for the safer vaccine development.

Results

Enhancement of HCVtcp production by adaptive mutations in E2, p7 and NS2 and by deleting sequences not essential for replication from replicon construct

In our HCVtcp system, the RNA polymerase I (Pol I)-driven replicon plasmid, which carries a dicistronic subgenomic luciferase reporter replicon of JFH-1 strain with a Pol I promoter and terminator (pHH/SGR-Luc), as well as a plasmid containing core-NS2 cDNA under the CAG promoter (pCAGC-NS2) were used (Masaki et al., 2010). In an effort to improve the yield of HCVtcp production, cell culture-adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) which were previously selected from serial passage of HCVcc (Russell et al., 2008) were introduced into the core-NS2 expression plasmid (Fig. 1A) (residues are numbered

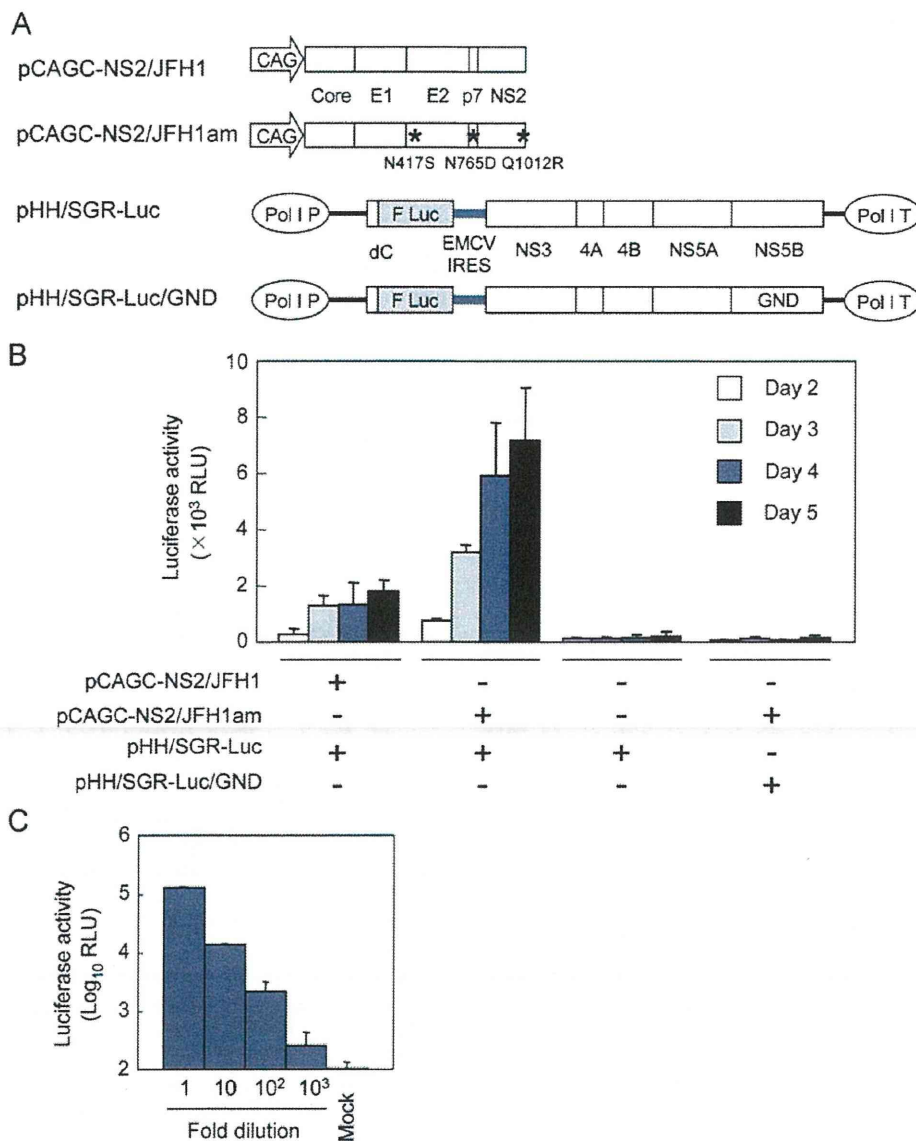


Fig. 1. HCVtcp production by two-plasmid transfection. (A) Schematic representation of plasmids is shown. HCV polyproteins derived from JFH-1 are indicated by white boxes. HCV UTRs are indicated by bold lines. The internal ribosomal entry site from encephalomyocarditis virus (EMCV IRES) is denoted as gray lines. Adaptive mutations are indicated as asterisks. F Luc: firefly luciferase gene; CAG: CAG promoter; Pol I P: RNA polymerase I promoter; Pol I T: RNA polymerase I terminator; GND: replication-deficient GND mutation. (B) Luciferase activity in Huh7.5.1 cells inoculated with supernatant from cells transfected with indicated plasmids at the indicated time points. Data are averages of triplicate values with error bars showing standard deviations. (C) Luciferase activity in cells inoculated with serially diluted HCVtcp.

according to positions within the JFH-1 polyprotein). Supernatants of cells transfected with plasmids (Fig. 1A) were collected and were used to infect Huh7.5.1 cells, which were analyzed by luciferase assay. Introduction of adaptive mutations (pCAGC-NS2/JFH1am) resulted in more than 4-fold higher production of HCVtcp at 5 day post-transfection, as compared to wild-type (WT) (pCAGC-NS2/JFH1) (Fig. 1B), indicating that the adaptive mutations contribute to enhancing HCVtcp production. To confirm that luciferase activity levels in HCVtcp-infected cells are correlated with the number of infectious particles, Huh7.5.1 cells were inoculated with serial dilutions of HCVtcp. Luciferase activity was well correlated with viral load (Fig. 1C), indicating that luciferase assay in HCVtcp-infected cells can be used to quantify HCV infection.

In order to further explore the efficient production of HCVtcp, we generated replicon constructs that lack the luciferase gene or include the partial coding sequences for structural proteins instead of reporter (Fig. 2A). Replication of each replicon in plasmid-transfected cells was then assessed by Western blotting (Fig. 2B). Among the constructs tested, NS5B levels were lowest in cells expressing pHH/SGR-Luc. NS5B levels in cells replicating other replicons appeared to be comparable. Cells were infected with supernatants of cells transfected with each replicon plasmid, along with pCAGC-NS2/JFH1am, followed by infectious unit assay (Fig. 2C). The highest production of HCVtcp was obtained from cells transfected with pHH/SGR, where the luciferase sequence was deleted from pHH/SGR-Luc, thus suggesting that deletion of the sequence not essential for RNA replication in the replicon may contribute to enhancing HCVtcp production.

Production of chimeric HCVtcp by providing heterologous core-p7

In order to elucidate whether *trans*-encapsidation of JFH-1 replicon can be achieved by providing core-p7 from other HCV strains, core-NS2 plasmids were constructed (Fig. 3A). In these plasmids, core through the N-terminal 33 aa of NS2, which contains transmembrane domain 1 of NS2, was derived from either H77c (genotype 1a), THpa (genotype 1b), Con1 (genotype 1b) or J6 (genotype 2a) strain. Residual NS2 was derived from JFH-1, as described previously (Pietschmann et al., 2006). HCVtcp was efficiently produced by core-p7 of J6 and THpa strains, but its production was less efficient in the case of Con1 strain. *Trans*-packaging was not detectable when core-p7 of H77c strain was used (Fig. 3C). Among HCV strains tested, difference in luciferase activity levels in HCVtcp-infected cells (Fig. 3C) were in agreement with that in the viral RNA levels in the culture supernatants of the transfected cells (Fig. 3B). Although the efficacy of *trans*-complementation was variable among strains, chimeric HCVtcp can be generated by providing genotype 1b-derived core-p7 in addition to intragenotypic viral proteins, and was used in subsequent studies.

ApoE- and CD81-dependent infection by HCVtcp

There is accumulating evidence that apolipoproteins, particularly ApoE, contribute to HCV production and infectivity (Chang et al., 2007; Owen et al., 2009). To determine whether ApoE is involved in infection of target cells by HCVtcp, we infected cells in the presence of increasing concentrations of anti-ApoE antibody.

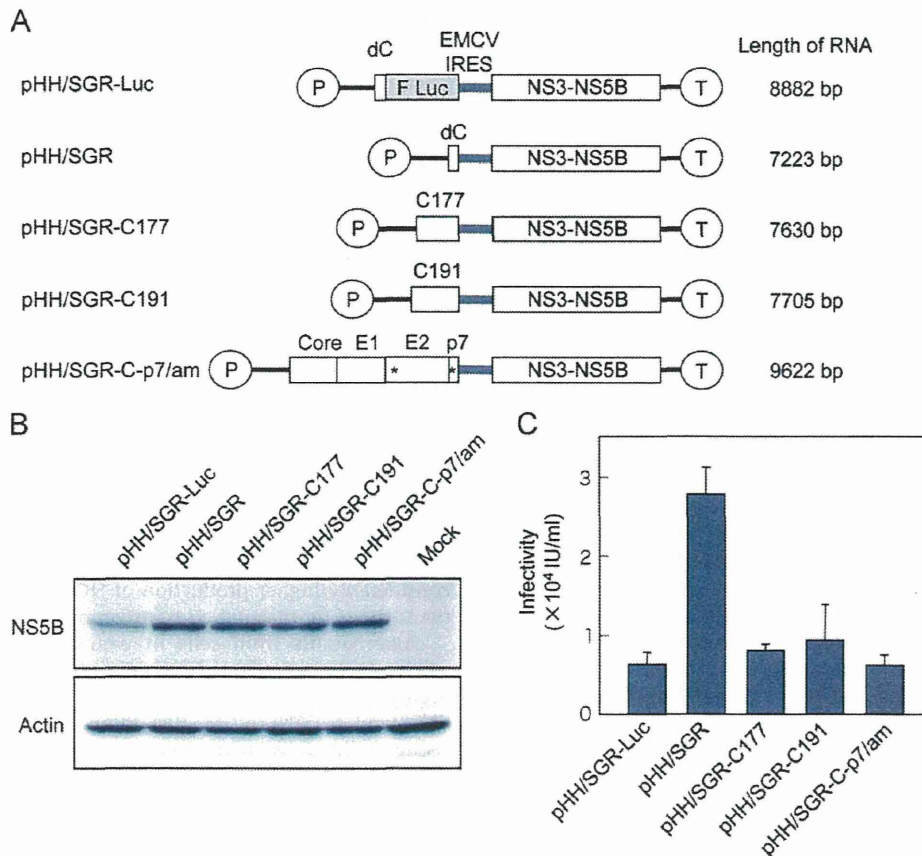


Fig. 2. Production of HCVtcp with different replicon constructs. (A) Schematic representation of plasmids used for production of HCVtcp. Deduced length of transcribed RNA from each construct is shown on the right. HCV polyproteins from JFH-1 strain are indicated by open boxes. HCV UTRs are indicated by bold lines. The EMCV IRES is denoted by gray bars. Adaptive mutations are indicated by asterisks. F Luc: firefly luciferase gene; P: RNA polymerase I promoter; T: RNA polymerase I terminator. (B) Detection of NS5B and actin in Huh7.5.1 cells transfected with indicated plasmids at 4 day post-transfection. (C) Infectivity of culture supernatants from cells transfected with indicated replicon plasmids along with pCAGC-NS2/JFH1am at 4 day post-transfection.

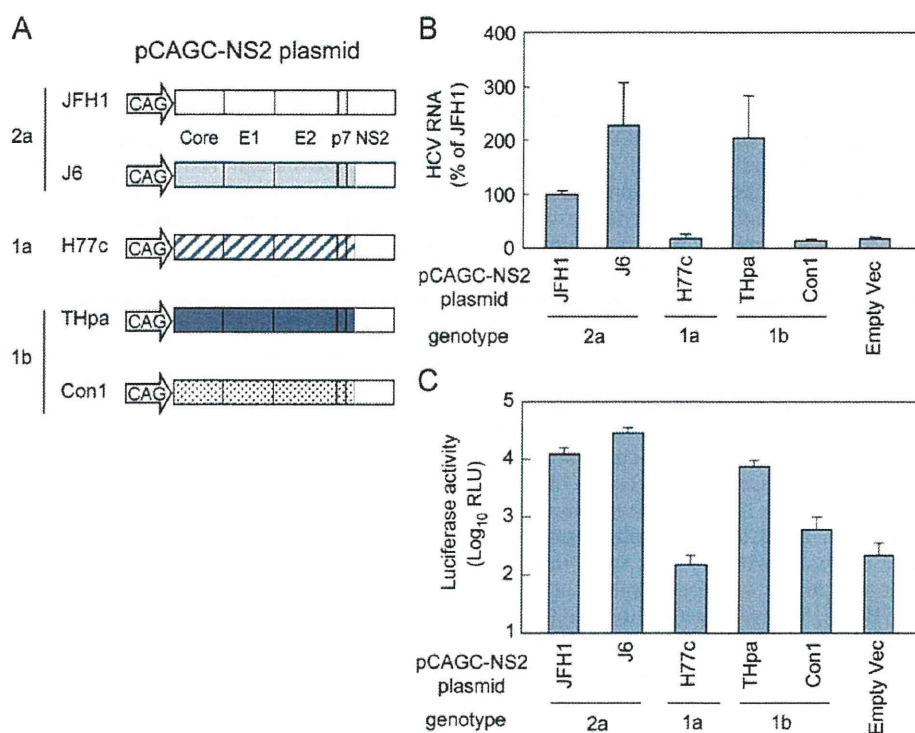


Fig. 3. HCVtcp production with structural proteins from various strains. (A) Schematic representation of plasmids used. HCV polyproteins of JFH-1, J6, H77c, THpa and Con1 strain are shown in the open box, bright gray box, box with diagonal lines, dark gray box and dotted box, respectively. (B) Relative levels of HCV RNA in the supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc at 4 day post-transfection. (C) Luciferase activity in cells inoculated with supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc at 4 day post-transfection.

pCAGC-NS2/THpa and pCAGC-NS2/JFH1am were used as core-NS2 plasmids for HCVtcp production carrying core-p7 derived from genotypes 1b and 2a (HCVtcp-1b and HCVtcp-2a, respectively). HCVpp derived from JFH-1 and VSVpp were generated and used for comparison. Infection with HCVtcp-1b or HCVtcp-2a was blocked by anti-ApoE antibody in a dose-dependent manner. In contrast, anti-ApoE antibody did not affect infection with HCVpp and VSVpp (Fig. 4A).

The CD81 dependence of infection was also compared between HCVtcp and HCVpp (Fig. 4B). Anti-CD81 antibody inhibited the entry of HCVtcp-1b, HCVtcp-2a, and HCVpp in a dose-dependent manner. The antibody had no effect on VSVpp infection. HCVtcp infection appears to be more sensitive to anti-CD81 antibody when compared with HCVpp infection; more than 60% inhibition was observed at 0.08 $\mu\text{g}/\text{mL}$ anti-CD81 antibody for HCVtcp-1b and HCVtcp-2a, whereas approximately 50% inhibition was observed for HCVpp at 2 $\mu\text{g}/\text{mL}$ antibody. Neutralization of HCVcc by anti-ApoE and anti-CD81 antibodies was also determined. Antibodies blocked HCVcc infection (Fig. 4C and D), as observed with HCVtcp. These results suggest that ApoE, as well as CD81, play an important role in HCVtcp infection. Thus, HCVtcp may be more useful for evaluating the HCV entry process than HCVpp.

Identification of novel culture-adaptive mutation in NS3 by serial passage of HCVtcp in packaging cells

The HCVtcp system was further applied to analyses of genetic changes during serial passages in target cells. As an initial attempt, supernatants of cells co-transfected with pCAGC-NS2/JFH1am and pHH/SGR were inoculated into Huh7.5.1 cells transiently transfected with pCAGC-NS2/JFH1am. However, infectious titer was lost after repeated inoculation, likely due to low HCVtcp titers and

low efficiency of plasmid transduction (data not shown). To overcome this, we utilized recombinant adenovirus vectors (rAdVs) to provide core-NS2. As we were not able to obtain rAdV directly expressing core-NS2, conditional transgene expression based on a Cre-loxP strategy was employed (Kanegae et al., 1995). We constructed an rAdV containing core-NS2 gene downstream of a stuffer DNA flanked by a pair of loxP sites (AxCALNLH-CNS2). When cells were doubly infected with AxCALNLH-CNS2 and the Cre-expressing rAdV, AxCANCre (Kanegae et al., 1995), the Cre-mediated excisional deletion removed the stuffer DNA, resulting in core-NS2 expression under control of the CAG promoter (Fig. 5A). As expected, tightly regulated production of HCVtcp was observed. The cells infected with AxCANCre and AxCALNLH-CNS2 along with transduction of pHH/SGR-Luc produced HCVtcp at high levels. Production of HCVtcp was undetectable when either AxCANCre or AxCALNLH-CNS2 was not infected (Fig. 5B). The Cre-mediated rAdV expression system appears to have yielded considerably higher production of HCVtcp when compared with the settings for plasmid co-transfection.

Supernatants from cells in which core-NS2 was expressed using rAdVs and the subgenomic RNA derived from pHH/SGR replicated were inoculated into cells infected with AxCALNLH-CNS2 and AxCANCre (Fig. 6A). Blind passage was performed by sequentially transferring culture supernatants to cells infected with the above rAdVs. The two independent 10 blind passages (p10) showed virus titers of $> 1 \times 10^6$ IU/mL, which were markedly higher than those of the passage 0 (p0) stock cultures (4×10^4 IU/mL). Side-by-side infection analysis revealed that the HCVtcp p10 #1 achieved a virus titer approximately 36 times higher than that of HCVtcp p0 on the packaging cells at 6 day post-infection (Fig. 6B). Sequencing of the entire replicon in the supernatants at p10 in two independent experiments revealed

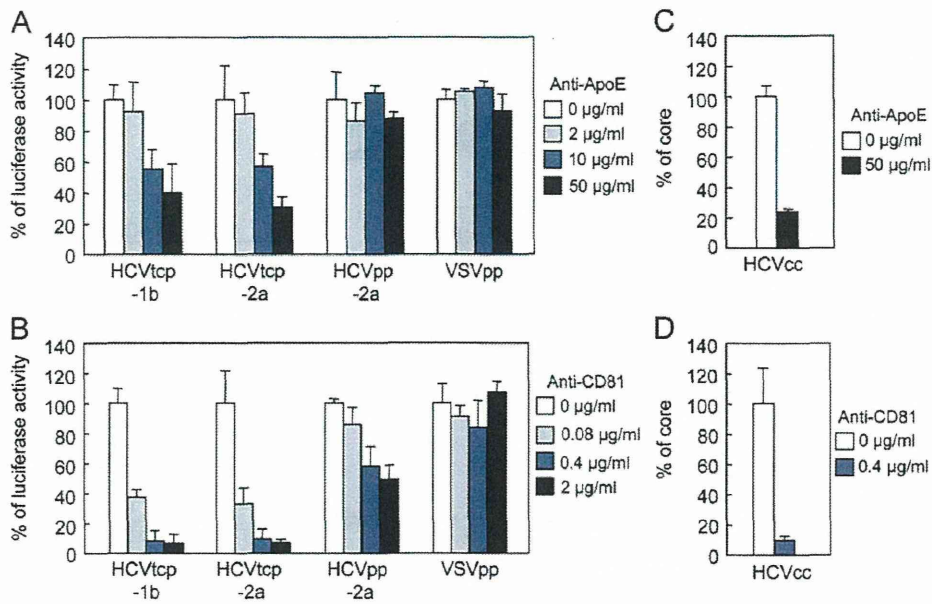


Fig. 4. Effects of anti-ApoE and anti-CD81 antibodies on HCV entry. (A) Aliquots of virus sample were incubated with increasing concentrations of anti-ApoE antibodies for 1 h and were then added to Huh7.5.1 cells. Luciferase activity was determined at 72 h post-infection and is expressed relative to activity without antibodies (white bar). (B) Huh7.5.1 cells were preincubated for 1 h with increasing concentrations of anti-CD81 antibodies, followed by inoculating virus samples. Luciferase activity was determined and expressed as shown in (A). (C) Aliquots of HCVcc were incubated with anti-ApoE antibodies for 1 h and were then added to Huh7.5.1 cells at an MOI of 0.05. Intracellular core levels were quantitated at 24 h post-infection and are expressed relative to levels without antibodies (white bar). (D) Huh7.5.1 cells were preincubated for 1 h with anti-CD81 antibodies. HCVcc infection and measurement of core proteins were performed as indicated in (C).

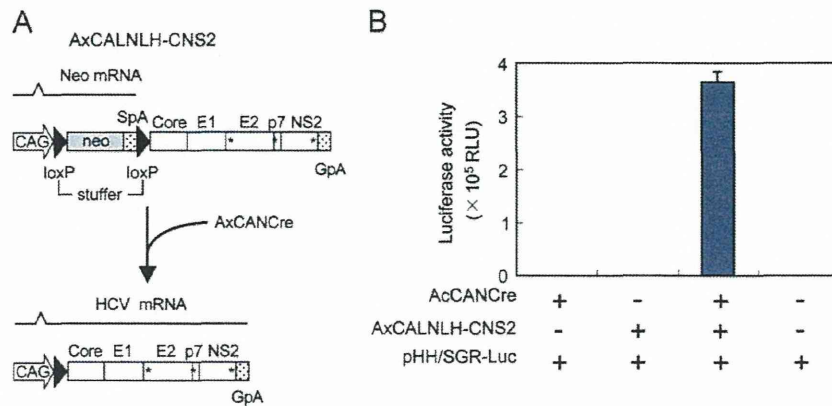


Fig. 5. Transgene activation mediated by rAdVs expressing Cre recombinase under control of CAG promoter. (A) Cre recombinase expressed by AxCANCre recognizes a pair of its target sequences loxP in AxCALNLH-CNS2, and removes the stuffer region resulting in expression of HCV core-NS2 polyprotein by CAG promoter. CAG: CAG promoter; SpA: SV40 early polyA signal; GpA: rabbit b-globin poly(A) signal. (B) Luciferase activity in Huh7.5.1 cells inoculated with 4-day post-transfection culture supernatant from cells transfected with pHH/SGR-Luc, and then infected with indicated rAdVs.

that both passaged HCVtcp had an identical nonsynonymous mutation in the NS3 region (N1586D) (Fig. 6C).

In order to examine the role of NS3 mutation identified on HCV RNA replication and on HCVtcp production, the N1586D mutation was introduced into pHH/SGR-Luc. Luciferase activities of the N1586D-mutated replicon were apparently lower than those of the WT-replicon, thus suggesting that the NS3 mutation reduced viral RNA replication (Fig. 7A). HCV RNA levels in the supernatants of cells transfected with WT- or mutant replicon plasmid along with pCAGC-NS2/JFH1am and luciferase activity in cells inoculated with supernatants from the transfected cells were then determined (Fig. 7B). The viral RNA level secreted from cells replicating the N1586D-mutated replicon was lower than that from cells replicating WT replicon (Fig. 7B, left). By contrast, a significantly higher infectivity of HCVtcp produced from the mutant replicon-cells was observed, as compared to WT replicon-cells (Fig. 7B, right),

suggesting that the adaptive mutation increased the specific infectivity (almost 9-fold) of the virus particles. To further determine whether the N1586D mutation affects infectious viral assembly and/or virus release, we used the CD81-negative Huh-7 subclone, Huh7-25 (Akazawa et al., 2007), which may produce infectious particles, but is not susceptible to HCV entry due to a lack of CD81 expression, therefore allowing us to examine viral assembly and release without the influence of reinfection by produced HCVtcp. Measurement of intracellular and extracellular HCVtcp indicated that Huh7-25 cells replicating the N1586D-mutated replicon produced more infectious virus than WT in both supernatants and cell lysates (Fig. 7C). Thus, it can be concluded that the N1586D mutation contributes to enhanced infectious viral assembly, not RNA replication. We could not exclude the possibility that N1586D mutation affects virus release, since the mutation enhanced extracellular virus titers more than did the intracellular titer.