

IL-6-mediated intersubgenotypic variation of interferon sensitivity in hepatitis C virus genotype 2a/2b chimeric clones

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ABSTRACT

Mechanisms of difference in interferon sensitivity between hepatitis C virus (HCV) strains have yet to be clarified. Here, we constructed an infectious genotype2b clone and analyzed differences in interferon-alpha sensitivity between HCV-2b and 2a-JFH1 clones using intergenotypic homologous recombination. The HCV-2b/JFH1 chimeric virus able to infect Huh7.5.1 cells and was significantly more sensitive to IFN than JFH1. IFN-induced expression of MxA and 25-OAS was significantly lower in JFH1 than in 2b/JFH1-infected cells. In JFH1-infected cells, expression of SOCS3 and its inducer, IL-6, was significantly higher than in 2b/JFH1-infected cells. The IFN-resistance of JFH1 cells was negated by siRNA-knock down of SOCS3 expression and by pretreatment with anti-IL6 antibody. In conclusion, intergenotypic differences of IFN sensitivity of HCV may be attributable to the sequences of HCV structural proteins and can be determined by SOCS3 and IL-6 expression levels.

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Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality (Alter, 1997). There is no therapeutic or prophylactic vaccine available for HCV and type I interferons have been the mainstay of HCV therapeutics (Hoofnagle and di Bisceglie, 1997). Antiviral therapeutic options against HCV are limited and yield unsatisfactory responses (Fried et al., 2002). Given these situations, gaining a detailed understanding of the molecular mechanisms of interferon resistance has been a high priority in academia and industry.

Molecular studies of HCV have been hampered by the lack of efficient *in vitro* and *in vivo* models of infection, which has been partly overcome by the development of HCV subgenomic replicons (Blight et al., 2000; Kato et al., 2003; Lohmann et al., 1999) and the HCV-JFH1 cell culture system

(Wakita et al., 2005). HCV-JFH1 is an isolate of HCV genotype 2a that was obtained from a patient with fulminant hepatitis C. The full-length JFH1 genome has been shown to produce infectious particles in cell culture. Simultaneously, a robustly replicating intragenotypic chimera has been reported, which consists of the structural region of a genotype 2a, J6-clone and nonstructural region of JFH-1 (Lindenbach et al., 2005).

HCV isolates are classified into seven major genotypes and multiple subtypes (Gottwein et al., 2009). In infected individuals, HCV exists as quasispecies of closely related genomes (Bukh et al., 1995). A number of studies have suggested that the outcome of HCV infection, as well as the response to interferon treatment, depends on the genotype or quasispecies with which the patient is infected. However, it is not clear how these subtle genetic differences of HCV affect viral replication, infectivity and host responses. Thus, it is important to establish multiple cell culture-permissive strains of different genotypes and isolates of the same genotype for their potential value for characterizing the virus life cycle, drug sensitivity and virus-related cell signaling.

Our present work describes the generation of chimeric viruses with their structural regions from genotype 2b and non-structural genes from the HCV-JFH1 strain. The intergenotypic 2b/JFH1 viruses were compared in terms of intracellular replication, infectious virus production and sensitivity to interferon-alpha. Here we show that the differences in sensitivity to interferon are attributable to upregulated expression of the cellular interferon signal attenuator, SOCS3, and that this upregulation is caused by overexpression of interleukin-6 (IL-6).

Abbreviations: HCV, hepatitis C virus; TLR, toll-like receptor; FBS, fetal bovine serum; ISG, interferon-stimulated gene; IFN, interferon; SOCS, suppressor of cytokine signaling; IL, interleukin; ALT, alanine aminotransferase; UTR, untranslated region; CLEIA, chemiluminescence enzyme immunoassay; PVDF, polyvinylidene fluoride; STAT, signal transducer and activator of transcription; IFNAR, interferon alpha/beta receptor.

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Results

In vitro and in vivo infectivity analyses of HCV-2b and 2b/JFH1 intragenotypic chimeras

First, we investigated the infectivity of the full-length genotype 2b clone *in vitro* and *in vivo*. The full-length genotype 2b HCV clone was infectious after direct injection of RNA transcribed *in vitro* into the livers of human hepatocyte engrafted albumin-uPA/SCID mice (see the Supplementary Fig. 1). However, transfection of the HCV RNA into Huh7.5.1 cells did not lead to replication or secretion of virions. Knowing that the full-length genotype 2b HCV was not infectious *in vitro*, we constructed genotype2b/JFH1 intergenotypic recombinants. We constructed three recombinant clones of 2b/JFH1 (Fig. 1A), which were joined between E2 and p7 (JE31F), NS2 and NS3 (JE39F), and within NS2 at nt. 2867 (JEC3F). After transfection of these chimeric HCV RNAs and JFH-1 RNAs into Huh7.5.1 cells, all four clones expressed detectable amounts of HCV core protein in the cells (Fig. 1B) and culture fluid (Fig. 1C). Among the four clones, JEC3F produced the highest level of core protein in the cells and culture fluid. Similarly, in the reinfection assays, JEC3F infected naïve cells most efficiently (Figs. 1D and E). We then compared the infectivity of JEC3F with the other chimeric viruses, genotype2a J6/JFH1 and the JFH1 clone (Supplementary Fig. 2). Transfection of the individual clones into Huh7.5.1 cells showed that JEC3F and the 2b/JFH1 chimera secreted core protein into the medium most efficiently (Fig. 1C). We measured HCV core antigen and HCV-RNA levels in culture supernatant of JEC3F and JFH-1 infected cells. As shown in Fig. 1F, the ratio between supernatant HCV core antigen and HCV-RNA between JEC3F and JFH1 was well correlated each other.

Comparisons of sensitivity to IFN between intragenotypic chimeras and JFH1

Next, we investigated the interferon- α sensitivity of the three 2b/JFH1 chimeric viruses with different junctions, JE31F, JE39F and JEC3F, as well as JFH1. The four viral RNAs were transfected separately into Huh7.5.1 cells and were treated with 0, 1, 3 or 9 IU/mL of interferon- α -2b. Seventy-two hours after addition of interferon, core antigen was measured in the culture fluid. As shown in Fig. 2, all 2b/JFH1 chimeric clones showed significantly higher responses to interferon than JFH1 ($p < 0.01$). These results indicate that the relative interferon sensitivity of 2b/JFH1 clones over JFH1 could be attributable to the sequences of HCV-2b-derived structural proteins, especially core, E1 or E2 protein.

Expression of IFN stimulated genes and STAT1 and 2 phosphorylation in HCV-infected cells

Knowing that the 2b/JFH1 chimeric clones are more sensitive to interferon than JFH1, we next analyzed the effects on cellular interferon signaling. We investigated the expression levels of the interferon-stimulated genes (ISGs), 250AS and MxA mRNAs that mediate antiviral effects (Itsui et al., 2009; Itsui et al., 2006). Induction of 250AS and MxA by IFN was significantly suppressed in cells infected with HCV-JFH1 and the JEC3F clones. Of note was that the induction of these ISGs was suppressed substantially in JFH1-infected cells compared to JEC3F-infected cells (Figs. 3A and B). We then detected IFN-induced phosphorylation of STAT1 and STAT2 to pSTAT1 and pSTAT2 in uninfected and JFH1- and JEC3F-infected cells. Phosphorylation of STAT1 and STAT2 occurs within minutes after addition of IFN and substantially decreased at time points later than 8 hours (Itsui, 2006 #1025). Thus, we detected pSTAT1 and pSTAT2 before and at 15 minutes after IFN treatment. As shown in Figs. 3C and D, production of pSTAT1 and pSTAT2 was decreased substantially in JFH1-infected cells, compared with uninfected and JEC3F-infected

cells. These finding indicated that the differences in sensitivity to interferon of JFH1 and JEC3F were closely associated with attenuation of the cellular IFN signaling pathway.

SOCS 3 is up-regulated in JFH1-infected, IFN-resistant cells

We next investigated the effects of HCV replication on the expression of SOCS1 and SOCS3 that suppress IFN receptor-mediated signaling (Song and Shuai, 1998; Vlotides et al., 2004). While SOCS1 mRNA expression did not differ significantly between uninfected and JFH1- and JEC3F-infected cells, the SOCS3 mRNA expression level was significantly higher in JFH1-infected cells than in uninfected and JEC3F-infected cells (Figs. 4A and B).

Knock down of the SOCS3 gene

To verify that SOCS3 was the key molecule determining the sensitivity to IFN, we performed siRNA knock down of SOCS3 in the virus-infected cells. A SOCS3-directed siRNA was cotransfected with HCV-JFH1 or -JEC3F RNA into Huh7.5.1 cells. Three days after transfection we measured SOCS3 mRNA expression in JFH1 and JEC3F-transfected cells with or without SOCS3-siRNA. Interestingly, SOCS3-knock down in JFH1-transfected cells restored sensitivity of IFN to the same levels as JEC3F-transfected cells (Figs. 5A and B).

Interleukin-6 is involved in SOCS-mediated interferon resistance

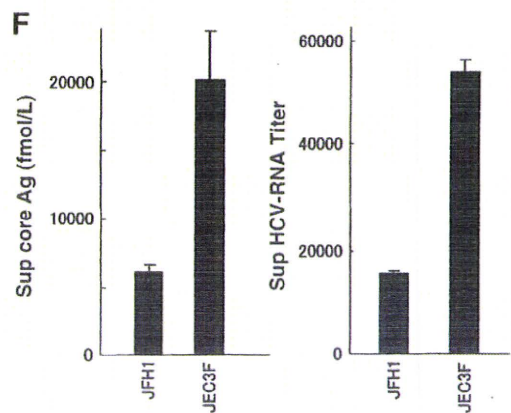
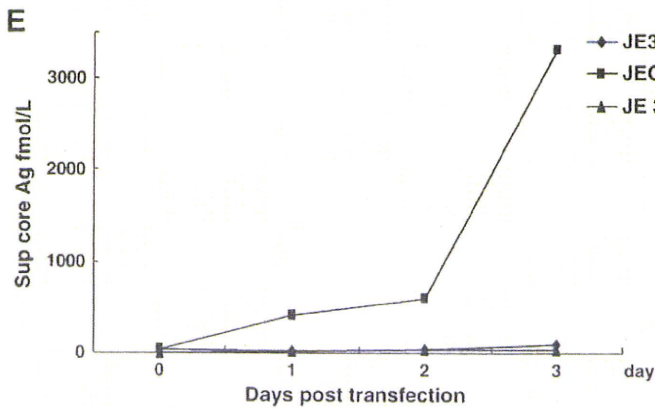
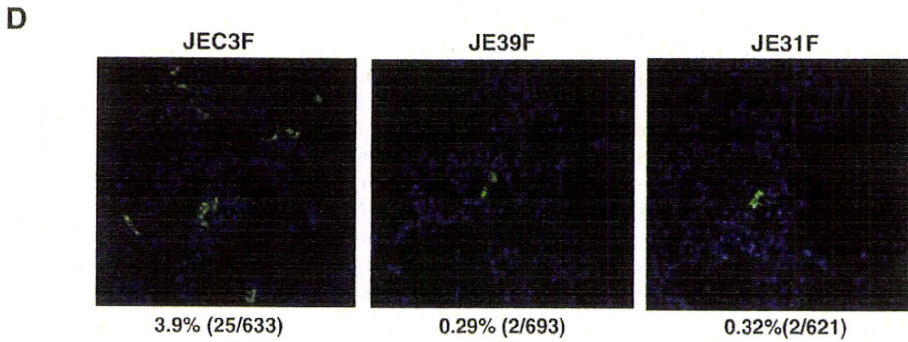
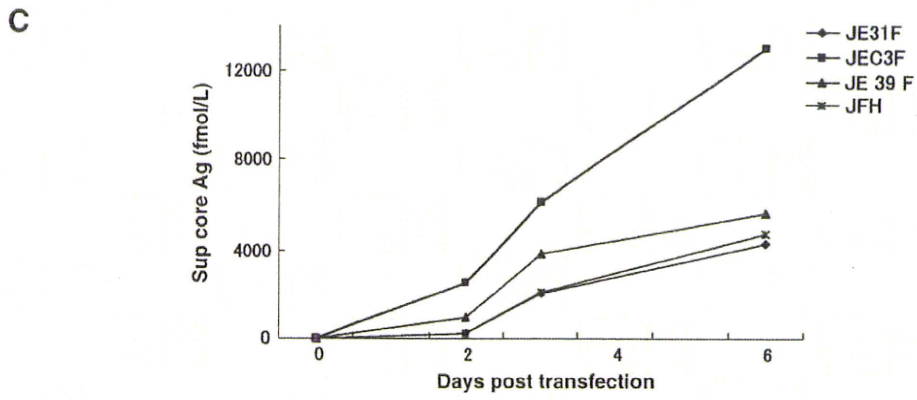
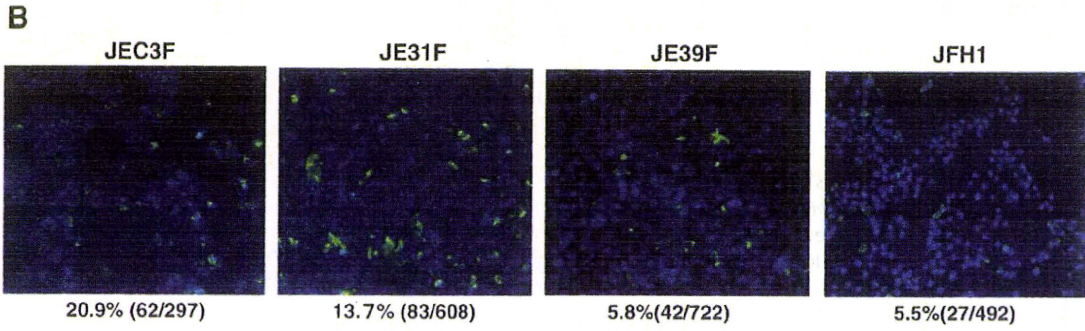
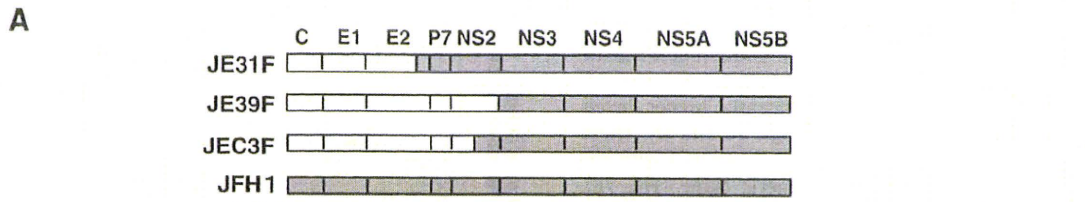
It has been reported that SOCS3 is induced principally by phosphorylated STAT3 (pSTAT3) (Hanada et al., 2003) and that interleukin-6 (IL-6) is a strong inducer of pSTAT3 via receptor-mediated Janus kinase activation in the liver (Ramadori and Christ, 1999). This background led us to investigate whether overexpression of SOCS3 is associated with overproduction of IL-6. We investigated Phosphorylated STAT3 (pSTAT3) expression and IL-6 mRNA expression in JFH1- and JEC3F-transfected Huh7.5.1 cells. Phosphorylated STAT3 level was significantly higher in JFH1-transfected cells than JEC3F-transfected cells and naïve Huh7.5.1 cell (Fig. 6A). Moreover IL-6 gene expression level was significantly higher in JFH1-transfected cells than JEC3F-transfected cells (Fig. 6B). Consistent with previous reports, treatment of the Huh7.5.1 cells with IL-6 induced expression of SOCS3 and SOCS1 mRNAs with SOCS3 being much stronger than SOCS1 (Fig. 6C).

Anti-IL-6 antibody restored IFN-resistance to HCV-infected cells

To investigate whether IL-6 is responsible for HCV infection-induced upregulation of SOCS and for resistance to interferon, JFH1 and JEC3F-infected Huh7.5.1 cells were pretreated with antibodies directed against IL-6 and subsequently treated with interferon. Interestingly, anti-IL-6-treated HCV-infected cells became significantly more susceptible to IFN treatment (Fig. 6D) without affecting viral expression levels in the absence of interferon (Fig. 6E). Cellular levels of SOCS3 mRNA were significantly lower in anti-IL-6-treated cells than untreated cells (Fig. 6F). These results strongly suggested that the interferon resistance of HCV-infected cells and the difference between the two viral strains are partly mediated by internal overproduction of IL-6 and subsequent upregulation of SOCS3.

Determination of the HCV structural region that induced SOCS3 and IL6

We studied further which part of HCV structural polyprotein is responsible for the difference in interferon-sensitivity. We constructed two additional chimeric clones between HCV-2b and JFH1. The 2bCoreJFH1 had the 2b-core region followed by the JFH1-structural and nonstructural regions. JCoreC3F was derived from JEC3F by exchanging the 2b-core with the JFH1-core (Fig. 7A). As



shown in Fig. 7B JFH1 and JCoreC3F, which had a JFH1-derived core region, were significantly more resistant to IFN than JEC3F and 2bCoreJFH1, with a 2b-derived core (Fig. 7B). Consistent with the interferon sensitivity results, JFH1 and JcoreC3F-infected cells expressed SOCS3 and IL6 mRNAs at significantly higher levels than JEC3F and 2bCoreJFH1-infected cells (Figs. 7C and D). These differences in gene expression were inversely associated with the cellular expression levels of each HCV chimeric clone (Fig. 7E). These results indicate that the amino acid sequence of the core protein is responsible for IL-6 and SOCS3-mediated interferon resistance.

Discussion

In this study, we succeeded in establishing a new genotype 2b infectious HCV clone and genotype 2b/JFH1 cell culture-competent intragenotypic chimeric viruses (Fig. 1). Relative interferon sensitivities of 2b/JFH1 chimeras, compared with HCV-JFH1 virus (Fig. 2), led us to conduct a series of assays to investigate the molecular mechanisms of IFN-related response pathways. We found that IFN-alpha receptor-mediated cellular responses were more attenuated in HCV-JFH1- and 2b/JFH1 chimera-infected than in uninfected Huh7.5.1 cells, but more potently for HCV-JFH1. Precise intragenotypic recombination analyses showed that the amino acid sequence of the HCV core protein is responsible for the differences in interferon sensitivity (Figs. 2, 7). The differences in the interferon-mediated antiviral effects were demonstrated further by the different rates of induction of interferon-inducible MxA and 25-OAS mRNAs (Figs. 3A and B) and IFN induced phosphorylation of STAT1 and STAT2 (Figs. 3D and E). We have demonstrated further that the expression of an interferon signal attenuator, SOCS3, was significantly higher in JFH1 than in 2b/JFH1-infected cells (Song and Shuai, 1998; Vlotides et al., 2004). Indeed, the siRNA-knock down of SOCS3 in JFH1 and 2b/JFH1-infected cells resulted in responsiveness to IFN (Fig. 5). Moreover, cellular expression of IL-6, which increases cytoplasmic phospho-STAT3 (Fig. 6A) and induces SOCS3 expression (Ramadori and Christ, 1999) was significantly higher in JFH1 transfected cells (Fig. 6B). Furthermore, by pre-treatment with anti-IL-6 antibody, JFH1- and 2b/JFH1-infected cells partially recovered elevation of SOCS3 expression and unresponsiveness to IFN (Fig. 6D). Taking all these things together, it is strongly suggested that the differences in IFN sensitivity between genotypes or isolates could be explained by SOCS3-mediated attenuation of interferon responses and, more importantly, IL-6 may constitute a molecular target to reverse such cellular interferon resistance.

Vast numbers of studies have failed to construct infectious HCV clones, other than HCV-JFH1. Murayama, et al. have conducted intragenotypic homologous recombination analyses between HCV-J6 and -JFH1 and have reported that that the NS3 protease and NS5B polymerase are essential for replication of the recombinant virus (Murayama et al., 2007). Up to now, several JFH1-based chimeric viruses have been reported, which include genotypes 4a (Scheel et al.,

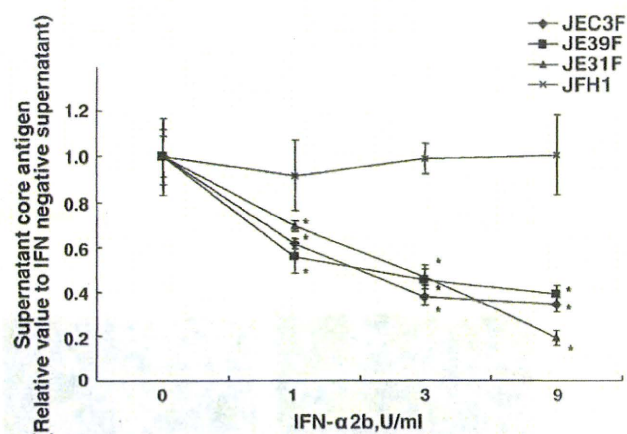


Fig. 2. Comparison IFN-alpha sensitivity among 2b/JFH1 chimeric viruses and JFH1. Ten μ g of JE31F, JE39F, JEC3F, JFH1 RNA were transfected into 5×10^6 Huh7.5.1 cells. The transfected cells were divided into 12 wells. Forty eight hours after transfection, cells were washed twice with PBS and treated with 0, 1, 3 and, 9 U/ml IFN-alpha-2b. Seventy-two hours after IFN-alpha 2b addition, quantification of HCV core antigen in culture fluids was conducted. The experiments were conducted twice by using Huh 751 cells of different passage, and a representative data was shown. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05.

2008), genotype 1a, 1b, 2a (Pietschmann et al., 2006), genotype 3a (Gottwein et al., 2007), genotype 5 a (Jensen et al., 2008) and, genotype 2b, 6a, 7a (Gottwein et al., 2009). Gottwein, et al. constructed intergenotypic chimeric HCV from JFH1 and genotypes 1 through 7 and analyzed differences in sensitivity to antiviral drugs (Gottwein et al., 2009). However, intergenotypic differences in sensitivity to IFN-alpha and the molecular mechanisms involved have not been well characterized. In this study, we constructed several chimeric virus clones between HCV-2b and HCV-JFH1 (2a), which showed variable sensitivity to IFN and confirmed that the core region is responsible for such IFN sensitivity. This study may support the feasibility of such inter and intragenotypic homologous recombination approaches to characterize differences in viral kinetics and drug responses.

Type I IFNs and their responsive ISGs are the principal mediators of host defense against virus infections, including HCV (Chang et al., 1991; Kalvakolanu, 2003; Ronni et al., 1998). On binding of IFNs to their receptors, IFNAR1 and IFNAR2, Janus kinases-1 and -2 phosphorylate STAT1 and STAT2 to form ISGF-3, which translocates to the nucleus and activates transcription of ISGs (Samuel, 2001; Taniguchi et al., 2001; Taniguchi and Takaoka, 2002). Members of the SOCS family are potent inhibitors of type I and type III IFN-induced activation of the Jak-STAT pathway and subsequent expression of ISGs (Vlotides et al., 2004). In HCV subgenomic replicon-expressing cells, expression levels of SOCS3 were inversely correlated with sensitivity to IFN to suppress viral RNA replication (Zhu et al., 2005).

Fig. 1. Replication and infection competency of HCV-2b/JFH1 chimeric viruses A. Genomic structures of HCV-JFH1, HCV-2b and 2b/JFH1 chimeric viruses. Intergenotypic homologous recombination was conducted between the HCV-2b and JFH1 (2a) clones and three chimeric clones were constructed that were joined between NS2-NS3 (JE39F), and within E2 at nt2541 (JE31F) and NS2 at nt. 2867 (JEC3F). B. Immunocytochemistry of HCV core. HCV RNA-transfected Huh7.5.1 cells were plated onto 22 mm-round micro cover glasses. Immunocytochemistry was performed 4 days after transfection using mouse-anti-core antibody (green) and DAPI (blue). C. Time courses of 2b/JFH1- and JFH1-transfected cells. *In vitro* transcribed HCV RNAs were transfected into Huh7.5.1 cells by electroporation and HCV core levels of culture fluids were sampled at the time points indicated and core antigen levels were measured. The experiment was done three times with similar results independently. Panel C shows representative date. D. Immunocytochemistry of HCV core. HCV RNA-infected Huh7.5.1 cells using Panel B supernatant that have same amount of HCV core antigen were plated onto 22 mm-round micro cover glasses. Immunocytochemistry was performed 4 days after infection using mouse-anti-core antibody (green) and DAPI (blue). Numbers at the bottom denote percentages of HCV core-positive cells. E. Time courses of 2b/JFH1 infected cells. JE31F, JE39F, JEC3F RNA-transfected cell culture fluids were used to infect naïve Huh7.5.1 cells in 60 mm-diameter plates at density of 3×10^5 cells per plate. Quantification of HCV core antigen in culture supernatants was carried out at 24 hours, 48 hours, 72 hours and 144 hours after inoculation. The experiment was done three times with similar results independently. Panel E shows representative date. F. Comparison between JFH1 and JEC3F supernatant HCV-RNA titer and core antigen. Four days after JFH1 and JEC3F RNA transfection, culture supernatant was harvested and subjected to both HCV core antigen assay and realtime RT-PCR of HCV-RNA. Assays were done in triplicate and the data are shown as mean \pm sd.

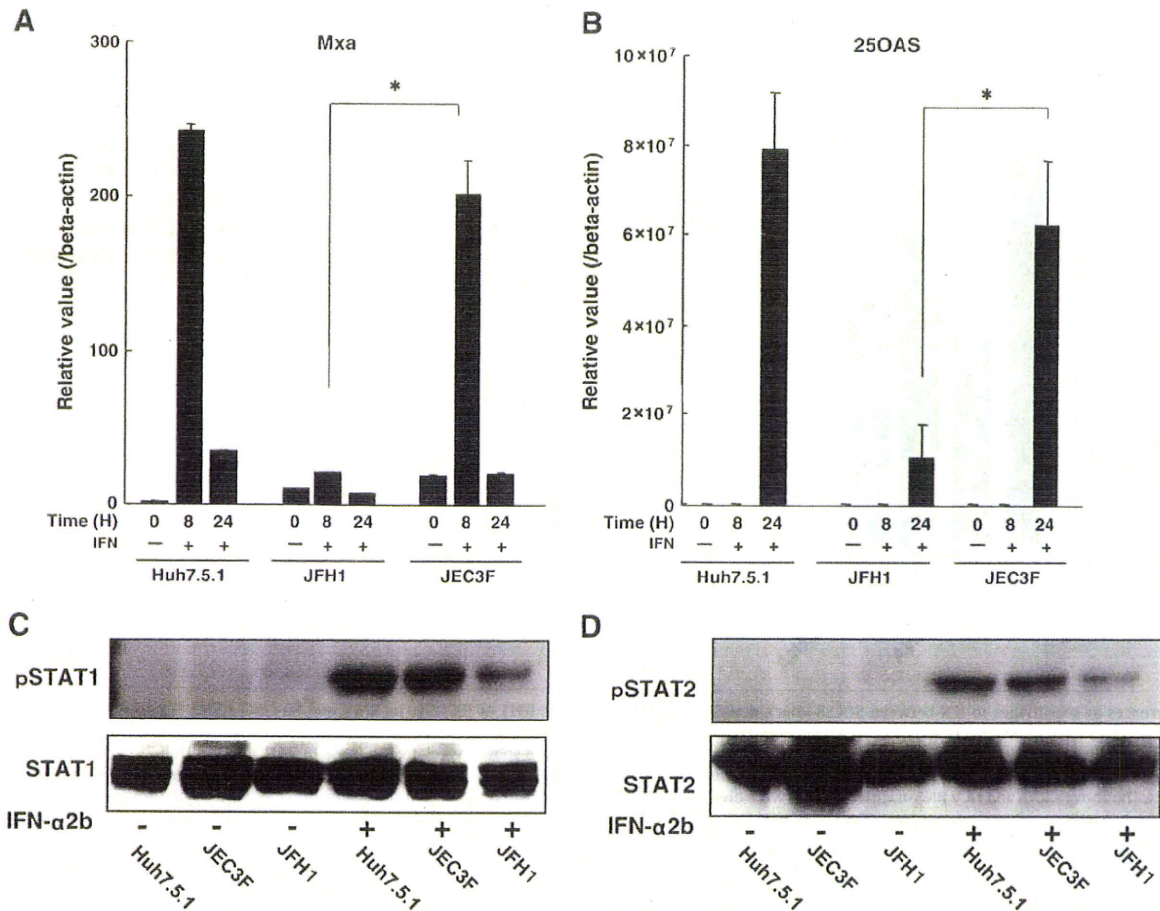


Fig. 3. Induction by interferon of the interferon-inducible genes, MxA (panel A), 25-OAS (panel B) and phosphorylated STAT1 (panel C) and STAT2 (panel D). JEC3F and JFH1 10 μ g RNA was transfected into Huh7.5.1 cells. Forty-eight hours after transfection, the cells were treated with 25 U/ml IFN- α 2b. Total cellular RNA was isolated before and 8 and 24 hours after IFN treatment. Relative gene expression levels of MxA (panel A) and 25-OAS (panel B) were determined by real-time PCR at the time points indicated. JEC3F and JFH1 RNA and MOCK was transfected into Huh7.5.1 cells. Forty eight hours after transfection, the cells were treated with 25 U/ml IFN- α 2b. Total cellular protein was isolated before and 15 minutes after IFN treatment. Ten μ g of extracted protein were used for analysis of phosphorylated STAT1, STAT2 protein and STAT1, STAT2 protein as controls. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05.

HCV, on the other hand, counteracts such IFN-mediated antiviral pathways. The NS5A and E2 proteins interfere with the action of IFN by inhibiting the activity of PKR (He and Katze, 2002; Taylor et al., 1999). NS5A also induced expression of IL-8 and attenuated expression of ISGs (Polyak et al., 2001). HCV core protein has been reported to bind the STAT1-SH domain (Lin et al., 2006) or destabilize STAT1 (Lin et al., 2005) to block IFN signaling. It also has been reported that overexpression of core protein upregulated SOCS3 expression (Bode et al., 2003). In this study, we used full-length HCV cell culture and found, for the first time, that SOCS3 expression is upregulated differently depending on the genetic sequences of HCV strains and that these differences in SOCS3 expression are associated with sensitivity to IFN. Moreover, overexpression and knock down of SOCS3 expression were closely associated with the IFN sensitivity of the HCV-infected cells. These results indicate that interferon-resistance of HCV-infected cells is directed by overexpression of SOCS3, which may be upregulated by HCV proteins as reported (Bode et al., 2003) (Kawaguchi et al., 2004). A sequence comparison of our HCV2b and JFH1 clones has found 16 amino acid differences. These structural differences of the core protein might affect cellular responses to interferon (see the Supplementary Fig. 4).

It has been reported that IL-6 is the principal activator of STAT3 in hepatocytes through binding its receptor (Hanada et al., 2003; Ramadori and Christ, 1999). Furthermore, plasma IL-6 levels are elevated in

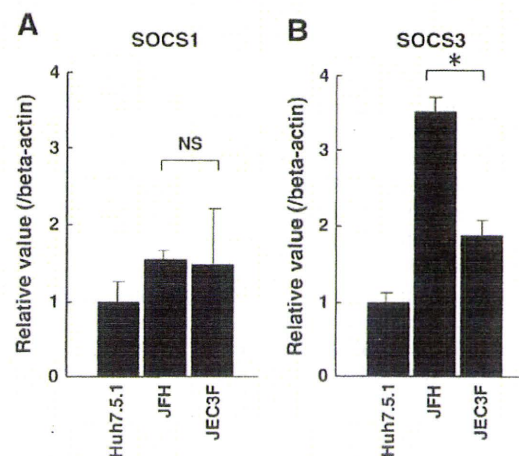


Fig. 4. Expression of SOCS1 mRNA (panel A), SOCS3 mRNA (panel B). Forty-eight hours after transfection of JEC3F, JFH1 10 μ g RNA or mock transfection into Huh7.5.1 cells, total RNA and total protein were isolated. Relative gene expression levels of SOCS1 (panel A) and SOCS3 (panel B) and were determined by real time PCR. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05.

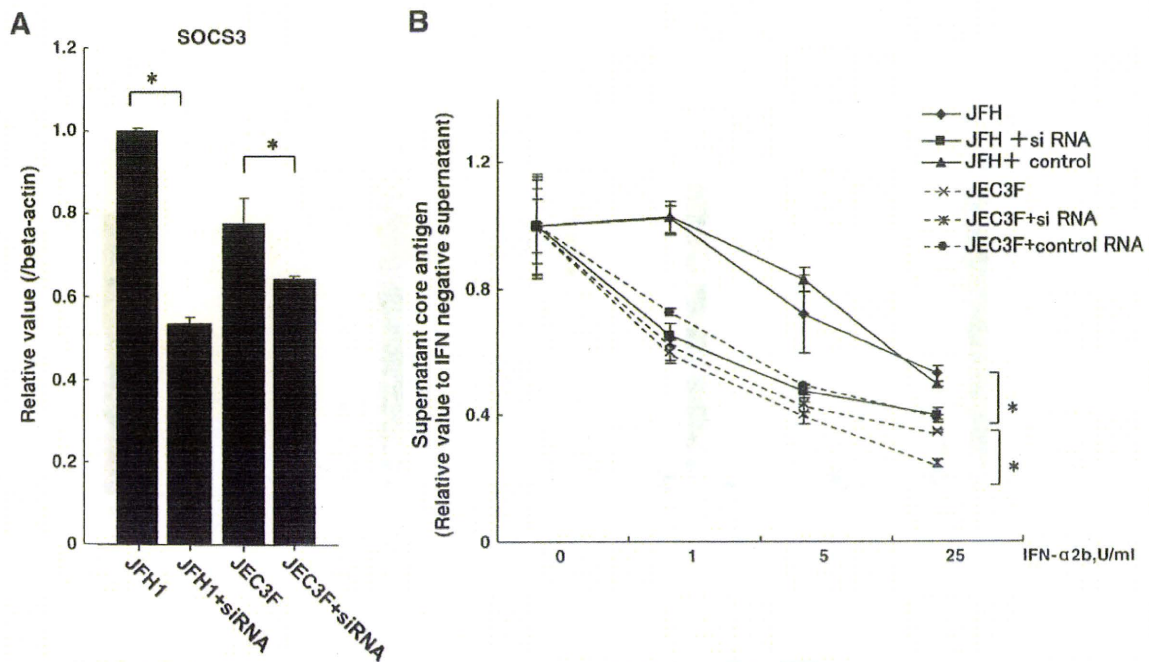


Fig. 5. Differences in sensitivities to IFN between SOCS3-knock down, HCV transfected cells. JFH1 or JEC3F 10 μ g RNA, and 80 pmol siRNA SOCS3-HSS113312 or MOCK were electroporated into 5×10^6 uninfected Huh7.5.1 cells. **A.** Expression of SOCS3 mRNA in uninfected and HCV-infected Huh7 cells. Forty-eight hours after transfection, total RNA was isolated. Relative gene expression level of SOCS3 were determined by real time PCR. Values are shown as relative to those of JFH1 infected Huh 751 cells. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05. **B.** Dose-dependent suppression of HCV replication by IFN in SOCS3-knock-down, HCV-infected cells. The above siRNA and HCV RNA-transfected cells were divided into 12 wells. Forty eight hours after transfection, the cells were treated with 0, 1, 5 and 25 U/ml of IFN- α 2b. Seventy two hours after treatment, quantification of HCV core antigen in culture fluids was carried out. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05.

chronic hepatitis C patients (Malaguarrera et al., 1997). Consistent with those reports, we found that IL-6 strongly induced SOCS3 expression in Huh7.5.1 cells (Fig. 6C). More importantly, cellular IL6 expression levels were in the order of uninfected < JEC3F < JFH1-infected cells, which correlated well with SOCS3 expression (Fig. 4) and with cellular responses to IFN (Fig. 2). In addition, the IFN-resistant JcoreC3F, in which the core region of JEC3F had been re-substituted by the JFH1-core, induced comparatively higher levels of IL-6 and SOCS3 mRNA to JFH1 (Fig. 7). Taken together, our results indicate that the amino acid sequence of the core protein determines IL-6 and SOCS3 expression levels and, as a consequence, resistance to IFNs.

It remains to be clarified what are the inducers of IL-6. There are reports that HCV core protein activates toll-like receptor (TLR)-2 in Huh7 cells and in adult human hepatocytes (Hoffmann et al., 2009; Mozer-Lisewska et al., 2005). TLRs are known to activate downward NF- κ B signaling that upregulates IL-6 expression. Alternatively, IL-6 may be secreted in response to cellular steatosis and insulin resistance. HCV patients with obesity or insulin resistance are refractory to IFN treatments. Such patients have higher levels of hepatic SOCS3 expression than those without obesity or insulin resistance (Miyasaki et al., 2009; Walsh et al., 2006). More recently, Sabio, et al have reported that fatty acid-induced secretion of IL-6 from adipocytes upregulates hepatic SOCS3, leading to insulin-resistance (Sabio et al., 2008).

In conclusion, our study demonstrates that HCV intragenotypic and inter-strain differences in IFN sensitivity can be, in most part, attributable to the amino acid sequence of the HCV core protein and that such IFN sensitivities are determined by cellular expression levels of SOCS3 and IL-6. Therapeutic targeting of IL-6 potentially may be a key to targeting IFN-resistance and improving antiviral chemotherapeutics against HCV.

Materials and Methods

Reagents and antibodies

Recombinant human interferon alpha-2b was from Schering-Plough (Kenilworth, NJ). Anti-CD 81 antibody (JS-81) was from BD Biosciences (Franklin Lakes, NJ) (Morikawa et al., 2007), anti-IL6 receptor antibody was from Chugai pharmaceutical Co (Tokyo, Japan), anti-SOCS3 was from Cell Signaling (Beverly, MA), and anti-IL6 antibody was from R&D Systems (Minneapolis, MN).

Cloning of HCV cDNA from patient serum

A serum sample was obtained from a 32-year-old male who developed acute hepatitis after intravenous drug injection. Serum was obtained one week after the onset of symptoms. Total RNA was extracted from 150 μ l of serum using ISOGEN (Nippon Gene, Osaka, Japan). cDNA was synthesized using SuperScript II (Invitrogen, Carlsbad, CA) reverse transcriptase. PCR primers, based on a genotype 2b prototype sequence, HC-J8 (accession number: D10988), were used to amplify 14 fragments of HCV cDNA covering nt. 13–9478 (nucleotide numbers corresponded to HC-J8) by PCR. All amplicons were purified and cloned into the pGEM-T EASY vector (Promega, Madison, WI) and nucleotide sequences were determined using Big Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems, Foster City, CA) and an automated DNA sequencer (ABI PRISM $\text{\textcircled{R}}$ 310 Genetic Analyzer; Applied Biosystems). The consensus sequence of five clones was adopted for each region. Each consensus sequence segment of HCV was assembled into pJFH1-full (Wakita et al., 2005) by substituting the insert sequence of pJFH1-full.

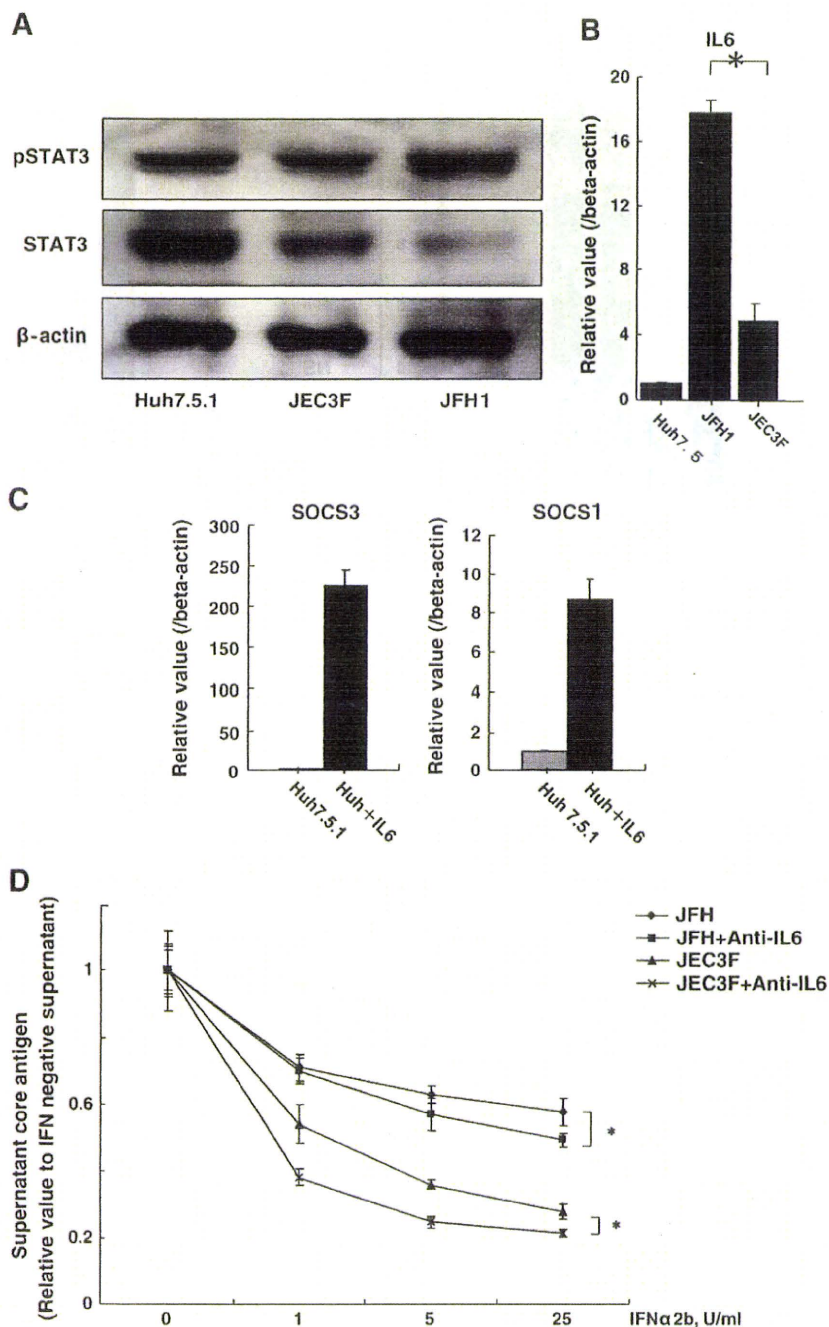


Fig. 6. IL-6 expression in HCV infected cells and change in IFN sensitivity by treatment with anti-IL6 antibody. **A.** Expression of cytoplasmic phospho-STAT3 in uninfected and HCV-infected Huh7 cells. JEC3F, JFH1 10 μ g RNA and MOCK was transfected into Huh7.5.1 cells. Forty eight hours total cellular protein was isolated. Ten μ g of extracted protein were used for analysis of phosphorylated STAT3, STAT protein and β -actin as controls. **B.** Expression of Interleukin-6 mRNA in uninfected and HCV-infected Huh7 cells. Forty-eight hours after transfection, total RNA was isolated. Relative gene expression level of IL6 were determined by real time PCR. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean \pm sd. **C.** IL-6 induces SOCS3 strongly in uninfected Huh7.5.1 cells. Uninfected Huh7.5.1 cells were treated with 10 ng/ml recombinant human IL6 (PEPRO TEC EC, London, England). Fifteen minutes after treatment, total RNA was isolated. Relative gene expression levels of SOCS1 and SOCS3 were determined by real time PCR. Uninfected Huh7.5.1 cells that were not treated with IL6 were used as a control. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean \pm sd. **D.** Dose-dependent suppression of HCV replication by IFN in HCV-infected cells pre-treated with anti-IL6 antibody. Immediately after electroporation, HCV RNA-transfected cells were divided into 12 wells and pretreated with 1 μ g/ml anti-IL6 antibody. Forty eight hours after transfection, the cells were washed with PBS and treated with 0, 1, 5 and 25 U/ml of IFN- α 2b. Seventy two hours after treatment, quantification of HCV core antigen was carried out in culture fluids. Assays were done in triplicate and the data are shown as mean \pm sd. **E.** Core protein secretion levels following treatment of HCV-transfected cells with anti-IL6 antibody. After treatment with anti-IL-6 antibody, HCV RNA-transfected cells were divided into 12 wells. Five days after transfection, quantification of HCV core antigen was carried out in culture fluids. Assays were done in triplicate and the data are shown as mean \pm sd. **F.** Expression of SOCS3 mRNA in uninfected and HCV-infected Huh7 cells. Forty-eight hours after transfection, total RNA was isolated. Relative SOCS3 gene to beta-actin gene expression were determined by real time PCR. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05.

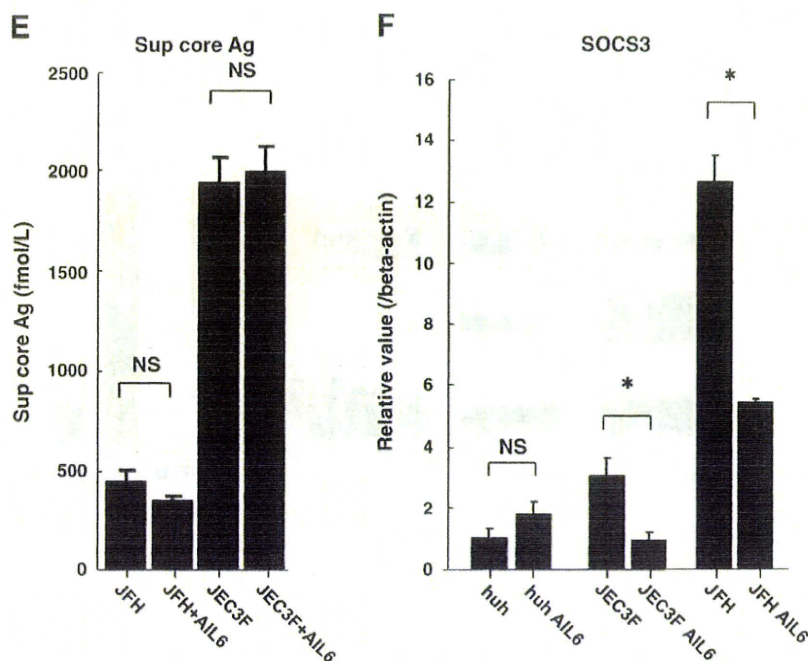


Fig. 6 (continued).

Construction of 2b/JFH-1 based intragenotypic chimeras and transfection

Chimeric HCV constructs of HCV-2b and JFH1 were shown in Figs. 1A and 7A. To construct 2b/JFH1-based intragenotypic chimera, JE31F, the 2b sequence of core through E2 (nt. 342–2541) was fused to the EcoRI-JFH1–5′-untranslated region (UTR) DNA by fusion PCR. The fused 5′UTR-E2 fragment and JFH1-E2-NS3 (nt2541 through 5324) were assembled by fusion PCR and cloned into pGEM-T EASY. The product was digested by EcoRI and AfeI and insert into pJFH1. Plasmids pJE39F, pJEC3F, pJcoreC3F and p2bcore JFH1 were constructed using a similar procedure. Plasmids pJEC3F and pJE39F were joined between NS2 and NS3, and within NS2 at nt. 2867, respectively. Plasmid pJcoreC3F was made by substitution of the core region of 2b/JFH1 with that of JFH1. The plasmid p2bcoreJFH1 was made by substitution of the core region of JFH1 with that of 2b/JFH1.

Cells and cell culture

Huh7.5.1 cells were maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum at 37 °C under 5% CO₂.

HCV cell culture system

Full-length HCV expression plasmids were as follows: pJFH1-full (Wakita et al., 2005), pJE31F, pJE39F, pJEC3F, pJcoreC3F, p2bcoreJFH1, and pFL-H77/JFH1, pFL-J6/JFH1 (Lindenbach et al., 2005). These plasmids were linearized at their 3′ ends and used as templates for HCV RNA synthesis using the RiboMax Large Scale RNA Production System (Promega, Madison, WI). After DNase I (RQ-1, RNase-free DNase, Promega) treatment, the HCV RNA was purified using ISOGEN (Nippon Gene, Tokyo, Japan). For the RNA transfection, Huh7.5.1 cells were washed twice with PBS, and 5 × 10⁶ cells were suspended in Opti-MEM I (Invitrogen Carlsbad, CA) containing 10 μg of HCV RNA, transferred into a 4 mm electroporation cuvette and finally subjected to an electric pulse (1,050 μF and 270 V) using the Easy Jet system (EquiBio, Middlesex, UK). After electroporation, the cell suspension

was left for 5 min at room temperature and then incubated under normal culture conditions in a cell culture dish.

Quantification of HCV core antigen in culture supernatants

Culture supernatants of HCV RNA transfected Huh7.5.1 cells were collected on the days indicated, passed through a 0.45 μm filter (MILLEX-HA, Millipore, Bedford, MA) and stored at –80 °C. The concentrations of core antigen in the culture supernatants were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan).

Re-infection analyses

Titer-adjusted supernatants (including 0.03 fmol HCV core antigen) from HCV RNA-transfected cells were inoculated onto naïve Huh7.5.1 cells plated on a 6 cm plate at a density of 3 × 10⁵ cells per plate. Forty-eight hours after inoculation, anti-core immunostaining was carried out with mouse anti-HCV core protein monoclonal antibody and the numbers of infected cells were counted. HCV core antigen in culture supernatants was measured at 24 hours, 48 hours, 72 hours and 144 hours after inoculation.

Real-time RT-PCR analysis

For the detection of HCV RNA in culture supernatant, supernatant was passed through a 0.45 μm filter (MILLEX-HA, Millipore, Bedford, MA) and stored at –80 °C until use. Protocol and primers for the realtime RT-PCR analysis of HCV-RNA has been described previously (Sekine-Osajima et al., 2008). For the detection of endogenous mRNAs, total cellular RNA was isolated using ISOGEN (Nippon Gene). Two micrograms of total cellular RNA were used to generate cDNA from each sample using SuperScript II. Expression of mRNA was quantified using the TaqMan Universal PCR Master Mix and the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City CA). Some primers have been described (Sekine-Osajima et al., 2008). SOCS3; forward, 5′-CAC ATG GCA CAA GCA CAA GAA G-3′ and reverse,

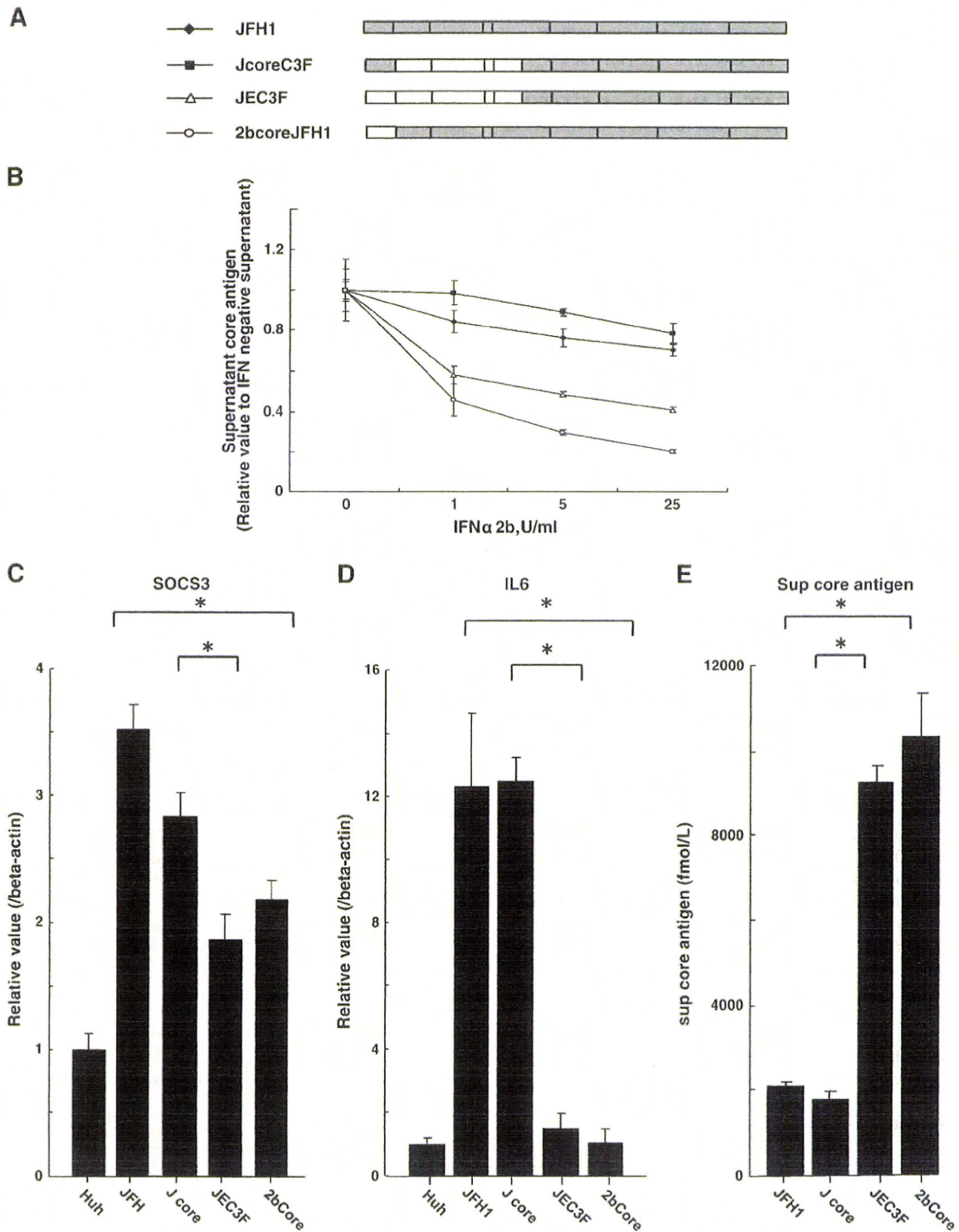


Fig. 7. Replacement of the HCV-2b-core region with JFH1-core causes upregulation of SOCS3 and IL-6 and restores resistance to IFN. A. Genome maps of JFH-1, JEC3F, J core C3F, 2b core JFH1 recombinant cDNA. J core C3 F was made by substitution of the core region of 2b/JFH1 with that of JFH1. The 2b core JFH1 was made by substitution of the core region of JFH1 with that of 2b/JFH1. B. Comparison of IFN-alpha sensitivity among JFH1 and JEC3F and core region substitution chimeric viruses. Ten μ g of J core C3F, 2b core JFH1, JEC3F, JFH1 RNA were transfected into 5×10^6 Huh7.5.1 cells and were divided into 12 wells. Forty eight hours after transfection, the cells were treated with 0, 1, 5 and 25 U/ml of IFN-alpha 2b. Seventy two hours after treatment, quantification of HCV core antigen was carried out in culture fluids. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05. C, D. Core substitution leads to SOCS3 and IL-6 mRNA over-expression. Forty eight hours after transfection into cells, total RNA was isolated. Relative gene expression level SOCS3 (panel C) and IL6 (panel D) were determined by real time PCR. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05. E. Change of secretion of core protein following core protein substitution. HCV RNA-transfected cells were divided into 12 wells. Five days after transfection, quantification of HCV core antigen was carried out in culture fluids. Assays were done in triplicate and the data are shown as mean \pm sd. Asterisks indicate p-values of less than 0.05.

5'-GGA GAA GCT GGA GAC TCA GGT G-3', SOCS1; forward, 5'-CAC TTC CGC ACA TTC CGT TCG-3' and reverse, 5'-GAG GCC ATC TTC ACG CTA AGG-3', IL6; forward, 5'-GGT ACA TCC TCG ACG GCA TCT-3' and reverse, 5'-GTG CCT CTT TGC TGC TTT CAC-3', 25OAS; forward, 5'-CCA CCT TGG AAA GTG CCG ACA ATG CAG ACA-3' and reverse, 5'-CGA GTC TTT AAA AGC GAT TGC CAG ATG ATC -3', MxA; forward, 5'-GCC AGC AGC TTC AGA AGG CCA TGC TGC AGC -3' and reverse, 5'-GGG CAA GCC GGC GCC GAG CCT GCG TCA GCC -3'.

The siRNAs

The siRNAs directed against SOCS3 were designed as follows: SOCS3-HSS113312 stealth (sequence 5'-CCC AGA AGA GCC UAU UAC AUC UAC U-3' and 5'-AGU AGA UGU AAU AGG CUC UUC UGG G-3', Invitrogen) was used. 10 µg in vitro-synthesized HCV-RNA and 80 pmol siRNA SOCS3-HSS113312 or MOCK or control siRNA (negative universal control Med #2, Invitrogen) were electroporated into 5 × 10 naïve Huh7.5.1 cells using the protocol described in *HCV cell culture system*. Forty-eight hours after transfection, expression levels of SOCS3 mRNA were measured by real-time PCR. The difference in IFN sensitivity between SOCS3 knock down HCV infected cells and control HCV infected cells was determined by measuring supernatants HCV core antigen 72 hours after addition of IFN.

Immunohistochemistry for HCV core

HCV-JFH1 transfected or infected Huh7.5.1 cells were cultured on 22 mm-round micro cover glasses (Matsunami, Tokyo, Japan). For detection of HCV core, cells were fixed with cold acetone for 15 min. The cells were incubated with the primary antibodies for 1 hour at 37 °C, and with Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Cells were mounted with VECTA SHIELD Mounting Medium and DAPI (Vector Laboratories, Burlingame, CA) and visualized by fluorescence microscopy (BZ-8000, KEYENCE, Osaka, Japan).

Western blot analysis

Western blotting was performed as described (Tanabe et al., 2004). Briefly, 10 µg of total cell lysate was separated by SDS-PAGE, and blotted onto a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and was visualized by chemiluminescence using the ECL Western Blotting Analysis System (Amersham Biosciences, Buckinghamshire, UK).

Statistical analyses

Statistical analyses were performed using Student's *t*-test; *p*-values of less than 0.05 were considered statistically significant.

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Appendix A. Supplementary data

Supplementary Fig. 1. Infectivity of the full-length 2b HCV RNA and 2b/JFH1 chimeric virus, JEC3F. A. Challenge of human liver-engrafted albumin-uPA/SCID mice with culture fluid from JFH1 and JEC3F cells. Cell culture fluids from the JFH1 clone and JEC3F were injected

intravenously into human liver engrafted albumin-uPA/SCID mice. Serum samples were obtained from the mice every 2 weeks after injection and the HCV RNA titer was determined. B. Fig. 1B Challenge of human liver-engrafted albumin-uPA/SCID mice by intrahepatic injection of in vitro synthesized, full-length 2b HCV RNA. Five hundred µl of RNA solution containing 30 µg of in vitro synthesized full-length 2b HCV RNA was injected into the livers of anesthetized chimeric mice through a small abdominal incision. Serum samples were obtained from the mice every 2 weeks after injection and the HCV RNA titer was determined.

Supplementary Fig. 2. Comparisons of replication efficiency of JFH1 and J6/JFH1, 2b/JFH1 chimeras after transfection into Huh7.5.1-cells. A. Structures of the J6/JFH1 and 2b/JFH1 genomes. J6 is joined between NS2 and NS3 with JFH1. 2b-HCV is joined with JFH1 within NS2 at nt. 2867. B. Measurements of core protein in cell culture fluids. Ten µg of JFH1, J6/JFH1, 2b/JFH1 RNA were transfected into 5 × 10⁶ Huh7.5.1 cells and the cells were cultured in 100 mm-diameter plates. The culture fluids from JFH1, J6/JFH1, H77/JFH1 or 2b/JFH1-transfected Huh7.5.1 cells were collected separately on the days indicated and the levels of core antigen were measured. These experiments were done three times with similar results independently. Panel B shows representative data.

Supplementary Fig. 3. Inhibition of infection by blocking CD81. Huh 7.5.1 cells were plated into a 6 well plate at 1.4 × 10⁵ cells per well. After 48 hours, the cells were incubated with anti-CD81 or isotypematched control antibody at the concentration indicated for 1 hour. Subsequently, cells were infected with 1 ml of JEC3F stock cell culture fluids at day 2 for 4 hours and washed with PBS. 48 hours after inoculation, anti-core immunostaining was performed with mouse anti-HCV core protein monoclonal antibody (Panels B and C). Quantification of HCV core antigen was carried out in culture fluids at 48 hours after infection (Panel A).

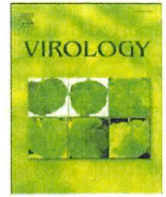
Supplementary Fig. 4. Comparison between 2b and JFH-1 core amino acid sequence.

Note: Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2010.07.041.

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Cell culture and in vivo analyses of cytopathic hepatitis C virus mutants ^{☆,☆☆}

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ABSTRACT

HCV-JFH1 yields subclones that develop cytopathic plaques (Sekine-Osajima Y, et al., Virology 2008; 371:71). Here, we investigated viral amino acid substitutions in cytopathic mutant HCV-JFH1 clones and their characteristics in vitro and in vivo. The mutant viruses with individual C2441S, P2938S or R2985P signature substitutions, and with all three substitutions, showed significantly higher intracellular replication efficiencies and greater cytopathic effects than the parental JFH1 in vitro. The mutant HCV-inoculated mice showed significantly higher serum HCV RNA and higher level of expression of ER stress-related proteins in early period of infection. At 8 weeks post inoculation, these signature mutations had reverted to the wild type sequences. HCV-induced cytopathogenicity is associated with the level of intracellular viral replication and is determined by certain amino acid substitutions in HCV-NS5A and NS5B regions. The cytopathic HCV clones exhibit high replication competence in vivo but may be eliminated during the early stages of infection.

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Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality (Alter, 1997). Antiviral therapeutic options against HCV have been limited to type I interferons and ribavirin and have yielded unsatisfactory responses (Fried et al., 2002). Given this situation, a precise understanding of the molecular mechanisms of interferon resistance has been a high priority of research in academia and industry.

Molecular analyses of the HCV life cycle, virus–host interactions, and mechanisms of liver cell damage by the virus are not understood

completely, mainly because of the lack of cell culture systems. These problems have been overcome to some extent by the development of the HCV subgenomic replicon (Lohmann et al., 1999) and HCV cell culture systems (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The HCV-JFH1 strain, which is a genotype 2a clone derived from a Japanese fulminant hepatitis patient and can replicate efficiently in Huh7 cells (Kato, 2001; Kato et al., 2003), has contributed to the establishment of the HCV cell culture system. Furthermore, the Huh7-derived cell lines, Huh-7.5 and Huh-7.5.1 cells, allow production of higher viral titers and have a greater permissivity for HCV (Koutsoudakis et al., 2007; Lindenbach et al., 2005; Zhong et al., 2005). The HCV-JFH1 cell culture system now allows us to study the complete HCV life cycle: virus–cell entry, translation, protein processing, RNA replication, virion assembly and virus release.

HCV belongs to the family *Flaviviridae*. One of the characteristics of the *Flaviviridae* is that they cause cytopathic effects (CPE). The viruses have positive strand RNA genomes of ~10 kilo-bases that encode polyproteins of ~3000 amino acids. These proteins are processed post-translationally by cellular and viral proteases into at least 10 mature proteins (Sakamoto and Watanabe, 2009). The viral non-structural proteins accumulate in the ER and direct genomic replication and viral protein synthesis (Bartenschlager and Lohmann, 2000; Jordan et al., 2002; Mottola et al., 2002). It has been recently

Abbreviations: HCV, hepatitis C virus; CPE, cytopathic effect; ER, endoplasmic reticulum; RdRp, RNA dependent RNA polymerase.

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reported that HCV-JFH1 transfected Huh-7.5.1 cells die when all of the cells are infected and intracellular HCV RNA reaches maximum levels (Zhong et al., 2006). These findings suggest HCV-induced cytopathogenicity. However, the mechanisms have not been well documented.

In a previous study, we investigated the cellular effects of HCV infection and replication using the HCV-JFH1 cell culture system and we reported that HCV-JFH1 transfected and infected cells show substantial CPE that are characterized by massive apoptotic cell death with expression of several ER stress-induced proteins. Taking advantage of the CPE, we developed a plaque assay for HCV in cell culture and isolated subclones of HCV that showed enhanced replication and cytopathogenicity (Sekine-Osajima et al., 2008). We have demonstrated that these viral characters were determined by mutations at certain positions in the structural and nonstructural regions of the HCV genome, especially the NS5A and NS5B regions.

In this study, we investigated the mechanisms and viral nucleotide sequences involved in HCV-induced cytopathic effects using HCV-JFH1 cell culture and a newly developed cytopathic plaque-forming assay. We demonstrated that introduction of NS5A and NS5B mutations into the JFH1 clone resulted in a higher replication efficiency, although introduction of these mutations into the JFH1 subgenomic replicon has no effect on viral replication. These mutations do not affect virion entry or release of viral particles but regulate virus replication, and high levels of virus replication result in cytopathogenicity.

Results

Development of cytopathic plaques by HCV infection of Huh-7.5.1 cells

A plaque assay was performed to investigate the morphological CPE following HCV-JFH1 infection (see [Materials and methods](#)). Culture supernatants from JFH1-transfected cells were diluted serially and inoculated onto uninfected Huh-7.5.1 cells. The cells were subsequently cultured in medium containing agarose. On 9 days after the inoculation, viable cells were stained and plaques were visualized (Fig. 1A). HCV-inoculated cell cultures developed plaques as unstained areas, accompanied by rounded cells in the periphery (Fig. 1B). The formation of cytopathic plaques was not observed in a parental Huh7 cell line (data not shown). Those results were consistent with our previous study (Sekine-Osajima et al., 2008).

Introduction of mutations in the NS5A and NS5B regions of the JFH1 clone augmented its cytopathic effects

Among the amino acid substitutions that developed in the plaque-derived HCV-JFH1 strains, 6 of the 9 amino acid changes appeared redundantly among 5 independently isolated plaques, and clustered in the C terminal part of the NS5A and NS5B regions. To investigate the phenotype of each amino acid substitution, we constructed mutant JFH1

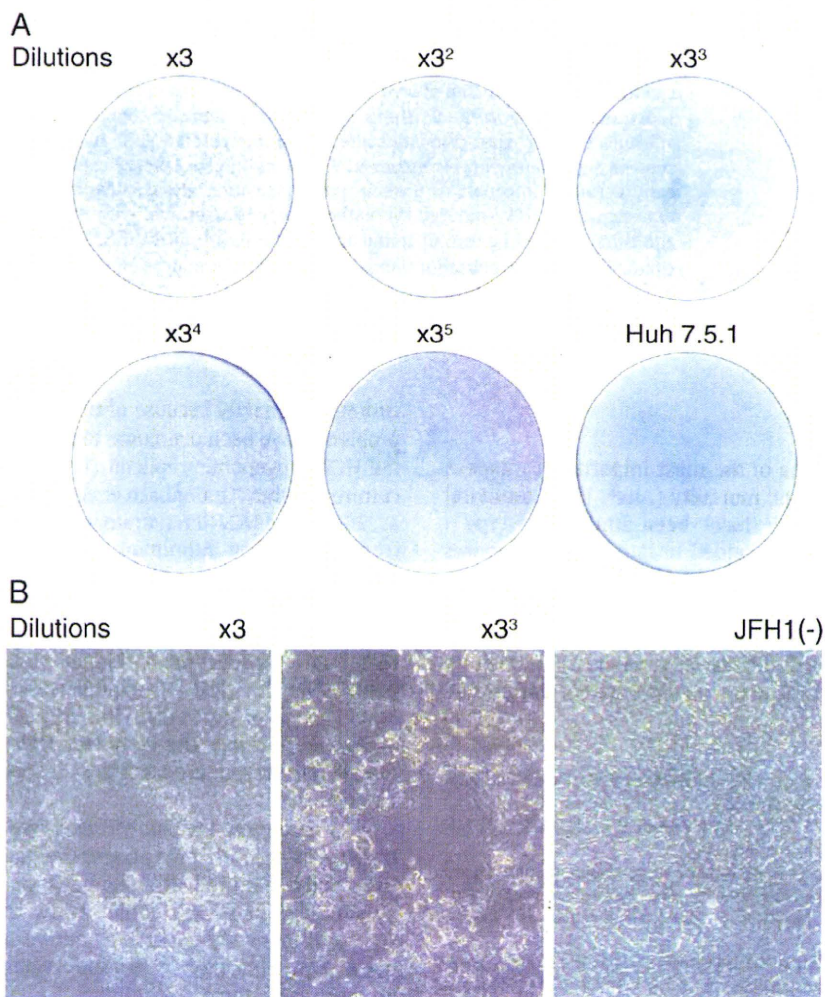


Fig. 1. The cytopathic effects of HCV-JFH1 *in vitro*. A. Plaque assay. Huh-7.5.1 cells were seeded in collagen-coated 60mm-diameter plates at density of 4×10^5 cells per plates and were incubated at 37 °C under 5.0% CO₂ (as described above). After overnight incubation, HCV-infected culture supernatants were serially diluted in a final volume of 2 ml per plates and transferred onto the cell monolayers. After ~5 h of incubation, the inocula were removed and the infected cells were overlaid with 8 ml of culture medium containing 0.8% methyl-cellulose and incubated under normal conditions. After 7 days culture, formation of cytopathic plaque was visualized by staining with 0.08% crystal violet solution. B. The cytopathic plaques were observed by phase-contrast microscopy at day 7 after HCV-JFH1 infection.

clones in which we introduced separately one amino acid substitution in NS5A and five substitutions in NS5B (Fig. 2A) and transfected the mutant HCV RNAs into Huh-7.5.1 cells. To compare the electroporation efficiencies of viral RNAs, Huh-7.5.1 cells were harvested 8 h after transfection and the levels of intracellular core antigen were measured. There was no difference in the efficiencies of electroporation (Fig. 2D). The substitutions G2964D, H3004Q, and S3005N did not lead to cytopathic effects but three mutant subclones (C2441S, P2938S and

R2985P) produced much more cell death compared to the wild type JFH1 (Fig. 2B). To assess the quantitative cytopathic effect seen in host cells for each of the mutants, we also performed MTS assay at 6 days post transfection. It showed that Huh-7.5.1 cells transfected with the triple mutants (C2441S, R2938S, or R2985P) induced apparently much more cytopathic effect compared to the parental JFH1 and other mutant clones, although the three mutant clones encoding the substitutions C2441S, P2938S, or R2985P did not show significant difference but

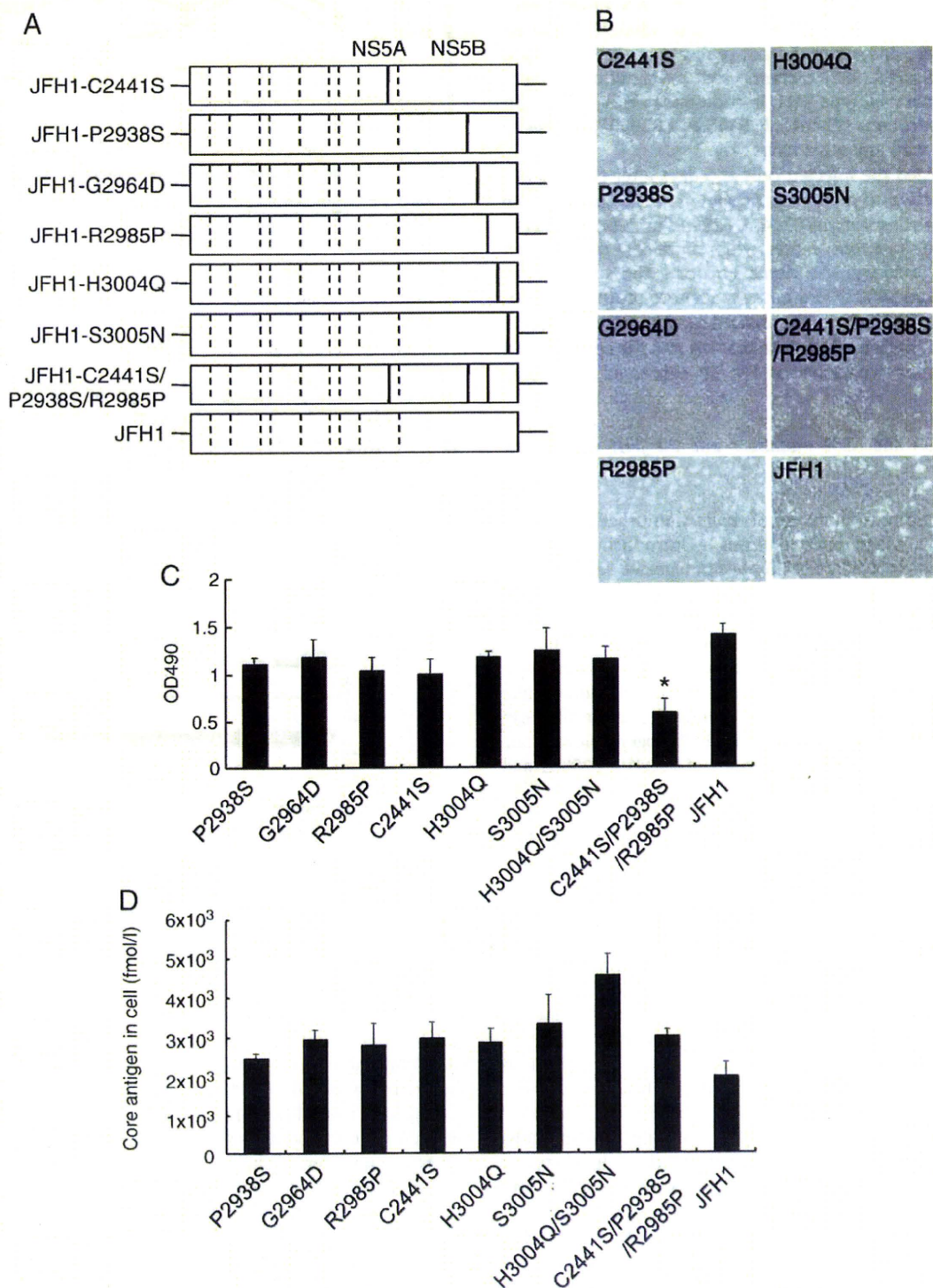


Fig. 2. Introduction of mutations into the NS5A and NS5B regions of JFH1. **A.** The mutations identified in the cytopathic plaque were introduced individually into the parental JFH1. Each JFH1 mutant was transfected into Huh-7.5.1 cells by electroporation. **B.** Huh-7.5.1 cells transfected with JFH1-mutants were observed by phase-contrast microscopy at day10 after transfection. **C.** MTS assay was performed to assess the quantitative cytopathic effect seen in Huh-7.5.1 cells for each of the mutants 6 days post transfection. Asterisks indicate p-values of less than 0.05 as compared with JFH1. **D.** Huh-7.5.1 cells were harvested at 8 h after transfection and the levels of intracellular core antigen were measured.

showed tendency to introduce more cytopathic effect than the parental JFH1 and the mutant clones encoding the substitutions G2964D, H3004Q, and S3005N (Fig. 2C).

Introduction of NS5A and NS5B mutations into the JFH1 clone led to a greater replication efficiency

To compare the expression levels of each mutant subclone, each HCV RNA was transfected and core antigen was detected subsequently in the culture medium. Similar to Fig. 2B, HCV clones with individual substitutions G2964D, H3004Q and S3005N produced significantly less core antigen or did not replicate at all. In contrast, the C2441S, P2938S and R2985P mutants produced significantly more core antigen than the wild type JFH1. In addition, an HCV clone with all 3 adaptive substitutions (C2441S, P2938S and R2985P) produced more core antigen than any other clone (Fig. 3A).

Next, we harvested the infected cells at 5 days after electroporation and performed western blotting. As shown in Fig. 3B, the three clones encoding the substitutions C2441S, P2938S, or R2985P, and the clone with all three mutations, expressed far more core protein than the parental JFH1, although the clones encoding the substitutions G2964D, H3004Q and S3005N did not express core protein. We also transferred culture media from the mutant clones onto uninfected Huh-7.5.1 cells and performed western blotting and the cells infected with the same mutant subclones as Fig. 3B expressed more core protein (Fig. 3C).

Introduction of NS5A and NS5B mutations into the JFH1 subgenomic replicon

To investigate the primary phase of replication of JFH1 mutants, we constructed JFH1 subgenomic replicons by introducing individually the six mutations in NS5A and NS5B. We transfected each replicon RNA into Huh7 cells and compared their replication levels according to the luciferase activities. Consistently with the mutant viruses, the subgenomic replicon encoding the changes C2441S, P2938S or R2985P, which produced higher amounts of core antigen, did replicate at higher levels than the other subgenomic replicons with single mutation, G2964D, H3004Q and S3005N. However, none of these mutants replicated at higher than the parental JFH1 subgenomic replicon. Furthermore, replicon with triple mutations of C2241S, P2938S and R2985P did not replicate (Fig. 4).

Introduction of NS5A and NS5B mutations into the JFH1 clone had no effect on the production of infectious virions

We sought to investigate the effects of the NS5A and NS5B mutations on virus replication and virion secretion independent of re-infection and spread of the viruses produced. Therefore, we used the S29-subclone of Huh7 cells, which cannot be infected by HCV because of a defect in CD81 expression but does support viral genomic replication and releases infectious HCV particles after transfection (Russell et al., 2008). The Huh7-S29 cells enabled us to evaluate a single cycle of infection and production of virions. Those cell lines did not show apparent cytopathic effects after transfection with HCV RNAs (data not shown). To analyze HCV particle production from cells transfected with the viral genomic RNAs transcribed in vitro, we harvested culture media and cells at 72 h post transfection and measured the core antigen levels in culture media and intracellular HCV RNA by real-time RT-PCR. The C2441S, P2938S, and R2985P mutants produced significantly greater amounts of core antigen in the culture medium than the wild type JFH1. The HCV clone carrying all three mutations produced the greatest amount of core antigen (Fig. 5A, top). Consistent with the core antigen levels in the culture media, intracellular HCV RNA levels were also higher in the cells transfected with the mutated genomes encoding separately C2441S,

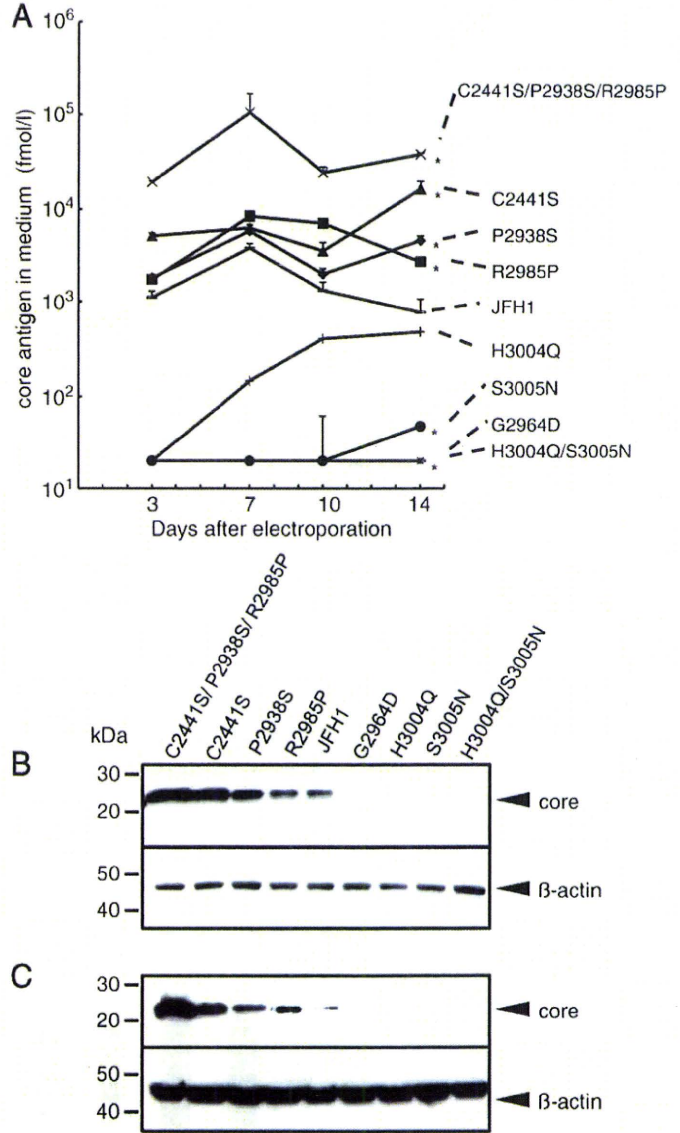


Fig. 3. Replication competences of HCV subclones with NS5A and NS5B mutations. A. Levels of core antigen in the culture medium. The culture media from transfected cells were collected on the days indicated and the levels of core antigen were measured. Asterisks indicate p-values of less than 0.05 as compared with JFH1. B. Huh-7.5.1 cells transfected with JFH1 mutants were harvested at 5 days after transfection and western blotting was performed. C. The culture media from Huh-7.5.1 cells transfected with JFH1 mutants were transferred onto uninfected Huh-7.5.1 cells. The cells were harvested at 3 days after infection. Western blotting was performed using anti-core and anti-beta-actin. kDa: kilo dalton.

P2938S, and R2985P, and that with all three mutations (Fig. 5A, middle), indicating that these mutations affected virus replication. Fig. 5A bottom shows the efficiency of infectious viral particle release from each transfectant, this being expressed as the core antigen level in the culture medium adjusted by dividing by the levels of intracellular HCV RNA. There was no difference in the efficiency of release of virions by the wild type JFH1 and the genomes carrying the C2441S, P2938S or R2985P changes. These results indicated that these three mutations in NS5A and NS5B did not affect virion entry or viral particle release but did regulate virus replication, and a high level of viral replication induces cytopathogenicity. Similarly, as shown in Fig. 3B, the three clones with C2441S, P2938S or R2985P, or all three mutations expressed much higher levels of core protein than the parental JFH1, while clones with G2964D, H3004Q or S3005N mutations did not express detectable amounts of core protein (Fig. 5B).

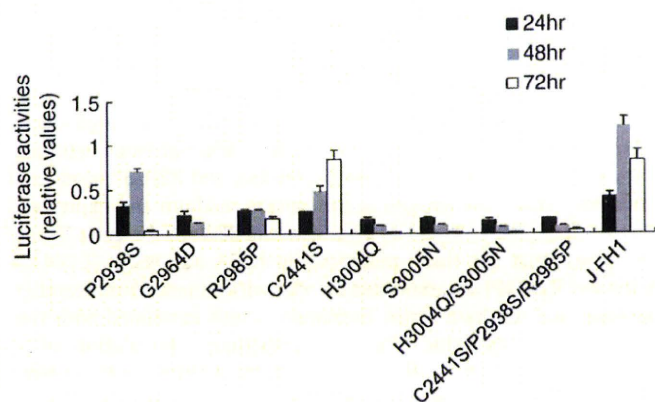


Fig. 4. Luciferase assay of the cytopathic JFH1-subgenomic replicon. Mutations were introduced into 2a-Feo subgenomic replicon and transcribed RNA for each replicon was transfected into Huh7 cells by electroporation. The cells were harvested at 24 h, 48 h and 72 h after electroporation and were used for Luciferase assay. Values are relative values to those of 8 h.

Mutations of NS5A and NS5B are associated with replication competence at earlier stages in vivo

We next used human hepatocyte chimeric mice to investigate the infectivity of the triple mutant of NS5A and NS5B. We confirmed the mouse liver chimerism greater than 70% by immunohistochemical analysis (data not shown). Culture media of the parental JFH1 and the mutant subclone with three mutations (C2441S, P2938S, and R2985P), were collected following transfection of Huh-7.5.1 cells, concentrated, and inoculated intravenously into human hepatocyte chimeric mice. We confirmed that the three mutations in NS5A and NS5B were conserved in the virus genome sequence of cell culture supernatants that were used for inoculation (data not shown). Two mice were inoculated with JFH1 and three were inoculated with the mutant virus. HCV RNA and human albumin in the sera of the mice were detected sequentially.

We repeated the same exam twice and confirmed consistency of the results. In the early phase post inoculation, the concentration of HCV RNA in serum was significantly higher in mice inoculated with the culture medium from the mutant subclone (Fig. 6A), suggesting that the mutations in NS5A and NS5B (C2441S, P2938S, and R2985P) are associated with virus replication in vivo. However, there was no difference in the level of HCV RNA in later period. The disparity of viral production at early time point could be influenced by the disparate numbers of infectious virus between the 2 initial inoculums. However, the sharp elevation of serum HCV RNA at day 5 after dropping at day 3 indicates that the mutants (C2441S, P2938S plus R2985P) are more replication competent at early stages in vivo. Serum levels of human albumin remained constant throughout the observed periods and showed no significant differences between wild and mutant-infected mice (Fig. 6B).

We also investigated expression of ER stress-related proteins, the glucose regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP), in liver of chimeric mice infected with JFH1 or the mutant in the early phase post inoculation. Human hepatocyte chimeric mice were inoculated in the same way as described above, and we verified that the level of virus titer in serum of each mouse was same as presented in Fig. 6A (data not shown). We sacrificed one each mouse that was infected with wild type or mutant JFH1 at 5 day of infection and investigated hepatic expression of GRP78 and CHOP. Liver histology showed no sign of inflammation or cytopathic cell death. However, as shown in Fig. 7, the expression level of both GRP78 and CHOP was higher in mice inoculated with the mutant viruses than the parental JFH1. There was no apparent difference in percents of hepatic chimerism between each mouse. These finding suggested that ER stress-related proteins were upregulated in the liver of HCV-infected

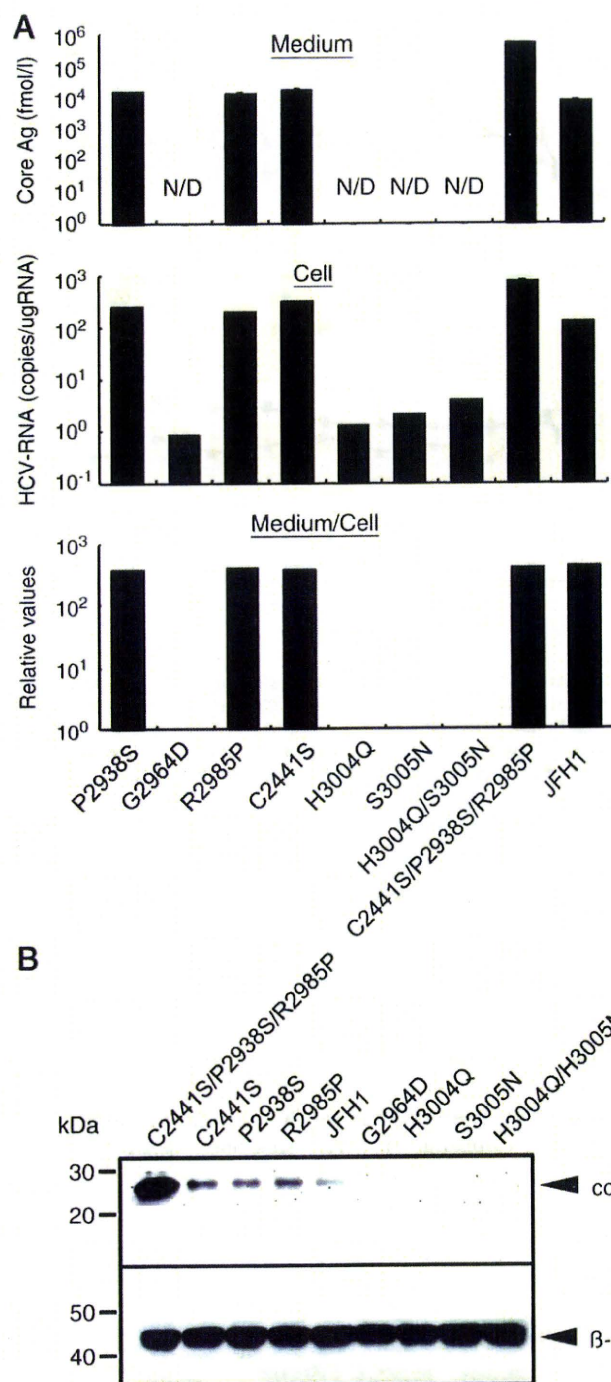


Fig. 5. Analysis of viral replication and production of viral particles using a single-cycle assay. A. Levels of core antigen in the culture media 3 days after transfection of JFH1 mutants into CD81-deficient Huh7-S29 cells (top). Levels of intracellular HCV RNA were quantified by real-time RT-PCR 3 days after transfection of JFH1 mutants into Huh7-S29 cells (middle). To determine the efficiency of infectious viral particle release from Huh7-S29 cells transfected with JFH1 mutants, the levels of core antigen in the culture media were adjusted by dividing by the levels of intracellular HCV RNA (bottom). Core Ag: Core antigen, N/D: not detectable. B. Huh7-S29 cells were harvested at 3 days after transfection of JFH1 mutants and western blotting was performed using anti-core and anti-beta-actin. kDa: kilo dalton.

mouse and that these responses were more strongly induced in the liver of mutant-infected mouse.

Highly adapted cytopathic mutations reverted to wild type in vivo

Finally, we analyzed the serum viral sequence at the specified time points. On days 1 and 5, the HCV genomic sequences of the mice

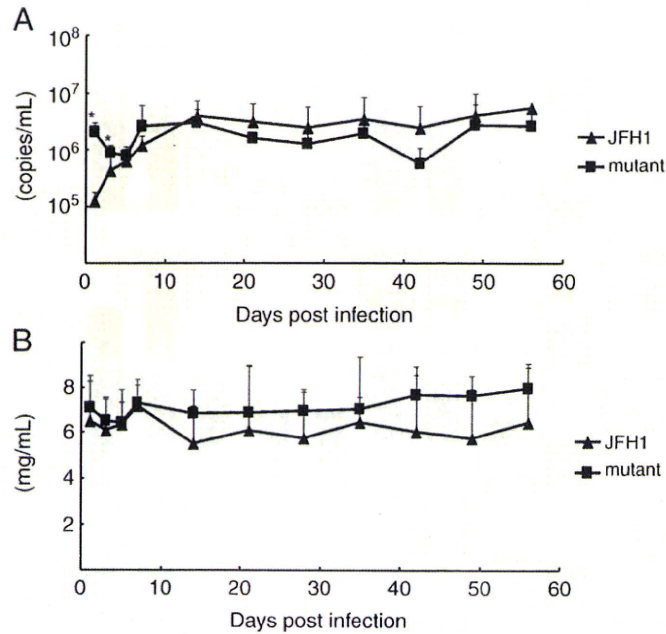


Fig. 6. In vivo analysis of cytopathic JFH1 mutants using human hepatocyte chimeric mice. A. Serial changes in HCV RNA in the sera of mice inoculated with the culture media from JFH1 mutants. The data shows the average of 2 mice for JFH1, and 3 mice for the mutant. Asterisks indicate p-values of less than 0.05 as compared with JFH1. B. Levels of human albumin in the sera of mice inoculated with the culture media from JFH1 mutants.

inoculated with the cytopathic mutant virus showed conservation of the mutations in codons 2441, 2938 and 2985. However, on days 21 and later, the mutation at codon 2985 had reverted to the wild type JFH1 sequence in all the mutant-injected mice and the mutation at codon 2938 had reverted to the wild type JFH1 sequence in two of the three mice. The C2441S mutation was more stable in the mutant-injected mice, but one mouse had lost it at day 56 (Fig. 8).

Discussion

In this study, we investigated the significance of genetic mutations in plaque-purified, cytopathic HCV-JFH1 subclones. Genetically engi-

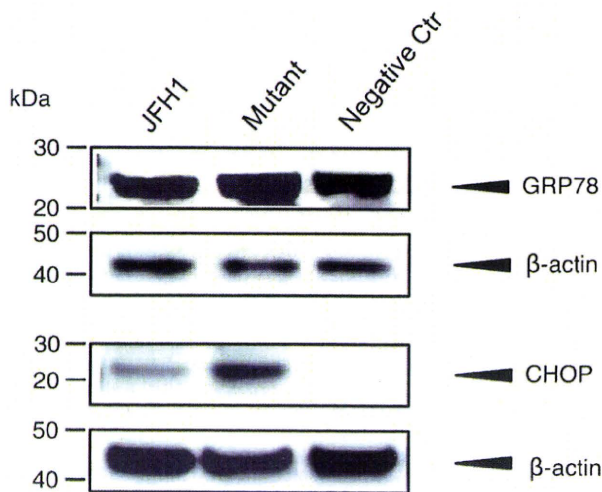


Fig. 7. Expression of ER stress-related proteins in human hepatocytes of chimeric mice infected with JFH1 or the mutant in the early phase. Western-blot analysis of the liver tissues of infected chimeric mice using anti-GRP78 goat monoclonal antibody, anti-GADD153/CHOP rabbit polyclonal antibody and anti-beta-actin. Liver samples were obtained at 5 days after inoculation. The negative control liver samples for this study was from uninfected human hepatocyte chimeric mouse.

neered JFH1-mutants encoding C2441S, P2938S, and R2985P led to much more cell death than the wild type JFH1, and also produced significantly higher amounts of core antigen in the culture medium and inside the cells than the parental JFH1 clone. In the single-cycle production assay, which exploited a receptor-deficient Huh7 cell line, the three JFH1-mutants, JFH1-C2441S, P2938S, and R2985P produced significantly more core antigen in the culture medium and expressed equivalently higher amounts of viral genomic RNA in the cells. These data suggest that the three mutations in NS5A and NS5B (C2441S, P2938S, and R2985P) are associated directly with enhanced intracellular replication and resultant virion formation, which correlated with the extent of the cytopathic effects. Interestingly, inoculation of a cytopathogenic mutant, JFH1-C2441S/P2938S/R2985P, into human hepatocyte chimeric mice produced significantly higher plasma HCV RNA concentrations than JFH1 at ~7 days post inoculation. At a later phase of infection, however, the mutations in this mutant HCV reverted partially to the wild type sequences. Taking all things together, it is suggested that in vitro-isolated, genetically modified cytopathic HCV subclones replicate robustly in the acute phase of in vivo infection but are eliminated rapidly and substituted by in vivo adapted clones.

Four of the five NS5B mutations appeared independently in several isolated subclones. This made us speculate that these amino acid substitutions may affect the enzymatic activity of RdRp. Mapping of the amino acid substitutions in the RdRp tertiary structure revealed that amino acid 2441 is located on the finger domain, and three amino acids, 2938, 2964, and 2985, are on the outer surface of the thumb domain, which corresponds to the opposite side of the nucleotide tunnel. The other substitutions, 3004 and 3005, are within the domain of the polypeptide linking the polymerase to the membrane anchor (Lesburg et al., 1999). Our preliminary study has shown that the NS5B mutations, P2938S and R2985P, did not affect cell-free enzymatic activities of the RNA polymerase. Thus, it is speculated that these mutations may affect the stability of the HCV replicase complex by altering surface affinity to other nonstructural proteins.

There are several reports on cell culture adaptive mutations in the HCV-JFH1 genome that gave more vigorous and consistent virus expression. Most studies involved prolonged cell culture of HCV-JFH1 or multiple rounds of successive passage onto naive cells. Zhong et al. detected the E2-G451R mutation after culture for more than 60 days. The mutation led to more efficient production of infectious viral particles than wild type JFH1 (Zhong et al., 2006). Delgrange et al. conducted successive virus infections of naive cells and identified the E2-N534K mutation that facilitated virus-CD81 attachment, and core-F172C and -P173S that increased secretion of virions (Delgrange et al., 2007). Using a similar method, Russell et al. identified E2-N417S that improved virus-cell attachment, and p7-N765D and NS2-Q1012R that increased virion production (Russell et al., 2008). Kaul et al. reported the NS5A-V2440L mutation, that was close to the C terminus and increased virion production (Kaul et al., 2007). Yi et al. used a chimeric virus of genotype 1a and JFH1 and identified the NS3-Q1251L mutation that resulted in enhanced virus production, possibly through improved interactions between NS2 and NS3 that were required for virion formation (Yi et al., 2002). Han et al. used EGFP-tagged virus and identified the mutually dependent mutations, NS3-M1290K and NS5A-T2438I, which improved virus production synergistically (Han et al., 2009).

Of note is that all of the mutations reported above promoted virion secretion or virus-cell surface interaction and none of them showed any effect on intracellular replication of viral RNA or translation of virus proteins. None of the adaptive mutations reported above overlapped with our cytopathogenic mutations. The mutations that we have identified conferred enhanced virus replication and protein expression in the early/acute stages of infection and subsequently led to massive cell death. Our data and the reports of other groups suggest that the HCV genome evolves to adapt to the host cell environment. Mutations that optimize virus secretion or virus-cell entry may be

	JFH1wt	2437	2446	2934	2943	2981	2990
	Mutant	DTTVCCSMSY	-----S-----	LGAPPLRVWK	-----S-----	LPEARLLDLS	-----P-----
Mutant	#1	Day 1	-----S-----	-----S-----	-----P-----	-----P-----	-----P-----
		Day 21	N/D	-----S-----	-----P-----	-----P-----	-----P-----
		Day 49	N/D	-----S-----	-----P-----	-----P-----	-----P-----
		Day 56	-----S-----	-----S-----	-----P-----	-----P-----	-----P-----
	#2	Day 5	-----S-----	-----S-----	-----P-----	-----P-----	-----P-----
		Day 49	-----S-----	-----S-----	-----P-----	-----P-----	-----P-----
		Day 56	-----S-----	-----S-----	-----P-----	-----P-----	-----P-----
	#3	Day 1	N/D	-----S-----	-----P-----	-----P-----	-----P-----
		Day 56	-----S-----	-----S-----	-----P-----	-----P-----	-----P-----
JFH1	#1	Day 1	-----S-----	-----S-----	-----P-----	-----P-----	-----P-----
		Day 56	-----S-----	-----S-----	-----P-----	-----P-----	-----P-----
	#2	Day 1	-----S-----	-----S-----	-----P-----	-----P-----	-----P-----
		Day 56	-----S-----	-----S-----	-----P-----	-----P-----	-----P-----

Fig. 8. Nucleotide sequence analysis of virus genomes circulating in the sera of infected mice. We extracted RNA from the sera of mice inoculated with culture media from JFH1 or JFH1-mutants and analyzed the viral sequence at the specified time points. N/D is not detectable. Wt: Wild type.

required for persistent infection in vitro, while those that affect cellular viral RNA replication may possibly promote viral genetic evolution and host cell damage.

The results of in vivo experiments using human hepatocyte chimeric mice were consistent with those of virus cell culture (Figs. 5, 6 and 7). The mutant JFH1 clones showed markedly higher levels of replication than the parental JFH1 in the acute phases. However, the serum HCV titers subsequently leveled out after two weeks of infection, concomitant with reversal of some cytopathic mutations to wild type sequences. Bukh et al. reported that inoculation of the HCV-1b genome into chimpanzee liver resulted in persistent infection, although the mutation reverted rapidly to wild type (Bukh et al., 2002). In this study, the NS5A-C2441S mutation was preserved in 2 of 3 mice, while NS5B-P2938S reverted to the wild type sequences in 2 of 3 mice and NS5B-R2985P reverted to wild type sequences in all 3 mice. These results suggest that the highly adapted JFH1 genome is infectious and viable in vivo, but is not as fit in vitro.

It is not clear why the subgenomic replicons with C2441S, P2938S or R2985P mutations did not show differences in replication levels compared to the wild type JFH1 subgenomic replicon. One may speculate that this discrepancy between the results using full-length HCV genomes and replicons might be the presence or absence of the HCV structural proteins. In addition, three individual substitutions G2964D, H3004Q and S3005N did not enhance viral replication as compared with the parental JFH1 nor did express detectable amounts of core protein. It is speculated that these mutants exist in host cells through co-infection with replication-competent viral clones resulting in enhanced replication.

There is clinical evidence that suggests the pathological outcomes of hepatitis C result from the immune response of the host rather than the direct cytopathic effects of the virus (Cerny and Chisari, 1999). However, several clinical studies have shown that fulminant hepatic failure (FHF, the HCV-JFH1 strain was isolated from such a case) featured massive hepatocyte apoptosis, as characterized by caspase activation and Fas-FasL expression (Leifeld et al., 2006; Mita et al., 2005; Ryo et al., 2000). The ER stress markers, GRP78 and ATF6 are upregulated in HCV-infected liver tissue as the histological grade advances (Shuda et al., 2003). This background and our results in vitro and in vivo suggest that HCV strains with highly infectious and cytopathic gene signatures may replicate aggressively in the acute phase of infection and that certain defects in innate or adaptive immune responses against the virus could lead to severe and persistent liver damage due to cytopathic effects induced directly by

HCV. Such mechanisms might explain some rare clinical features of HCV infection, such as fulminant hepatic failure and post-transplantation severe fibrosing cholestatic hepatitis (Delladetsima et al., 1999; Dixon and Crawford, 2007).

In conclusion, we identified three substitutions in cytopathic HCV-JFH1 subclones derived from plaque assay. These substitutions directly enhanced virus replication in the early phases of virus infection in vitro and in vivo. This highly enhanced replication induced ER stress-mediated apoptosis and resulted in cytopathogenicity. Further analyses of cellular effects on HCV replication may elucidate the pathogenesis of HCV infection and may define novel host factors as targets of antiviral chemotherapeutics.

Materials and methods

Cells and cell culture

Huh-7.5.1 cells (Zhong et al., 2005) (kindly provided by Dr Francis V. Chisari) and CD81 deficient Huh7-S29 cells (Russell et al., 2008) (kindly provided by Dr Rodney S. Russell and Dr Robert H. Purcell) were maintained in Dulbecco's modified minimal essential medium (DMEM, Sigma, St. Louis, MO) supplemented with 2 mmol/L L-glutamine and 10% fetal bovine serum at 37 °C under 5.0% CO₂.

Sequence analysis

The cDNA from the isolated JFH1-plaque was amplified from cytopathic virus-infected Huh-7.5.1 cells by RT-PCR and subjected to direct sequencing.

In vitro RNA synthesis and transfection

A plasmid, pJFH1full (Wakita et al., 2005), which encodes full-length HCV-JFH1 sequence, was used. In vitro RNA synthesis and transfection were conducted as previously described (Sekine-Osajima et al., 2008). Briefly, HCV RNA was synthesized from linearized pJFH1 plasmid as template and transfected into Huh-7.5.1 cells by electroporation. The transfected cells were split every 3 to 5 days. The culture media were subsequently transferred onto uninfected Huh-7.5.1 cells and Huh7-S29 cells. The levels of HCV replication and viral protein expression were detected by real-time PCR and western blotting.

Plaque assay

HCV plaque assays were performed as reported previously (Sekine-Osajima et al., 2008). Huh-7.5.1 cells were seeded in collagen-coated 60 mm-diameter plates. After overnight incubation, HCV-infected culture media were serially diluted in a final volume of 2 ml per plate and transferred onto the cell monolayer. After ~5 h of incubation, the inocula were removed and the cell monolayer was overlaid with 8 ml of culture medium containing 0.8% methylcellulose (Sigma). After 7 to 12 days culture, cytopathic plaques were visualized by staining with 0.08% crystal violet solution (Sigma). The levels of cytotoxicity were evaluated by counting the plaques and calculating the titer (plaque-forming unit/ml).

Establishment of mutant JFH1 clones

In order to introduce various mutations into the NS5A and NS5B region of JFH1, plasmid pJFH1 was digested with HindIII and the DNA fragment encompassing nt. 8231 to 9731 was subcloned into the pBluescript II SK+ phagemid vector (Stratagene, La Jolla, CA). Mutations were introduced into the DNA fragment in the subcloning vector by site-directed mutagenesis (Quick-Changell Site-Directed Mutagenesis Kit, Stratagene) to generate the following codon changes: P2938S, G2964D, R2985P, H3004Q and S3005N. Finally, the HindIII–HindIII fragments were subcloned back into the parental plasmid, pJFH1. A PCR fragment (nt. 7421–7839) was subcloned into the pGEM-T Easy plasmid vector (Promega, Madison, WI) and digested with RsrII and BsrGI. Finally, after introducing the codon change C2441S, the RsrII–BsrGI fragment was reinserted into the parental plasmid.

Quantification of HCV core antigen in the culture medium

The culture media from JFH1-RNA transfected Huh-7.5.1 cells and Huh7-S29 cells were collected on the days indicated, passed through a 0.45 µm filter (MILLEX-HA, Millipore, Bedford, MA), and stored at –80 °C. The levels of core antigen in the culture media were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan).

Western blotting

Western blotting was carried out as described previously (Itsui et al., 2009). Briefly, 10 µg of total cell lysate were separated by SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) Western Blotting membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and visualized by chemiluminescence using the ECL Western blotting Analysis System (Amersham Bioscience, Buckinghamshire, UK). The antibodies used were anti-core mouse monoclonal antibody (Abcam, Cambridge, MA), anti-GRP78 goat monoclonal antibody, anti-GADD153/CHOP rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-beta-actin antibody (Sigma).

HCV subgenomic replicon constructs

The HCV subgenomic replicon plasmid, pRep-Feo, was derived from the HCV-N strain, pHCV1bneo-dels (Tanabe et al., 2004; Yokota et al., 2003). The replicon RNA was synthesized from pRep-Feo and transfected into Huh7 cells.

Luciferase reporter assay

Luciferase activity was measured using a 1420 Multilabel Counter (ARVO MX, Perkin Elmer, Waltham, MA) with a Bright-Glo Luciferase

Assay System (Promega) (Tasaka et al., 2007). Assays were carried out in triplicate and the results expressed as means ± SD.

MTS assays

To evaluate cell viability, dimethylthiazol carboxymethoxy-phenyl sulfophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega), as described previously (Sakamoto et al., 2007).

Real-time RT-PCR analysis

Total cellular RNA was isolated using an RNeasy Mini Kit (QIAGEN, Valencia, CA). Two micro-grams of total cellular RNA were used to generate cDNA from each sample using SuperScript II (Invitrogen) reverse transcriptase. Expression of mRNA was quantified using TaqMan Universal PCR Master Mix (Applied Biosystems) and the ABI 7500 Real-Time PCR System (Applied Biosystems). The primers used were as follows: HCV-JFH1 sense (positions 285 to 307; 5'-GGT-CTGCCTGATAGGGTGCTT-3'), HCV-JFH1 antisense (positions 349 to 375; 5'-TGGTTTTTCTTTGAGGTTTAGGATTC-3'), GAPDH sense (5'-CTCCCGCTTCGCTCTCT-3'), and GAPDH antisense (5'-GCTGGCGACG-CAAAAGA-3').

HCV RNA inoculation into human hepatocyte chimeric mice

Housing, maintenance, and care of the mice used in this study conformed to the requirement for the humane use of animals in scientific research as defined by Animal Care and Use Committee of our institute. The culture media of Huh-7.5.1 cells transfected with parental JFH1 and JFH1 mutants were collected 10 days after transfection and passed through a 0.45 µm filter. The three mutations introduced in NS5A and NS5B were confirmed to conserve by the sequence analysis of virus genome of cell culture supernatants before inoculation. Filtrated culture medium was then pooled and concentrated using Amicon Ultra-15 (100,000 molecular weight cutoff, Millipore). 100 µl of each culture medium was injected intravenously into human hepatocyte chimeric mice (PXB mice, Phenix Bio, Hiroshima, Japan) (Mercer et al., 2001). The rate of liver chimerism of these human hepatocyte chimeric mice was confirmed more than 70% by immunohistochemical analysis. After infection, blood samples were taken serially and levels for HCV RNA and human albumin were quantified using real-time RT-PCR and an enzyme immunoassay, respectively. RNA was extracted from serum samples and subjected to direct sequence determination.

Protein extraction from human hepatocyte chimeric mice and expression of ER stress-related proteins

5 days post inoculation, mice were sacrificed and proteins were extracted from liver samples with complete Lysis-M Reagent Kit (Roche Applied Science, Indianapolis, IN). One Mini Protease Inhibitor Cocktail Tablet was dissolved into 10 ml of Lysis-M Reagent and 500 µl of this fluid was added to 50 µg of each liver sample and homogenized. The lysate was transferred to a microcentrifuge tube and centrifuged at 14,000×g for 5 min. The supernatant containing soluble protein was transferred to a new reaction tube and 20 µg of each protein was used for western blotting to detect ER stress-related proteins.

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