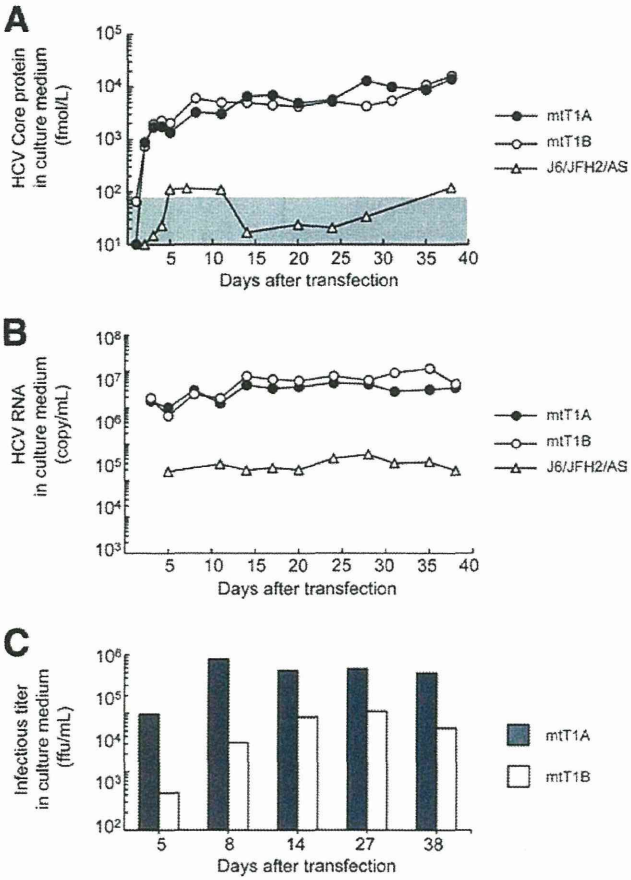

FIG 10 Continuous passage of J6/JFH2/AS cell culture-adapted virus RNA-transfected cells. Full-length HCV RNA was synthesized from the J6/JFH2/AS, J6/JFH2/AS/mtT1A (mtT1A), and J6/JFH2/AS/mtT1B (mtT1B) constructs. RNA-transfected cells were serially passaged until 38 days after transfection, and culture supernatants were harvested at the indicated time points. HCV core protein (A) and HCV RNA (B) levels in the culture media were determined. The data in the gray area were below the detection limit of the assay to detect HCV core protein. (C) Infectivity in the culture medium was determined by focus formation assay at 5, 8, 14, 27, and 38 days after transfection.

also used for electron microscopy analysis. After the density gradient purification, spherical viral particles were detected (Fig. 7, right panel). After the core protein levels plateaued, naive Huh-7.5.1 cells were inoculated with the culture medium, as described above. When the core protein levels plateaued again after the third inoculation of T3 and T4 cells, we sequenced the viral genome in the culture medium (T3i3 and T4i3, respectively) to determine the adaptive mutation. We found the following nonsynonymous mutations: 414IT in E2, 1510EG and 1617RQ in NS3, 2006KQ, 2233AV and 2234NS in NS5A, and 2695TI in NS5B of T3i3; and 387VG in E1, 828VA in NS2, 1225RQ and 1283RG in NS3, 1883VA in NS4B, 2206SA, 2279KN, and 2441CR in NS5A, and 2695TI in NS5B of T4i3 (Fig. 3B). We introduced these mutations into the pJFH2/AS plasmid (pJFH2/AS/mtT3 and pJFH2/AS/mtT4). Synthesized RNA from pJFH2/AS/mtT3 and pJFH2/AS/mtT4 and the related control plasmids was transfected into Huh-7.5.1 cells. HCV core protein levels, HCV RNA levels, and infectivity were monitored in the culture medium of the transfected cells until 96 h after transfection (Fig. 9A to C). JFH2/AS/mtT3 (mtT3) and JFH2/AS/mtT4 (mtT4) secreted similar levels of HCV core protein, RNA, and infectious virus with J6/JFH2/AS/mtT1A and J6/JFH2/AS/mtT1B. Although JFH2/AS/mtT3 secreted slightly higher levels of HCV core protein and RNA than JFH2/AS/mtT4, the secreted infectious virus titers were similar for both viruses. JFH2/AS/mtT3 and JFH2/AS/mtT4 RNA-transfected cells were also serially passaged, and the HCV core proteins were secreted immediately after transfection (Fig. 14A). However, JFH2 and JFH2/AS RNA-transfected cells did not secrete significant amounts of HCV core protein into the culture medium. HCV RNA levels in the culture medium of the RNA-transfected cells were at similar levels for JFH2/AS/mtT3 and JFH2/AS/mtT4 (around 10^7 copy/ml) (Fig. 14B). Infectivity was also detected as higher than 10^4 FFU/ml even at 3 days after the RNA transfection, and this level of infectious titer was maintained during the cell passages (Fig. 14C). We also analyzed JFH2/AS/mtT3 and JFH2/AS/mtT4 culture media by density gradient assay (Fig. 14D). The density profiles with HCV core protein and RNA levels and infectious titers in the fractions were basically similar to those of J6/JFH2/AS-adapted viruses (Fig. 6E and 11D). Taken together, the results described in this section indicate infectious virus was also recovered from the full-length JFH-2 construct with the 2217AS mutation.

Mechanistic analysis of adaptive mutations introduced in the J6/JFH2/AS and JFH2/AS cell culture-adapted viruses. To elucidate the mechanisms of adaptive mutations discovered in J6/JFH2/AS and JFH2/AS virus genomes, we transfected JFH-2 and J6/JFH2 constructs along with possible control constructs into Huh7-25 cells (2) (Fig. 15), which are CD81 defective. The transfection of JFH-1 RNA into Huh7-25 cells results in infectious HCV production, but there was no reinfection into Huh7-25 cells because the cell surface expression of CD81 is essential for HCV infection (10). HCV core protein levels were measured in the culture medium and cell lysate to monitor virus particle secretion and intracellular virus genome replication, respectively (Fig. 15A and B). JFH2/AS, JFH2, J6/JFH2, JFH1/GND, and J6/JFH2/GND RNA-transfected cells did not show increased levels of intracellular core protein expression. However, other RNA-transfected cells showed increased intracellular core protein expression. The cellular core protein level was especially increased at 72 and 96 h after transfection with J6/JFH2/AS RNA, which suggests a higher replication efficiency than J6/JFH; however, core protein secretion was not detected with J6/JFH2/AS, which suggests defective virus particle formation or secretion. Other adaptive mutations in J6/JFH2/AS/mtT1A and J6/JFH2/AS/mtT1B further increased virus genome replication and core protein secretion. In the case of JFH2/AS RNA transfection, cellular core protein expression was not detected, suggesting a lower replication efficiency than that of J6/JFH2/AS. This lower replication efficiency of JFH2/AS may be due to the presence of different sequences in the region of core protein to NS2. However, core protein expression in the cell lysate and culture medium was detected with both JFH2/AS/mtT3 and JFH2/AS/mtT4 RNA transfection. Thus, adaptive mutations in mtT3 and mtT4 are necessary to increase viral genome replication and efficient core protein secretion. JFH-1 and J6/JFH-1 had intracellular core protein expression levels that were similar and high. From the intracellular core protein data, it is clear that J6/JFH2/AS/mtT1A, J6/JFH2/AS/mtT1B, JFH2/AS/mtT3, and JFH2/AS/mtT4 constructs obtained higher replication capacities by

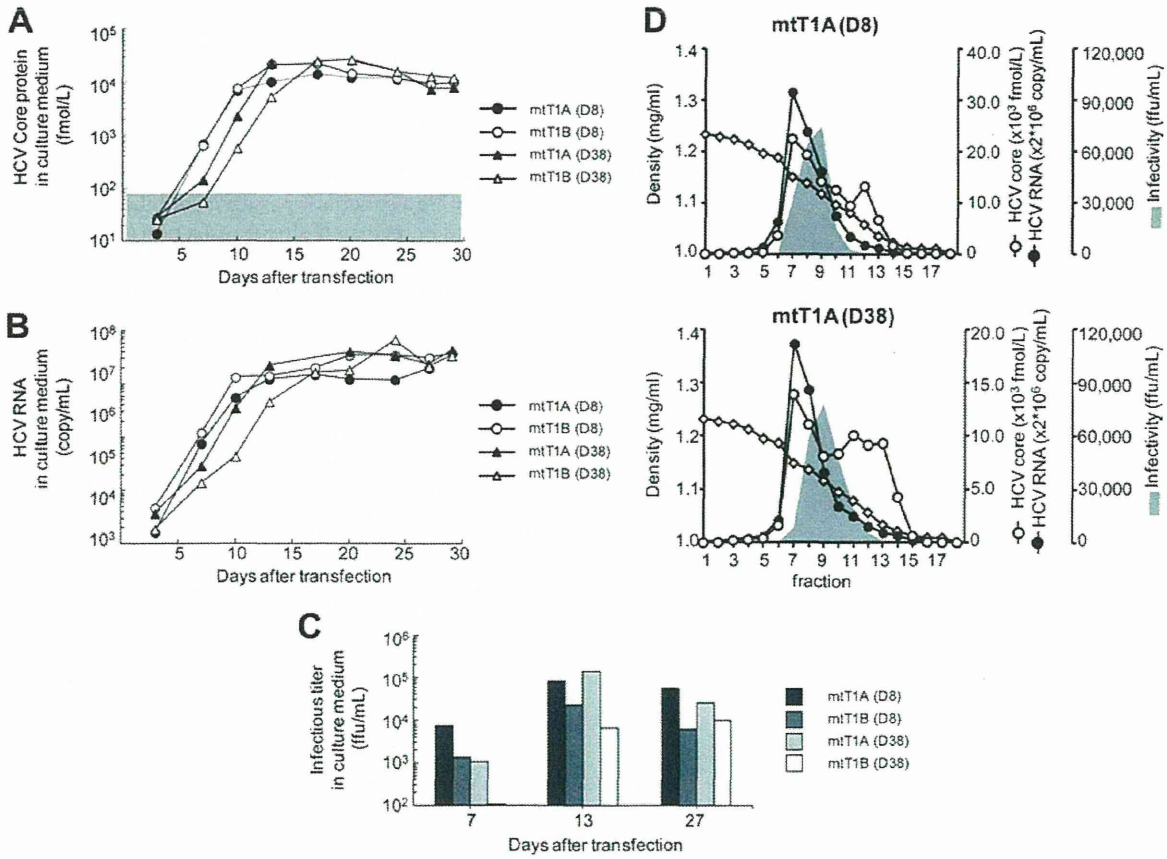

FIG 11 Serial passages of J6/JFH2/AS cell culture-adapted virus infected cells. Naive Huh-7.5.1 cells were inoculated with culture medium of the RNA-transfected cells at an MOI of 0.01. Inoculated cells were serially passaged, and culture supernatants were harvested at the indicated times. HCV core protein (A), HCV RNA (B), and infectivity (C) levels in the culture media were determined. (D) Density gradient analysis of culture supernatant from J6/JFH2/AS/mtT1A cell-culture adapted virus-infected Huh-7.5.1 cells. Culture supernatant of infected Huh-7.5.1 cells with mtT1A (day 8 [D8] D38 posttransfection) was harvested at 20 days after inoculation. Assays were performed as described in the legend of Fig. 6E. Open diamond, buoyant density.

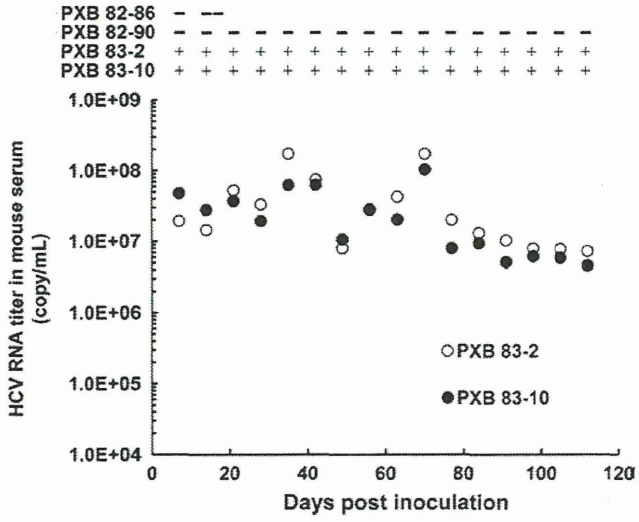

FIG 12 Serum HCV RNA titer in human hepatocyte-transplanted uPA/SCID mice inoculated with JFH-2 serum and cell culture-adapted virus. Mice PXB 82-86 and PXB 82-90 were inoculated with the cell culture-adapted J6/JFH2/AS HCV particles, and mice PXB 83-2 and PXB 83-10 were inoculated with JFH-2 patient serum. Upper and lower panels indicate the results of the RT-PCR and the quantitative detection RT-PCR of HCV RNA in mouse serum, respectively.

adaptive mutations; however, their replication levels are lower than those of JFH-1 and J6/JFH1.

We also analyzed the ratio of extracellular protein to total core protein to analyze the virus secretion efficiency (Fig. 15C). J6/JFH-1 secreted a higher percentage of core protein into culture

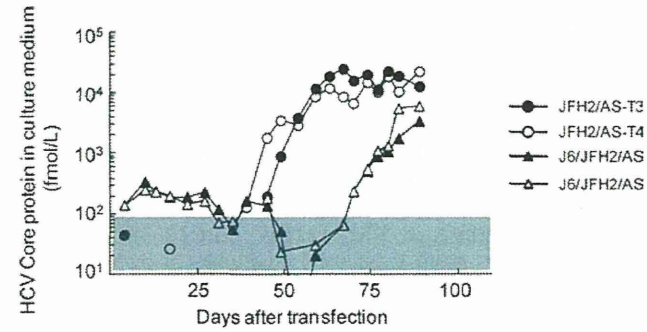

FIG 13 Propagation of a full-length JFH2 virus. Huh-7.5.1 cells were transfected with the transcribed RNA from pJFH2/AS and pJ6/JFH2/AS. Two independently JFH2/AS RNA-transfected cell lines (T3 and T4) were independently passaged. At each time point, culture medium was harvested and analyzed for the presence of HCV core protein.

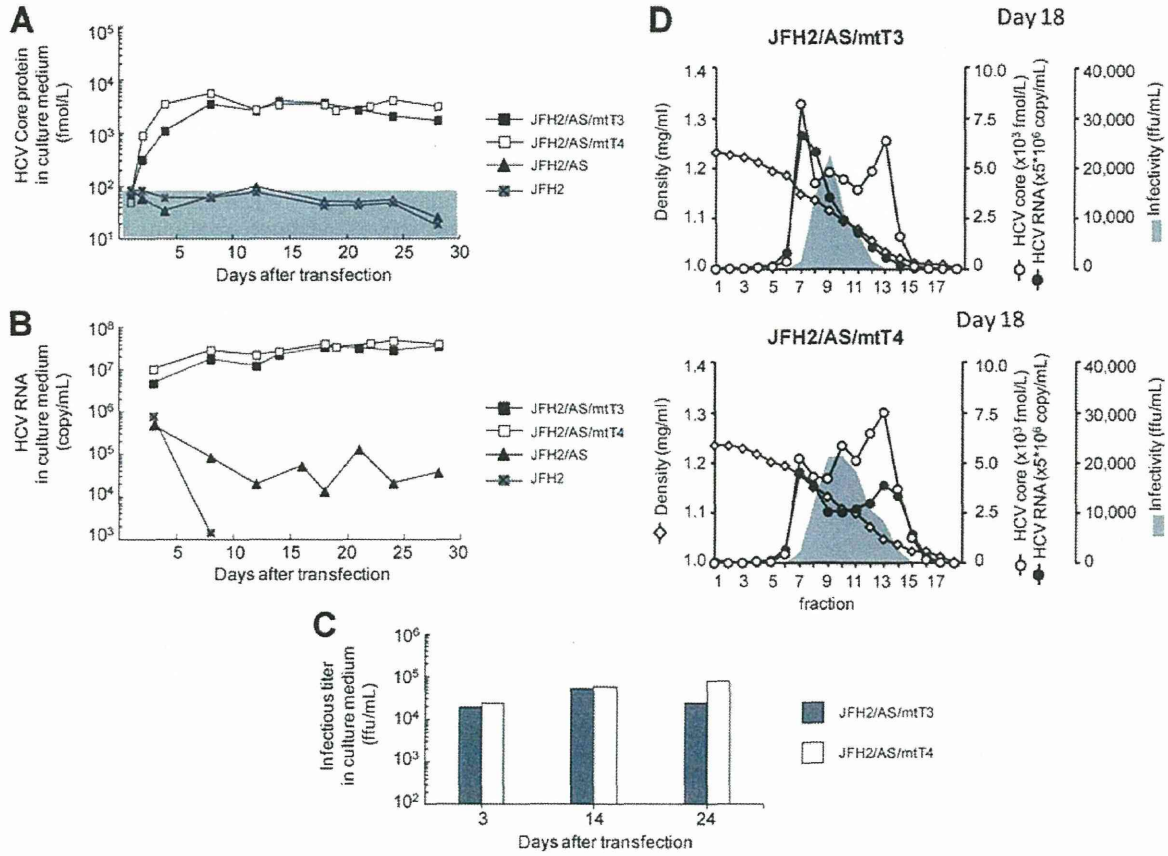

FIG 14 Full-length RNA was synthesized from the JFH2 construct and its derivatives with mutations. RNA-transfected cells were serially passaged, and culture supernatants were harvested at the indicated time points. HCV core protein (A), HCV RNA (B), and infectivity (C) levels in the culture media were determined. The data in the gray area were below the detection limit of the assay to detect HCV core protein. (D) Density gradient analysis of culture supernatant from JFH2/AS cell-culture adapted virus-infected Huh-7.5.1 cells. Culture supernatants of Huh-7.5.1 cells infected with JFH2/mtT3 and JFH2/mtT4 viruses were harvested 18 days after inoculation. Assays were performed as described in the legend of Fig. 6E. Open diamond, buoyant density.

medium than JFH-1. J6/JFH2/AS/mtT1A, J6/JFH2/AS/mtT1B, JFH2/AS/mtT3, and JFH2/AS/mtT4 RNA-transfected cells showed different percentages of secreted core protein. mtT1A and mtT1B constructs showed similar replication levels (Fig. 15B), but mtT1B showed a higher percentage of core protein secretion than mtT1A (Fig. 15C). mtT3 and mtT4 showed similar percentages of core protein secretion, which are higher than the level of JFH1 (Fig. 15C). Because J6/JFH2/AS RNA-transfected cells did not secrete core protein despite intracellular core protein expression (Fig. 15A and B), the adaptive mutant constructs obtained core protein (or virus particle) secretion phenotypes. Thus, during the adaptation process, the viruses obtained both higher replication capacity and core protein secretion capacity by their adaptive mutations.

Other HCV constructs with the 2217AS mutation. The alanine residue at amino acid position 2217 is located in the ISDR of NS5A, and it is conserved among HCV strains including genotype 1 and 2 strains. Because the 2217AS mutation in NS5A is the key mutation for the production of cell culture-adapted HCV, we introduced this mutation into other wild-type HCV constructs, i.e., H77 (genotype 1a), Con1 (genotype 1b), and J6CF (genotype 2a). Synthetic RNAs including the 2217AS mutation were electroporated into Huh-7.5.1 cells, and then the transfected cells were serially passaged. HCV core protein secretion was measured in the culture medium of transfected cells. However, we could not observe the increment of HCV core levels in the culture medium (data not shown). Therefore, we concluded that the 2217AS mutation does not always induce cell culture adaptation in HCV isolates.

DISCUSSION

In previous studies, we have isolated cell culture-infectious HCV, the JFH-1 strain, from a patient with fulminant hepatitis (14, 38). In this report, we isolated another HCV cDNA, named JFH-2, also from a fulminant hepatitis patient. We constructed a subgenomic replicon with the JFH-2 sequence, but its replication efficiency was low. Among the mutations found in the replicon genome, the 2217AS mutation in the ISDR exhibited the strongest adaptive effect. Interestingly, the full-length chimeric or wild-type JFH-2 genome with adaptive mutations could replicate and produce infectious virus particles. Virus infection efficiency was sufficient for autonomous virus propagation in cultured cells.

Several full-length HCV cDNAs have been cloned, and their infectivity has been confirmed *in vivo* with chimpanzee models (18, 39). However, it has been difficult to produce recombinant viral particles and test their infectivity by using cell culture

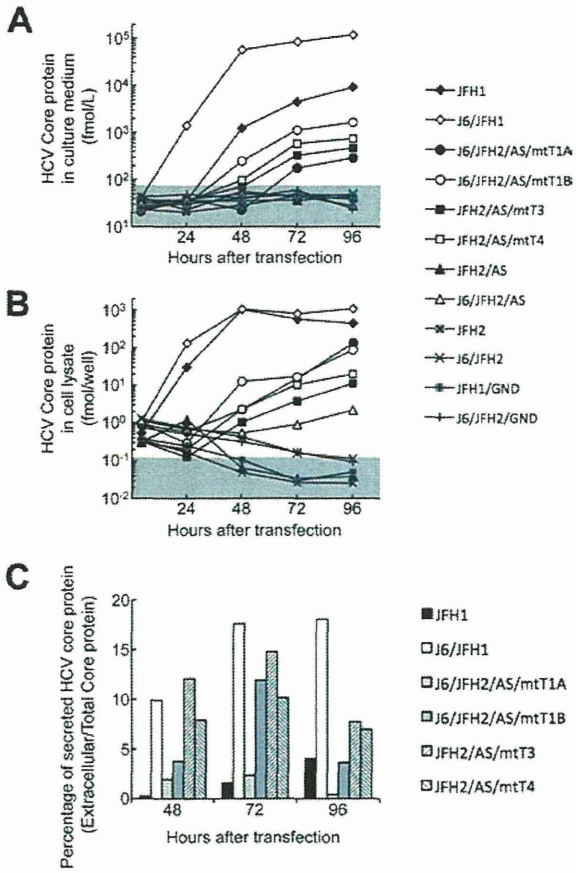

FIG 15 Transient virus production assay of J6/JFH2- and JFH2-related constructs with CD81-defective Huh7-25 cells. Full-length HCV RNA was synthesized from the JFH1, J6/JFH1, JFH2, and J6/JFH2 constructs and their derivatives with mutations and transfected into Huh7-25 cells. (A) HCV core protein levels in culture medium were determined at 4, 24, 48, 72, and 96 h after transfection. The data in the gray area were below detection limit. (B) HCV core protein levels in the cell lysate were determined at 24, 48, 72, and 96 h after transfection. (C) Percentages of secreted HCV core protein from the transfected cells were determined at 48, 72, and 96 h after transfection. Percentages of secreted HCV core protein were calculated only for the indicated viruses. All assays were performed in duplicate, and the data represent average values.

systems (4, 28). Only the JFH-1 strain efficiently replicates in HuH-7 cells and other hepatic and nonhepatic cell lines in subgenomic replicon form (20, 38, 41). Full-length wild-type JFH-1 RNA and chimeric JFH-1 RNA can replicate in HuH-7 cells and produce infectious virus. Since the JFH-1 strain was isolated from a patient with fulminant hepatitis, we assumed that virus strains that cause fulminant hepatitis may replicate efficiently in cultured cells. To identify more HCV clones that can replicate in cultured cells, we isolated the JFH-2 strain from another fulminant hepatitis patient (15). Interestingly, the JFH-2 strain showed a low level of replication in cultured cells in the initial subgenomic replicon experiment. This result may suggest that HCV strains isolated from fulminant hepatitis patients are able to replicate more efficiently than strains from chronic hepatitis patients; however, this hypothesis should be confirmed by testing more HCV strains from patients with fulminant hepatitis. The JFH-2 patient received a course of betamethasone therapy and developed fulminant hepatitis after the withdrawal of betamethasone. It is thus possible that the JFH-2 strain obtained its higher replication capacity in the immune-suppressed host environment. To confirm this hypothesis, we must test the replication efficiency of HCV strains isolated from other immune-suppressed patients, such as patients who are coinfected with HIV, patients who are reinfected after a transplant, and patients who are treated with immunosuppressive agents.

In previous reports, adaptive mutations have been found to enhance viral RNA replication at the expense of virus particle formation efficiency (28). A highly cell culture-adapted Con1 strain can replicate in cultured cells, but it cannot produce infectious virus particles. Interestingly, a highly adapted Con1 strain was not infectious for chimpanzees, while moderately adapted Con1 was infectious. However, the virus recovered from the infected animal was wild-type Con1 virus (5). This result clearly suggests that HCV strains with lower replication efficiencies are favorable for *in vivo* infection. However, we must note that the "replication efficiency" is determined in cultured cells. In the case of JFH-2, we found several adaptive mutations in the subgenomic replicon clones, and the most adaptive mutation, 2217AS, was tested in full-length HCV replication and virus production. After the RNA transfection of J6/JFH2/AS, we could not detect substantial virus secretion for about 30 days. However, after 30 days, significant levels of infectious virus particles were secreted into the culture medium. Naive Huh-7.5.1 cells were inoculated three times with the cell culture-adapted virus. This virus adaptation was also tested with full-length JFH2/AS, and we successfully obtained infectious JFH2/AS virus. Both the J6/JFH2/AS and JFH2/AS viruses acquired the ability for autonomous virus expansion in Huh-7.5.1 cells, and several additional mutations were found in their genomes. Interestingly, the 2695TI mutation in NS5B was commonly found in all of the adapted virus genomes, and isoleucine at amino acid position 2695 is also found in the JFH-1 strain. However, the introduction of only the 2695TI mutation into the J6/JFH2/AS or JFH2/AS virus genome did not restore robust virus production (data not shown). After repeated virus passages, mutations were found throughout the viral genome (in J6/JFH2/AS-T1Ai3 and -T1Bi3 and in JFH2/AS-T3i3 and -T4i3), and we are currently investigating which mutations or combinations of mutations are most important for this adaptation. From the comparisons of cell culture-adapted viruses and their parental virus constructs, adaptive mutations are necessary to increase both viral genome replication and virus particle assembly/secretion efficiency (Fig. 15). The procedure to produce cell culture-adapted HCV was thus established. The adaptive mutations found from the subgenomic replicon assay were introduced into the full-length genome, and the cells transfected with virus RNA were repeatedly passaged until the virus particles were produced.

In vivo infectivity may be inversely related to the replication efficiency in cultured cells, as discussed above. The original JFH-2 patient serum was infectious in human liver-transplanted mice; however, cell culture-adapted J6/JFH2/AS virus was not infectious. The JFH-1 virus was infectious not only for cultured cells but also for chimpanzees and human liver-transplanted mice (10, 38). However, the JFH-1 infection in chimpanzees was only mild and transient without any liver pathology. Thus, the J6/JFH2/AS and JFH2/AS viruses are more cell culture-adapted and attenuated than the JFH-1 virus. It may be worthwhile to test this cell culture-

adapted strain as a live attenuated vaccine candidate to induce protective immunity. However, for ethical reasons, the necessary chimpanzee experiments are not appropriate to perform. Therefore, we should wait for the establishment of immunocompetent small-animal models susceptible to HCV infection to perform this kind of study. Furthermore, future studies should examine the *in vivo* infectivity of the adapted J6/JFH2 and JFH2 viruses isolated in the present study.

The 2217AS mutation is located in the ISDR. In the previous study of the genotype 1b subgenomic replicon, mutations introduced into the ISDR enhanced the colony formation efficiency of the HCV replicons (17, 23). However, mutations in the ISDR impaired the genotype 1b HCV replication in human liver-transplanted mice (9). The exact mechanism of the ISDR is still not clear although the number of mutations in the ISDR is related to the efficacy of interferon therapy (8). Our results in this study also support the concept that the 2217AS mutation in the ISDR enhances replicon replication efficiency although the J6/JFH2/AS virus did not infect human liver-transplanted mice. Further studies are necessary to understand the molecular mechanism of the effects of adaptive mutations in the ISDR.

In the present study, we established a cell culture-adapted HCV strain, JFH-2. The virus could be passaged continuously in naive Huh-7.5.1 cells. This approach may be applicable to the establishment of new infectious HCV clones. Novel antiviral drugs are under development, and some of them will be used in the clinical setting. However, most of them target genotype 1 HCV strains. To eradicate other genotypes of HCV, it is important to establish their replicons and infectious virus culture systems.

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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.

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

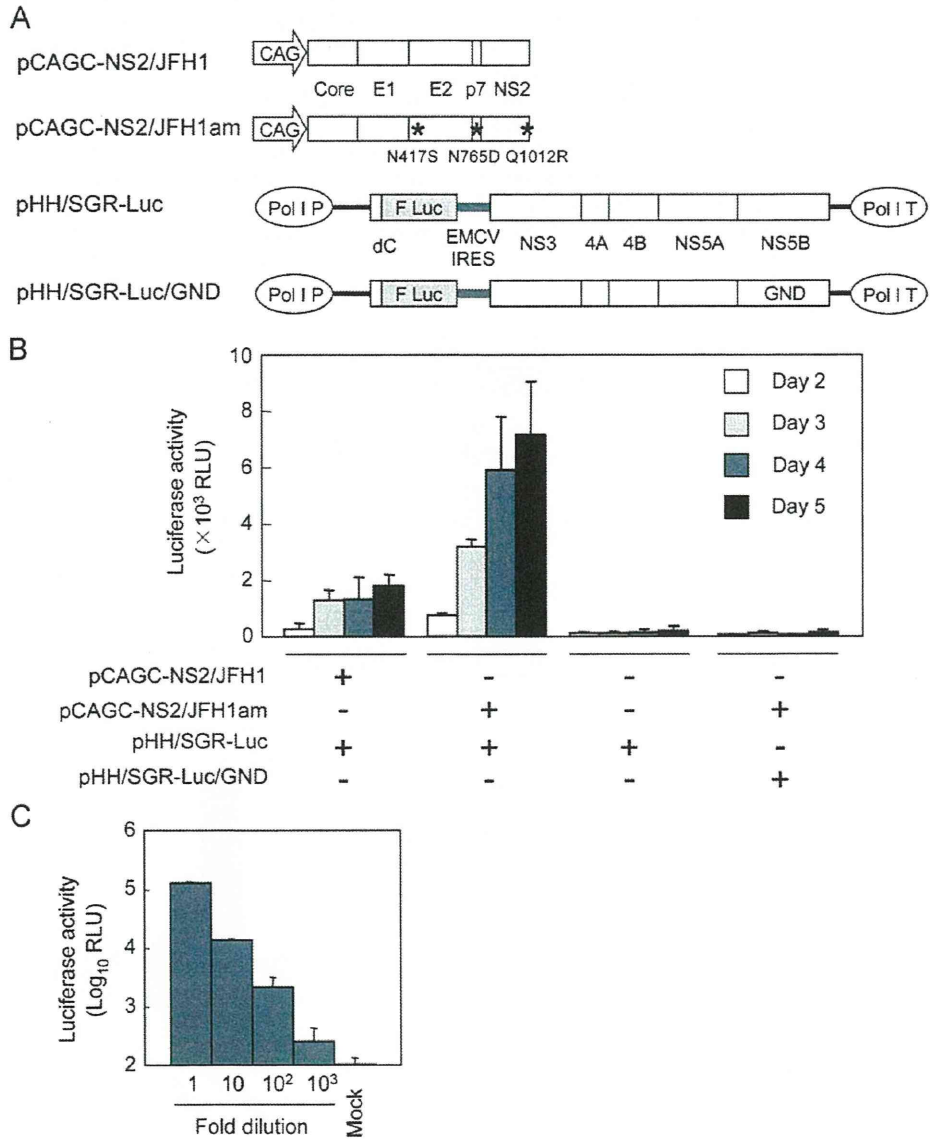

Fig. 1. HCVtcp production by two-plasmid transfection. (A) Schematic representation of plasmids is shown. HCV polyproteins derived from JFH-1 are indicated by white boxes. HCV UTRs are indicated by bold lines. The internal ribosomal entry site from encephalomyocarditis virus (EMCV IRES) is denoted as 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.

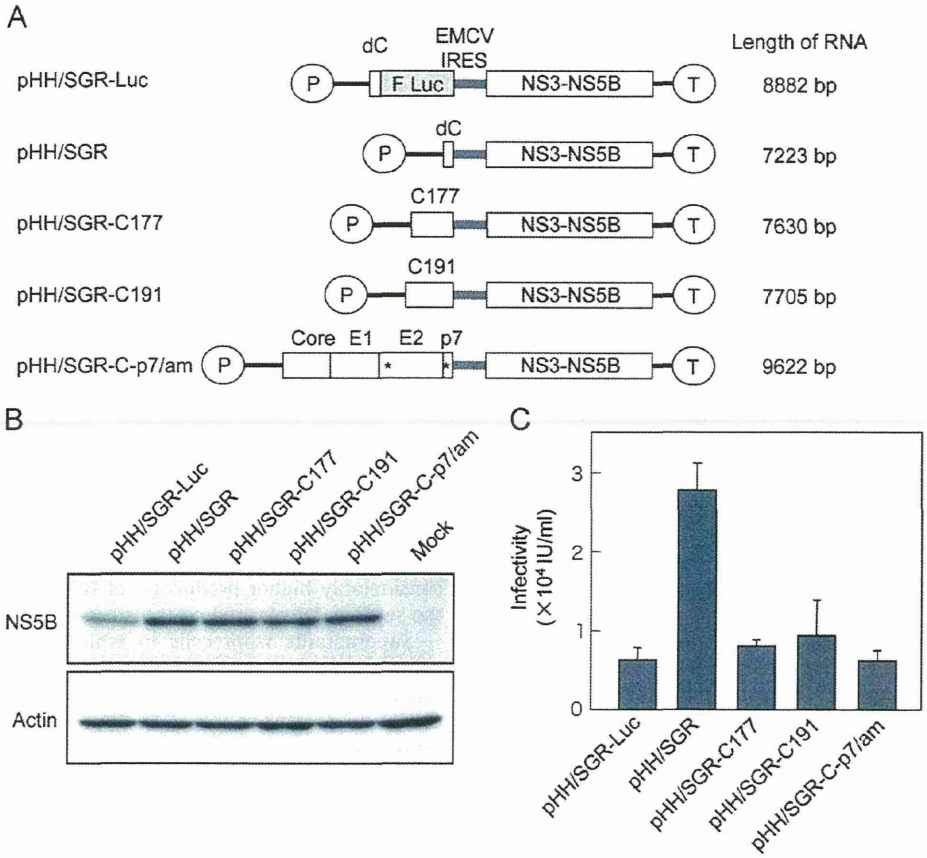

Fig. 2. Production of HCVtcp with different replicon constructs. (A) Schematic representation of plasmids used for production of HCVtcp. Deduced length of transcribed RNA from each construct is shown on the right. HCV polyproteins from JFH-1 strain are indicated by open boxes. HCV UTRs are indicated 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.

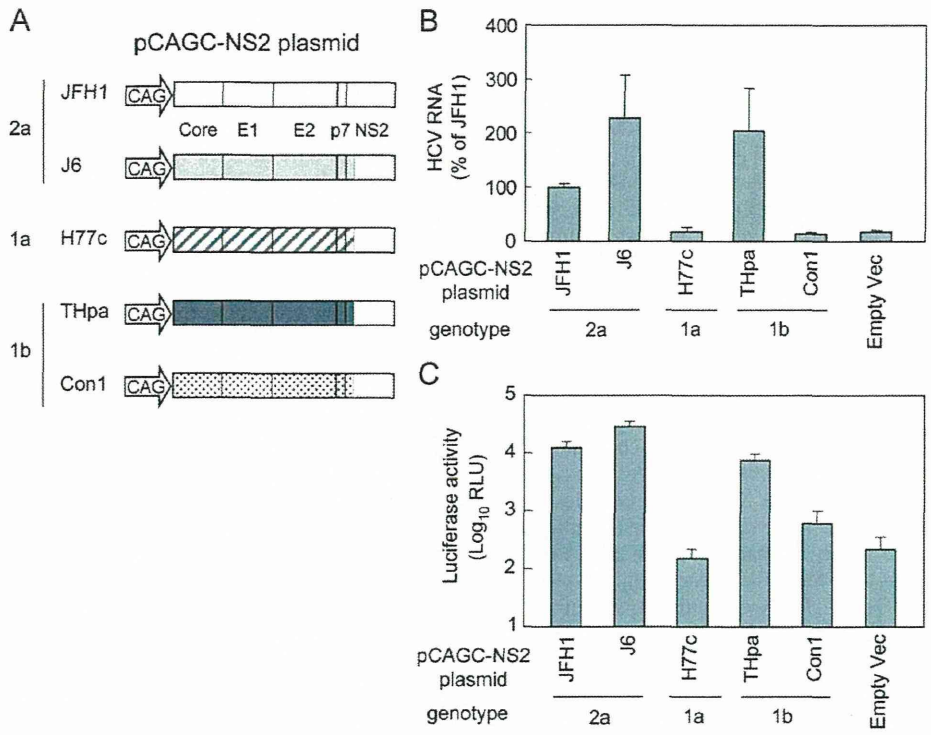

Fig. 3. HCVtcp production with structural proteins from various strains. (A) Schematic representation of plasmids used. HCV polyproteins of JFH-1, J6, H77c, THpa and Con1 strain are shown in the open box, bright gray box, box with diagonal lines, dark gray box and dotted box, respectively. (B) Relative levels of HCV RNA in the supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc. (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 μg/mL anti-CD81 antibody for HCVtcp-1b and HCVtcp-2a, whereas approximately 50% inhibition was observed for HCVpp at 2 μg/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