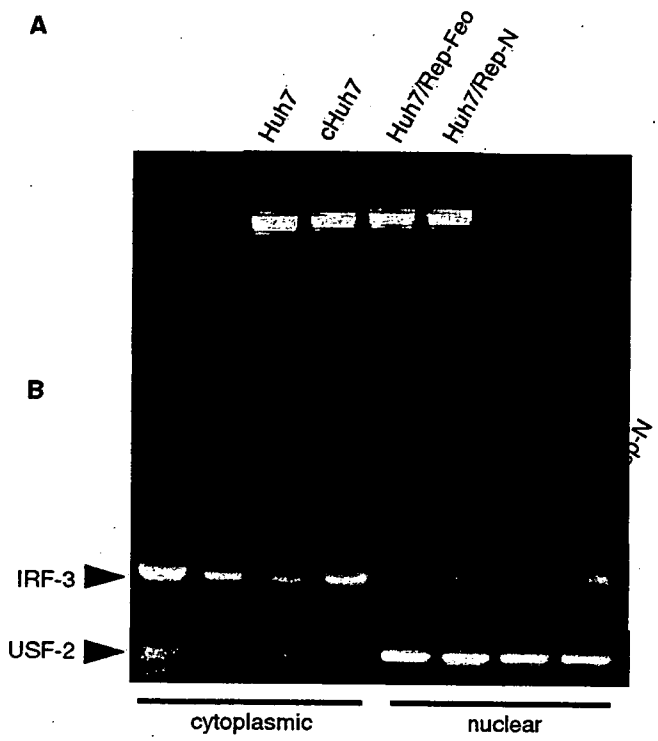

Fig. 2A,B. Expression of endogenous interferon regulatory factor-3 (*IRF-3*) in cells with and without expression of the HCV replicon. **A** Western blotting. Whole cell lysates from Huh7 and Huh7/Rep-Feo were prepared. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotting, the expression of IRF-3 protein was detected by a monoclonal anti-IRF-3 antibody. **B** To detect IRF-3 translocated to the nucleus, we prepared the cytoplasmic and the nuclear fractions of cell lysates from naïve Huh7, Huh7/rep-Feo, Huh7/Rep-N, and cured Huh7 (*cHuh7*) cell lines, and detected IRF-3 expression by Western blotting. The purity of cellular fractionation was tested by immunoblotting for USF-2. The differences in the IRF-3 expression levels were due to different yields from the preparation of cytoplasmic and nuclear fractions. However, the ratio of nuclear to the respective cytoplasmic IRF-3 remained equal among the cell lines tested

nificant difference in expression levels of IRF3 between Huh7 and Huh7/Rep-Feo (Fig. 2A). Similarly, levels of IRF-3 mRNA were not significantly different between Huh7 and Huh7/Rep-Feo.

IRF-3, once activated by site-specific phosphorylation, translocates into the nucleus. To examine the nuclear translocation of the activated IRF-3, we prepared nuclear and cytoplasmic fractions of cell lysates from naïve Huh7, cured Huh7, and two Huh7 that expressed the replicon, Huh7/Rep-N and Huh7/Rep-Feo. Western blotting of the cell fractions showed that most IRF-3 protein was localized in the cytoplasm in each cell line, and that there was no obvious increase in nuclear IRF-3 in any of the cell lines, nor were there differences in the nuclear IRF-3 levels between cells with and without the HCV replicon (Fig. 2B). Similarly, the immunocytochemistry analysis showed that IRF-3 was mainly localized in the cytoplasm, and there were no differences in the patterns of IRF-3 staining (data not shown).

Overexpression of IRF-3 and effects on ISRE activity and HCV replication

Because IRF-3 is a strong inducer of ISGs on activation, a slight change in the IRF-3 activation level could affect ISRE enhancer activity. Thus, we examined ISRE reporter activities of cells with and without the replicon, and evaluated the effects of IRF-3 by overexpression. Transfection of an IRF-3 expression plasmid, pcDNA-IRF-3, resulted in expression of 6xHis-tagged IRF-3, which was confirmed by Western blotting using an anti-IRF-3 antibody (Fig. 3A) and an anti-His antibody (Fig. 3B). Immunohistochemistry showed cytoplasmic expression of the transfected IRF-3 (Fig. 3C). Cotransfection of pcDNA-IRF-3 with an ISRE-luciferase reporter plasmid, pISRE-TA-luc, into Huh7, cHuh7, and Huh7/Rep-N cell lines resulted in a significant increase of ISRE activity in cells in which IRF-3 was overexpressed (Fig. 3D). Interestingly, the cell line expressing the replicon, Huh7/Rep-N, showed a significantly higher ISRE induction ratio by IRF-3 overexpression than naïve Huh7 or cured Huh7 (28.7-fold vs. 8.9- or 11.7-fold, $P < 0.01$), suggesting partial activation of IRF-3 in the replicon-expressing cells. Concomitantly with the ISRE activation, transfection of pcDNA-IRF-3 into Huh7/Rep-Feo resulted in a significant decrease of internal luciferase activities to $19.6 \pm 1.8\%$ of control, indicating suppression of cellular HCV replication by IRF-3 overexpression (Fig. 3E). MTS assays of the IRF-3 transfected cell lines showed no obvious effects on cell growth or viability, indicating that these effects of IRF-3 overexpression were not due to nonspecific effects or to cytotoxic cell death (Fig. 3F).

Effect of IRF-3 siRNA oligonucleotides on ISRE-luc and HCV Feo-replicon cells

To investigate the effects of suppression of IRF-3 synthesis on HCV replication, three synthetic siRNAs, siRNA1, siRNA2, and siRNA3, were used. Western blotting showed that transfection of each siRNA into Huh7 cells resulted in a decrease of the IRF-3 protein level, by 39.5%, 57.8%, and 37.4%, respectively. To study the effects of IRF-3 suppression on HCV replication, siRNAs were transfected into Huh7/Rep-Feo cells, and a luciferase assay was done after 4 days of transfection. The siRNAs upregulated HCV replication to various extents (Fig. 4A). Thus, we used siRNA3, which was the most efficient, for the following assays. Cotransfection of ISRE-TA-Luc with siRNA3 or a

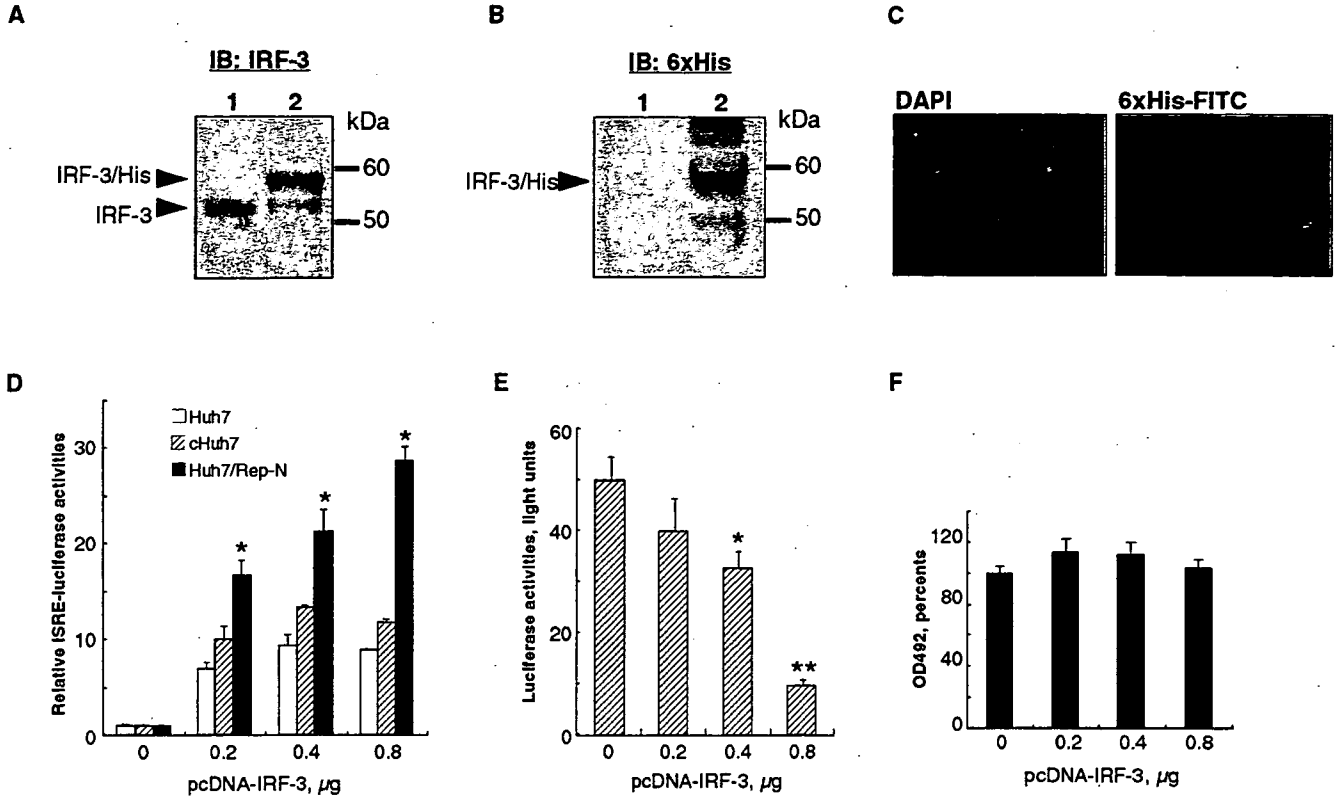

Fig. 3A–F. Effects of IRF-3 overexpression on the regulation of interferon-stimulated response element (*ISRE*) activity and HCV replication. An IRF-3-expression plasmid, pcDNA-IRF-3, was transfected into Huh7 cells (*lane 2*), and Western blotting analyses were performed using monoclonal anti-IRF-3 antibody (**A**) and anti-His antibody (**B**). Untransfected Huh7 is shown as a control (*lane 1*). **C** Fluorescence microscopy. The pcDNA-IRF-3 was transfected into Huh7 cells, and the cells were stained with 4 ,6-diamidino-2-phenylindole (*DAPI*) and with anti-His antibody followed by fluorescein isothiocyanate (*FITC*)-labeled secondary antibody. The figure shows DAPI staining for nuclei (*left panel*) and transgenic IRF-3 expression (*right panel*). Magnification, ×40. **D** Effects of transgenic IRF-3 expression on ISRE reporter activity. The pcDNA-IRF-3 and ISRE-TA-luc reporter plasmids were cotransfected into Huh7 (*white bars*), cured Huh7 (*gray bars*), and Huh7/Rep-N cells (*black bars*), and luciferase activities were measured 24 h after the transfection. *Error bars* denote means + SD (*$P < 0.01$ relative to Huh 7 cells and cured Huh7 cells; Student-Newman-Keuls test). **E** Effects of transgenic IRF-3 expression on the levels of HCV replication. The indicated amounts of pcDNA-IRF-3 were mixed with empty pcDNA plasmid to adjust the total amount of DNA, mixed with Lipofectamine 2000, and transfected into Huh7/Rep-Feo cells seeded onto 24-well culture plates, and the luciferase activities were measured 24 h after transfection. *Error bars* denote means + SD (*$P = 0.006$; **$P = 0.0001$ relative to transfection with the empty vector). **F** MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays. The indicated amounts of pcDNA-IRF-3 were transfected into Huh7/Rep-Feo cells with the same conditions described above, and MTS assays were performed. *Error bars* indicate means + SD

control siRNA into Huh7 and Huh7/Rep-N resulted in significant suppression of ISRE-luciferase activities, by 50% in both Huh7 cells and Huh7/Rep-N (Fig. 4B). To study the relation between the suppression level of IRF-3 by siRNA3 and HCV replication, siRNA3 was transfected into Huh7/Rep-Feo cells, and a luciferase assay was done after 2 days of transfection. siRNA3 upregulated HCV replication in a dose-dependent manner (Fig. 4C).

Discussion

Persistent virus replication in host cells is the function of the interplay between the cellular antiviral system and the counteraction of the virus to evade host antiviral responses.[17] In our present study, even though IRF-3 expression levels were mostly similar between cells with and without HCV replication (Fig. 2A), overexpression (Fig. 3A–C) and knockdown (Fig. 4A) of IRF-3 were associated with up- and downregulation of ISG expression, as indicated by ISRE reporter activities (Figs. 3D and 4B), and were inversely correlated with HCV subgenomic replication levels (Figs. 3E and 4C). These

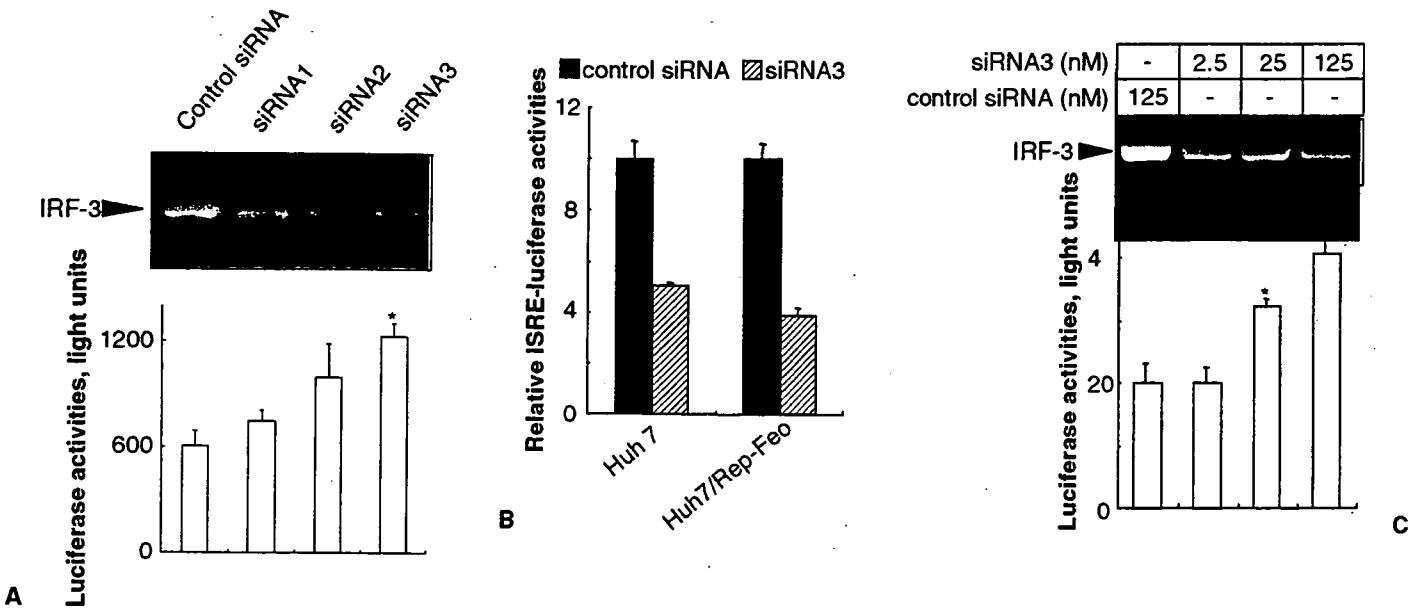

Fig. 4A–C. Suppression of IRF-3 expression by siRNA and effects on ISRE activity and HCV replication. **A** Suppression of endogenous IRF-3 expressions by IRF-3-directed siRNAs. Three siRNAs, siRNA1, siRNA2, and siRNA3, were transfected into Huh7 cells, and IRF-3 were detected by Western blotting using monoclonal IRF-3 antibody. Transfection of the three siRNAs substantially inhibited expression of IRF-3 protein. Three siRNAs and a control siRNA were transfected into Huh7/Rep-Feo cells that was plated onto 24-well plates. Graph: luciferase activities of the corresponding samples. *Error bars* denote means + SD (*$P = 0.0007$ relative to transfection with a control siRNA). Suppression of IRF-3 by siRNA increased HCV replication. **B** Effects of an IRF-3-directed siRNA, siRNA3, on ISRE-reporter activity. The siRNA and a control siRNA were individually transfected with pISRE-TA-luc reporter plasmid into Huh7 and Huh7/Rep-Feo cells, and luciferase activities were measured 24 hours after transfection. *Error bars* denote means + SD (*$P < 0.001$ relative to transfection with a control siRNA). **C** The correlation between the suppression level of IRF-3 by siRNA and HCV replication. The indicated amounts of siRNA3 or a control siRNA were transfected into Huh7/Rep-Feo cells, which were plated onto 24-well plates. The Western blotting shows dose-dependent suppression of IRF-3. *Graph*: luciferase activities of the corresponding samples. *Error bars* denote means + SD (*$P = 0.003$; **$P = 0.0007$ relative to transfection with a control siRNA)

results are consistent with a previous report that IRF-3 mediates expression of the antiviral gene via ISRE.[12] Furthermore, the replicon-expressing cells showed a significantly higher rate of ISRE activation than naïve or cured cells when IRF-3 was overexpressed (Fig. 3D). These results suggest different activation levels of the IRF-3-mediated pathway by replication of the HCV subgenome. Because IRF-3 is a strong inducer of interferon- production,[4,12,13] it is possible that the effects of IRF-3 on HCV replication were predominantly mediated by interferon production, which led to activation of ISRE-dependent transcription. Collectively, our results suggest that replication of the HCV subgenome was closely correlated with expression and activation levels of IRF-3 and that IRF-3 was a key cellular factor controlling ISRE-regulated ISG expression and cellular antiviral responses.

Foy et al.[18] reported that the HCV NS3/4A fusion protein substantially blocks phosphorylation and nuclear translocation of IRF-3 by experimental virus infection. We have also confirmed that double-stranded RNA-induced activation of the IRF-3 pathway was abolished in cells expressing the HCV replicon (unpublished data). Our present results reinforce the reports that IRF-3 is a key molecule of the cellular innate immune responses against HCV and that it may constitute a target of antiviral strategies.

Although our findings suggest activation of the cellular IRF-3-IFN pathway along with HCV replication, there are still unsolved questions: Which molecule is the sensor of HCV? Which viral component is the target of the detection? How is the triggered signal transduced to the IRF-3 activation leading to IFN responses? Studies of the virus-induced IFN signaling pathway are making progress in the discovery and elucidation of these issues. Several molecules have been recently identified that are involved in innate immune responses against various pathogens, including viruses: toll-like receptor (TLR) families, which recognize viral components of double- or single-stranded RNAs and lipoproteins;[27] two kinases, IKKe and TBK1, which catalyze phosphorylation of IRF-3;[28,29] and TRIF,[30] which mediates Myd88-independent TLR signaling. TLR3 has been reported to recognize double-stranded RNA and to activate IRF3-

mediated IFN signal transduction, suggesting that TLR3 could be a candidate receptor for innate immune responses against viruses.[8,11] However, our preliminary studies have shown that treatments with polyinosinic polycytidylic acid [poly(I-C)] and lipopolysaccharides, which are ligands of TLR3 and TLR4, respectively, have no effect on cellular ISRE activities or on HCV subgenomic replication (data not shown), a part of which is consistent with the findings of previous studies.[31] More recently, a DExD-box helicase, RIG-I, has been identified as a cytoplasmic receptor molecule that recognizes double-stranded RNA.[32] Speculatively, unknown molecules may recognize HCV genomic replication in cells and activate the IRF-3-mediated antiviral pathway.

Because our present study was based on the HCV subgenomic replicon system, which expresses only viral nonstructural proteins and not structural proteins, our results may have limited implication for the association between HCV infection and the innate immune system. Moreover, Huh7 cells, which are the host of the HCV replicon, are of human hepatoma origin.[33] Most hepatomas arise from chronic viral hepatitis and liver cirrhosis.[1] Although little information is available on what Huh7 cells were derived from, it is possible that these cells have been primed by past HCV infection, which could modify the cellular innate immunity continuously. To address these possibilities, further investigation using other cell lines that stably support HCV replication may be warranted.

Although in vitro HCV replication is highly sensitive to exogenous IFN, in clinical settings, a majority of HCV-infected patients are resistant to IFN treatments.[2] Our results suggest that an IRF-3-mediated innate immune system response might be activated by HCV infection in hepatocytes. This initial reaction in the host cells against the virus may determine the activities of the cellular and humoral immune responses that follow, and the clinical course of the infection thereafter. At present, few reports correlate clinical features with the function of IRF-3 in the HCV-infected liver. Castelruiz et al.[33] have reported that patients with chronic hepatitis C show a significant increase in IFN- mRNA in liver tissue. Thus, one of our next objectives is to elucidate how the innate immune system participates in the whole clinical process of HCV infection, and whether individual differences in the innate immune response influence clinical features.

In conclusion, our results demonstrate that IRF-3 negatively regulates HCV replication in vitro, possibly through IRF3-mediated ISG expression pathways. Therefore, IRF-3 might be a key molecule not only as a mediator of the host antiviral responses against HCV but also as a potential therapeutic target to control HCV replication.

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Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis C virus replication

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SUMMARY. Type-I interferons (IFNs) and the interferon-stimulated genes (ISGs) play a major role in antivirus responses against hepatitis C virus (HCV) infection. In this study, we studied expression profiles of ISGs in cells supporting subgenomic HCV replication (Huh7/Rep), and screened their activities to suppress HCV replication. Real-time PCR analyses showed that the expression levels of 23 ISGs were significantly lower in Huh7/Rep than naive Huh7 cells due to transcriptional suppression of the interferon-stimulated response element (ISRE). Furthermore, the expression level of ISGs was also decreased in the cured Huh7 cells in which replicon had been eliminated (cHuh7), indicating adaptation of the cells to support HCV replication by downregulating ISGs. On the other hand, expression of HCV replicon was significantly suppressed by overexpression of several ISGs including PKR, MxA, IRF-9, GBP-1, IFI-6-16, IFI-27, 25OAS and IRF-1. Knock down of GBP-1, IFI-6-16 and IFI-27 by short hairpin RNA resulted in increase of HCV replication. Thus, we conclude that downregulation of ISG expression is required in the host cells supporting HCV replication and that several ISGs directly suppress HCV replication. The search for ISGs that regulate HCV replication may help to elucidate the cellular antiviral defence mechanisms against HCV infection.

Keywords: guanylate binding protein-1, hepatitis C virus, interferon-induced protein 6–16, interferon-inducible protein-27, interferon-stimulated gene, replicon.

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Abbreviations: HCV, Hepatitis C virus; IFN, interferon-alpha; ISG, interferon-stimulated gene; 25OAS, 2′, 5′-oligoadenylate synthetase; MxA, myxovirus resistance 1; PKR, double-stranded RNA-dependent protein kinase R; IFI-56K, interferon-induced protein 56; IRF, interferon regulatory factor; GBP-1, guanylate binding protein-1; IFI-6-16, interferon-induced protein 6–16; IFI-27, interferon-inducible protein 27; ISGF-3, interferon-stimulated gene factor-3; TAP1, transporter ATP-binding cassette, major histocompatibility complex 1; IFP35, interferon inducible protein 35kD; PLSCR1, phospholipid scramblase 1; LMP7-E1, major histocompatibility complex encoded proteasome subunit LMP7-E1; eIF2-alpha, eukaryotic initiation factor-2 alpha; eIF3, eukaryotic initiation factor-3; STAT, signal transducer and activator of transcription; NS5A, nonstructural protein 5A; Fluc, firefly luciferase; ISRE, interferon-stimulated response element; GAS, interferon-gamma activation site; AP1, activator protein 1; NF-kappa B, nuclear factor-kappa B; Rluc, renilla luciferase; shRNA, short hairpin RNA; IRES, internal ribosome entry site.

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INTRODUCTION

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality [1,2]. HCV is characterized by persistent infection in the liver that leads to the development of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Type-I interferon (IFN) plays a central role in eliminating viruses not only as therapeutic applications [3] but also as natural cellular antiviral defence mechanisms [4,5]. DNA microarray analysis of chimpanzee liver experimentally inoculated with HCV revealed that expression of the interferon-stimulated genes (ISGs), including cytokines and chemokines, was the principal reaction during the course of the viral infection and its clearance and that a considerable proportion of the genes were inducible by IFNs [6].

Interferons are naturally produced in response to virus infection, and to cellular exposure to IFN itself [7]. The expressional control of the ISGs is directed by receptor-mediated stimuli of type-I IFNs [8]. Binding of the IFNs onto their receptors activate receptor associated janus kinases, which phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2. The phosphorylated STATs 1 and 2 recruit IFN regulatory factor-9 (IRF-9) to form a

complex of IFN-stimulated gene factor-3 (ISGF-3), which translocates to the nucleus, binds the IFN-stimulated response element (ISRE) located in the promoter/enhancer region of ISGs, and activates expression of ISGs [4,5,9].

Interferons induce expression of a variety of ISGs, several of which show antiviral function by limiting virus replication at multiple points within the replication cycle [7,10]. At least four ISGs have been reported to direct antiviral activity through distinct cellular pathways of translational control; 2′, 5′-oligoadenylate synthetase (OAS), double-stranded RNA-dependent protein kinase R (PKR), myxovirus resistance 1 (MxA), and interferon-induced protein 56 (IFI-56K). Transcriptional induction of 2′, 5′-OAS activates ribonuclease L (RNase L) which leads to translational suppression though the cleavage and subsequent inactivation of 28S rRNA [11]. PKR is activated by viral double-stranded RNA, and catalyses the phosphorylation of eukaryotic initiation factor-2 alpha (eIF2-alpha). The phosphorylated eIF2-alpha blocks translation initiation by reducing the cellular pool of functional eIF2 and by disrupting the critical delivery of methionyl-tRNA to the 40S ribosome [12]. MxA protein results in degradation of cellular RNA, general repression of protein synthesis and apoptotic cell death [13]. IFI-56K binds eukaryotic initiation factor-3 (eIF3) and suppresses translation [10,14]. However, cells in which of PKR, MxA, or 25OAS are knocked down still retain IFN responses to suppress HCV replication, suggesting that there may be unidentified ISGs that show antiviral activities [15].

An HCV replicon system is an *in vitro* model that simulates stable and noncytopathic cellular autonomous replication of HCV genomic RNA [16,17]. There has been a lack of adequate HCV replication models, although this problem has been partially overcome by the introduction of the HCV replicon system. Replication of HCV replicon can be abolished by treatment with small amounts of type-I and type-II IFNs [15,18,19], suggesting intact IFN receptor-mediated cellular responses. In the present study, we analysed the expression profiles of ISGs by using the HCV replicon system which expresses chimeric luciferase reporter protein [20,21], and by using overexpression analysis, we identified several ISGs that suppress HCV replication.

MATERIALS AND METHODS

Cells and cell culture

Huh7 and 293T cells were maintained in Dulbecco's modified minimal essential medium (Sigma, St Louis, MO, USA) supplemented with 10% foetal calf serum at 37 C under 5% CO_2. To maintain cell lines carrying the HCV replicon (Huh7/Rep or Huh7/Rep-Feo cells), G418 (Nakalai Tesque, Kyoto, Japan) was added to the culture medium at a final concentration of 500 μg/mL.

HCV replicon constructs and transfection

Hepatitis C virus replicon plasmid, pRep-Feo was derived from the HCV-N strain, pHC1bneo/delS [19]. The pRep-Feo expressed chimeric reporter protein of firefly luciferase (Fluc) and neomycin phosphotransferase [20,21]. The replicon RNA synthesis and transfection have been described (Huh7/Rep-Feo) [17].

Establishment of the cured Huh7 cells

Cured Huh7 cells (cHuh7) were established by eliminating replicon from Huh7/Rep-Feo cells by treatment with 100 U/mL of IFN-alpha for 14 days. Clearance of replicon RNA was confirmed by RT-PCR and by the loss of resistance to G418.

Reverse transcription and Light Cycler-based PCR assay:

Total cellular RNA was extracted from Huh7, Huh7/Rep, and cHuh7 cells using Isogen (Wako, Osaka, Japan). Two micrograms of total cellular RNA was used to generate cDNA from each sample using SuperScript II (Invitrogen, Carlsbad, CA, USA) reverse transcriptase. The mRNA expression levels were measured with a Light Cycler PCR and detection system (Roche Applied Science, Indianapolis, IN, USA). Thermocycling was done in a final volume of 10 μL containing 1 μL cDNA sample or calibrator; 1.25 mM $MgCl_2$; 0.5 μM of each primer and 1 μL of LightCycler FastStart DNA Master SYBR Green 1 mix (Roche). Cycle numbers of the logarithmic linear phase were plotted against the logarithm of the concentration of template DNA. The concentrations of DNA in the samples were calculated by comparing the cycle numbers of the logarithmic linear phase of the samples with the external standards. Genes assayed were IP10, IFI-56K, MxA, GBP-1, IFI-6-16, TAP1, 9-27, IFP35, PLSCR1, LMP7-E1 and PKR (Table 1).

Construction of plasmids expressing ISG and the reporter assay

We constructed plasmids expressing 18 ISGs, which were expressed in the liver during acute HCV infection [6] and induced by IFN-alpha treatment of Huh7 cells (Table 2). The full-length human ISGs were amplified by PCR from Huh7, HeLa, or SuperScript cDNA library human liver (Invitrogen) and cloned into pcDNA3.1 or pcDNA3.1D/V5-His-TOPO (Invitrogen) to yield the mammalian expression construct, pcDNA-ISG. Each ISG-expression plasmid, pcDNA-ISG was transfected into Huh7/Rep-Feo cells, and the replication level of HCV replicon was analysed by luciferase assay. A plasmid, pcDNA3.1D/V5-His/lacZ (Invitrogen) was used as a control plasmid vector for mock transfection.

Table 1 List of interferon-stimulated genes analysed by the RT-PCR

Category/gene	Gene accession number
Cytokines/Chemokines	
IP10	X02530
MK	M94250
IL8	NM000584
Antiviral genes	
MxA	M30817
PKR	M35663
GBP-1	NM002053
IFI-56 K	M24594
25OAS	NM003733
Transcription factors	
IFP35	U72882
IRF-9 (ISGF3gamma)	XM033291
STAT1a	M97935
STAF50	X82200
Interferon inducible genes	
IFI-6-16 (G1P3)	BT006850
ISG15	M13755
IFI-27 (ISG12)	X67325
Apoptosis-related genes	
PLSCR1	AF098642
TRAIL	U37518
XAF1	X99699
Proteasome	
LMP7-E1	Z14982
MECL1	X71874
RING4 (TAP1)	X57522
Antiproliferative genes	
9–27	J04164
Immune modulation	
Mac2BP	L13210
Unknown	
RIG-G (IFIT4)	U52513
NP (IFI41,75)	L22342
HCV microtubul	D28915
IRF family	
IRF-1	NM002198
Cytoskeletal	
Beta-actin	X00351

Plasmids for signal transduction and the reporter assays

We analysed the effects of IFN on signal transduction of ISRE, interferon-gamma activation site (GAS), activator protein 1 (AP1), and nuclear factor-kappa B (NF-kappa B). Plasmids, pISRE-TA-Luc, pGAS-TA-Luc, pAP1-TA-Luc, and pNF-kappaB-Luc (Clontech Laboratories, Franklin Lakes, NJ, USA) contained consensus motifs upstream of the firefly luciferase gene. A plasmid, pTA-Luc (Clontech), which lacks the enhancer element, was used for background determination. The reporter plasmid of IFI-56K promoter was constructed. IFI-56K natural promoter (IFI-56K promoter -250-+93; gene accession number; X06559) was cloned from genomic DNA by PCR. The DNA product was inserted into pGL3 basic (Promega, Madison, WI, USA) as instructed (IFI-56K-Luc). Plasmid, pRL-CMV (Promega), which expresses the renilla luciferase protein, was used for normalization of transfection efficiency [22].

Plasmids for HCV-IRES and reporter assay

To measure the effect of ISG-expression on the HCV-IRES-mediated translational efficiency, reporter assays using HCV-IRES-luciferase construct were performed. A plasmid, pCIneo.Rluc-IRES-Fluc, expressed bicistronic RNA from which renilla luciferase was translated in a cap-dependent manner and firefly luciferase was translated in an HCV-IRES-dependent manner. A plasmid, pCIneo.Rluc-IRES-Fluc was tranfected with the ISG-expression plasmid, pcDNA-ISG into naive Huh7 cells. Huh7 cells were seeded at 8×10^4 per well in 24-well plates on the day before transfection. A total of 200 ng of pCIneo.Rluc-IRES-Fluc were cotransfected with 200 ng of pcDNA-ISG or pUC19-shRNA331 into each well by using 1 μL of lipofectamine 2000 (Invitrogen) according to the manufacture's protocol. At 48 h after transfection, the cell lysates were collected and dual luciferase assays were performed. Plasmid, pUC19-shRNA331, which was directed towards the HCV-5′-untranslated region (UTR) and inhibited translation of HCV, was transfected into naive Huh7 cells as a positive control which suppressed the activity of HCV-IRES.

Synthetic shRNA and shRNA-expression plasmid

The ISG-directed short hairpin RNA (shRNA)-expression vectors (pUC19-shRNA-ISG) were designed and constructed as described previously [21]. Briefly, oligodeoxyribonucleotides encoding shRNA sequences were synthesized and cloned just downstream of human U6 promoter in the plasmid pUC19. To avoid problems due to structural instability of DNA strands arising from the tight palindrome structure to transcribe shRNA, several point mutations were introduced in the sense strand of the shRNA sequences, which fully retained silencing activity of the shRNA [21,23]. Sequences of the shRNAs were as follows; shRNA-IFI-6-16; 5′-TGA AGC CCA GCG CGG GCA GCC CGG CGA CTT TGG AGT CGC CGG GCT GCC CGT GTT GGG CTT TAT TTT TT -3′, shRNA-IFI-27; 5′- CGA TTC CCG CCG CAG TGA AGC CCA TGG CAT TGG TGC CAT GGG CTT CAC TGT GGC GGG AAT TGT TTT TT -3′, and shRNA-GBP-1; 5′-CGA GGC CCG TTG ACC TGG ATG CCT CCT GAC CAA TCA GGA GGC ATC CAG GTT AAC GGG CTT TGT TTT TT -3′. Two control shRNA vectors were used: pUC19-shRNA-control expressed shRNA directed towards an unrelated target, the Machado-Joseph disease gene, and an HCV shRNA directed towards the 5′-untranslated region of HCV genome, pUC19-shRNA331 which significantly suppressed HCV replication [21].

Table 2 Expression profiles of interferon-stimulated genes in naive Huh7, Huh7/Rep and cured Huh7 cells

Gene	Fold induction by IFN in Huh7	Fold induction by IFN in Huh7/Rep	Basal expression in Huh7/Rep	Basal expression in cHuh7
IP10	101.5	7.16	0.13*	0.58*
RIG-G	88.2	–	0.01	0.39
IFI-27	73.1	–	0.72*	–
IFI-56 K	71.8	24.7	0.41*	0.21*
MxA	46.5	12.6	0.49*	0.06*
IRF-9	36.8	–	0.49*	0.65*
GBP-1	14.8	5.51	0.38*	0.68
IFI-6-16	12.1	18.7	0.48*	0.26*
HCV microtubul	10.6	–	0.70	–
RING4	10.2	–	0.89	0.80
STAF50	10.1	–	0.98	–
TRAIL	9.37	–	0.58	–
9-27	9.87	124.6	0.56*	0.49*
IFP35	9.79	6.98	0.70*	0.73*
PLSCR1	6.70	–	0.62*	0.65*
STAT1a	6.33	–	0.92	–
NP	4.75	–	0.40*	–
LMP7	3.26	5.60	0.36*	0.16*
PKR	2.35	–	0.47*	–
Mac2BP	1.57	–	0.63*	0.06*
MECL1	1.34	–	1.50	0.56*
MK	0.99	–	0.70*	0.87
IL8	0.87	1.44	0.22*	0.60*
IRF-1	–	–	0.54*	0.13

IFN, interferon; ISGs, interferon-stimulated gene.
*P-values of <0.05.
Relative induction levels of ISGs by treating Huh7 or Huh7/Rep cells with 100 U/mL of interferon-alpha.
Values represent relative expression levels in comparison with those of naive Huh7 cells.

Luciferase assays

Luciferase activity was measured with a Lumat LM9501 luminometer (Promega) using a Bright-Glo Luciferase Assay System (Promega) or a Dual-Luciferase Reporter Assay System (Promega).

MTS assays

To evaluate cell growth and cell viability, MTS assays were performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega).

Western blot analysis

Western blotting was performed as described [20]. 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-labelled anti IgG antibody, and visualized by chemiluminescence using the ECL Western blotting Analysis System (Amersham Biosciences, Buckinghamshire, UK). Antibodies used were anti-6xHis, anti-V5 (Invitrogen), anti-PKR, anti-IRF-9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NS5A antibodies (kindly provided by Dr Kohara), and anti-beta-actin antibodies (Sigma, St Louis, MO, USA).

Statistical analyses

Statistical analyses were performed using Student's t-test; P-values of <0.05 were considered statistically significant.

RESULTS

Decreased expression levels of ISGs in cells expressing HCV replicon and in the cured Huh7 cells.

Type-I IFNs stimulate the expression of numerous ISGs [24]. We studied a set of these genes, which are overexpressed in the early phase of acute HCV infection, and elimination of virus from the liver of a chimpanzee that had been

experimentally inoculated with HCV (Table 1) [6]. Basal expression levels of the ISGs were quantified in naive Huh7 cells, HCV replicon-expressing cells (Huh/Rep), and in the cured Huh7 cells from which the replicon had been eliminated (cHuh7) (Table 2). Of 24 ISG tested, 22 ISGs were overexpressed by IFN-alpha treatment of naive Huh7 cells. The induction velocities of the ISGs were similar between Huh7/Rep and naive Huh7 cells. On the contrary, basal expression levels of the ISGs in the Huh7/Rep cells were significantly lower than in the naive Huh7 cells. Furthermore, the expression levels of ISGs were similarly decreased in the cHuh7 cells. These findings suggested that the decrease of ISGs in the replicon-expressing cells was not because of functional suppression by the replication of HCV genome or by the expression of the virus nonstructural proteins but because of the adaptation of cells in which ISGs were down-regulated, thereby enabling a higher level of HCV genomic replication.

Downregulation of IFI-56K promoter and ISRE promoter activity in replicon-expressing cells

To verify whether the decreased expression of the ISG in replicon expressing cells (Huh7/Rep) and in cured cells (cHuh7) was because of transcriptional suppression, reporter assays were performed using reporter constructs that directed ISRE-, IFI-56K-, NF-kappa B- and AP1-dependent promoters: i.e. pISRE-TA-Luc, IFI-56K-Luc, pNF-kappa B-Luc and pAP1-TA-Luc (Fig. 1). The luciferase reporter activities of pIFI-56K-Luc and pISRE-TA-Luc were significantly lower in Huh7/Rep than in the naive Huh7 (19.3 ± 1.46% and 15.1 ± 0.450%, respectively, Fig. 1a,b). Conversely, there was no difference in NF-kappa B and AP1-reporter activities between Huh7/Rep and naive Huh7 cells (Fig. 1c,d). These results suggest that the decrease of ISGs in Huh7/Rep cells is due to down regulation of the ISRE-dependent transcriptional regulatory domain.

The effect of ISG over-expression on HCV replication

Based on the above results demonstrating an overall decrease of ISG expression levels in the replicon-harbouring cells, we next conducted the following studies to screen ISGs, which were suppressed in the replicon-expressing cells, for their activities in suppressing intracellular HCV replication. To conduct the study, we constructed 18 plasmid vectors expressing respective ISG (pcDNA-ISGs), and analysed their anti-HCV activities by overexpression. Among the genes that were overexpressed in IFN-alpha treatment of Huh7 cells, 18 genes were subcloned into mammalian expression plasmids.

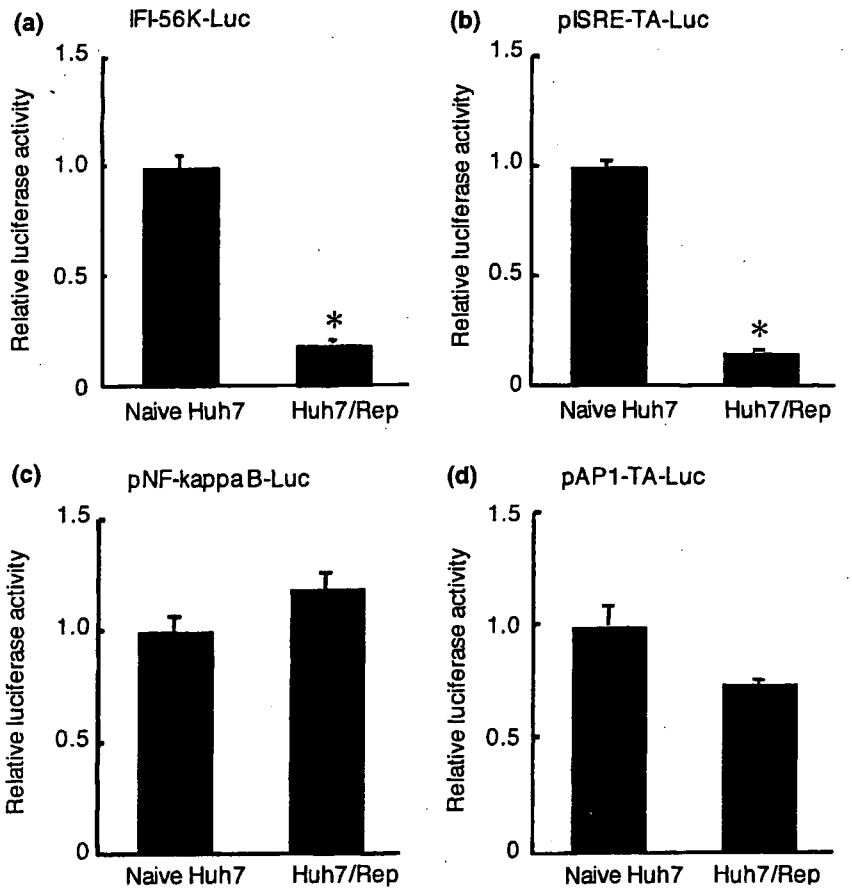

Fig. 1 Suppression of interferon-induced protein (IFI)-56K promoter and interferon-stimulated response element (ISRE) promoter activities in cells expressing hepatitis C virus replicon. Promoter activities of IFI-56K (panel A), ISRE (panel B), NF-kappa B (panel C), and AP1 (panel D) were measured by luciferase reporter assays. Reporter plasmids, IFI-56K-Luc, pISRE-TA-Luc, pNF-kappaB-TA-Luc, pAP-1-TA-Luc, and pTA-Luc were respectively transfected into naive Huh7 and Huh7/Rep cells together with pRL-CMV to normalize transfection efficiency. After 48 h of transfection, dual luciferase assays were performed. Error bars indicate mean + SD. *P-values of <0.05.

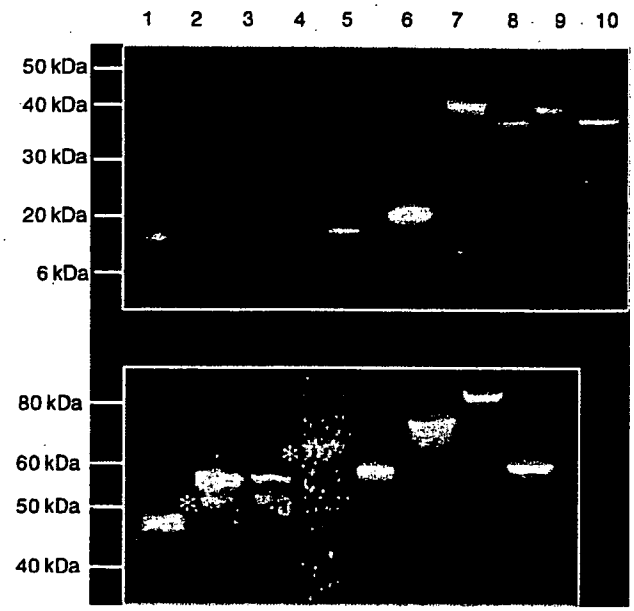

Fig. 2 Western blotting analysis of cells transfected with the interferon-stimulated gene (ISG)-expression plasmids. ISG expression vectors were respectively transfected into Huh7 cells or into 293T cells. The cells were harvested at 48 h after transfection. Ten micrograms of cell lysate was separated by SDS-PAGE and blotted onto a nylon membrane. The membrane was immunoblotted with anti-6xHis antibodies, anti-V5 antibodies, anti-protein kinase R (PKR) antibodies, or anti-interferon regulatory factor (IRF)-9 antibodies and detected by chemiluminescence reaction. Lane 1: IP10, lane 2; IL8, lane 3; IFI-27, lane 4; 9-27, lane 5; IFI-6-16, lane 6; ISG15, lane 7; PLSCR1, lane 8; LMP7-E1, lane 9; IFP35, lane 10; TRAIL, lane 11; IRF-1, lane 12; IRF-9, lane 13; IFI-56K, lane 14; PKR, lane 15; RIG-G, lane 16; GBP-1, lane 17; MxA and lane 18; 25OAS. *Expected size of IRF-9 in lane 12 or PKR in lane 14, respectively.

Transfection of each ISG-expression plasmid into Huh7 cells and Western blotting showed that each ISG-expression plasmid yielded a protein of the expected size (Fig. 2). We then transfected the pcDNA-ISG plasmids into Huh7/Rep-Feo cells, in which the expression levels of the replicon can be monitored by the luciferase assay. Transfection analyses showed that the replication level of HCV replicon was significantly suppressed by plasmid vectors expressing PKR (48.7 ± 7.2%), MxA (46.8 ± 5.6%), IRF-9 (44.8 ± 4.4%), GBP-1 (36.3 ± 7.5%), IFI-6-16 (37.4 ± 19.2%), IFI-27 (28.4 ± 1.2%), 25OAS(25.6 ± 4.1%) and IRF-1 (8.64 ± 1.13%) (Fig. 3a).

MTS assays of the cells transfected with pcDNA-ISG plasmids showed no significant effects on cell growth and viability, demonstrating that the effects of ISG transfection on the expression of the replicon were not because of cytotoxicity (Fig. 3b).

Similarly, Western blotting showed that the expression of NS5A protein was decreased by the overexpression of the ISGs (Fig. 3c).

The effects of ISGs on cellular signal transduction pathways

It has been reported that expressional levels of several host proteins affect the functions of various cellular signal transduction pathways. IRF-1, for instance, binds directly not only to IRF-E but also to ISRE and positively regulates the expression of ISGs [25,26]. To examine whether the ISGs that significantly suppressed HCV replication affect on cellular signal transduction pathway, the ISG-expression plasmids were respectively cotransfected with reporter plasmids, pISRE-TA-Luc, pGAS-TA-Luc, pNF-kappa B-TA-Luc, or pAP1-TA-Luc into Huh7 cells. An IRF-1 expression plasmid, pcDNA-IRF-1, was transfected as a positive control to activate ISRE, and interferon-gamma was used as a positive control to activate GAS. After the transfection, the expression of each ISG did not show any significant effects on ISRE-, AP1-, NF-kappa B, or GAS-luciferase reporter activities (Fig. 4).

The effects of GBP-1, IFI-6-16 and IFI-27 on the translational activity of HCV

Among the genes that have shown suppressive activities on HCV replication (Fig. 3), the antiviral activities of GBP-1, IFI-6-16 and IFI-27 have not been widely reported [27]. Therefore, we conducted further investigations on those genes. To verify that overexpression of the genes influences HCV-IRES-mediated translation, a reporter assay using HCV-IRES-luciferase plasmid was performed. A plasmid, pClneo-Rluc-IRES-Fluc was cotransfected with pcDNA-GBP-1, pcDNA-IFI-6-16, and pcDNA-IFI-27, respectively into Huh7 cells. Luciferase assay after 48 h of transfection showed that the IRES-dependent Fluc activity was not significantly changed by overexpression of GBP-1, IFI-6-16, and IFI-27 (Fig. 5), suggesting that GBP-1, IFI6-16 and IFI-27 had little effect on the expression of HCV proteins.

The effects of knock down of GBP-1, IFI-6-16 and IFI-27 on HCV replication

We subsequently investigated effects of suppression of GBP-1, IFI-6-16, or IFI-27 expression on HCV replication. To conduct the study, we used shRNA expression-plasmid vectors, pUC19-shRNA-GBP-1, pUC19-shRNA-6-16 and pUC19-shRNA-IFI-27, which expressed shRNA that targeted corresponding genes. The shRNA-expressing plasmids were cotransfected with plasmids expressing respective target genes into Huh7 cells. Western blots showed that the expression level of each protein was significantly suppressed

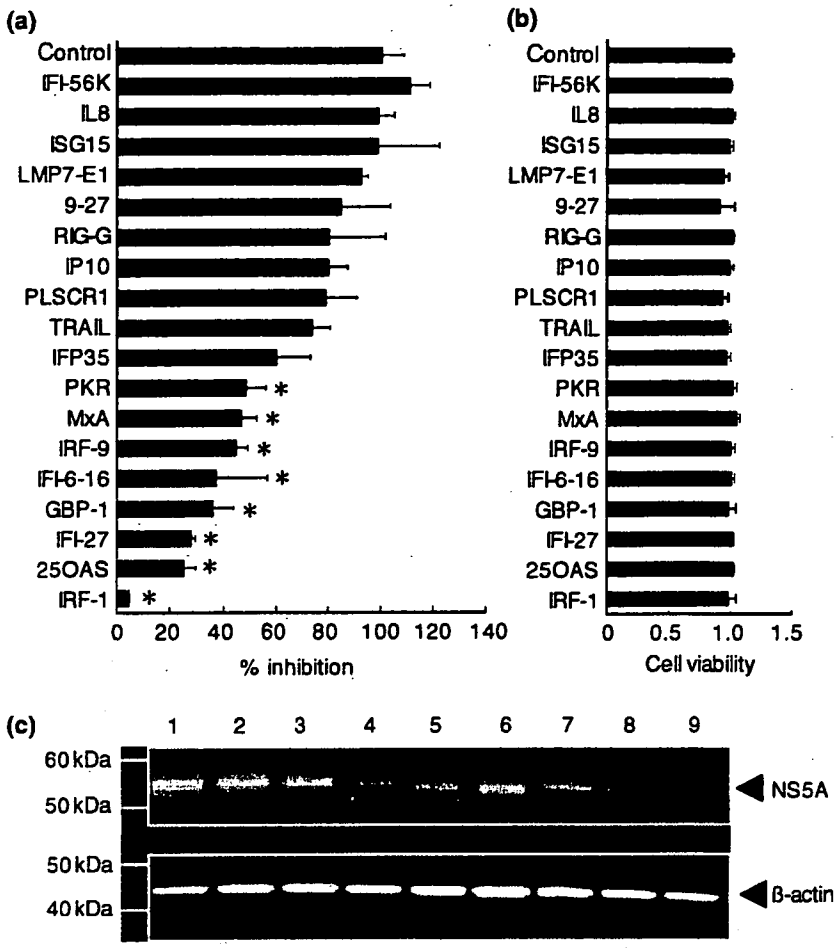

Fig. 3 The effect of ISG overexpression on *in vitro* hepatitis C virus replication. (a) The indicated interferon-stimulated gene (ISG)-expression plasmids were transfected into Huh7/Rep-Feo cells. At 48 h after transfection, luciferase activities were measured. The values were displayed as percentages of luciferase activities relative to that of Huh7/Rep-Feo transfected pcDNA3.1D/V5-His/lacZ (control). Error bars indicate mean + SD. *P-values of <0.05. (b) MTS assays of the cells transfected ISG-expression plasmids. (c) ISG-expression plasmids were transfected into Huh7/Rep cells. Forty-eight hours after transfection, the cell lysates were subjected Western blotting by using anti-NS5A antibody (upper panel) or anti-beta-actin antibodies (lower panel). Lane 1; pcDNA3.1D/V5-His/lacZ (control), lane 2; pcDNA-PKR, lane 3; pcDNA-MxA, lane 4; pcDNA-IRF-9, lane 5;pcDNA-IFI-6-16, lane 6; pcDNA-GBP-1, lane 7; pcDNA-IFI-27, lane 8; pcDNA-25OAS and lane 9; pcDNA-IRF-1.

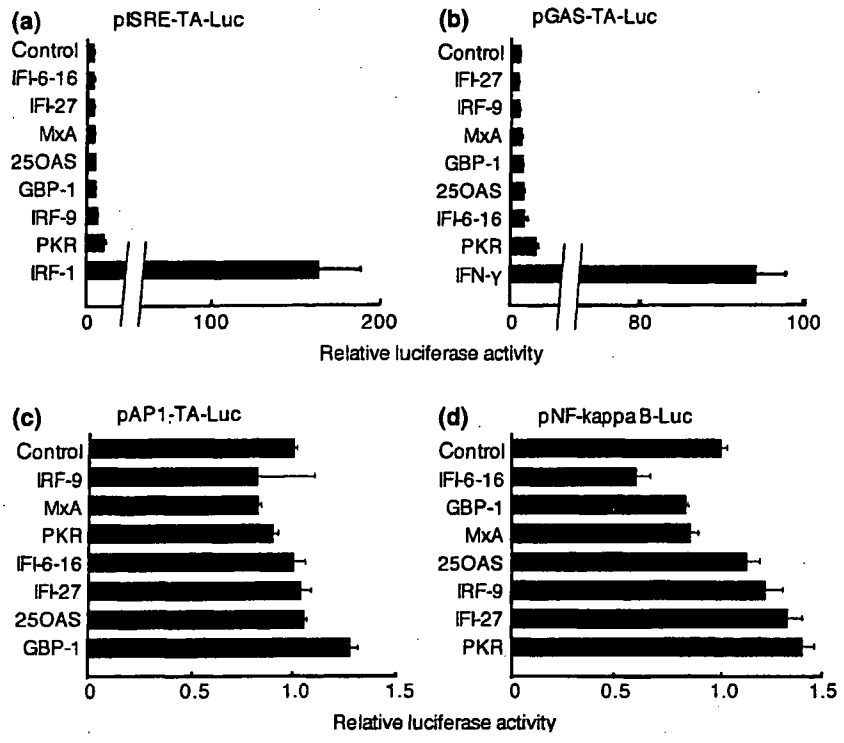

Fig. 4 The effects of interferon-stimulated genes (ISGs) on cellular signal transduction pathways. Reporter plasmids, pISRE-TA-Luc (panel A), pGAS-TA-Luc (panel B), pAP1-TA-luc (panel C), or pNF-kappa B-TA-luc (panel D) were cotransfected into Huh7 cells with the indicated ISG-expression plasmid vectors. At 48 h after transfection, the internal luciferase activities were measured. The values are displayed as relative to those in cells transfected with a control plasmid, pcDNA3.1D/V5-His/lacZ (control). Error bars indicate mean + SD. *P-values of <0.05.

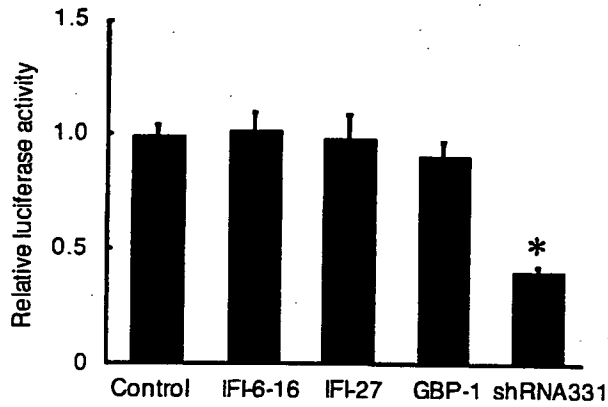

Fig. 5 The effect of guanylate binding protein (GBP)-1, interferon-induced protein (IFI)-6-16 and IFI-27 expression on hepatitis C virus (HCV)-internal ribosome entry site (IRES). HCV-IRES-luciferase construct, pClneo.Rluc-IRES-Fluc was cotransfected with the indicated ISG-expression plasmid, pcDNA-ISG or pUC19-shRNA331 into naive Huh7 cells. Luciferase activities were measured at 48 h after transfection. The values are displayed as relative to those in cells transfected with a control plasmid, pcDNA3.1D/V5-His/lacZ (control). Values were displayed as mean + SD. *P-values of <0.05.

by the respective shRNA-expressing plasmids (Fig. 6a). Thus, we next transfected the shRNA-expression plasmid into Huh7/Rep-Feo cells. Luciferase assays 48 h after transfection showed that expression levels of HCV replicon were significantly increased in the shRNA-transfected cells (46.2 ± 10.6% for shRNA-GBP-1, 34.0 ± 10.3% for shRNA-6-16, and 48.1 ± 28.1% for shRNA-IFI-27, respectively, Fig. 6b).

DISCUSSION

Persistence of virus replication in host cells is a function determined by the cellular antiviral system and by the counteraction of the virus to evade the antiviral responses [28]. In this study, we have found that the expression levels of individual ISGs were substantially decreased in HCV replicon-expressing cells (Huh7/Rep) compared with naive Huh7 cells (Table 2) which was because of a transcriptional suppression by the exclusive decrease of ISRE activities in Huh7/Rep (Fig. 1).

Activation of ISRE is mediated by ISGF-3 or by IRFs. However, in the absence of IFN stimuli, ISGF-3 is not the main activating factor for ISRE [29]. Thus, the IRFs, including IRF-1, IRF-3 and IRF-7, are potential regulators of basal ISRE activity. The attenuation of the cellular IFN-mediated transcriptional network has been reported by Geiss *et al.* [30]. We have further reported that expression of interferon regulatory factor-1 (IRF-1) is decreased in cells expressing replicon which may contribute to the enhanced replication of the viral genome [22]. Similarly in the nonhepatocyte HeLa cell lines which support replication of HCV replicon, ISRE activity was significantly lower in the replicon-expressing cells than in the naive cells. These findings suggest that the decreased baseline ISRE activities may contribute to the high permissiveness of HCV replicon.

The actions of IFN are mediated by expressional induction of various ISGs which have a wide spectrum of activities. The decreased ISG expression levels in the replicon-expressing cells and the enhanced HCV replication in the cured cells, in which ISG expression had been decreased, have led us to speculate that certain ISGs which were down-regulated in the replicon-expressing cells may have direct effects on viral replication. Thus we have investigated the IFN-induced proteins through [1] the generation of cells overexpressing IFN-induced gene products and [31] the generation of cells in which the expression of IFN-inducible gene products have been knocked down. Our overexpression analyses using the cell lines expressing HCV replicon (Huh7/Rep-Feo) identified eight genes that inhibited HCV replication and expression of the virus proteins; PKR, 2'5'OAS, IRF-1, IRF-9, MxA, GBP-1, IFI-6-16 and IFI-27 (Fig. 3a,c). These genes did not activate ISRE, GAS, NF-kappa B, or AP1 promoter activities (Fig. 4a–d), suggesting that the genes directly mediate antiviral effects without activating aberrant signal transduction pathways which may induce aberrant antiviral actions. Among the genes, the antiviral activities of PKR, 2'5'OAS, IFI-56K and MxA have been well documented [10,11,13,14], while the effects of GBP-1, IFI-6-16 and IFI-27 on virus replication have not been reported, with the exception of one study on IFI-6-16 [27]. Overexpression of GBP-1, IFI-6-16 and IFI-27 did not influence HCV-IRES-mediated translation efficiency (Fig. 5); shRNA-directed knock down of the genes caused a significant increase in the HCV replication level (Fig. 6). These results suggest that GBP-1, IFI-6-16 and IFI-27, which we have identified as having antiviral activities, may contribute to the IFN-induced cellular antiviral responses.

GBP-1 is thought to belong to a group of large GTP-binding proteins such as Mx and dynamin [32–34]. There is one report that GBP-1 mediates antiviral effects against vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) in HeLa cells [31]. Dong *et al.* [35] have recently reported antiviral action of phospholipids scramblase 1 (PLSCR1) on VSV replication. In our present study, overexpression of GBP-1 significantly suppressed replication of HCV replicon and suppression of GBP-1 caused HCV replication levels to increase, while overexpression of PLSCR1 did not affect HCV replication (Figs 3 and 6). These findings suggest that GBP-1 may suppress intracellular HCV replication and demonstrate that the expression of GBP-1 in the absence of IFN treatment results in inhibition of viral replication. However, little is known about the underlying antiviral mechanisms. Our preliminary data showed that overexpression of GBP-1 did not suppress EMCV-IRES-mediated translation efficiency (data not shown). It is poss-

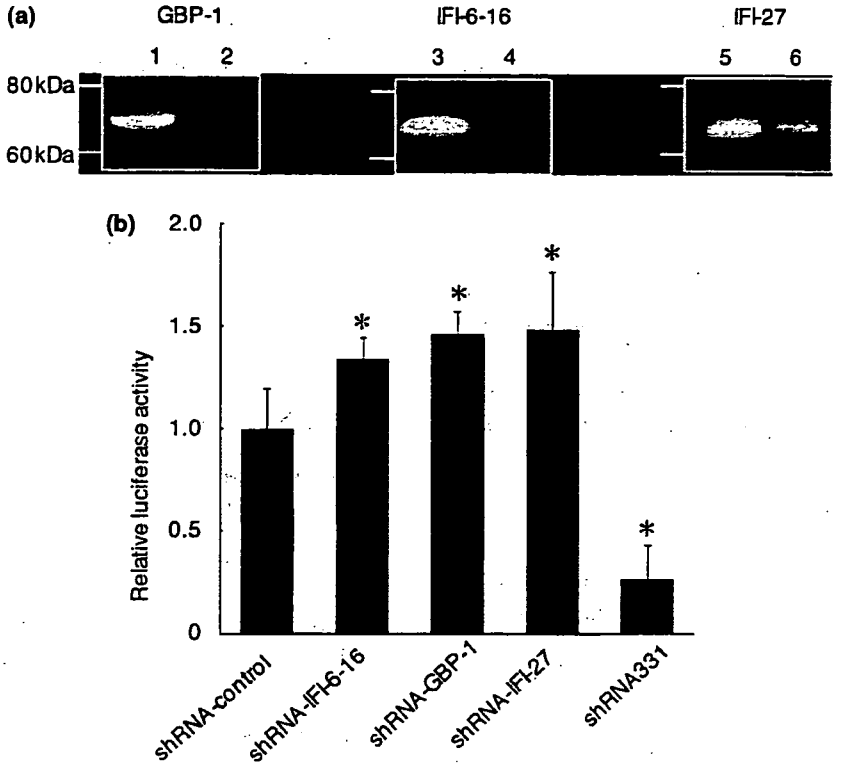

Fig. 6 Effects of shRNA-directed suppression of guanylate binding protein (GBP)-1, interferon-induced protein (IFI)-6-16 and IFI-27 expression on hepatitis C virus (HCV) replication. (a) The indicated interferon-stimulated gene (ISG)-expression plamids, pcDNA-GBP-1 (Lanes 1 and 2), pcDNA-IFI-6-16 (Lanes 3 and 4), or pcDNA-IFI-27 (Lanes 5 and 6) were cotransfected with shRNA-expressing plasmid, pUC19-shRNA-control (Lanes 1, 3 and 5) or pUC19-shRNA-GBP-1 (Lane 2), pUC19-shRNA-IFI-6-16 (Lane 4), pUC19-shRNA-IFI-27 (Lane 6) into Huh7 cells. Cells were harvested at 48 h after transfection and Western blotting was performed using anti-6xHis or anti-V5 antibodies. (b) Effects of ISG suppression on HCV replication. Plasmids, pUC19-shRNA-ISG, pUC19-shRNA331, or pUC19-shRNA-control were respectively transfected into Huh7/Rep-Feo cells, and luciferase activities were measured after 48 h. The values are displayed as relative to those in cells transfected with a control plasmid, pcUC19-shRNA-control. Values were displayed as mean + SD. *P-values of <0.05.

ible that GBP-1 has functional homology with MxA which shows GTPase activity and mediates degradation of cellular RNA, general repression of protein synthesis and apoptotic cell death [13]. It has also been reported that a Th1 cytokine, IFN-gamma, suppressed replication of HCV replicon [15]. As GBP-1 is a major IFN-gamma-induced proteins in human cells [36], GBP-1 might also contribute to IFN-gamma-directed inhibition of HCV replication.

A group of small ISGs including IFI-27 and IFI-6-16 remains uncharacterized, and their functions are still basically unknown [37–42]. IFI-6-16 is a hydrophobic protein and thought to be present in the membrane. It is postulated to be a precursor protein but its signal peptide region has not been confirmed. Little is known about molecular functions of IFI-6-16 except that its promoter region contains one ISRE [43]. One study has reported on the antiviral effect of IFI-6-16 on HCV replication, however, the mechanism of action remained uncharacterized [27]. The study reported that the expression of IFI-6-16 protein enhanced IFN-mediated antiviral activity, while transfection of IFI-6-16 alone did not cause significant inhibition of HCV replication. IFI-27 (also referred to as ISG12) is a putative highly hydrophobic protein of 122 amino acids which has a 33% overall sequence similarity to IFI-6-16. IFI-27 is unique in that it is the only protein found thus far to localize to the nuclear envelope, although its function in the nuclear envelope has not yet been explored [44]. It has also been reported that ISG12 exerts protective effects during lethal Sindbis virus infection and that ISG12 is the only ISG with greater expression in the disease recovery phase than the acute phase of the viral infection [45]. In our present study, overexpression of IFI-6-16 or IFI-27 inhibited replication of HCV replicon (Fig. 3), and the shRNA-knock down of each gene caused HCV replication to increase (Fig. 6). These findings also suggest that the expression of IFI-6-16 or IFI-27 without IFN treatment inhibits HCV-replication. In a previous DNA microarray study in an HCV infection model, IFI-6-16 gene expression was highly induced at the early stage of infection, while IFI-27 was induced in both the acute and recovery phases of infection [6]. Because neither of the proteins suppressed HCV-

IRES-directed translation, their actions against HCV may not involve translation of the virus protein, but rather the inhibition of post-translational processing, or blockage of the assembly of nonstructural proteins to form replicase complex.

In conclusion, the downregulation of ISG expression levels in cells supporting HCV subgenomic replication, which we have demonstrated in this study, indicates that cellular interferon responses are essential for the regulation of HCV replication. The response of IFN is directed by a synergistic action of various ISGs, including the gene that we have newly identified, each of which mediates antiviral actions. Further investigation of their antiviral actions may help elucidating the cellular defence mechanisms against virus infection and will provide new direction for investigating how IFNs protect cells against viral infection.

ACKNOWLEDGEMENTS

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Site-specific mutation of the interferon sensitivity-determining region (ISDR) modulates hepatitis C virus replication

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SUMMARY. The number of amino acid substitutions in the interferon sensitivity-determining region (ISDR) in the non-structural 5A (NS5A) gene of hepatitis C virus (HCV) is closely associated with the interferon (IFN) response and viral load. Several HCV replicon-based studies have reported that ISDR sequences had an influence on viral replication *in vitro*. However, it is unclear as to how different ISDR sequences affect HCV replication. Various clinically observed ISDR sequences were introduced into HCV replicons and their contribution to viral replication was investigated using a colony formation assay and/or a transient replication assay. A mapping study of the ISDR was performed to identify the amino acid positions that critically affect replication. While no colonies were formed in the colony formation assay using HCV replicons with few mutations (0, 1 and 3) in the ISDR, numerous colonies (>200) appeared when using constructs with six mutations. Introduction of various distinct ISDR sequences with multiple mutations resulted in replication enhancement in transient assays. A mapping study identified several specific sites in the ISDR that critically affected replication, including codon 2209 which, in patients, was closely associated with a strong response to IFN. ISDR sequences associated with a clinical IFN response and viral load modulated the replication of HCV replicons, suggesting the importance of the ISDR sequence in HCV infection.

Keywords: HCV replicon, NS5A, ISDR.

INTRODUCTION

Hepatitis C virus (HCV) is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma worldwide [1]. Interferon (IFN)-alpha remains the key anti-viral agent against HCV, even in the era of combination therapy with pegylated-IFN and ribavirin [2,3]. However, because the eradication rate does not exceed half of the patients treated overall, the molecular mechanism(s) enabling HCV to survive during IFN treatment should be elucidated to enable the development of more effective therapeutic modalities.

The interferon sensitivity-determining region (ISDR) at position 2209–2248 of NS5A in the HCV genome was originally identified as the viral genomic element in which codon changes were closely related to the clinical IFN response, i.e. the more substitutions in the ISDR, the more favourable the IFN response [4–6]. The close correlation of the ISDR mutations with IFN responses has been confirmed by other researchers and by a recent meta-analysis [7–10]. Amino acid substitutions in the ISDR were also associated with serum HCV-RNA levels, indicating that ISDR sequences have an important role in HCV replication. Because HCV clearance was strongly associated with the expression of numerous IFN-stimulated genes induced by endogenous IFN in the natural course of infection, the IFN-response and viral replication capacity cannot be considered separately [11]. It is thought that clearance and replication of HCV is controlled by a tug of war between IFN and HCV. Thus, the NS5A protein might perturb this process according to the ISDR sequence.

A variety of putative NS5A functions have been postulated to date on the basis of studies *in vitro*. These include binding to TRADD [12], Grb2 [13], p21 [14,15], amphiphysin II [16] and other proteins that may influence the pathogenesis of HCV through their anti-viral effects, modulating apoptosis, signal transduction, or regulating the cell

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Abbreviations: ISDR, interferon sensitivity-determining region; NS5A, nonstructural 5A; HCV, hepatitis C virus; IFN, interferon.

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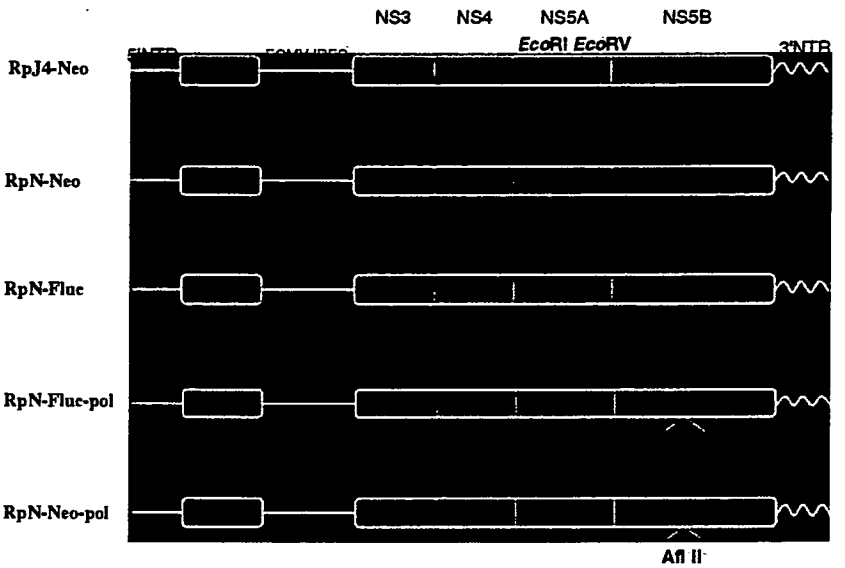

Fig. 1 Construction of HCV replicons. HCV replicons derived from HCV-N and HC-J4 isolates, and with the reporter genes *Neo* or *Fluc*, were used in this study. Using *Eco*RI and *Eco*RV digestion sites for HC-J4, and *Bam*HI and *Eco*RV sites for HCV-N, ISDR sequences could be substituted by mutated sequences, as demonstrated.

cycle [15,17–20]. Meanwhile, studies focusing on ISDR sequences were quite restricted and most of these reports emphasized the importance of PKR as a binding counterpart of NS5A, because this protein binds to NS5A in an ISDR-dependent manner [21,22]. However, more recent studies could not prove any effect of NS5A on the activity of PKR either in Huh7 or HeLa cells [23,24], and the role of PKR remains unclear.

The subgenomic HCV replicon developed recently simulates HCV replication and is a valuable model for NS5A protein analysis, because NS protein function is required for replication in this system [25]. However, the HCV replicon needs to adapt to the cell culture environment and these adaptive mutations are considered to be detrimental to HCV infection *in vivo* [26,27]. Importantly, even though these adaptive mutations were observed throughout the subgenome, most were found to be clustered in the central region of NS5A. This finding demonstrates the significance of NS5A sequences for HCV replication *in vitro* as well as *in vivo*, although they have opposing effects on replication in the two systems [26,28]. In NS5A, the serine cluster region has been much focused upon as the target for adaptive mutations because of its strong adaptive mutational effect and its influence on NS5A protein phosphorylation [26,29–31]. Although the ISDR was reported to be the target of adaptive mutations [26,32,33], and is located just downstream of the serine cluster region, its role in the replication of HCV replicons has not been investigated in detail.

On the other hand, we have recently conducted a clinical study of 334 patients with HCV-1b who were treated with IFN and found a close correlation between the IFN response and several individual mutations in the ISDR, suggesting that the effect of mutations in the ISDR varies according to the site [34]. This correlation also was reported in a meta-analysis study [10]. Because the tertiary structures of the NS5A protein determine their functions and affinity with host or other viral proteins, it may be speculated that the ISDR function is not simply regulated by the number of mutations, but by the resultant NS5A protein conformation as determined by the respective ISDR amino acid sequences.

Therefore, using the HCV replicon system, we investigated how a variety of ISDR sequences from various clinical backgrounds influence replication, and whether there exist site-specific mutations critically affecting replication, as predicted by the clinical findings.

MATERIALS AND METHODS

Cell culture

Human hepatoma Huh7 cells were grown in Dulbecco's modified minimal essential medium (Sigma, St Louis, MO, USA) supplemented with 2 mM L-glutamine, non-essential amino acids, 100 IU of penicillin, 100 mg/L of streptomycin, and 10% foetal bovine serum at 37 C under 5.0% CO_2. G418 (Wako, Osaka, Japan) was added to the culture medium at a final concentration of 200 μg/mL for cells carrying HCV replicons. The cells were split twice weekly at 1 : 5 to 1 : 6, depending on their confluence.

Plasmid constructions

Two replicon-harbouring plasmids were used as starting materials: a plasmid 1bneo/delS containing the replicon construct derived from the chimpanzee-infectious clone HCV-N [35] (GenBank accession no. AF139594) and a plasmid pRpJ4 containing the replicon construct derived from the chimpanzee-infectious clone HC-J4 [33,36] (Fig. 1). Both HCV isolates were classified as genotype 1b. Into these plasmids, the firefly luciferase gene (*Fluc*) or the neomycin phosphotransferase gene (*Neo*) was introduced as a reporter

of replication. Next, in order to introduce various ISDR sequences into 1bneo/delS, a *Bam*HI site [nucleotide (nt.) 5302 to 5307 of 1bneo/delS] and an *Eco*RV site (nt. 5335 to 5340 of 1bneo/delS) were introduced into the NS5A region by site-directed mutagenesis (Quick-Change Multi Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA). Mutagenesis primers were as follows: ISDR *Bam*HI-mutagenesis, 5′-CGTAGGTTGGCCAGGGGATCCCCCCCCTCCTTGGCC-3′, and ISDR *Eco*RV-mutagenesis, 5′-GAGGGGGATGAGAATGAGATATCCATTGCGGCGGAG-3′. Digestion with *Bam*HI and *Eco*RV excised a DNA fragment encompassing nt. 5304 to 5537 of 1bneo/delS, including the ISDR. The DNA fragment was subcloned into the pLITMUS 28i vector (New England Biolabs, Beverly, MA, USA) and various ISDR mutations were introduced by site-directed mutagenesis of the pLITMUS 28i vector. Finally, these ISDR-mutated *Bam*HI–*Eco*RV fragments were subcloned back into the parental plasmid. From pRpJ4, digestion with *Eco*RI and *Eco*RV excised a DNA fragment encompassing nt. 5084 to 5528, including the ISDR, without mutagenesis. Likewise, various mutations were introduced into this DNA fragment in the subcloning vector pLITMUS28i. As replication-deficient controls, HCV-N derived replication-deficient replicons were constructed by introducing an NS5B frameshift. In order to construct this NS5B frameshift, plasmid 1bneo/delS was digested with *Afl*II, then subjected to blunting using a DNA Blunting Kit (Takara, Tokyo, Japan) and finally self-ligated (RpN-Fluc-polΔ as a replication-deficient control for RpN-Fluc and RpN-Neo-pol$\not{\text{G}}$ as a replication-deficient control for RpN-Neo).

In vitro transcription and transfection

Synthesis and transfection of the replicon RNA were performed as described previously. Briefly, the replicon RNA was synthesized from the linearized replicon plasmid using the RiboMax Large Scale RNA Production System (Promega, Madison, WI, USA). Transcription was terminated by the addition of 1 U of DNase and the transcribed RNA was purified by ISOGEN (Wako) according to the manufacturer's protocol. The replicon RNA was transfected into Huh7 cells by electroporation. Cells suspended at a density of 5×10^6 cells in 500 μL were mixed with 10 μg of replicon RNA and subjected to an electric pulse of 1050 μF and 270 V in an electroporation cuvette with a 4-mm gap width, using the EasyJect system (EqiBio, Middlesex, UK).

Luciferase assays

After transfection of the replicon RNAs expressing the luciferase reporter protein, the cells were harvested at 4 and 96 h, and the luciferase activities of the cell lysates were measured using a luminometer (TD-20/20; Turner Designs, Sunnyvale, CA, USA) with the Bright-Glo kit (Promega).

Colony formation assay

After transfection of the replicon RNAs expressing neomycin phosphotransferase, cells were cultured in the presence of 200 μg/mL G418. Three weeks after the transfection, G418-resistant cell colonies were stained with neutral red (Sigma-Aldrich Corp., St Louis, MO, USA), and colony-forming units (CFU) were determined.

Analyses of clinical data of IFN-treated patients

In order to associate the results of HCV replication *in vitro* with the clinical response to IFN, we used our previously reported database of 334 patients who had received IFN monotherapy between 1994 and 1998 [34].

RESULTS

HCV replication in vitro is closely associated with the total number of amino acid substitutions in the ISDR

In the previous study using an HCV-N-derived replicon, four amino acid substitutions plus four amino acid insertions into the ISDR enhanced replication dramatically, suggesting that the ISDR has an important role in determining replication [32]. Therefore, in order to determine how ISDR sequences modulate HCV replication *in vitro* according to the number of mutations in other HCV isolates, we first made replicons with different numbers of mutations in the ISDR: RpJ4-Neo ISDR(0), RpJ4-Neo ISDR(1), RpJ4-Neo ISDR(3) and RpJ4-Neo ISDR(6) using a HC-J4-derived replicon with a *Neo* reporter gene (Fig. 2a). Consistent with the original reports [34], clinical analysis of 334 patients treated with IFN revealed that ISDR sequences with few mutations (zero, one, or three mutations) were associated with a low IFN response because the CR rate was low (8%, 13% and 0%, respectively), while those with six mutations were associated with a high IFN response (CR rate: 100%). As demonstrated in Fig. 2b, while RpJ4-Neo ISDR(0), RpJ4-Neo ISDR(1), and RpJ4-Neo ISDR(3) did not form any G418-resistant colonies, RpJ4-Neo ISDR(6) formed numerous G418-resistant colonies (>200). The data demonstrated that replication of the HC-J4-derived replicon is associated with the ISDR sequence, and might be enhanced by an increase in the number of ISDR mutations, irrespective of the source of the HCV isolate.

Next, we investigated how ISDR sequences with different mutational patterns affect replication *in vitro*. Several distinct ISDR sequences with multiple mutations, mirroring those observed in patients, were introduced into the HCV-N-derived replicon, RpN-Fluc (six substitutions, four substitutions plus four insertions, seven substitutions plus one insertion, and total ISDR-deletion; Fig. 3a), and the replication capacities were measured as luciferase activities. Some patients received

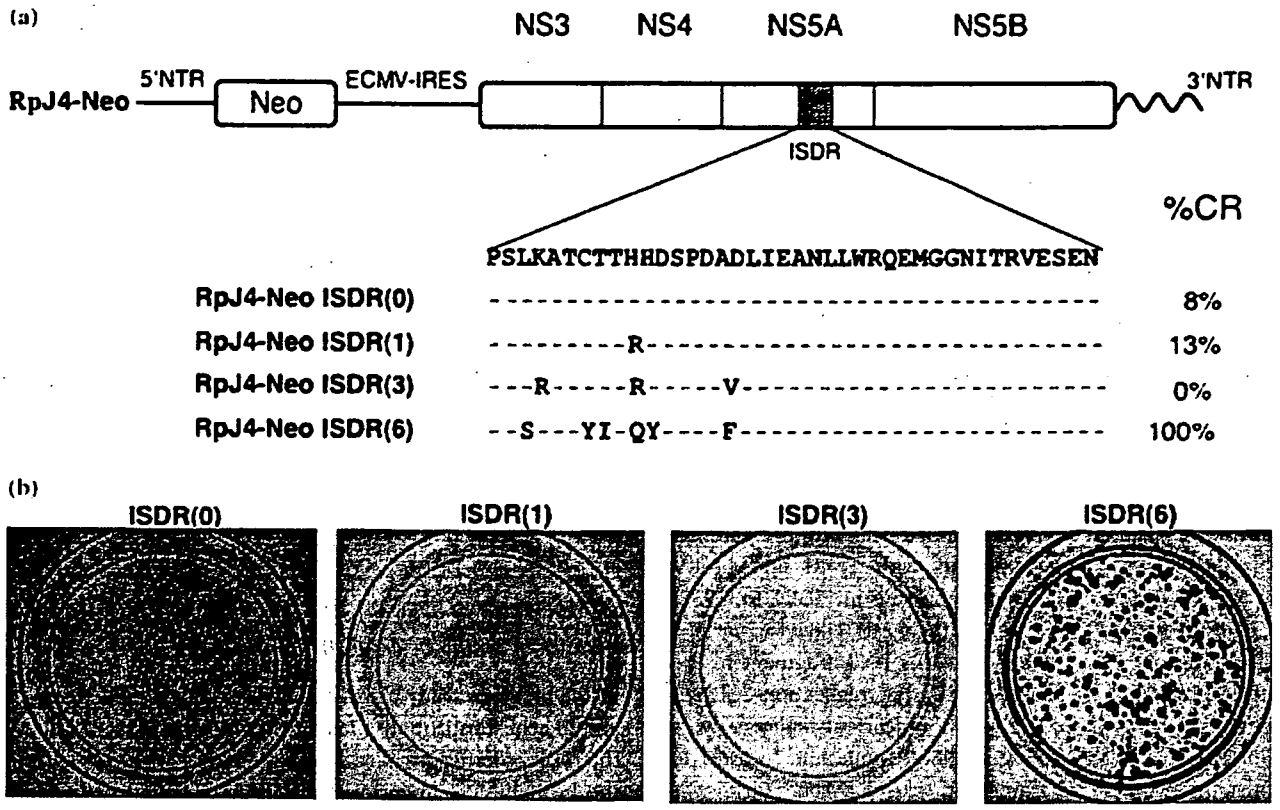

Fig. 2 HCV replication *in vitro* is regulated by the total number of amino acid substitutions in the product of the ISDR. (a) Alignment of ISDR sequences in HC-J4 replicons with different numbers of mutations in the ISDR. These ISDR sequences were obtained from patients with various IFN responses. As indicated to the right, the CR rate for IFN monotherapy was low in patients with few mutations (8% for 0, 13% for 1, 0% for 3). In contrast, the CR rate was 100% in patients with six mutations, according to analysis of 337 patients receiving IFN monotherapy. (b) In the colony formation assay, replicons with few mutations (0–3) in the ISDR could not form any colonies. In contrast, a replicon with six codon changes formed numerous colonies (>200), suggesting the replication capacity is enhanced according to the number of mutations in the ISDR.

IFN therapy, and their response rates are shown in Fig. 3a. In order to optimize the assay, we introduced an additional mutation (S2201del), one of the cell culture-adaptive mutations, into the NS5A serine cluster region of these replicons to enhance baseline replication. It is known that serine cluster mutations and ISDR mutations enhance the replication of replicons synergistically [32,33]. As demonstrated in Fig. 3b, intracellular replication was greatly enhanced by the introduction of multiple mutations into the ISDR, even though the mutational patterns were all different. In contrast, deletion of the entire ISDR completely abolished replication capacity. The data indicate that multiple mutations in the region enhance replication, irrespective of different ISDR sequences. At the same time, however, enhanced replication competency was not completely proportional to the number of amino acid mutations. Specifically, RpN-Fluc ISDR(6) was more replication-competent than RpN-Fluc ISDR(4s + 4i) or RpN-Fluc ISDR(7s + 1i), also suggesting that the mutational effect enhancing replication might be different according to the positions of the mutations.

HCV replication in vitro is influenced by site-specific substitutions in the ISDR

We have reported previously that clinical outcomes of IFN therapy were not only determined by the total number of ISDR mutations, but also by the sites of those mutations. Among them, codons 2209 ($P = 0.02$), 2216 ($P = 0.01$), and 2227 ($P = 0.02$) were significantly associated with a higher response than was found in the absence of these mutations (Fisher's exact probability test), while codons 2218 and 2224 were associated with low IFN efficacy [34]. These findings led us to speculate that specific amino acid substitutions in the domain of NS5A specified by the ISDR may be associated with the functions of that protein and consequently with the efficiency of replication of the viral genome.

Thus, we subsequently investigated the replication capacity of replicon constructs possessing a single codon change at various sites in the ISDR. Six individual amino acid substitutions found at high frequency in the clinical study were

