DNA

pCMV-Luc encoding firefly luciferase gene was constructed, as described previously.[27] pDNA was purified using an Endo-free plasmid Giga kit (Qiagen, Valencia, CA). For the cellular association experiments, pDNA was radiolabelled with [α-^{32}P]dCTP by nick translation.[28] For the activation experiments, all DNA samples were extensively purified with Triton-X-114, a non-ionic detergent, to minimize the activation by contaminated lipopolysaccharide (LPS). Extraction of endotoxin from pDNA, EC DNA, and CT DNA samples was performed according to reviously published methods[29,30] with slight modifications. DNA samples were purified by extraction with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and ethanol precipitation. Then, 10 mg DNA was diluted with 20 ml pyrogen-free water, followed by the addition of 200 μl Triton-X-114 and mixing. The solution was placed on ice for 15 min and incubated for 15 min at 55°. Subsequently, the solution was centrifuged for 20 min at 25°, 600 *g*. The upper phase was transferred to a new tube, 200 μl Triton-X-114 was added, and the previous steps were repeated at least three times. The activity of LPS was measured by *Limulus* amoebocyte lysate (LAL) assay using the Limulus F Single Test kit (Wako, Tokyo, Japan). After purification using the Endo-free plasmid Giga kit, 1 μg/ml pDNA contained 0·01–0·05 EU/ml endotoxin. After Triton-X-114 extraction, the endotoxin levels of the DNA samples could no longer be determined by LAL assay, i.e. 1 μg/ml DNA contained less than 0·001 EU/ml. Without extraction of endotoxin by Triton-X-114, 100 μg/ml naked pDNA, which contains 1–5 EU/ml endotoxin, could release 521 ± 73 pg/ml TNF-α at 24 hr from peritoneal macrophages.

Cationic liposome formation

Lipofectin complexes were prepared according to the manufacturer's instructions. In brief, DNA was diluted in 100 μl Opti-MEM per 1 μg DNA (solution A) and 5 μl Lipofectin reagent was diluted in another 100 μl Opti-MEM (solution B). Then solutions A and B were combined and mixed gently. After a 15 min incubation at room temperature, complex was added to the cells.

Cellular association experiments

DC2.4 cells cultured in 24-well plates were washed three times with 0·5 ml Hanks' balanced salt solution (HBSS) without phenol red and 0·5 ml HBSS containing 0·1 μg/ml naked [^{32}P]pDNA or 0·1 μg/ml [^{32}P]pDNA/Lipofectin complex was added. After incubation at 37 or 4° for a specified time, the HBSS was removed and the cells were washed five times with ice-cold HBSS and then solubilized with 1·0 ml 0·3 N NaOH with 0·1% Triton-X-100. Aliquots of the cell lysate were taken for the determination of ^{32}P radioactivity using an LSA-500 scintillation counter (Beckman, Tokyo, Japan) and the protein content was measured using the modified Lowry method with BSA as a standard.

Confocal microscopy

DC2.4 cells were washed three times with 1·0 ml HBSS and incubated with HBSS containing fluorescein-labelled pDNA (FL-pDNA) or FL-pDNA/Lipofectin complex. After a 3 hr incubation, the cells were washed five times and fixed with 4% paraformaldehyde for 10 min.

Cytokine secretion

BMDC or DC2.4 cells cultured in 24-well plates were washed three times with 0·5 ml RPMI-1640 before use. Naked DNA was diluted in 0·5 ml Opti-MEM. The cells were incubated with the naked DNA solution continuously for 8 hr. In the case of DNA/Lipofectin complexes, cells were incubated for 2 hr with 0·5 ml of the solutions containing the complexes. Then, the cells were washed with RPMI-1640 and incubated with RPMI-1640 with 10% FBS. After a 6 hr incubation, the supernatant was collected for ELISA and kept at −80°. The levels of TNF-α, IL-6, and IL-12p70 in the supernatants were determined by the OptEIA Set (BD Biosciences, San Diego, CA).

Results

Uptake of DNA with cationic lipid complexes is not saturated, although normal uptake is saturated in GM-CSF DC

TLR9 exists in the endosomal-lysosomal compartment.[13,14] The amount of naked DNA in the compartment can be limited because naked DNA is supposed to be taken up by DC via receptor-mediated endocytosis.[15] However, DNA/cationic lipid complexes are supposed to be taken up by DC via a non-specific mechanism based on electrostatic interaction, so-called adsorptive endocytosis. Therefore, cationic lipid Lipofectin was used to deliver DNA efficiently to the compartment. To examine the binding and uptake of naked pDNA and pDNA/cationic lipid complexes in DC, we carried out cellular uptake experiments using naked [^{32}P]pDNA and [^{32}P]pDNA/Lipofectin complexes. As expected, the uptake of naked [^{32}P]pDNA by DC2.4 cells at 37° was increased up to 2 hr (Fig. 1a). Following an incubation of 2–5 hr, the amount of DNA remained unchanged, probably due to continued uptake and degradation.[15] On the other hand, complexation with cationic lipids enhanced the DNA uptake. Cationic lipids enhanced [^{32}P]pDNA binding and

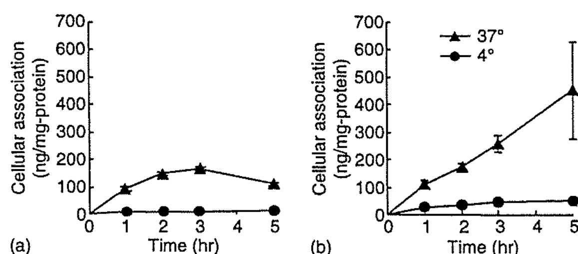

Figure 1. Cellular association time courses of naked [^{32}P]pDNA (a) or [^{32}P]pDNA/Lipofectin complex (b) in DC2.4 cells. Cells were incubated at 37° (closed triangle) or 4° (closed circle). Each point represents the mean ± SD (n = 3).

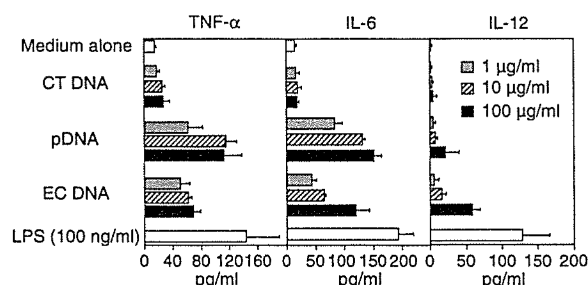

Figure 3. Cytokine secretion induced by naked DNA from GM-CSF DC. The cells were incubated with EC DNA, pDNA, or CT DNA for 8 hr. The supernatants were collected and the amount of TNF-α, IL-6, and IL-12 secreted from the cells was determined by ELISA. Each result represents the mean ± SD (n = 3).

uptake in DC2.4 cells and the amount of [^{32}P]pDNA increased in a time-dependent manner (Fig. 1b).

Next, we examined the localization of fluorescence-labelled DNA (FL-pDNA). In the confocal microscopy experiments, the fluorescence derived from naked FL-pDNA is bound to the cellular membrane at 4° (Fig. 2a). At 37°, FL-pDNA was observed inside the cells after 1 hr and it appeared to accumulate in the nucleus after a 3 hr incubation. On the other hand cationic lipids completely changed the localization of DNA. The fluorescence of the FL-pDNA/Lipofectin complex was observed in a punctuated pattern at 1 hr, then diffused into the cells after a 3 hr incubation (Fig. 2b).

The activation of GM-CSF DC by DNA

Next, cytokine production from DC by naked DNA was examined. Plasmid DNA and *E. coli* DNA were used as models of bacterial CpG DNA, and calf thymus DNA was used as a model of vertebrate DNA. As shown in Fig. 3, naked bacterial plasmid DNA and *E. coli* DNA with replete immunostimulatory CpG motifs induced TNF-α, IL-6 and IL-12 secretions from bone marrow-derived DC. The results are consistent with previous studies demonstrating that plasmid DNA stimulates GM-CSF DC to induce TNF-α and IL-12.[18] Vertebrate calf thymus DNA (CT DNA) containing less CpG motifs did not. LPS induced small amounts of cytokines, probably because of relatively short-term incubation (8 hr). Similar results were observed in the experiment using DC2.4 cells, although the cells released a higher amount of cytokines (Fig. 4). These results demonstrate that the cytokine secretion from the DC corresponds to the difference between endogenous DNA and exogenous DNA.

Next, cellular activation in DC by DNA/cationic lipid complexes was examined. The *E. coli* DNA/Lipofectin complexes stimulated GM-CSF cultured DC to produce cytokines, TNF-α, IL-6 and IL-12 in a dose-dependent manner (Fig. 5). Similar results were observed with pDNA/Lipofectin complex. The amounts of cytokines released from the DC were significantly increased by complex formation with cationic lipids compared with naked DNA (Fig. 3). The DC were unable to produce a significant amount of pro-inflammatory cytokines following

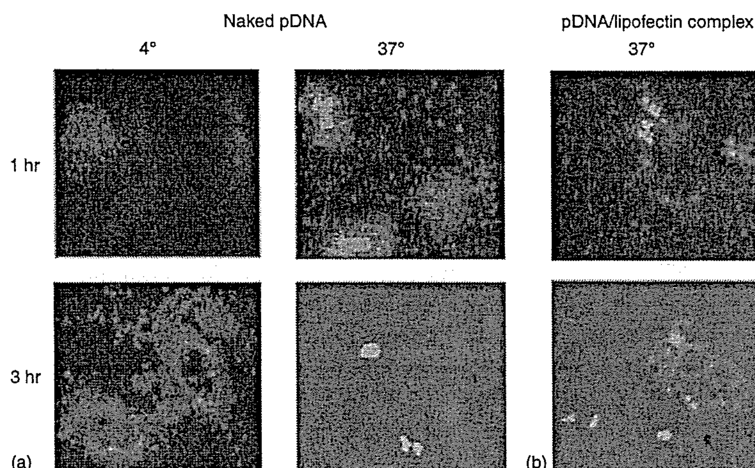

Figure 2. Uptake of naked FL-pDNA (a) or FL-pDNA/Lipofectin complex (b) by DC2.4 cells. The cells were incubated with 5·0 μg/ml naked FL-pDNA or 30 μg/ml FL-pDNA/Lipofectin complex.

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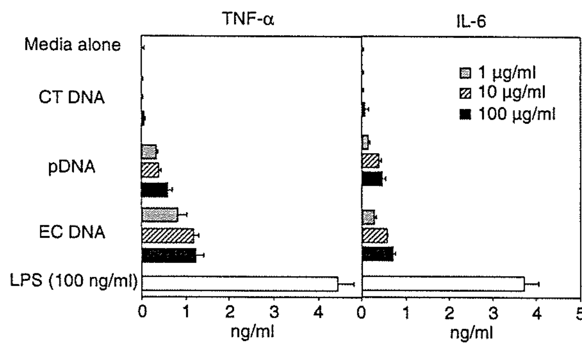

Figure 4. Cytokine secretion induced by naked DNA from DC2.4 cells. The cells were incubated with EC DNA, pDNA, or CT DNA for 8 hr. The supernatants were collected and the amount of TNF-α and IL-6 secreted from the cells was determined by ELISA. Each result represents the mean ± SD ($n = 3$).

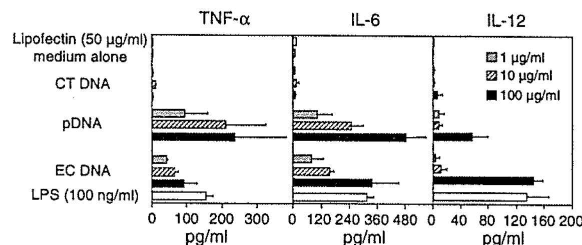

Figure 5. Cytokine secretion induced by DNA/Lipofectin complex from GM-CSF DC. The cells were incubated with EC DNA, pDNA, or CT DNA/Lipofectin complex (5 μl Lipofectin per 1 μg DNA). After a 2 hr incubation, liposomes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 hr after the incubation with liposomes. The amount of TNF-α, IL-6, and IL-12 secreted from the cells was determined by ELISA. Each result represents the mean ± SD ($n = 3$).

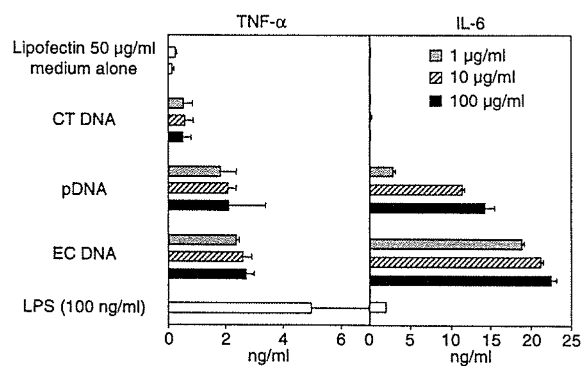

Figure 6. Cytokine secretion induced by DNA/Lipofectin complex from DC2.4 cells. The cells were incubated with EC DNA, pDNA, or CT DNA/Lipofectin complex (5 μl Lipofectin per 1 μg DNA). After a 2 hr incubation, liposomes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 h after the incubation with liposomes. The amount of TNF-α and IL-6 secreted from the cells was determined by ELISA. Each result represents the mean ± SD ($n = 3$).

stimulation with vertebrate calf thymus DNA (CT DNA) containing less CpG motifs when DNA is complexed to Lipofectin. Lipids alone were unable to stimulate the DC sufficiently to release pro-inflammatory cytokines. Similar results were obtained in DC2.4 cells (Fig. 6). These results demonstrate that GM-CSF DC discriminate between bacterial DNA and mammalian DNA.

Discussion

The most important role of immune system is to distinguish between 'self' and 'non-self'. Although the TLR9 subfamily (TLR7, 8 and 9) recognizes non-self nucleic acids[31] under special conditions, such as systemic lupus erythematosus, these TLRs are stimulated in response to self nucleic acids. For example chromatin–immunoglobulin complexes trigger DC activation in a TLR9-dependent and TLR9-independent manner.[32] Recently, Barton *et al.* have demonstrated that the fusion protein of TLR4/9, which is delivered to cellular membranes, is activated by vertebrate DNA.[33] One proposed hypothesis is that compartmentalization of TLR9 prevents the response induced by endogenous DNA.

In the present study, we have demonstrated that GM-CSF-derived DC activation is triggered by exogenous naked DNA. Bacterial DNA induces cytokine secretion from DC, although vertebrate DNA does not. Flt-3 L cultured murine DC (Flt-3 L DC) also induce activation of TLR9 in response to naked bacterial DNA, but not naked vertebrate DNA.[24] Therefore, these studies imply that both GMCSF-DC and Flt-3 L DC can discriminate between bacterial non-self DNA and vertebrate self DNA.

On the other hand, these characteristics are different from murine macrophages.[22] Primary macrophages do not respond to naked DNA in spite of TLR9 expression, although the macrophage-like cell line RAW264.7 cells do. Both primary macrophages and DC take up DNA via a similar mechanism.[15–17] The mechanism of unresponsiveness of macrophages to DNA has not been elucidated, although TLR9 is present in the cells. Macrophages have deoxyribonuclease II (DNase II) in the lysosomal compartment, and they are responsible for apoptotic cell engulfment, DNA digestion and erythroid cell differentiation.[34] In erythropoiesis, macrophages take up nuclei and digest DNA. In DNase II-deficient mice, undigested DNA in macrophages causes IFN-β production via unknown receptors.[35] The cytokine production is mediated by the TLR9/MyD88 pathway and novel pathways that have been identified recently.[36,37] Therefore, the mechanism of the unresponsiveness of macrophages to naked DNA may involve the limited uptake and degradation by DNase II. However, further investigation is required.

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The TLR4/9 fusion protein on the cell membrane is activated by vertebrate DNA.[33] This research indicates that compartmentalization into cells avoids TLR9 responses to endogenous DNA. Therefore, we forced DNA to internalize into cells using cationic lipids. In fact, vertebrate DNA/cationic lipid complexes can induce cytokine secretion from murine macrophages and Flt-3 L DC.[23,24] Following enhancement of DNA uptake by cationic lipids, these cells cannot distinguish between 'self' and 'non-self' DNA. In peritoneal macrophages, complexation of calf thymus DNA with cationic lipids elicited a similar level of inflammatory cytokine production to that obtained with bacterial *E. coli* DNA using cationic lipids.[23] In addition, calf thymus DNA with cationic lipid DOTAP causes a high degree of IFN-α release from murine Flt-3 L cultured DC or human peripheral blood mononuclear cells.[24] The amount of IFN-α induced by calf thymus DNA with DOTAP is similar to that induced by bacterial plasmid DNA. However, the result with GM-CSF DC is different from that in these cells. The cells only recognize bacterial DNA. Vertebrate DNA/cationic lipid complexes do not stimulate GM-CSF DC, although bacterial DNA does. There are two possibilities to explain these observations. One is the possibility that different types of cationic lipids lead to different forms of delivery of DNA, and result in different responses. For example, murine macrophages release inflammatory cytokines in response to the addition of vertebrate CT DNA/cationic lipid complexes.[23] Lipofectamine was used for this research. Synthetic double-stranded DNA containing no CpG motif can stimulate macrophage cell lines when DNA is complexed with the cationic lipid Fugene 6.[38] In addition, vertebrate CT DNA/cationic lipid Lipofectamine complexes induce macrophage activation via TLR9-dependent and -independent mechanisms.[39] Flt-3 L cultured DC (Flt-3 L DC) also responds to vertebrate DNA/cationic lipid DOTAP complexes via TLR9-dependent and -independent pathways.[24] TLR9-independent activation is also observed following transfection using Lipofectamine 2000.[37] Honda *et al.* showed that different cellular distributions of DNA result in different cytokine responses.[40] CpG-B ODN normally do not induce IFN-α release from plasmacytoid DC. However, following complexation with DOTAP, the same ODNs trigger IFN-α. Confocal microscopy reveals that DOTAP retains DNA in early endosomes, although ODNs without DOTAP are immediately transferred to lysosomal vesicles. Taken together, enhancement of the DNA uptake may not explain the response of TLR9 to vertebrate DNA and TLR9 may be present in specific compartments.

The other possibility is that GM-CSF DC, Flt-3 L DC and macrophages may contribute to the immune systems in different ways, by producing different types or degrees of induction. TLR9 is mainly expressed in B cells and plasmacytoid DC in humans.[31] On the other hand, mouse TLR9 is also present in myeloid DC and macrophages. Although further studies are required to clarify the contribution of DC or macrophages to immune responses *in vivo*, the present study suggests that DC are the main cells that respond to naked bacterial DNA, although both DC and macrophages will release inflammatory cytokines after the administration of bacterial DNA/cationic lipid complexes.

Very recently Martin *et al.* have shown that GM-CSF DC release type I IFN upon stimulation of mammalian DNA complexed with Fugene, another kind of lipid for transfection.[41] Interestingly, the cells do not produce TNF-α, IL-6 or IL-12. The activation is independent of TLR9 because GM-CSF DC from TLR9$^{-/-}$ deficient mice respond to mammalian DNA/Fugene complexes to secrete type I IFN. Another group has also demonstrated that non-CpG DNA/lipofectamine complexes stimulate GM-CSF DC to induce type I IFN.[42] The activation is not dependent on the MyD88 or TRIF pathways. Based on these observations, one can hypothesize that, distinct from Flt-3 L DC, GM-CSF DC respond to only bacterial or viral DNA via TLR9-dependent pathway, and release cytokines, such as TNF-α IL-6 and IL-12. However when mammalian DNAs are translocated into cells, GM-CSF DC may not induce these cytokines. Instead, the cells may release IFN-α through a TLR9-independent pathway. Further studies are required for these TLR9-dependent and -independent mechanisms.

In conclusion, the present study has demonstrated that murine GM-CSF DC or the DC cell line, DC2.4, produce pro-inflammatory cytokines following stimulation with CpG-containing DNAs and this production is increased when the DNAs are added to the cells in a complex form with cationic lipids. These findings form an important basis for future DNA-based therapies, including gene therapy and DNA vaccination.

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Negative regulation of intracellular hepatitis C virus replication by interferon regulatory factor 3

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Editorial on page 814

Background. Interferon regulatory factor (IRF)-3 plays an important role in initiating cellular interferon-stimulated gene-mediated antiviral responses. In the present study, we evaluated the effects of IRF-3 expression and activation on intracellular hepatitis C virus (HCV) replication using an HCV replicon system. ***Methods.*** An HCV replicon was constructed that expressed a neomycin-selectable chimeric firefly luciferase reporter protein. A small interfering (si) RNA oligonucleotide directed against IRF-3 mRNA was designed and synthesized. A eukaryote expression plasmid vector was constructed that expressed IRF-3 mRNA under control of the cytomegalovirus early promoter/enhancer. To evaluate transcriptional activity of the interferon-stimulated genes, a reporter vector was used that expressed firefly luciferase under control of the interferon-stimulated response element (ISRE). ***Results.*** The baseline expression of IRF-3 did not significantly differ between cells with and without expression of the replicon. Transfection of an IRF-3 expression plasmid into the cells raised the ISRE-luciferase activities. The increase of ISRE activity was significantly more potent in the replicon-expressing cells than in cells without replicon expression. Concomitantly, the overexpression of IRF-3 suppressed HCV replication levels. In contrast, siRNA knockdown of IRF-3 suppressed ISRE activity by 38% ± 2%. Interestingly, the suppression of IRF-3 resulted in a significant increase of HCV replication, by up to twofold, depending on the IRF-3 suppression levels. ***Conclusions.*** IRF-3 negatively regulated intracellular HCV replication, and was partially activated in cells that expressed the HCV replicon. Thus, IRF-3 is a key molecule controlling HCV replication through modulation of host interferon gene responses.

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Key words: hepatitis C virus, interferon regulatory factor 3

Introduction

Hepatitis C virus (HCV) is a worldwide health-care problem causing a spectrum of liver disease ranging from an asymptomatic carrier state to liver cirrhosis and hepatocellular carcinoma.[1] Currently available anti-HCV treatments are based on high-dose administration of a major antiviral cytokine, interferon (IFN)- . However, even with the most efficient regimen of pegylated interferon in combination with ribavirin, almost half of all cases are refractory to the treatment and fail to eradicate the virus.[2] Without the IFN therapy, HCV is associated with persistent infection and replication in the liver in spite of intact host immune systems; these features lead us to speculate that HCV escapes from or attenuates host antiviral responses.

Type I IFN plays a central role in eliminating viruses not only in therapeutic applications but also as a na-tural cellular antiviral defense mechanism.[3,4] IFNs mediate antiviral responses by inducing expression of interferon-stimulated genes (ISGs), including those encoding 2,5-oligoadenylate synthetase, double-stranded RNA-dependent protein kinase R, and MxA proteins, resulting in the degradation of cellular RNA, general repression of protein synthesis, and apoptotic cell death.[5] Also, a DNA microarray analysis of chimpanzee liver experimentally inoculated with HCV revealed that expression of ISGs, including those encoding 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]

The expressional control of ISGs is directed by receptor-mediated stimuli of type I IFNs.[7] Binding of

the IFNs onto their receptors activates receptor-associated janus kinases, which phosphorylate signal transducer and activator of transcription (STAT) 1 and STAT2. The phosphorylated STATs, 1 and 2, recruit IFN regulatory factor (IRF)-9 to form a complex with IFN-stimulated gene factor-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.[3,4,8]

Other than by type I IFNs, expression of ISGs is controlled by binding ISRE with other molecules, including IRF-1, IRF-3, and IRF-7. Among them, IRF-3 is a transducer of virus-mediated signaling and plays a critical role in the induction of cellular antiviral responses.[8-11] IRF-3, which is ubiquitously expressed in the cytoplasm, is subjected to phosphorylation by virus infection, double-stranded RNA, and bacterial lipopolysaccharides. The phosphorylated IRF-3 forms a homodimer, translocates to the nucleus, and predominantly activates expression of the IFN- gene and certain ISGs.[4,12,13]

The IRF-3-mediated IFN pathway might be a target of viruses to counteract antiviral responses and to promote virus replication in the infected cells. Ebola virus, bovine diarrhea virus, and influenza A virus interfere with the activation of IRF-3 through interactions of their virus-encoded proteins.[14-16] In the case of HCV, some reports suggest interaction of virus proteins with the cellular IFN system. The viral NS5A protein has been reported to interfere with cellular IFN signaling.[17] It has recently been reported that HCV NS3/4A fusion protein blocks virus-induced activation of IRF-3.[18] Taken together, these findings indicate that IRF-3 is not only a key molecule of cellular innate immune responses but also might be a target of antiviral strategies. However, the mechanisms of IRF-3 activation by HCV infection in hepatocytes have not been explored yet, nor have the effects of the activated IRF-3 on HCV been satisfactorily studied.

An HCV subgenomic replicon is an in vitro model that simulates cellular autonomous replication of HCV genomic RNA. The development of the replicon system has partly overcome the problem of a lack of HCV replication models.[19] Replication of the HCV replicon can be abolished by treatment with small amounts of type I and type II IFNs,[20-22] suggesting intact IFN receptor-mediated cellular responses. However, in the absence of the exogenous interferon, persistent and high-level expression of the replicon has caused us to speculate that intracellular virus-induced antiviral responses become attenuated or malfunction as a result of the expression of viral proteins. We have previously reported that the baseline activity of ISG expression is substantially decreased in cells expressing replicon and that this decrease is partly attributable to the transcriptional suppression of IRF-1.[23] In the present study, we extended our observations by investigating the effects of IRF-3 expression and activation on HCV replication.

Materials and methods

Cell culture

A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO, USA) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in medium containing 300 μg/ml G418 (Wako, Osaka, Japan).

HCV replicon constructs and transfected cell lines

An HCV subgenomic replicon plasmid, pHCVIbneo-delS (designated pRep-N), was derived from an infectious HCV clone, HCV-N, genotype Ib.[22] The replicon pRep-N was reconstructed by replacing the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotransferase genes (pRep-Feo, Fig. 1).[24,25] RNA was synthesized from pRep-N and pRep-Feo using T7-polymerase (Promega, Madison, WI, USA) and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/Rep-N and Huh7/Rep-Feo, respectively). We have previously reported that firefly luciferase activities of Feo-replicon-expressing cells correlate well with HCV NS3, NS4A, and NS5A protein expression levels and with the replicon RNA expression levels.

Cured Huh7 cells

To establish cured Huh7 cells (cHuh7), from which replicon RNA was eliminated, Huh7/Rep-Feo was treated

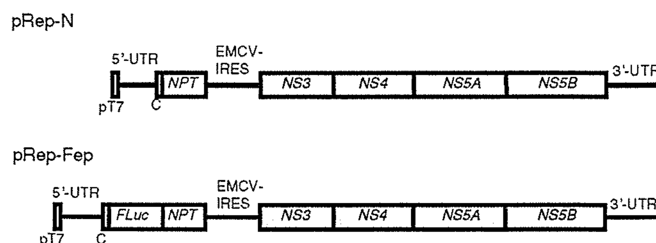

Fig. 1. Structures of the hepatitis C virus (HCV) replicon plasmids. *UTR*, untranslated region; *pT7*, T7 promoter; *C*, truncated HCV core region (nucleotides 342–377); *EMCV*, encephalomyocarditis virus; *Fluc*, firefly luciferase gene; *NPT*, neomycin phosphotransferase gene; *NS3*, *NS4*, *NS5A*, and *NS5B*, genes that encode HCV nonstructural proteins

with 100 U/ml of IFN- for 14 days. The absence of replicon RNA was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) and by the loss of resistance to G418.[23]

Small interfering RNAs

Three small interfering RNAs (siRNAs) directed against IRF-3 were synthesized: siRNA1 (5 -gug gga gac agg acg cug cTT-3), siRNA2 (5 -gcc aga cac cuc ucc gga cTT-3), and siRNA3 (5 -ggu ugu gcc cac gug ccu cTT-3). A control siRNA was used as previously described (5 -ucg ggg cac ugc uag auc cTT-3).[24]

Plasmids

The expression plasmid vector pcDNA3.1-IRF-3 expresses the human IRF-3 open reading frame, which was cloned from human hepatocyte mRNA by RT-PCR using primers IRF-3/5 (5 -CAC CAT GGG AAC CCC AAA GCC ACG GAT CCT-3) and IRF-3/3 (5 -GCT CTC CCC AGG GCC CTG GAA ATC CAT G-3). The PCR product was inserted into the pcDNA3.1 TOPO vector (Invitrogen, Carlsbad, CA, USA) as instructed, and the nucleotide sequence was confirmed. The plasmid pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. The plasmid pISRE-TA-Luc (Invitrogen) contained five copies of consensus ISRE motifs upstream of the firefly luciferase gene. pRL-CMV (Promega), which expressed *Renilla* luciferase protein, was used for correction of transfection efficiency.

Transient transfection

DNA and siRNA transfection was performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. To perform reporter assays to determine the effect of IRF-3 on ISRE in the cells, a total of 5×10^4 Huh7, cHuh7, and Huh7/Rep-N cells were subcultured onto 24-well plates the day before transfection. A total of 100 ng of pISRE-TA-Luc and various amounts of pcDNA3.1-IRF-3 with empty vector and 0.1 ng of pRL-CMV, to a total mass of DNA of 400 ng, were transfected by using 2 μl of Lipofectamine 2000.

Western blotting

Cytoplasmic and nuclear fractions of cell lysates were prepared as described elsewhere.[26] The purity of the cytoplasmic and nuclear fractions was monitored by immunoblotting using an antibody directed against a nuclear protein, USF-2 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Twenty micrograms of protein was separated using NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was immunoblotted with anti-IRF-3 (Santa Cruz) or anti-His antibodies (Invitrogen), and detected by chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche).

Immunocytochemical staining

Cells seeded onto tissue culture chamber slides were washed with phosphate-buffered saline (PBS) and fixed with 99% cold acetone for 10 min. After rinsing with PBS, cells were incubated with an anti-IRF-3 antibody at a dilution of 1/500 or an anti-His antibody at a dilution of 1/200 in PBS/3% goat serum. After 3 h, cells were washed three times with PBS, and incubated with fluorescein isothiocyanate (FITC)-labeled secondary antibodies. Cells were then washed and mounted with VectaShield mounting medium with 4 ,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Fluorescence microscopy was carried out with an Olympus BX50.

Luciferase reporter assays

Luciferase activity was measured by luminometer (Lumat LB9501; Promega) using a Bright-Glo Luciferase Assay System (Promega) or a Dual Luciferase Assay System (Promega). Assays were done in triplicate, and the results were expressed as means ± SD.

MTS assays

To evaluate cytotoxicity, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

Statistical analyses

Statistical analyses were performed using an unpaired, two-tailed Student's t test; P values less than 0.05 were considered statistically significant.

Results

Expression level of IRF-3 in cells with and without HCV replication

First, we evaluated the expression levels of endogenous IRF-3 in Huh7 cells with or without expression of the HCV replicon. Western blotting analysis showed no sig-

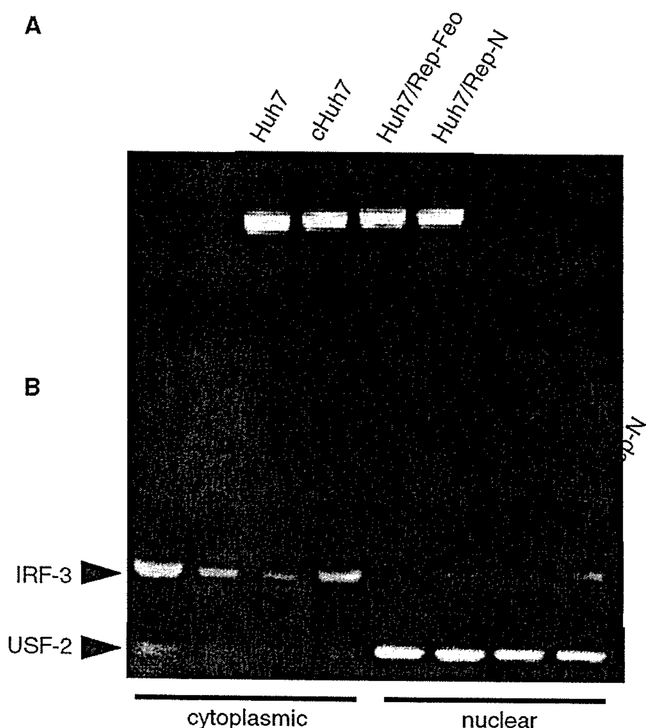

Fig. 2A,B. Expression of endogenous interferon regulatory factor-3 (*IRF-3*) in cells with and without expression of the HCV replicon. **A** Western blotting. Whole cell lysates from Huh7 and Huh7/Rep-Feo were prepared. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotting, the 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

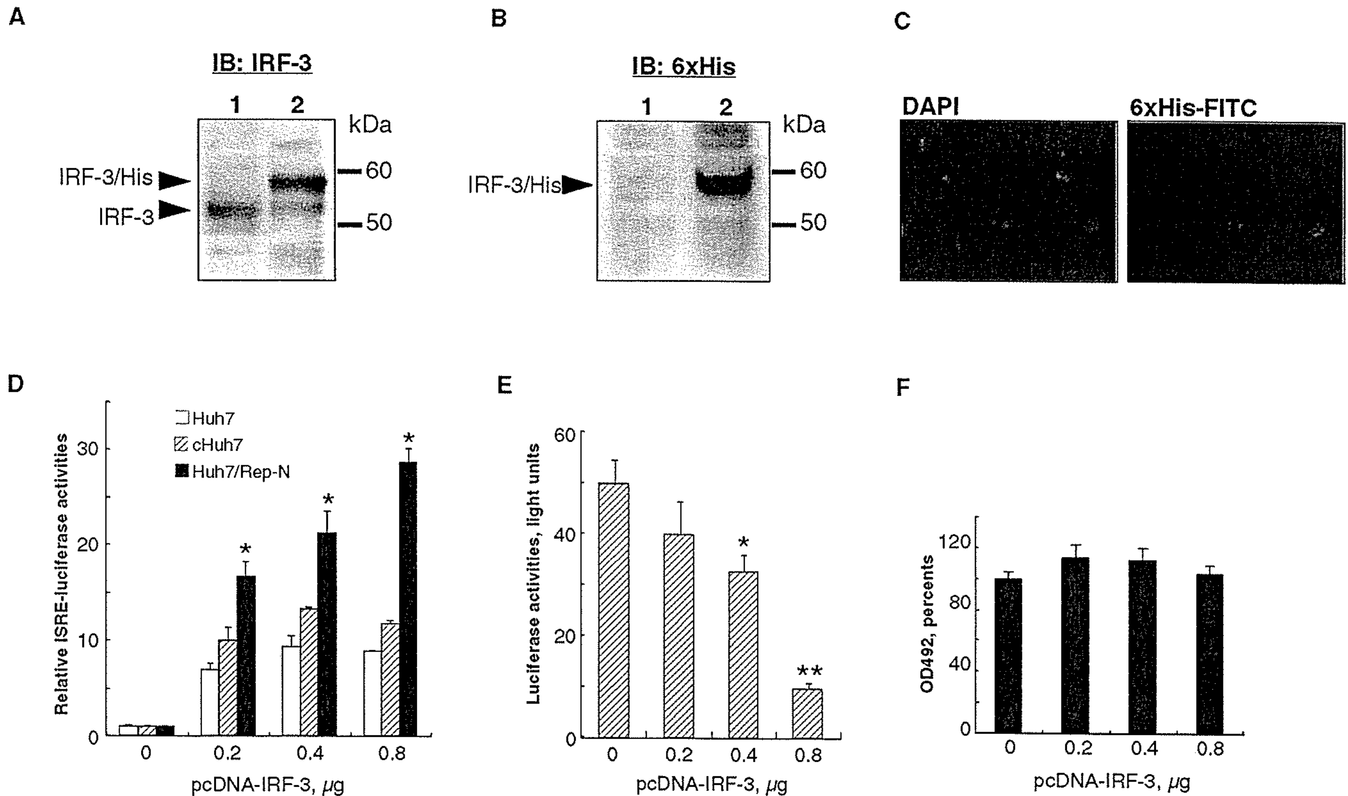

Fig. 3A–F. Effects of IRF-3 overexpression on the regulation of interferon-stimulated response element (*ISRE*) activity and HCV replication. An IRF-3-expression plasmid, pcDNA-IRF-3, was transfected into Huh7 cells (*lane 2*), and Western blotting analyses were performed using monoclonal anti-IRF-3 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 24h 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 24h 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

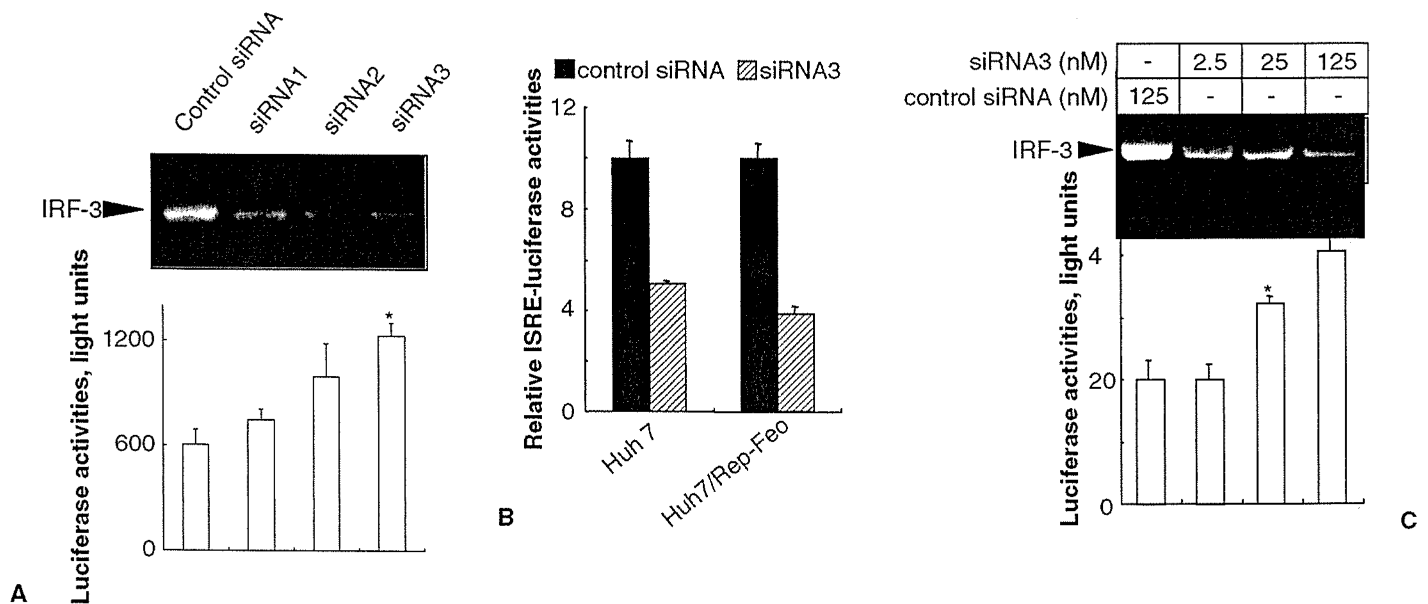

Fig. 4A–C. Suppression of IRF-3 expression by siRNA and effects on ISRE activity and HCV replication. **A** Suppression of endogenous IRF-3 expressions by IRF-3-directed siRNAs. Three siRNAs, siRNA1, siRNA2, and siRNA3, were transfected into Huh7 cells, and IRF-3 were detected by Western blotting using 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, pClneo.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, pClneo.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 pClneo.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.

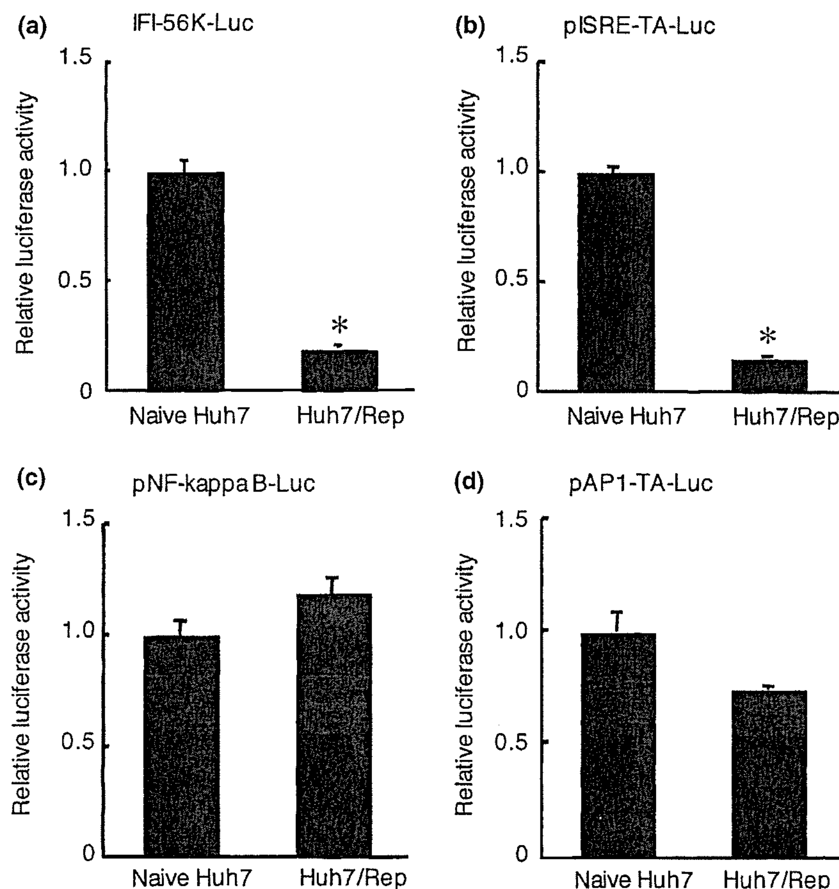

Fig. 1 Suppression of interferon-induced protein (IFI)-56K promoter and interferon-stimulated response element (ISRE) promoter activities in cells expressing hepatitis C virus replicon. Promoter activities of IFI-56K (panel A), ISRE (panel B), NF-kappa B (panel C), and AP1 (panel D) were measured by 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.

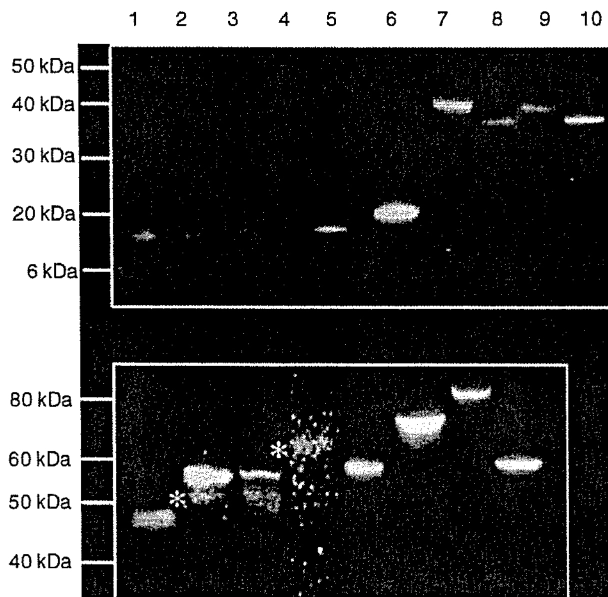

Fig. 2 Western blotting analysis of cells transfected with the interferon-stimulated gene (ISG)-expression plasmids. ISG expression vectors were respectively transfected into Huh7 cells or into 293T cells. The cells were harvested at 48 h after transfection. Ten micrograms of cell lysate was separated 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, pCIneo-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