3. Results

3.1. HCV protein expression in SGR, FGR, HCV-infected cells and those treated with IFN

Immunofluorescence analysis revealed that almost all the cells in SGR and FGR cultures, and >90% of the cells in the HCV J6/JFH1-infected culture were positive for HCV antigens (Fig. 2A). Western blot analysis also confirmed HCV protein expression in SGR, FGR and HCV-infected cells (Fig. 2B). In some experiments, HCV replication in SGR, FGR and HCV-infected cells was eliminated by IFN treatment for 10 days (Fig. 2A and B).

3.2. Selective suppression of cellular glucose uptake by HCV replication

2-Deoxygluose uptake levels in SGR, FGR and HCV-infected cells were significantly suppressed by about 50–60%, compared with the control Huh-7.5 cells (Fig. 3A and B). On the other hand, thymidine uptake, which was used as a control, did not significantly differ among all the cells tested (data not shown). Moreover, glucose uptake levels in SGR, FGR and HCV-infected cells were restored by IFN treatment (Fig. 3A and B). These results strongly suggest that cellular glucose uptake is selectively suppressed by HCV RNA replication.

3.3. Down-regulation of cell surface expression of GLUT2 and GLUT1 by HCV replication

GLUT2 is the principal glucose transporter of hepatocytes *in vivo* while GLUT1 is expressed in a wide variety of cultured cells. We therefore examined cell surface expression of GLUT2 and GLUT1 by flow cytometry analysis. As shown in Fig. 4A, cell surface expression of GLUT2 and GLUT1 was markedly down-regulated in SGR and FGR cells, compared with the control. On the other hand, cell surface expression of transferrin receptor was not significantly suppressed in SGR or FGR, compared with the control, with the result ensuring the specificity of the down-regulation of GLUT2 and GLUT1 cell surface expression in SGR and FGR (Fig. 4A). Moreover, treatment of SGR and FGR cells with IFN restored the surface expression of GLUT2 and GLUT1 (Fig. 4A). These results suggest that HCV RNA replication specifically mediates down-regulation of GLUT2 and GLUT1.

Down-regulation of GLUT2 surface expression was observed also in HCV-infected cells (Fig. 4B). On the other hand, down-regulation of GLUT1 surface expression was only marginal and, compared to that of GLUT2, less evidently observed in HCV-infected cells. As a control, cell surface expression of transferrin receptor did not differ at all between HCV-infected cells and the control. Again, treatment of HCV-infected cells with IFN restored surface expression of GLUT2 (Fig. 4B).

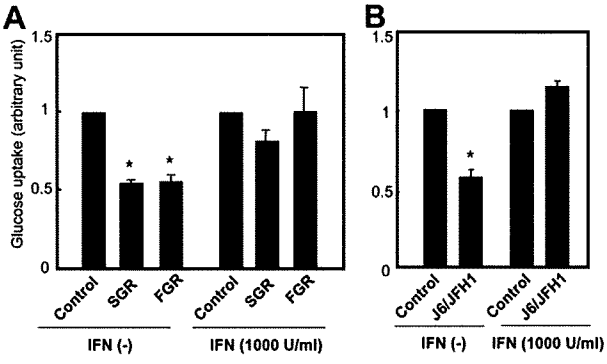

Fig. 3. Selective suppression of cellular glucose uptake by HCV replication. (A) Uptake of 2-deoxy-D-[1,2-^{3}H] glucose in SGR, FGR and HCV-negative control. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to glucose uptake analysis. Data represent mean ± SEM of four independent experiments and the values for the control cells were arbitrarily expressed as 1.0. $^*P < 0.01$, compared with the control. (B) Uptake of 2-deoxy-D-[1,2-^{3}H] glucose in J6/JFH-1-infected cells and the uninfected control. In parallel, cells at 5 days after infection were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to glucose uptake analysis.

3.4. Proteasomal degradation is not involved in the down-regulation of GLUT2 or GLUT1

Some viruses down-regulate cell surface molecules, such as immunoreceptors and intercellular adhesion molecules, through ubiquitination and proteasomal degradation of the target proteins [25]. To test this possibility, we treated SGR and FGR cells with lactacystin, a potent proteasome inhibitor. While lactacystin treatment enhanced cell surface expression of transferrin receptor, the same treatment did not increase cell surface expression of GLUT2 or GLUT1 in SGR or FGR cells (Fig. 5). This result suggested that down-regulation of cell surface expression of GLUT2 or GLUT1 in HCV-replicating cells was not due to increased degradation through the ubiquitin–proteasome system. The result rather implied the possible involvement of another mechanism(s), e.g., transcriptional suppression and/or impaired intracellular trafficking.

3.5. Transcriptional suppression of GLUT2, but not GLUT1, by HCV replication

To examine whether HCV RNA replication suppresses GLUT2 and GLUT1 expression at the transcriptional level, we measured mRNA expression levels by quantitative RT-PCR. The results obtained revealed that GLUT2 mRNA levels were reduced significantly in SGR, FGR and HCV-infected cells, compared to the control (Fig. 6A). It should be noted that the degree of GLUT2 mRNA suppression was greater in FGR

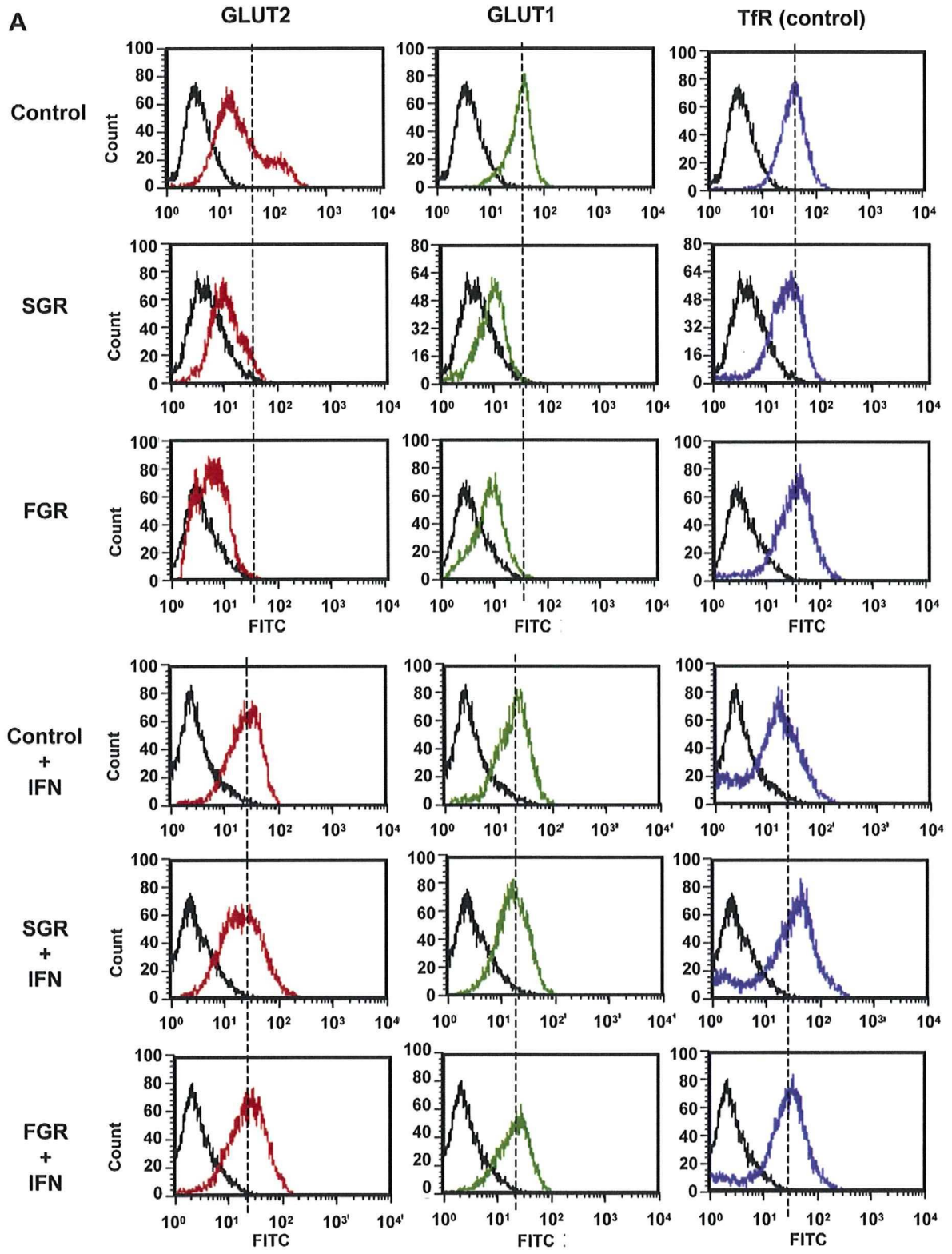

Fig. 4. Down-regulation of cell surface expressions of GLUT2 and GLUT1 by HCV replication. (A) SGR, FGR, the HCV-negative control cells were stained with specific antibodies, followed by FITC-conjugated second antibody (GLUT2, red line; GLUT1, green line) or stained with FITC-conjugated antibody alone (black line). Transferrin receptor (TfR) served as a control (blue line). In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to flow cytometry. (B) HCV-infected cells and the uninfected control were analyzed by flow cytometry as in (A). In parallel, cells at 5 days after infection were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to flow cytometry analysis.

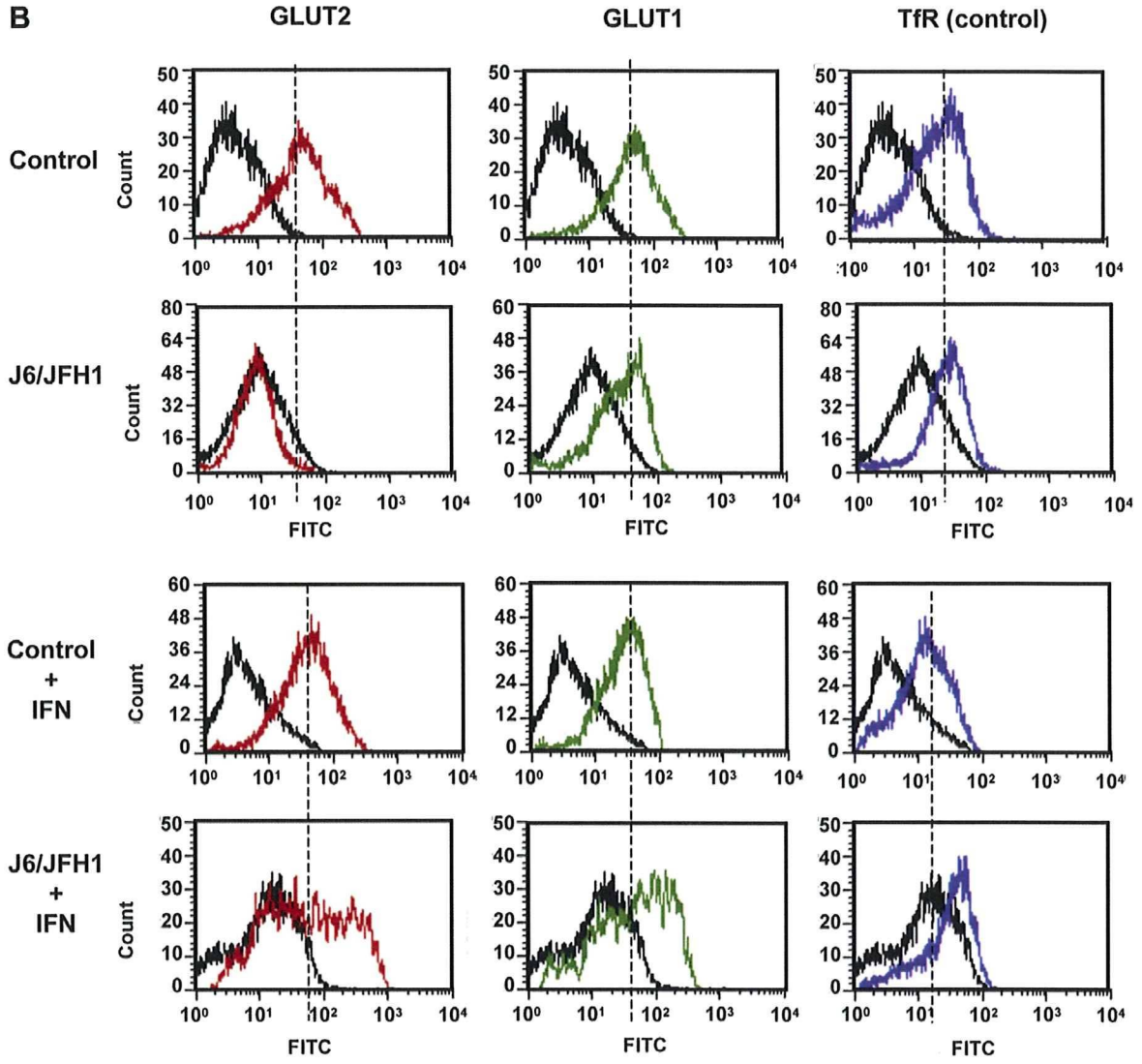

Fig. 4 (*continued*)

than in SGR cells. On the other hand, GLUT1 mRNA levels were not affected by HCV RNA replication (SGR and FGR) or HCV infection (Fig. 6B).

We also confirmed that GLUT2 mRNA expression levels in SGR, FGR and HCV-infected cells were restored by IFN treatment (Fig. 6A).

3.6. Suppression of GLUT2 promoter activity by HCV replication

Next, we performed luciferase reporter assay to examine the possible effect of HCV replication on GLUT2 promoter activities. The result obtained demonstrated that GLUT2 promoter activities were significantly suppressed in SGR, FGR and HCV-infected cells, compared to the control cells (Fig. 6C). Furthermore, GLUT2 promoter activities in SGR, FGR and HCV-infected cells were restored by IFN treatment. It is thus likely that HCV replication suppresses GLUT2 promoter activity, thereby decreasing GLUT2 mRNA levels.

3.7. Ectopically expressed GLUT1 or GLUT2 mediates increased glucose uptake in SGR, FGR and HCV-infected cells

We examined the possible effects of ectopically expressed GLUT1 and GLUT2 on glucose uptake in SGR, FGR and HCV-infected cells. Glucose uptake was significantly increased by ectopically expressed GLUT1 or GLUT2 in SGR, FGR and HCV-infected cells as well as in the control Huh-7.5 cells (Fig. 6D). It should be noted that, in this series of transient transfection experiments, only ca. 20% of the cells were ectopically overexpressing GLUT1 or GLUT2. These results collectively suggest the possibility that down-regulation

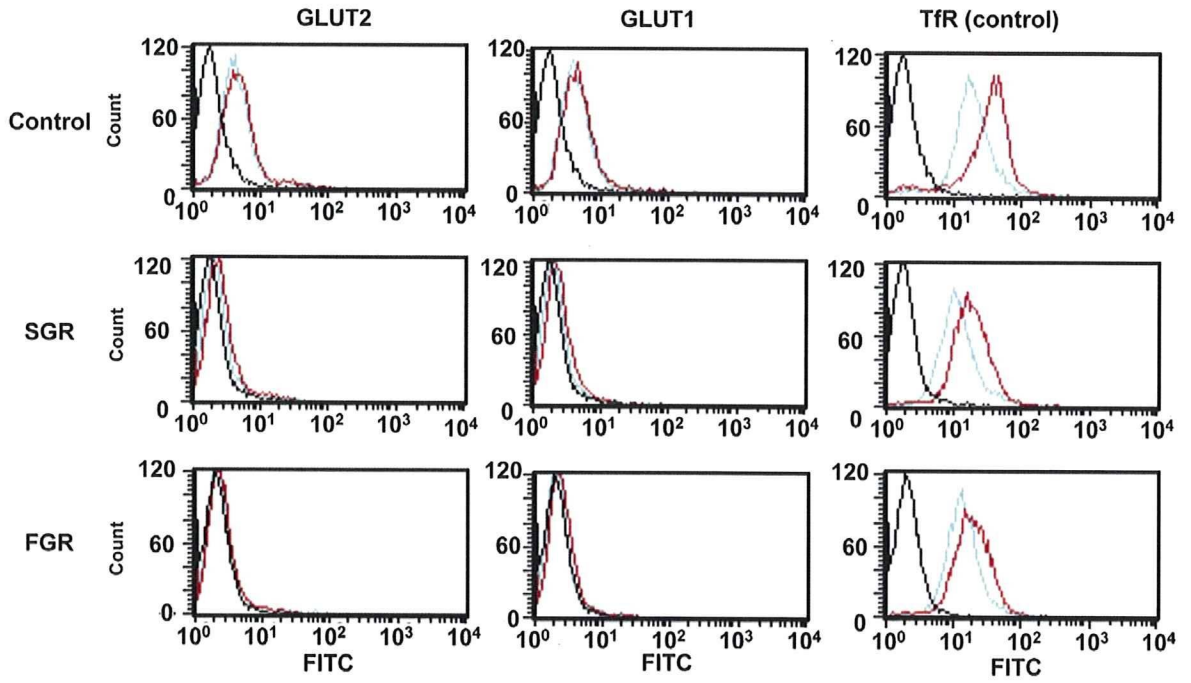

Fig. 5. Effects of lactacystin treatment on cell surface expression of GLUT2, GLUT1 and transferrin receptor (TfR). Cells were treated with lactacystin (10 μM) overnight to inhibit proteasomal degradation, and analyzed by flow cytometry. Cells treated with lactacystin are shown in red line and those left untreated in blue line. The negative controls stained with FITC-conjugated antibody alone are shown in black line.

of GLUT1 and GLUT2 expression is primarily involved in the decreased glucose uptake in SGR, FGR and HCV-infected cells.

3.8. Decreased GLUT2 expression in hepatocytes obtained from HCV-infected patients

GLUT2 is the principal glucose transporter expressed in hepatocytes *in vivo*. As shown in Fig. 7B, practically all hepatocytes obtained from patients without HCV infection showed positive staining for GLUT2, which was most evidently observed near the plasma membrane. On the other hand, hepatocytes obtained from HCV-infected patients showed markedly reduced GLUT2 staining in most, if not the entire, areas of the section, compared with the uninfected control (Fig. 7D). This heterogeneous staining pattern might reflect concomitant presence of areas comprising either virus-infected or uninfected hepatocytes in a tissue sample. Whereas all the sections obtained from 8 patients without HCV infection showed evenly positive staining for GLUT2, sections from 8 (89%) of 9 HCV-infected patients showed moderately to markedly reduced GLUT2 staining (Table 2). Reduced GLUT2 staining was observed also with hepatocytes in the liver tissues obtained from HBV-infected patients. However, the areas of reduced GLUT2 staining appeared to be more restricted in sections obtained from HBV-infected patients than in those from HCV-infected ones.

4. Discussion

HCV infection is known as an initiation and precipitating factor of type 2 diabetes [7–10,26,27]. Progression of liver fibrosis induced by persistent viral infection may induce diabetes [28]. Furthermore, it has been reported that the prevalence of diabetes is higher among patients with HCV-associated liver cirrhosis than in those with HBV-associated cirrhosis [7]. It is likely, therefore, that HCV infection itself is a risk factor of diabetes. Previous reports suggest that HCV infection directly causes insulin resistance that would cause the progression of diabetes [29–31]. However, the underlying mechanism(s) is not yet completely elucidated. In this study, we analyzed the effect of HCV infection on cellular glucose uptake and expression of glucose transporters.

We observed that glucose uptake was suppressed in cells harboring HCV RNA replicons (SGR and FGR) and those infected with HCV than in the control cells (Fig. 3). It has been reported that glucose disposal *in vivo* occurs through both insulin-dependent and insulin-independent mechanism [32]. We observed that treatment of SGR, FGR and the control Huh-7.5 cells with insulin (10^{-4} M to 10^{-9} M) increased glucose uptake by only about 50% from their basal levels (data not shown). Nevertheless, decreased glucose uptake by HCV-infected hepatocytes is a potential cause of hyperglycemia as the liver is a big organ accounting for 2% of the total body weight.

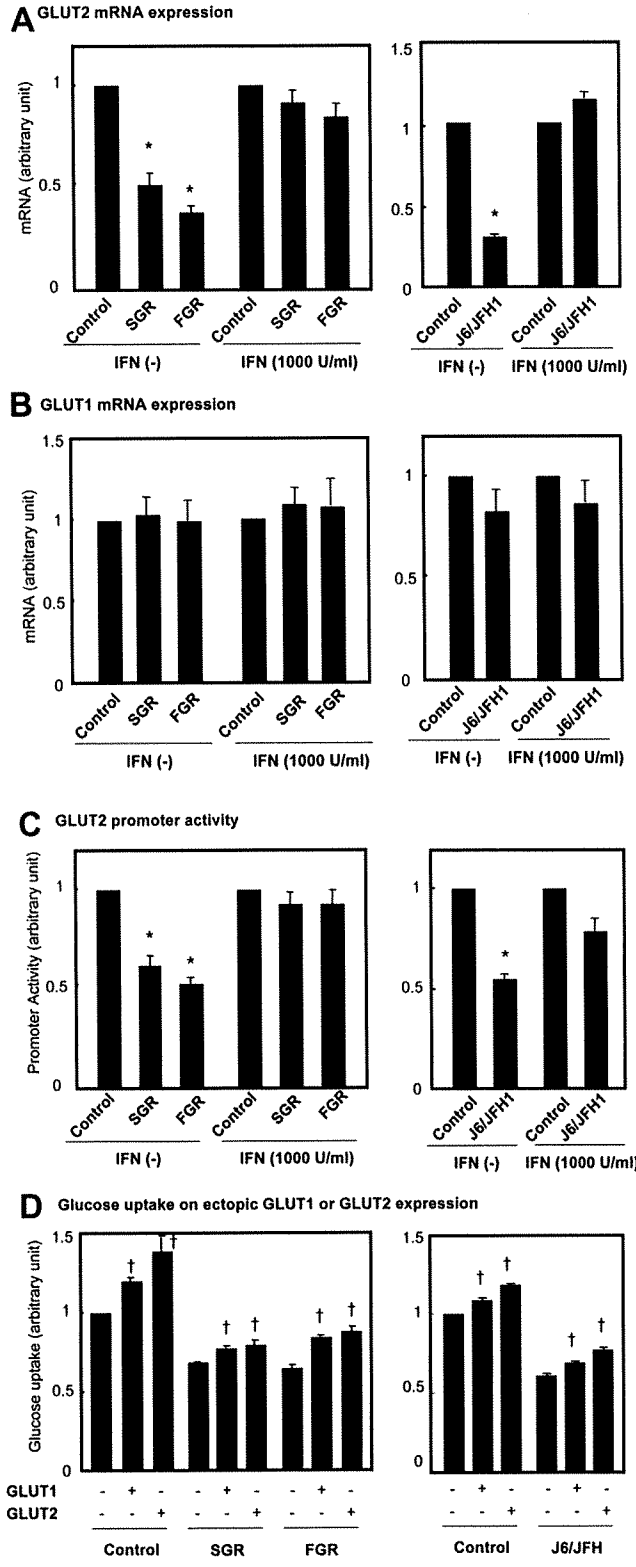

Fig. 6. Differential suppression of GLUT2 and GLUT1 mRNAs by HCV replication. (A and B) Quantitative RT-PCR analysis of mRNA for GLUT2 (A) and GLUT1 (B). mRNA expression levels of GLUT2 and GLUT1 in SGR, FGR and HCV-infected cells were determined and normalized with β-glucuronidase mRNA levels. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to quantitative RT-PCR analysis. Data represent mean ± SEM of three independent experiments. $^{*}P < 0.01$, compared with the control. (C) GLUT2 promoter activities in SGR and FGR, HCV-infected cells were analyzed using luciferase reporter assay. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to luciferase reporter assay. Data represent mean ± SEM of five independent experiments. $^{*}P < 0.01$, compared with the control. (D) Glucose uptake in cells ectopically expressing GLUT1 or GLUT2. Data represent mean ± SEM of two independent experiments. $^{\dagger}P < 0.01$, compared with mock transfected control.

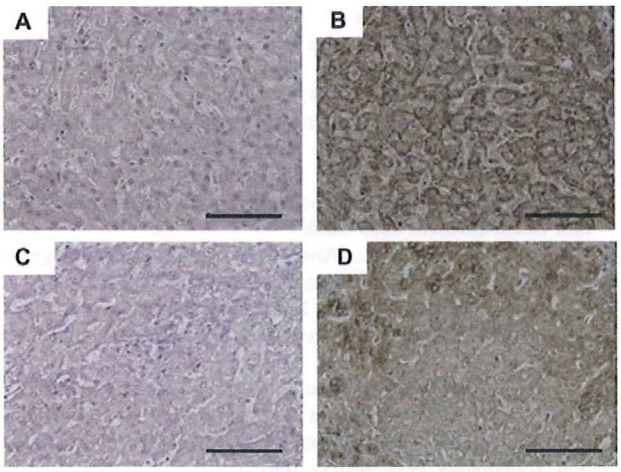

Fig. 7. Down-regulation of GLUT2 expression in HCV-infected human liver tissues *in vivo*. Normal human adult liver tissues (A and B) and HCV-infected, non-cancerous liver tissues (C and D) were fixed with formalin, sectioned and stained with normal rabbit IgG (A and C) or polyclonal anti-GLUT2 antibody (B and D). Scale bar = 100 μm.

Any proliferating cell requires energy sources, including glucose, and GLUTs play an important role in glucose uptake into the cell. In the liver, GLUT2 is the predominant glucose transporter, which regulates glucose metabolism by mediating a bidirectional transport, both entry and exit, of glucose into and from hepatocytes [13]. GLUT1, on the other hand, is known to be expressed in malignant cells including hepatocellular carcinoma [12,13] and a wide variety of cultured cells. In the present study we found that cell surface expression of GLUT2 and GLUT1 was markedly suppressed in SGR, FGR and HCV-infected cells compared to the control (Fig. 4A and B).

Table 2
Reduction of GLUT2 expression in hepatocytes of HCV-infected and HBV-infected human liver tissues.

Liver tissues	Sample No.	Reduction of GLUT2 expression
Uninfected	1	– *
	2	–
	3	–
	4	–
	5	–
	6	–
	7	–
	8	–
HCV-infected	9	1+ (Focal)[a]
	10	1+ (Focal)
	11	3+ (Diffuse)
	12	3+ (Diffuse)
	13	3+ (Diffuse)
	14	3+ (Focal)
	15	–
	16	2+ (Focal)
	17	3+ (Diffuse)
HBV-infected	18	–
	19	3+ (Diffuse)
	20	1+ (Focal)
	21	–
	22	2+ (Focal)
	23	1+ (Focal)
	24	2+ (Focal)

* -, no reduction; 1+, weak reduction; 2+, moderate reduction; 3+, strong reduction.

[a] Parentheses indicate either focal or diffuse appearance of the areas with reduced GLUT2 expression in each liver tissue sample.

GLUT2 expression is regulated at the transcriptional level, at least partly, by glucose [33]. It has been reported that hyperglycemia increases the GLUT2 mRNA and protein expression in an *in vivo* study [34]. Our present study demonstrated that GLUT2 mRNA expression was significantly suppressed in SGR, FGR and HCV-infected cells compared to the control (Fig. 6A). Consistent with this result, GLUT2 promoter activities, as measured by luciferase reporter assay, were suppressed in SGR, FGR and HCV-infected cells (Fig. 6C). In this connection, it was reported that GLUT2 promoter activities were up-regulated by sterol response element-binding protein (SREBP)-1c [35,36]. We confirmed in our study that GLUT2 promoter activities were up-regulated by over-expression of human SREBP-1c, and that the SREBP-1c-mediated GLUT2 promoter activities were suppressed significantly in SGR, FGR and HCV-infected cells (data not shown).

Unlike GLUT2 mRNA, GLUT1 mRNA was not suppressed by HCV RNA replication or HCV infection (Fig. 6B). Nevertheless, cell surface expression of GLUT1 was markedly down-regulated in SGR and FGR cells (Fig. 4A). As GLUT1 surface expression was not restored by treatment with lactacystin, a potent proteasome inhibitor (Fig. 5), it was unlikely that HCV-mediated suppression of GLUT1 surface expression was mediated through increased degradation by the ubiquitin-proteasome system. We assume that intracellular trafficking of GLUT1 (and possibly GLUT2 as well) is impaired by HCV RNA replication although we could not precisely prove it due mainly to the lack of an appropriate antibody that enables us to monitor GLUT1 trafficking. Further study is needed to elucidate the issue.

By means of immunohistochemical analysis, we confirmed that GLUT2 was strongly expressed in hepatocytes of the liver tissues obtained from all of 8 individuals without HCV infection (Fig. 7B and Table 2). More importantly, we demonstrated that GLUT2 expression was significantly down-regulated in hepatocytes obtained from 8 of 9 HCV-infected patients (Fig. 7D and Table 2). Interestingly, the areas where GLUT2 down-regulation was observed appeared to be scattered across the liver tissue sections. This may reflect the general observation that a group of hepatocytes in limited areas of the hepatic lobules, but not all the hepatocytes, are infected with HCV *in vivo*. By means of real-time quantitative PCR analysis, we found a tendency that levels of GLUT2 mRNA expression in liver tissues obtained from HCV-infected patients were lower than that obtained from uninfected controls although the dif-

ference was not statistically significant (data not shown). As stated above, not all the hepatocytes in the liver were infected with HCV and, therefore, the possible reduction of GLUT2 mRNA expression in HCV-infected hepatocytes might have been masked by the normal levels of expression in uninfected hepatocytes concomitantly present in the same tissue samples.

It should also be noted that GLUT2 staining was also reduced in hepatocytes obtained from HBV-infected patients, though to a lesser extent than that from HCV-infected ones (Table 2). We assume that inflammatory responses in the liver may trigger some intracellular event that leads to decreased GLUT2 expression in hepatocytes *in vivo*.

In conclusion, we have demonstrated for the first time that HCV replication inhibits cellular glucose uptake through down-regulation of cell surface expression of GLUT2 and possibly GLUT1. It is conceivable that the decreased glucose uptake by hepatocytes causes impaired glucose metabolism, leading eventually to the initiation and progression of diabetes mellitus during a prolonged period of HCV persistence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.12.029.

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Efficient production of infectious hepatitis C virus with adaptive mutations in cultured hepatoma cells

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Robust production of infectious hepatitis C virus (HCV) in cell culture was realized by using the JFH1 strain and the homologous chimeric J6/JFH1 strain in Huh-7.5 cells, a highly HCV-permissive subclone of Huh-7 cells. In this study, we aimed to establish a more efficient HCV-production system and to gain some insight into the adaptation mechanisms of efficient HCV production. By serial passaging of J6/JFH1-infected Huh-7.5 cells, we obtained culture-adapted J6/JFH1 variants, designated P-27, P-38 and P-47. Sequence analyses revealed that the adaptive mutant viruses P-27, P-38 and P-47 possessed eight mutations [four in E2, two in NS2, one in NS5A and one in NS5B), 10 mutations [two additional mutations in the 5′-untranslated region (5′-UTR) and core] and 11 mutations (three additional mutations in 5′-UTR, core and NS5B), respectively. We introduced amino acid substitutions into the wild-type J6/JFH1 clone, generated recombinant viruses with adaptive mutations and analysed their infectivity and ability to produce infectious viruses. The viruses with the adaptive mutations exhibited higher expression of HCV proteins than did the wild type in Huh-7.5 cells. Moreover, we provide evidence suggesting that the mutation N534H in the E2 glycoprotein of the mutant viruses conferred an advantage at the entry level. We thus demonstrate 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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INTRODUCTION

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 I (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 CO_2 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 *Xba*I 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, 6×10^6 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 4×10^4 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 2×10^5 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′-TCTGCGGAACCGGTGAGTA-3′ (sense) and 5′-TCAGGCAGTA-CCACAAGGC-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-

tions. PCR primers of between 20 and 26 bases, designed using the sequence of J6/JFH1, were used to amplify four fragments of HCV cDNA (nt 49–3517, 2582–5966, 5832–8038 and 7870–9286) to cover most of the HCV genome. In addition, the 5′-end sequence was amplified by using the 5′ RACE System for Rapid Amplification of 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 (6×10^4 cells per 24-well plate) were pretreated with anti-CD81 antibody (clone JS-81; BD Biosciences) or an isotype-matched control antibody (purified mouse IgG1, κ 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 6×10^4 f.f.u. ml^{-1} at 20 days post-infection. This culture supernatant was designated P-1.

To generate higher infectivity titres for HCV, naïve Huh-7.5 cells (3×10^5 cells per six-well plate) were infected with 1 ml virus stock of P-1 (6×10^4 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 7.0×10^3 f.f.u. ml^{-1} for P-27, 1.7×10^4 f.f.u. ml^{-1} for P-38 and 3.3×10^4 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

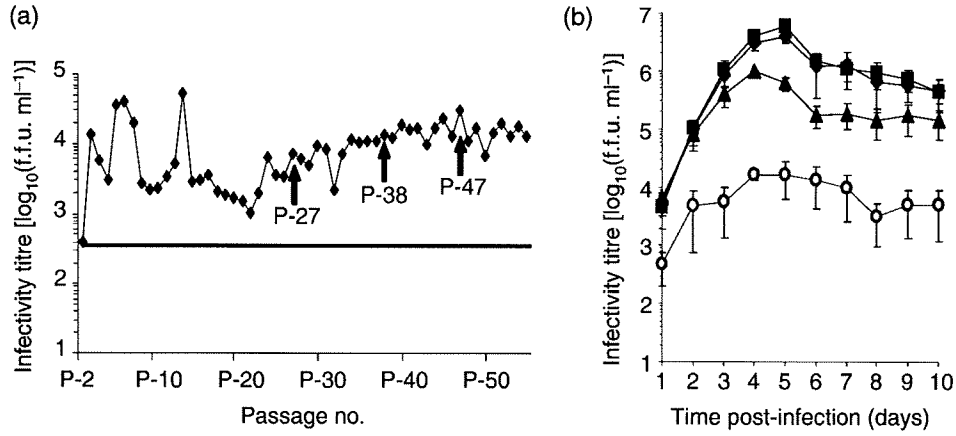
To examine the virus-production kinetics of these viruses in Huh-7.5 cells, naïve Huh-7.5 cells (3×10^4 cells per 24-well plate) were infected with each inoculum (6×10^3 f.f.u.) at an m.o.i. of 0.2. After infection, the culture supernatants were harvested each day for 10 days and assayed for infectivity titres (Fig. 1b). The P-1 virus showed a peak infectivity titre of 2.3×10^4 f.f.u. ml^{-1} at 4 days post-infection, whereas the P-27, P-38 and P-47 viruses showed peak titres of 1.0×10^6, 2.3×10^6 and 6.0×10^6 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



(c)

Virus	Position changed										
	5′-UTR	Core	E2				NS2		NS5A	NS5B	
	(nt 146)	78	396	416	534	712	852	879	2281	2876	2925
Wild-type	U	Lys	Thr	Thr	Asn	Ala	Tyr	Trp	Phe	Met	Thr
P-1	–	–	–	–	–	–	–	–	–	–	–
P-27	–	–	Ala	Ala	His	Val	His	Arg	Leu	Leu	–
P-38	A	Glu	Ala	Ala	His	Val	His	Arg	Leu	Leu	–
P-47	A	Glu	Ala	Ala	His	Val	His	Arg	Leu	Leu	Ala

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 (3×10^5 cells per six-well plate) were infected with 1 ml stock of wild-type J6/JFH1 virus (P-1) (6×10^4 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.

(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 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. 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.

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

Virus	HCV RNA copies [$\log_{10}$(GE ml^{-1})]	Infectivity titre [$\log_{10}$(f.f.u. ml^{-1})]	Specific infectivity (f.f.u.: GE)
P-1	6.7±0.1	4.6±0.1	1:133
P-27	7.3±0.1	6.0±0.2	1:21
P-38	7.4±0.1	6.4±0.0	1:10
P-47	7.3±0.1	6.3±0.2	1:10

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

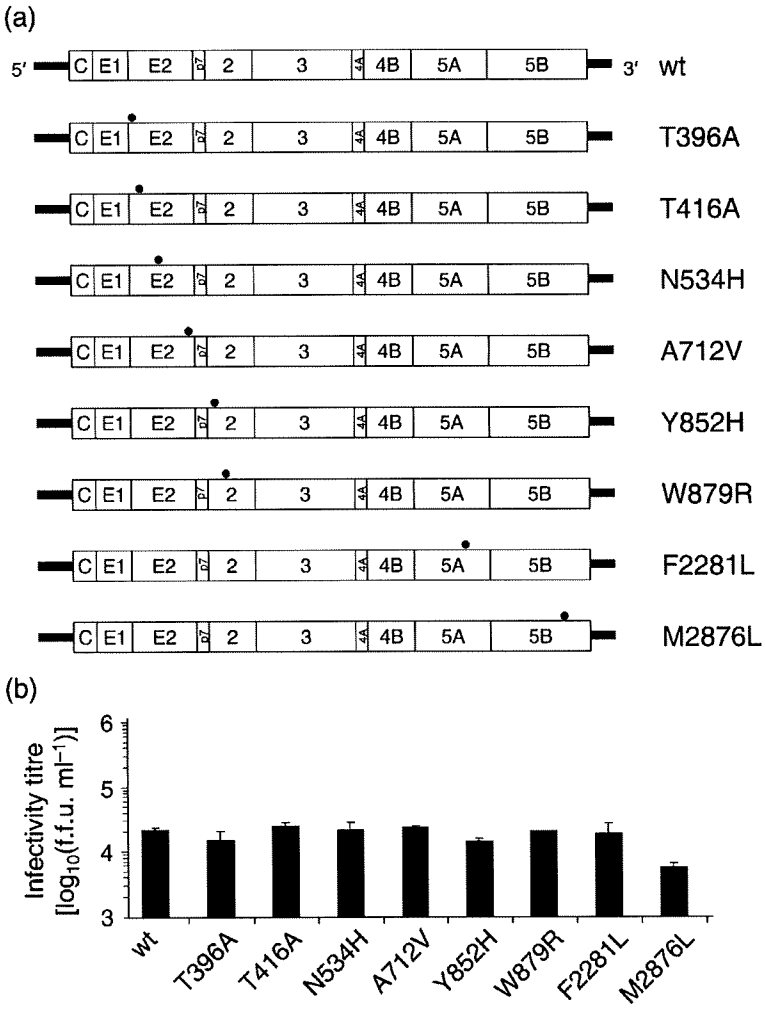


Fig. 2. Effects of individual mutations on the production of infectious HCV. (a) Schematic representation of the wild-type (wt) and mutant chimeric HCV J6/JFH1 genomes. HCV J6/JFH1 mutants with a single point mutation are shown. The adaptive mutations T396A, T416A, N534H, A712V, Y852H, W879R, F2281L and M2876L are indicated by ●. (b) The *in vitro*-transcribed mutant J6/JFH1 RNAs were electroporated into Huh-7.5 cells to generate recombinant mutant viruses. The infectivity titres of the culture supernatants were measured by titration assay. 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 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. Infectivity titres reached maximal levels at 10 days post-infection and the maximal infectivity titres were plotted. Error bars represent SD for triplicate measurements.

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 (6×10^4 cells per 24-well plate) were infected with the P-1, R-27, R-38 and R-47 viruses (1.2×10^4 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).

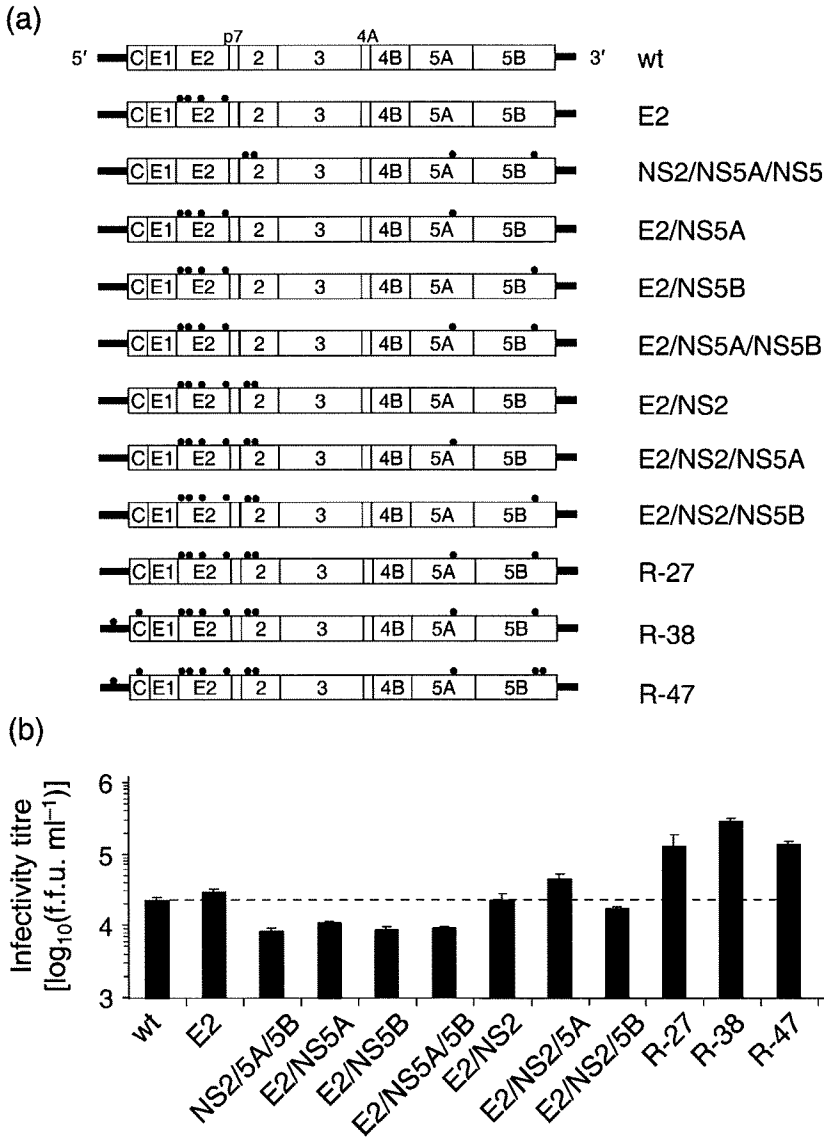


Fig. 3. Effects of combination of adaptive mutations on the production of infectious HCV. (a) Schematic representation of the wild-type (wt) and mutant chimeric HCV J6/JFH1 genomes. The HCV J6/JFH1 genomes with a combination of adaptive mutations at nt 146 (U to A) in the 5′-UTR and amino acid changes at K78E, T396A, T416A, N534H, A712V, Y852H, W879R, F2281L and M2876L are indicated by ●. (b) Recombinant mutant viruses with a combination of mutations were generated. 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 to release infectious virus particles was examined by titration assay. Infectivity titres reached maximal levels at 10 or 11 days post-infection and the maximal infectivity titres were plotted. Error bars represent SD for triplicate measurements.

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

Virus	HCV RNA copies [$\log_{10}$(GE ml^{-1})]	Infectivity titre [$\log_{10}$(f.f.u. ml^{-1})]	Specific infectivity (f.f.u. : GE)
P-1	6.6±0.1	4.3±0.1	1 : 197
R-27	6.8±0.1	51±0.2	1 : 46
R-38	6.9±0	15.4±0.1	1 : 35
R-47	6.9±0.1	5.1±0.1	1 : 54

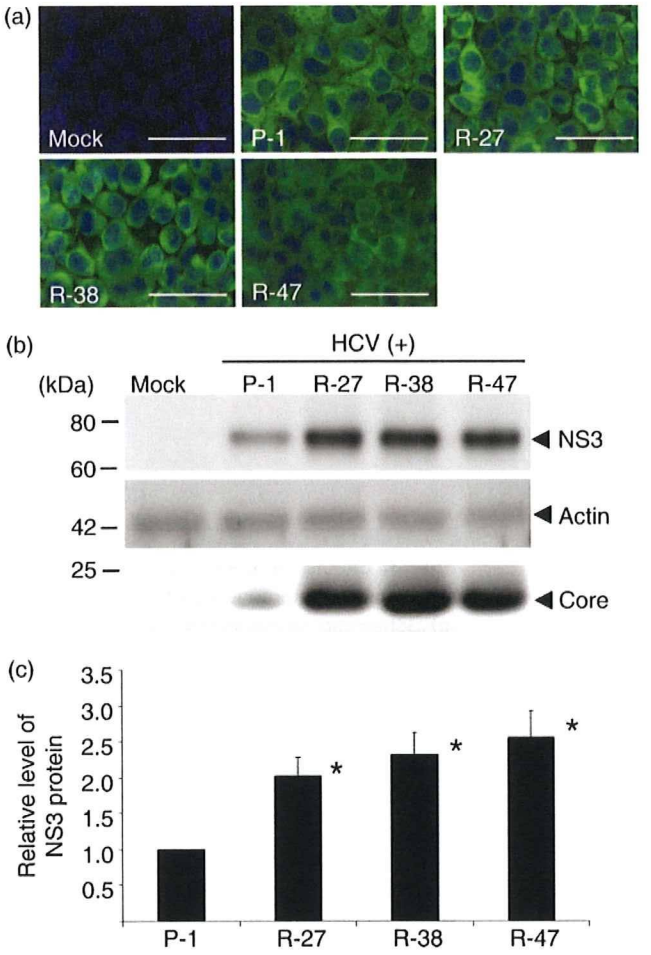


Fig. 4. Efficient expression of HCV proteins in Huh-7.5 cells infected with the adaptive mutants. Huh-7.5 cells (6×10^4 cells per 24-well plate) were infected with 200 µl P-1, R-27, R-38 or R-47 virus (6×10^4 f.f.u. ml^{-1}) at an m.o.i. of 0.2. (a) Cells were fixed 5 days post-infection and stained for immunofluorescence with anti-HCV-positive sera. Bars, 10 µm. (b) Immunoblot analysis of core and NS3 proteins in Huh-7.5 cells infected with R-27, R-38 and R-47 viruses. Data are representative of three independent experiments. (c) Quantification of the data shown in (b). Intensities of the gel bands were quantified by using the Scion Image for Windows program. The level of actin served as a loading control. Error bars represent SD for triplicate measurements. The difference between P-1 and the adaptive mutant (R-27, R-38 or R-47) was significant (*$P<0.05$ by Student's *t*-test).

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. Naïve 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 this *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

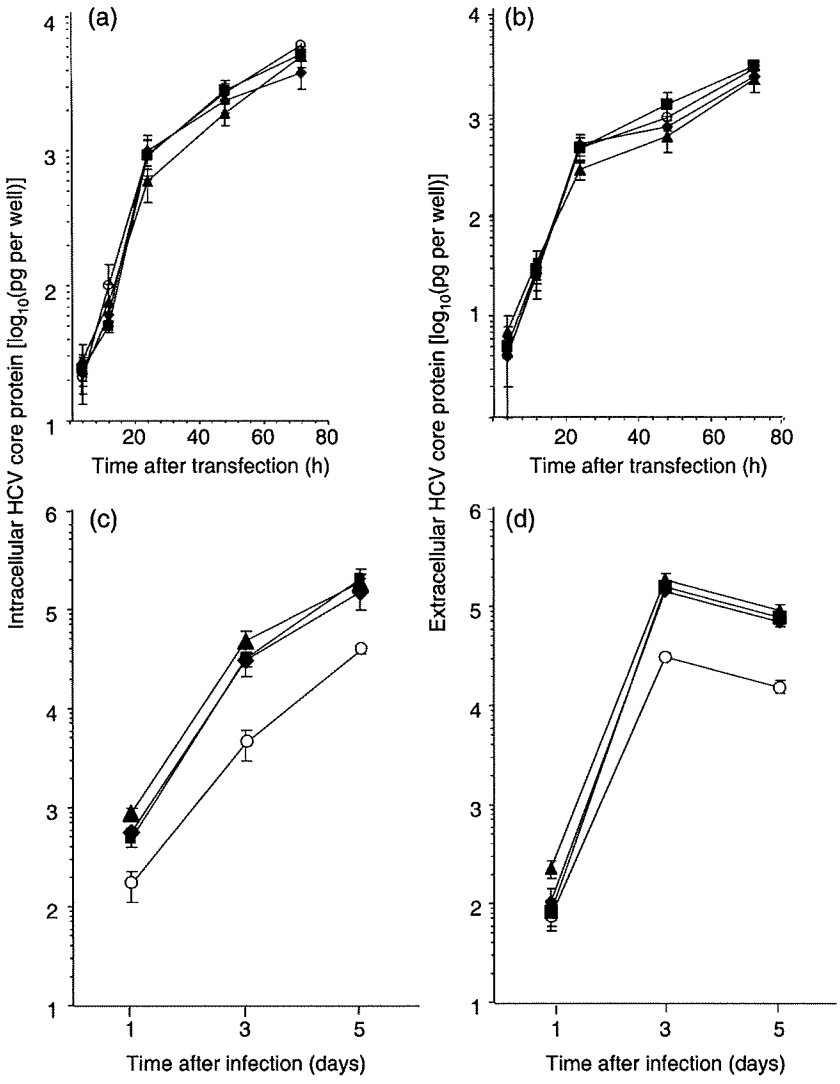


Fig. 5. Effects of adaptive mutations on the production of intracellular and extracellular core protein after transfection of *in vitro*-translated HCV RNAs or after infection of recombinant HCV. (a, b) After electroporation of 10 μg *in vitro*-translated HCV RNAs P-1 (○), R-27 (▲), R-38 (◆) and R-47 (■) into Huh-7.5 cells (5×10^6), 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 (1.2×10^5 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.

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 (Grakoui *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

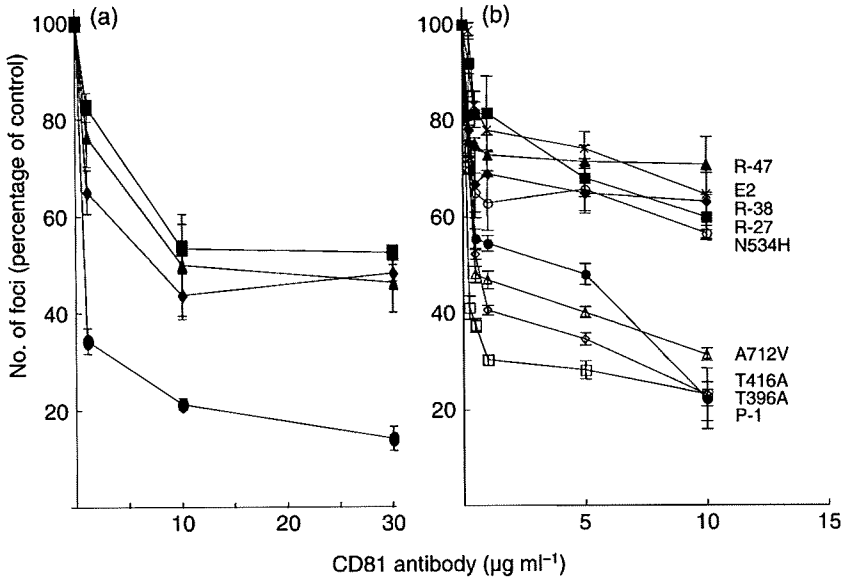


Fig. 6. Blocking of virus attachment and entry with anti-CD81 antibody. (a) Huh-7.5 cells (2×10^5 cells per six-well plate) were pretreated with 0, 1, 10 or 30 μg CD81 antibody (clone JS-81) ml^{-1} for 1 h and then infected with the wild-type (●, P-1) or recombinant mutant (■, R-27; ◆, R-38; ▲, R-47) 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 (2×10^5 cells per six-well plate) were pretreated 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.

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.

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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Role of Oxysterol Binding Protein in Hepatitis C Virus infection▽†

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Hepatitis C virus (HCV) RNA genome replicates within the ribonucleoprotein (RNP) complex in the modified membranous structures extended from endoplasmic reticulum. A proteomic analysis of HCV RNP complexes revealed the association of oxysterol binding protein (OSBP) as one of the components of these complexes. OSBP interacted with the N-terminal domain I of the HCV NS5A protein and colocalized to the Golgi compartment with NS5A. An OSBP-specific short hairpin RNA that partially downregulated OSBP expression resulted in a decrease of the HCV particle release in culture supernatant with little effect on viral RNA replication. The pleckstrin homology (PH) domain located in the N-terminal region of OSBP targeted this protein to the Golgi apparatus. OSBP deletion mutation in the PH (ΔPH) domain failed to localize to the Golgi apparatus and inhibited the HCV particle release. These studies suggest a possible functional role of OSBP in the HCV maturation process.

Hepatitis C virus (HCV) infection is one of the leading causes of chronic hepatitis. HCV infection is associated with cirrhosis, steatosis, and hepatocellular carcinoma (33). The HCV RNA genome of ~9.6 kb is translated via an internal ribosome entry site element on the rough endoplasmic reticulum (ER) as a polyprotein precursor of about 3,010 amino acids that is co- and posttranslationally processed by cellular and viral proteases into mature structural and nonstructural (NS) proteins (33). HCV replicates within ribonucleoprotein (RNP) complexes associated with modified ER membranous structures (15). Recent work implicated lipid droplets that emanate from the ER as sites of RNA replication (28, 44). Almost all of the HCV NS proteins along with a variety of cellular factors are associated with the RNP complexes engaged in viral RNA replication (37). It is likely that these NS proteins not only participate in replication process but also are involved in the various steps of virion morphogenesis and assembly. Membrane-associated RNP complexes are generally composed of viral proteins, replicating RNA, host proteins, and altered cellular membranes (1). In this respect, a growing body of evidence implicates the functional role of NS5A in early steps of virion assembly and morphogenesis (3, 27, 45). NS5A is a phosphoprotein that migrates in sodium dodecyl sulfate gels as 56-kDa (basally phosphorylated) and 58-kDa (hyperphosphorylated) forms of proteins. The C-terminal domain III region of NS5A and the phosphorylated residue (Ser^{457}) are important for virion maturation (3, 27, 45). NS5A domain III contains the binding site for viral core protein, indicating the possible involvement of NS5A protein in virus assembly (27). NS5A anchors to the ER membrane by an N-terminal hydrophobic α-helix, and this attachment is needed for its key role(s) in viral replication (10). Studies suggest that phosphorylation of NS5A plays a functional role in viral replication (12). The hyperphosphorylated NS5A reduces its interaction with the human vesicle-associated membrane protein-associated protein A (VAP-A) (12). VAP-A binds both NS5A and NS5B (13, 17). These associations are important for RNA replication (13, 17).

HCV alters lipid homeostasis to benefit its infectious processes. Host lipids and their synthesis affect viral infectious process (21, 40, 51, 57). HCV RNA replication can be induced by saturated and monounsaturated fatty acids and inhibited by polyunsaturated fatty acids (18, 21). HCV gene expression induces lipogenesis by stimulating the activation of the sterol regulatory element binding proteins, the master regulators of lipid/fatty acid biosynthetic pathways (51). Reagents that interfere with host lipid biosynthetic pathways abrogate viral replication (21, 57). It has been suggested that HCV utilizes the very-low-density lipoprotein (VLDL) secretion pathway for its viral particle release (14, 19). These studies collectively suggest that host lipid metabolism plays a key role in the viral life cycle including replication, virion assembly, and secretion (56).

In the present study, we focus on the functional role of oxysterol binding protein (OSBP) that was identified by proteomic analysis as one of the host factors associated with the HCV RNP complexes. OSBP belongs to a family of the OSBP-related proteins. Originally discovered as a major cytosolic receptor for oxidized cholesterols, it undergoes translocation from the cytosolic/vesicular compartment to the Golgi apparatus upon ligand (hydroxycholesterol) binding (38). OSBP also binds to VAP-A via its FFAT motif (53). Golgi apparatus translocation of OSBP is regulated by the pleckstrin homology (PH) domain. This domain also harbors binding sites for phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-biphosphate ($PI4,5P_2$) (25). OSBP and OSBP-related pro-

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