Hepatitis C virus (HCV) is the main cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990). As more than 170 million people worldwide are infected chronically with HCV (Poynard et al., 2003) and because the current antiviral therapy, interferon and ribavirin, produces sustained virus clearance in <50% of treated patients (Manns et al., 2007), HCV infection is clearly a problem of major proportions. HCV is a single-stranded, positive-sense RNA virus that is classified in the genus Hepacivirus in the family Flaviviridae. The approximately 9.6 kb HCV genome encodes one large open reading frame (ORF) that is flanked at the 5’ and 3’ ends by untranslated regions (UTRs) (Choo et al., 1991). The HCV polyprotein is processed into at least 10 proteins by viral proteases and cellular signalases (Grakoui et al., 1993; Hijikata et al., 1993a; McLauchlan et al., 2002). The structural proteins core, E1 and E2 are located in the N terminus of the polyprotein, followed by p7 and the non-structural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (Bartenschlager & Sparacio, 2007). Study of the HCV life cycle and virus-host interaction has been hampered severely by the lack of a robust in vitro cell-culture system and small-animal models of HCV infection (Bartenschlager & Sparacio, 2007). The development of HCV replicon systems has made an important contribution to the study of HCV translation and RNA replication in the human hepatoma cell line Huh-7 (Blight et al., 2000; Lohmann et al., 1999). Sequence analyses of multiple HCV replicons have revealed that several adaptive mutations enhance RNA replication to varying degrees (Bartenschlager & Sparacio, 2007; Blight et al., 2000; Lohmann et al., 2001). Such adaptive mutations were primarily identified in a central portion of the NS5A protein. Although the extent to which these adaptive mutations enhance RNA replication was subsequently studied by using various transient replication assays, the molecular mechanism underlying replication enhancement still remains elusive (Bartenschlager & Sparacio, 2007). The HCV replicons containing adaptive mutations do not produce infectious virus particles in culture and are severely attenuated (Blight et al., 2002; Pietschmann et al., 2002). Using recombinant HCV envelope glycoproteins
and HCV pseudoparticles, several cell-surface molecules have been shown to interact with HCV during virus binding and entry, including the tetraspanin CD81 (Bartosch et al., 2003; Pileri et al., 1998), the scavenger receptor class B member 1 (SR-BI) (Bartosch et al., 2003; Scarselli et al., 2002) and the tight junction protein claudin-1 (CLDN1) (Evans et al., 2007).

The major breakthrough was made by establishing an HCV-production system using HCV strain JFH1, a genotype 2a isolate, and Huh-7 cells (Wakita et al., 2005). Two other groups reported a robust production of infectious virus using a homologous chimeric FL-J6/JFH1 strain (Lindenbach et al., 2005) or using Huh-7.5.1 cells (Zhong et al., 2005) derived from the cell line Huh-7.5, which has a defect in the RIG-I pathway (Sumpter et al., 2005). Upon transfection of Huh-7 cells with the in vitro-transcribed HCV JFH1 genome or the chimera FL-J6/JFH1, infectious HCV particles were secreted in an envelope glycoprotein-dependent manner (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Using HCV-production systems, adaptive or compensatory mutations that promote the production of infectious virus from wild-type JFH1 (Delgrange et al., 2007; Kaul et al., 2007; Russell et al., 2008; Zhong et al., 2006) or chimeric viruses (Gottwein et al., 2007; Yi et al., 2006, 2007) have been identified. However, the molecular mechanisms of adaptive mutations are poorly understood.

In this study, we aimed to establish an efficient HCV-production system and to gain more insight into the determinants of efficient virus production. By serial passaging of Huh-7.5 cells infected with the HCV J6/JFH1 strain, we identified adaptive mutations in the clones and analysed the mutations by examining the production of the recombinant mutant viruses.

**METHODS**

**Cell culture.** Huh-7.5 cells (Blight et al., 2002), a highly HCV-permissive subclone of Huh-7 cells, were kindly provided by Dr C. M. Rice (Rockefeller University, New York, NY, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wako) supplemented with 10% fetal bovine serum (FBS; Biowest), 0.1 mM non-essential amino acids (Invitrogen), 100 IU penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ (Invitrogen). DMEM containing 10% FBS was designated complete DMEM. Cells were grown at 37°C in a CO₂ incubator.

**Antibodies.** The mouse monoclonal antibodies (mAbs) used in this study were anti-core (2H9) mAb (Wakita et al., 2005) and anti-HCV NS3 mAb (Chemicon). Goat anti-actin polyclonal antibody (C-11) (Santa Cruz Biotech) was used. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (MBL) and HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotech) were used as secondary antibodies.

**Plasmids.** Plasmid pFL-J6/JFH1 (Lindenbach et al., 2005) containing the full-length chimeric HCV genome was used to generate infectious HCV. Amino acid substitutions were introduced by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene). All PCR-amplified DNA fragments were verified extensively by using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The primer sequences used in this study are available from the authors upon request.

**HCV RNA transfection and virus production.** The pFL-J6/JFH1 plasmid was linearized with XbaI and in vitro-transcribed by using the T7 RiboMAX Express large-scale RNA production system (Promega) following the manufacturer’s instructions. The quality of synthesized RNA was examined by agarose gel electrophoresis. Cells were trypsinized and washed with serum-free DMEM. In total, 6 x 10⁶ cells were suspended in 500 μl serum-free DMEM and mixed with 10 μg in vitro-transcribed RNA in a 4 mm cuvette (Bio-Rad). The synthesized RNA was introduced into Huh-7.5 cells by electroporation using a Bio-Rad Gene Pulser system with a single pulse at 270 V, 975 μF. The cells were then plated in 10 cm culture dishes containing complete DMEM.

**Indirect immunofluorescence.** Immunofluorescence staining was performed essentially as described previously (Takigawa et al., 2004). Cells seeded on glass coverslips in a 24-well plate at a density of 4 x 10⁴ cells per well were infected with HCV. Cells were cultured, washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature, followed by permeabilization in 0.1% Triton X-100 in PBS for 10 min at room temperature. After being washed twice with PBS, cells were blocked with 5% goat serum in PBS and then incubated with the serum of an HCV-infected patient with a high titre of anti-HCV antibodies. Fluorescein isothiocyanate-conjugated goat anti-human IgG (MBL) was used as a secondary antibody. The cells were washed with PBS, counterstained with Hoechst 33342 solution (Molecular Probes) at room temperature for 10 min, mounted on glass slides and examined under a fluorescence microscope (BX51; Olympus).

**Virus titration.** Culture supernatants were diluted serially 10-fold in complete DMEM and used to infect 2 x 10⁵ naïve Huh-7.5 cells per well in 24-well plates. The inoculum was incubated with cells for 6 h at 37°C and then supplemented with fresh complete DMEM. The level of HCV infection was determined 1 day post-infection by immunofluorescence using anti-HCV polyclonal antibody. The virus titre was expressed in focus-forming units (ml supernatant)⁻¹ (f.f.u. ml⁻¹), as determined by the mean number of HCV-positive foci detected at the highest dilutions according to a previously described method (Zhong et al., 2005).

**Immunoblotting.** Immunoblotting was performed essentially as described previously (Muramatsu et al., 1997). To detect the expression of HCV proteins, the immune complexes were visualized by an ECL Western blotting detection kit (GE Healthcare) following the manufacturer’s instructions.

**HCV RNA quantification.** Total RNA was extracted by using RNAiso (TaKaRa) according to the manufacturer’s instructions. One microgram of isolated RNA was reverse-transcribed by using a QuantiTect reverse transcription kit (Qiagen) with random primers. RT-qPCR analysis was performed as described previously (Zhong et al., 2005). HCV RNA was monitored by using the PCR primers 5’-TCTGGCGAACCCTGTAGTA-3’ (sense) and 5’-TCAGCCAGTACCAACAGGC-3’ (antisense). HCV transcript levels were determined relative to a standard curve comprising serial dilutions of plasmid containing the HCV J6/JFH1 cDNA.

**HCV RNA genome sequencing.** HCV RNA was isolated from 140 μl viral supernatant by using a QIAamp Viral RNA Mini kit (Qiagen), and then used as a template to generate cDNA in a reverse-transcription reaction using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen) according to the manufacturer’s instruc-
amplified DNA were determined by using an ABI PRISM 3100-Avant 9 cDNA Ends (Invitrogen) and the 3′-end sequence was amplified by using a 3′-Full RACE Core set (TaKaRa). The sequences of the amplified DNA were determined by using an ABI PRISM 3100-Avant Genetic Analyzer.

**Quantification of HCV core protein.** HCV core protein in the cells or cell-culture supernatants was quantified by using a highly sensitive enzyme immunoassay (Ortho HCV antigen ELISA kit; Ortho Clinical Diagnostics). To determine intracellular amounts of core, cell lysates were prepared as described by Schaller *et al.* (2007).

**Blocking of virus attachment and entry with anti-CD81 antibody.** Blocking of virus attachment and entry with anti-CD81 antibody was performed essentially as described previously (Wakita *et al.*, 2005). Huh-7.5 cells (6 × 10⁴ cells per 24-well plate) were pretreated with anti-CD81 antibody (clone J-81; BD Biosciences) or an isotype-matched control antibody (purified mouse IgG1, k isotype control; BD Biosciences) as indicated for 1 h. Cells were then infected with the wild-type or mutant viruses at an m.o.i. of 0.5 or 0.01 for 6 h. The viruses were removed, and the cells were washed with PBS and then supplemented with complete DMEM. The efficiency of infection was monitored 1 day after infection by counting the number of HCV-positive foci by immunofluorescence.

**Statistical analysis.** A two-tailed Student’s *t*-test was applied to evaluate the statistical significance of differences measured from the datasets. A *P* value of <0.05 was considered to be statistically significant.

**RESULTS**

**Increase in HCV infectivity titres during serial passage**

To produce infectious HCV particles, *in vitro*-transcribed genomic J6/JFH1 RNA was electroporated into Huh-7.5 cells. Transfected Huh-7.5 cells were maintained and the infectivity titre of the culture supernatant reached 6 × 10⁴ f.f.u. ml⁻¹ at 20 days post-infection. This culture supernatant was designated P-1.

To generate higher infectivity titres for HCV, naive Huh-7.5 cells (3 × 10⁵ cells per six-well plate) were infected with 1 ml virus stock of P-1 (6 × 10⁴ f.f.u. ml⁻¹) at an m.o.i. of 0.2 and the infected cells were passaged serially every 3–4 days to maintain a subconfluent culture for 6 months. The culture medium was replaced with fresh complete DMEM every day. The extracellular infectivity titres fluctuated in the beginning after transfection and became lowest at the 22nd passage (Fig. 1a). Thereafter, the extracellular infectivity titres increased again and reached highest infectivity at the 47th passage. Therefore, we further examined the supernatants at the 27th, 38th and 47th passages, and the viruses were designated P-27, P-38 and P-47, respectively. The infectivity titres were determined to be 7.0 × 10⁶ f.f.u. ml⁻¹ for P-27, 1.7 × 10⁴ f.f.u. ml⁻¹ for P-38 and 3.3 × 10⁶ f.f.u. ml⁻¹ for P-47 (Fig. 1a). These viruses were used as inocula in the following experiments.

**Kinetics of virus production after infection with putative adaptive J6/JFH1 mutants**

To examine the virus-production kinetics of these viruses in Huh-7.5 cells, naive Huh-7.5 cells (3 × 10⁵ cells per 24-well plate) were infected with each inoculum (6 × 10³ f.f.u.) at an m.o.i. of 0.2. After infection, the culture supernatants were harvested each day for 10 days and assayed for infectivity titres (Fig. 1b). The P-1 virus showed a peak infectivity titre of 2.3 × 10⁴ f.f.u. ml⁻¹ at 4 days post-infection, whereas the P-27, P-38 and P-47 viruses showed peak titres of 1.0 × 10⁶, 2.3 × 10⁶ and 6.0 × 10⁶ f.f.u. ml⁻¹ at 4–5 days post-infection, respectively (Fig. 1b), suggesting that these three viruses produce infectious HCV particles more efficiently than the P-1 virus. The increased infectivity titres may have been due to an increase in the absolute number of released HCV particles or an increased proportion of infectious relative to non-infectious particles. To address this question, we compared the specific infectivities of the mutant viruses with those of the wild-type virus. The ratio of viral infectivity titre (f.f.u. ml⁻¹) to HCV RNA content [genome equivalents (GE) ml⁻¹] was determined as shown in Table 1. The mutant viruses, P-27, P-38 and P-47, had higher specific-infectivity titres (1 : 21, 1 : 10 and 1 : 10, respectively) than the wild-type virus P-1 (1 : 133), suggesting that the mutant viruses are more infectious than the wild type and that the mutant viruses possess adaptive mutations in the virus genomes.

**Sequence analysis of genetic mutations in the adaptive mutants**

To identify the genetic changes in the virus genomes that are responsible for the adaptation to Huh-7.5 cells, we sequenced the whole genomes of the viruses. No mutation was found in the P-1 virus, whereas several mutations were identified in the P-27, P-38 and P-47 viruses (Fig. 1c). The sequencing analysis of P-27 identified eight mutations that were located in the E2, NS2, NS5A and NS5B regions as follows: T396A, T416A, N534H and A712V in E2; Y852H and W879R in NS2; F2281L in NS5A; and M2876L in NS5B (Fig. 1c). P-38 possessed 10 mutations, the same mutations as in P-27 and two additional mutations. The additional mutations were found at nucleotide position 146 (U to A) in the 5′-UTR and an amino acid change, K78E, in the core region. P-47 contained 11 mutations, including the same 10 mutations as P-38 and one additional mutation, T2925A in NS5B. Thus, the first eight mutations were all present in the genomes of the three viruses, and the results suggested that these eight mutations contribute to the enhanced infectivity.

**Effects of individual mutations on the production of infectious HCV**

To determine which mutation is responsible for the enhancement of infectivity, recombinant genomes containing only one of the selected mutations were constructed.
(Fig. 2a). The in vitro-transcribed mutant J6/JFH1 RNAs were electroporated into Huh-7.5 cells and mutant viruses were generated. Then, naïve Huh-7.5 cells were infected with each virus at an m.o.i. of 0.01 and cultured for 12 days. The ability of each mutant virus to release infectious virus particles was examined by titration assay. As shown in Fig. 2(b), the recombinant viruses with single point mutations did not enhance the production of infectious virus particles, suggesting that a single point mutation is not enough for the enhanced infectivity.

**Effects of combination of adaptive mutations on the production of infectious HCV**

We then generated recombinant viruses with several mutations, as shown in Fig. 3(a). Naïve Huh-7.5 cells were infected with each virus at an m.o.i. of 0.01 and cultured for 12 days. The culture supernatant was collected every day from 1 to 12 days post-infection. The ability of each mutant virus to release infectious virus particles was examined by titration assay. The R-27, R-38 and R-47 viruses reached higher titres than the wild type and other mutant viruses, suggesting that all of the mutations in E2, NS2, NS5A and NS5B were important for the enhancement of infectivity (Fig. 3b). To determine the specific infectivities of the mutant viruses, the ratio of the viral infectivity titre (f.f.u. ml⁻¹) to the HCV RNA content (GE

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**Table 1. Specific-infectivity titres of the adaptive J6/JFH1 mutant viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>HCV RNA copies [log₁₀(GE ml⁻¹)]</th>
<th>Infectivity titre [log₁₀(f.f.u. ml⁻¹)]</th>
<th>Specific infectivity (f.f.u.: GE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1</td>
<td>6.7 ± 0.1</td>
<td>4.6 ± 0.1</td>
<td>1:133</td>
</tr>
<tr>
<td>P-27</td>
<td>7.3 ± 0.1</td>
<td>6.0 ± 0.2</td>
<td>1:21</td>
</tr>
<tr>
<td>P-38</td>
<td>7.4 ± 0.1</td>
<td>6.4 ± 0.0</td>
<td>1:10</td>
</tr>
<tr>
<td>P-47</td>
<td>7.3 ± 0.1</td>
<td>6.3 ± 0.2</td>
<td>1:10</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Increase in HCV infectivity titres during serial passage. (a) Serial passage of HCV J6/JFH1-infected Huh-7.5 cells. Huh-7.5 cells (3×10⁶ cells per six-well plate) were infected with 1 ml stock of wild-type J6/JFH1 virus (P-1) (6×10⁴ f.f.u. ml⁻¹) at an m.o.i. of 0.2, and the infected cells were passaged serially every 3–4 days to maintain a subconfluent culture for 6 months. The culture medium was replaced with fresh complete DMEM each day. The extracellular infectivity titres were determined by titration assay and are expressed as f.f.u. ml⁻¹. Arrows show the time points at which we collected the putative adapted viruses, designated P-27, P-38 and P-47. (b) Kinetics of virus production after infection with putative J6/JFH1 adaptive mutants in Huh-7.5 cells. Huh-7.5 cells were infected with the wild-type J6/JFH1 virus (○, P-1) or putative adaptive mutants (▲, P-27; ■, P-38; □, P-47) at an m.o.i. of 0.2. After infection, the culture supernatants were harvested every day until 10 days post-infection. Infectivity titres were measured by immunofluorescence assay and are expressed as f.f.u. ml⁻¹. Error bars represent SD for triplicate measurements. (c) Genetic mutations identified during passage. Numbers indicate the amino acid position where mutations were identified. The nucleotide position with mutation is given in parentheses.
ml⁻¹) was calculated as shown in Table 2. The recombinant mutant viruses, R-27, R-38 and R-47, had higher specific-infectivity titres (1:46, 1:35 and 1:54, respectively) than the wild-type virus P-1 (1:197), suggesting that the particles released from cells infected with the R-27, R-38 and R-47 viruses are more infectious than those released from cells infected with the wild-type J6/JFH1 virus.

Efficient expression of HCV proteins in Huh-7.5 cells infected with the adaptive mutants

To investigate further the mechanism of adaptive mutations, we performed immunofluorescence staining of the infected cells. Huh-7.5 cells (6 × 10⁴ cells per 24-well plate) were infected with the P-1, R-27, R-38 and R-47 viruses (1.2 × 10⁴ f.f.u.) at an m.o.i. of 0.2. Cells were fixed 5 days post-infection and stained for immunofluorescence. Approximately 90% of the cells were HCV-positive in the P-1-, R-27-, R-38- and R-47-infected cells (Fig. 4a). We next examined protein synthesis by immunoblotting for the HCV core and NS3 proteins. Immunoblot analysis of the cell lysates demonstrated that the levels of the core and NS3 proteins in cells infected with the R-27, R-38 and R-47 viruses were 2.0- to 2.5-fold higher than those in cells infected with the P-1 virus (Fig. 4b, c), suggesting that these mutant viruses have a replicative advantage.

Growth curves of infectious HCV after transfection of RNAs or infection with HCV

To determine whether the replicative advantage is at the level of entry or replication/translation of the genome, we examined one-step growth curves by transfecting equivalent amounts of RNAs of the wild-type and the mutant viruses into Huh-7.5 cells by means of electroporation (Fig. 5a, b). The intracellular and extracellular core protein levels were quantified by core protein-specific ELISA at the indicated times. The one-step growth curves showed that the intracellular and extracellular core protein levels increased with very similar kinetics in the cells transfected with the wild-type and adapted RNAs (Fig. 5a, b).
We next examined the growth curves of the core protein levels by infecting cells with the recombinant viruses. The intracellular and extracellular core protein levels in cells infected with the P-1, R-27, R-38 and R-47 viruses were quantified. Huh-7.5 cells (1.2 × 10^5 cells per 12-well plate) were infected with these viruses at an m.o.i. of 0.2. The intracellular core protein levels in cells infected with the R-27, R-38 and R-47 viruses were 3- to 5-fold higher at day 1 post-infection than those in the P-1-infected cells. The intracellular core protein levels in the cells infected with the mutant viruses were 7- to 11-fold higher at day 3 post-infection than those in the P-1-infected cells (Fig. 5c). The extracellular core protein levels in the P-1-infected cells were comparable to the levels in cells infected with mutant viruses at day 1 post-infection. However, the extracellular core protein levels in cells infected with the R-27, R-38 and R-47 viruses increased more rapidly and reached 4.4- to 5.8-fold higher at day 3 post-infection than those in cells infected with the P-1 virus (Fig. 5d). Taken together, these data suggest that the adaptive mutants have advantages at the entry level, rather than the virus replication/translation level.

Table 2. Specific-infectivity titres of the recombinant adaptive mutant viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>HCV RNA copies [log_{10}(GE ml^{-1})]</th>
<th>Infectivity titre [log_{10}(f.f.u. ml^{-1})]</th>
<th>Specific infectivity (f.f.u. : GE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1</td>
<td>6.6 ± 0.1</td>
<td>4.3 ± 0.1</td>
<td>1:197</td>
</tr>
<tr>
<td>R-27</td>
<td>6.8 ± 0.1</td>
<td>51 ± 0.2</td>
<td>1:46</td>
</tr>
<tr>
<td>R-38</td>
<td>6.9 ± 0.1</td>
<td>15.4 ± 0.1</td>
<td>1:35</td>
</tr>
<tr>
<td>R-47</td>
<td>6.9 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>1:54</td>
</tr>
</tbody>
</table>
Blocking of virus attachment and entry with anti-CD81 antibody

To determine whether the adapted mutant viruses have advantages at the entry level, we examined CD81-dependent entry into Huh-7.5 cells. Naive Huh-7.5 cells were incubated with CD81-specific or non-specific antibody prior to inoculation. We scored infection by immunofluorescence at 24 h post-infection. As shown in Fig. 6(a), the anti-CD81 antibody inhibited the entry of the mutant viruses R-27, R-38 and R-47, as well as the wild-type virus, in a dose-dependent manner, suggesting that interaction between CD81 and HCV E2 glycoprotein is crucial for virus entry for all of these viruses. However, infections by the mutant viruses R-27, R-38 and R-47 were less dependent on CD81 than the wild-type virus. This result suggests that the mutations in the E2 glycoprotein confer an advantage to the mutant viruses at the entry level. We further analysed the mutant viruses to determine which mutation(s) is important for the advantage at the entry level. We infected Huh-7.5 cells with mutant viruses with a single point mutation in the E2 glycoprotein, such as T396A, T416A, N534H or A712V, or with all of the four mutations in E2. Blocking of virus entry with the anti-CD81 antibody was examined as shown in Fig. 6(b). Infection by the mutant virus N534H, as well as the mutant viruses E2, R-27, R-38 and R-47, was less dependent on CD81 than infection by the wild-type virus, whereas the other mutant viruses T396A, T416A and A712V showed a similar pattern to the wild type. These results indicate that the N534H mutation in the E2 region confers an advantage to the adaptive mutant viruses at the entry level.

DISCUSSION

In this study, we established an efficient HCV-production system by serial passaging of Huh-7.5 cells infected with the chimeric HCV J6/JFH1. Sequence analyses revealed that the adapted viruses possessed more than eight non-synonymous mutations in the genomes. Reverse-genetics analysis revealed that the recombinant viruses R-27, R-38 and R-47 exhibited higher expression of the HCV proteins than the wild-type virus. Moreover, we demonstrated that the N534H mutation in the E2 glycoprotein confers an advantage to the mutant viruses at the entry level.

The adaptive mutant viruses possessed four mutations (T396A, T416A, N534H and A712V) in E2. Two of these mutations (T416A and N534H) are in the regions that are involved in E2–CD81 binding and are, therefore, the possible target for neutralizing antibodies inhibiting E2–CD81 interactions (Helle & Dubuisson, 2008). The blocking of virus attachment and entry with CD81-specific antibody in this study revealed that the infections by the E2 R-27, R-38, R-47 and N534H mutants were less dependent on the CD81 molecule than that by the wild type J6/JFH1, suggesting that the N534H mutation gives the mutant viruses a selective advantage at the entry level. The N534H mutation is located in the sixth of 11 N-glycosylation sites, and is predicted to remove N-glycosylation. The removal of N-glycosylation sites has been shown to have variable effects on CD81 binding and infectivity (Owsianka et al., 2006; Roccasecca et al., 2003). The glycans at positions 417, 532 and 645 (E2N1, E2N6 and E2N11) were shown to reduce the sensitivity of HCV pseudoparticles to antibody neutralization and to reduce the access of CD81 to its binding site on E2 (Goffard et al., 2005). JFH-1 virus with the N534K mutation spread faster than the wild-type
JFH-1 virus after two successive amplifications in naïve cells, although the numbers of infectious viruses in the supernatant of transfected cells were initially low (Delgrange et al., 2007). Our results in the growth curves of the viruses in the transfected cells and infected cells were consistent with their report. The CD81 inhibition assay in this study demonstrated clearly that the N534H mutation of the J6/JFH-1 virus confers a selective advantage for J6/JFH-1 at the entry level. To our knowledge, the present study is the first to prove that the mutation at site N534 gives infectious HCV a selective advantage at the entry level. These results raise two possibilities. One is that the N534H mutation in the E2 glycoprotein removes N-glycosylation and this mutant E2 glycoprotein possesses a higher affinity for the CD81 molecule, resulting in efficient entry to the cells. Another possibility is that the E2 glycoprotein with the N534H mutation gains higher affinity for other HCV receptors. Further investigation will be required to elucidate the mechanism of this adaptive mutation.

Our results showed that a combination of the mutations in E2, together with four additional mutations in NS2, NS5A and NS5B, resulted in higher infectivity of HCV, suggesting that the additional four mutations possess an advantage at different steps.

NS2 is a membrane-associated cysteine protease (Garakou et al., 1993; Hijikata et al., 1993b; Lorenz et al., 2006). The N terminus of NS2 consists of one or more transmembrane domains, whilst the C-terminal domain of NS2, together with the N-terminal one-third of NS3, forms the NS2–3 protease, an enzyme that catalyses a single cleavage at the NS2/NS3 boundary. The crystal structure of the C-terminal domain of NS2 has recently been determined and reveals a dimeric protease containing two composite active sites (Lorenz et al., 2006). Jones et al. (2007) showed that NS2 and p7 are essential for HCV infectivity. The Y852 and W879 residues are located in the hydrophobic region of NS2. Although the exact topology of NS2 is disputed, the Y852H and W879R mutations would be predicted to lie

Fig. 5. Effects of adaptive mutations on the production of intracellular and extracellular core protein after transfection of in vitro-translated HCV RNAs or after infection of recombinant HCV. (a, b) After electroporation of 10 μg in vitro-translated HCV RNAs P-1 (○), R-27 (▲), R-38 (●) and R-47 (■) into Huh-7.5 cells (5×10⁵), the cells were divided into five sets, replated into a six-well plate and cultured. The cells and culture supernatants were harvested at the time points given. Intracellular (a) and extracellular (b) core protein levels were quantified by core protein-specific ELISA. (c, d) After Huh-7.5 cells (1.2×10⁵ cells per 12-well plate) were infected with the P-1 (○), R-27 (▲), R-38 (●) and R-47 (■) viruses at an m.o.i. of 0.2, the cells and culture supernatants were harvested at the time points given. Intracellular (c) and extracellular (d) core protein levels were quantified by core protein-specific ELISA.
within the second and third transmembrane domains, respectively (Yamaga & Ou, 2002). Murray et al. (2007) demonstrated that the A880P mutation increased infectious virus production significantly in the context of the J6/JFH1 genome, suggesting that the mutations in the transmembrane domain of NS2 play an important role in HCV infectivity. It is possible that the Y852H and W879R mutations in the transmembrane domain affect the topology and localization of NS2, and thereby HCV infectivity. Interestingly, NS2 has been found to interact with all other HCV NS proteins in in vitro pull-down assays, as well as cell-based colocalization and co-immunoprecipitation experiments (Dimitrova et al., 2003; Hijikata et al., 1993b), suggesting a role for NS2 as part of the replication complex.

Sequence analyses of HCV replicon cells revealed that highly adaptive mutations lie within the NS4B, NS5A and NS5B coding regions, with the majority clustering in NS5A. However, the mechanism underlying the replication enhancement is not known (Bartenschlager & Sparacio, 2007). The mutant viruses possessed an F2281L mutation that was located in domain II of NS5A. NS5A is an RNA-binding phosphoprotein composed of three domains that are separated by trypsin-sensitive low-complexity sequences (LCS I and LCS II) and an N-terminal amphipathic α-helix that anchors the protein stably to intracellular membranes (Brass et al., 2002; Penin et al., 2004; Tellinghuisen et al., 2004). According to the X-ray crystal structure of domain I, it forms a dimer with a claw-like shape that can accommodate a single-stranded RNA molecule (Tellinghuisen et al., 2005). Domain III of NS5A plays an important role in virus assembly and the production of infectious particles (Appel et al., 2008; Masaki et al., 2008; Tellinghuisen et al., 2008). However, the role played by domain II of NS5A in the HCV replication cycle is unknown. Further examination will be required to clarify the effects of the F2281L mutation on the infectivity of the virus. Kaul et al. (2007) reported the V2941M mutation in NS5B in the context of the JFH1 genome. Lohmann et al. (2001) reported the R2884G mutation in the context of Con1-based replicon cells. Amino acid substitutions within NS5B may favour HCV replication and virus production in ways that remain to be determined.

Miyanari et al. (2007) proposed that HCV NS proteins and replication complexes are recruited to lipid droplet-associated membranes by the HCV core protein and that this recruitment is critical for producing infectious viruses. Cholesterol and sphingolipid associated with HCV particles are important for virion maturation and infectivity (Aizaki et al., 2008). We speculate that the additional four mutations in NS2, NS5A and NS5B may confer an advantage in the maturation of virus particles or modification of virus envelopes with cholesterol and sphingolipid. Further investigation will be necessary to elucidate the mechanism of the adaptive mutations in NS2, NS5A and NS5B.

Fig. 6. Blocking of virus attachment and entry with anti-CD81 antibody. (a) Huh-7.5 cells (2×10⁵ cells per six-well plate) were pre-treated with 0, 1, 10 or 30 μg CD81 antibody (clone JS-81) ml⁻¹ for 1 h and then infected with the wild-type (●, P-1) or recombinant mutant (■, R-27; ◊, R-38; ▲, R-47) viruses at an m.o.i. of 0.5. The cells were cultured for 24 h. The infection was monitored by HCV immunofluorescence and the numbers of HCV-positive foci were counted. Each result is expressed as a fraction of the number of foci observed in wells that received the control antibody instead of anti-CD81. Error bars represent SD for triplicate measurements. (b) Huh-7.5 cells (2×10⁶ cells per six-well plate) were pre-treated with 0, 0.25, 0.5, 1, 5 or 10 μg CD81 antibody ml⁻¹ for 1 h and then infected with the wild-type (●, P-1) or recombinant (■, R-27; ◊, R-38; ▲, R-47; ×, E2; □, T396A; ○, T416A; ○, N534H; △, A712V) viruses at an m.o.i. of 0.01. Blocking of virus entry with anti-CD81 antibody was examined. The infection was monitored by HCV immunofluorescence and the number of HCV-positive foci was counted.
In conclusion, we have developed an efficient HCV-production system by passaging HCV J6/JFH1-infected Huh-7.5 cells. We have demonstrated that an efficient HCV-production system could be obtained by introducing adaptive mutations into the J6/JFH1 genome. The J6/JFH1-derived mutant viruses presented here would be a good tool for producing HCV particles with enhanced infectivity and for studying the molecular mechanism of HCV entry.

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REFERENCES


