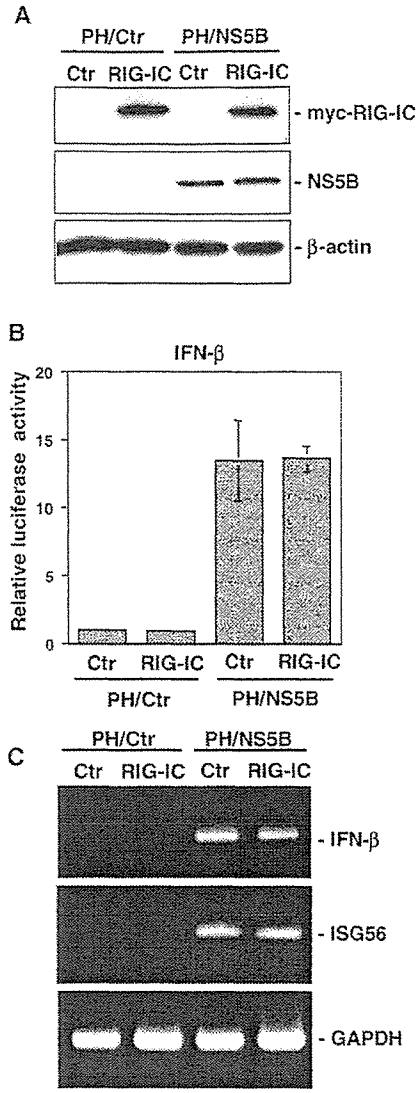

Fig. 7. Induction of IFN-β by NS5B is not mediated through the RIG-I signaling pathway. (A) Expression of RIG-IC and NS5B in PH5CH8 cells introduced by retrovirus-mediated gene transfer. Western blot analysis of PH/Ctr or PH/NS5B cells infected with pCXpur retrovirus encoding myc-tagged RIG-IC was performed. The pCXpur retrovirus was used as a control infection. Anti-myc, anti-NS5B, and anti-β-actin antibodies were used for the immunoblotting analysis. (B) Dual luciferase reporter assay of the IFN-β gene promoter. The cells shown in panel A were transfected with pIFN-β(-125)-Luc, and the dual luciferase assay was performed as indicated in Fig. 6B. (C) RT-PCR analysis of IFN-β and ISG56 mRNAs. The total RNAs were extracted from the cells shown in panel A and subjected to RT-PCR analysis using primer sets for IFN-β (341 bp), ISG56 (320 bp), and GAPDH (587 bp).

tagged RIG-IC was confirmed by Western blot analysis (Fig. 7A). Using PH/Ctr cells stably expressing myc-tagged RIG-IC, we confirmed that IFN-β production was markedly suppressed after infection with Sendai virus (data not shown), as initially observed in Newcastle disease virus infection (Yoneyama et al., 2004). This indicates that RIG-IC functions as a dominant negative inhibitor of RIG-I in PH5CH8 cells. We then performed a dual-luciferase reporter assay using an *IFN-β* gene promoter. The results revealed that the enhancement of luciferase activity in PH/NS5B cells was not suppressed regardless of RIG-IC expression (Fig. 7B). Furthermore, the mRNA expression levels for IFN-β and one of its target genes, ISG56, were also unchanged by the expression of RIG-IC (Fig. 7C). These results suggest that NS5B's induction of IFN-β is not mediated through the RIG-I signaling pathway.

NS5B does not interact with TLR3 adaptor protein

Since we showed that NS5B's induction of IFN-β was mediated through the TLR3 but not the RIG-I signaling pathway, we further examined the mechanism underlying IFN-β induction by testing the possibility of interaction between NS5B and the TLR3 adaptor protein TRIF (Yamamoto et al., 2002). We prepared HEK/NS5B cells stably expressing myc-tagged NS5A or myc-tagged TRIF and examined whether or not NS5B interacts with TRIF by an immunoprecipitation method following Western blot analysis. The results clearly showed that NS5B and myc-tagged NS5A were co-immunoprecipitated by anti-myc antibody as reported previously (Shirota et al., 2002). However, co-immunoprecipitation of NS5B and myc-tagged TRIF was clearly not observed (Fig. 8). This result suggests that the activation of the TLR3 signaling pathway by NS5B occurs through one or more factors other than TRIF.

Induction of IFN-β depends on RNA-dependent RNA polymerase (RdRp) activity of NS5B

Since dsRNA, an intermediate of viral replication, is known as a natural ligand for the activation of TLR3 (Alexopoulou et

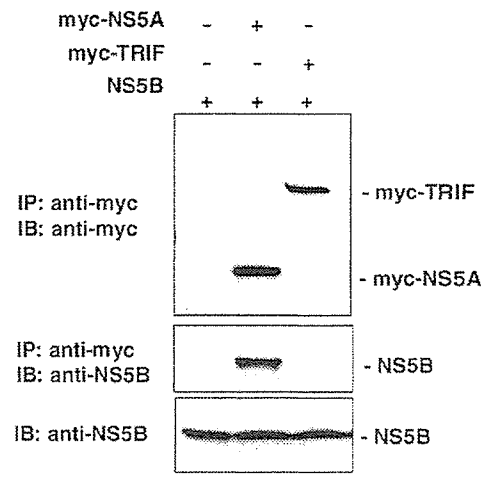

Fig. 8. NS5B does not interact with TRIF. HEK/NS5B cells were infected with pCXpur retrovirus encoding myc-tagged NS5A (middle lane) or myc-tagged TRIF (right lane). pCXpur retrovirus was used as a control infection (left lane). Cell lysate was immunoprecipitated (IP) with anti-myc antibody-conjugated agarose beads. The immunoprecipitates were resolved by SDS-PAGE, and anti-myc (upper panel) and anti-NS5B (middle panel) antibodies were used for the immunoblotting (IB) analysis. To confirm the expression level of NS5B, cell lysates were subjected to immunoblotting analysis using anti-NS5B antibody (lower panel).

al., 2001; Takeda et al., 2003), we next examined whether or not the induction of IFN-β in human hepatocytes expressing NS5B (591 amino acids; amino acids 2420 to 3010 in the HCV-1b genotype) (Kato et al., 1990) depends on NS5B's RdRp activity. Since this activity is already well characterized (Hagedorn et al., 2000), we constructed several NS5B mutants to evaluate this subject (Fig. 9A). One is the substitution mutant G2736V of the GDD motif (amino acids 2736–8) located in the catalytic site, and the other is the deletion mutant Δ2575–7 (R2753T, K2754S, and Δ2575–7) at the priming and interrogation sites, all of which are essential for NS5B's RdRp activity (Behrens et al., 1996; Bressanelli et al., 2002). We also constructed three carboxyl-truncated forms (ΔC21, ΔC56, and ΔC97, lacking 21, 56, and 97 amino acids, respectively) of NS5B. These truncated mutants of NS5B lack the last 21 hydrophobic amino acids, which are necessary and sufficient to target NS5B to the cytosolic side of the endoplasmic reticulum (ER) membrane (Schmidt-Mende et al., 2001; Yamashita et al., 1998). Although ΔC21 and ΔC56, but not ΔC97, possess RdRp activity in vitro, ΔC56 shows higher RdRp activity than ΔC21 because only the latter possesses a regulatory motif inhibiting RNA binding and polymerase activity (Leveque et al., 2003). We prepared PH5CH8 cells stably expressing these NS5B mutants and then performed cell cycle analysis using these

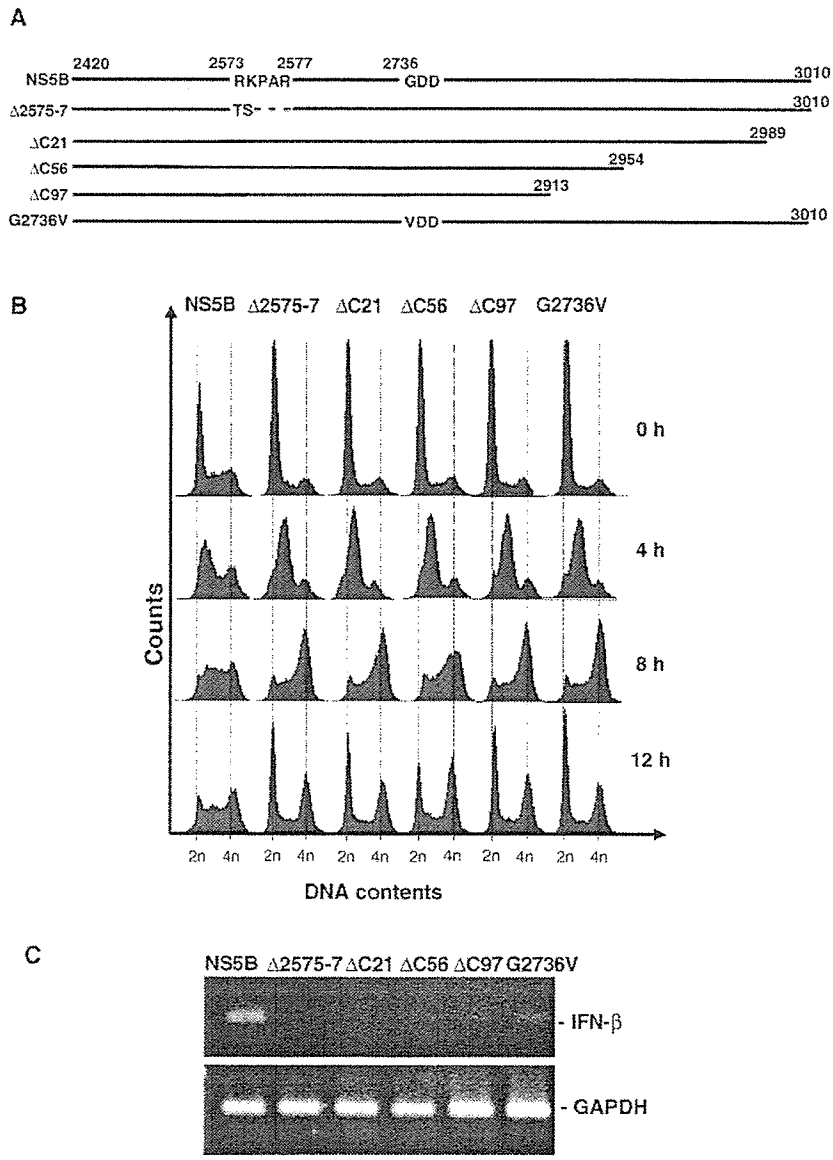

Fig. 9. The RdRp activity of NS5B anchoring on ER membrane is required for induction of IFN-β and following the delay of S phase progression. (A) Schematic presentation of the NS5B mutants used. Only amino acid sequences in the mutated regions of NS5B are indicated. (B) Cell cycle analysis of PH/NS5B and PH5CH8 cells expressing NS5B mutants. Cell cycle distribution was analyzed as described in Fig. 1B. (C) RT-PCR analysis of IFN-β mRNA in PH/NS5B and PH5CH8 cells expressing NS5B mutants. RT-PCR analysis was performed as described in Fig. 3A.

prepared cells. The results revealed no effect on S phase progression in the PH5CH8 cells expressing NS5B mutants (Fig. 9B), although PH5CH8 cells expressing ΔC56 showed a slight delay of S phase progression. Induction of IFN-β mRNA was also not observed in the PH5CH8 cells expressing NS5B mutants (Fig. 9C). These results revealed that the delay of S phase progression and the induction of IFN-β depend on the RdRp activity of NS5B, and these effects are coupled with ER membrane anchorage of NS5B in cells.

To examine the activation of IRF3, a factor specifically induced by stimulated TLR3 or TLR4, by the expression of NS5B and its mutants, we performed a dual-luciferase reporter assay using a synthetic promoter having five repeats of the consensus ISRE, which was the same as the IRF3 target sequence in the *IFN-β* gene promoter (Fig. 10A) and an intrinsic *IFN-β* gene promoter (Fig. 10B). The results showed that the luciferase activity was enhanced approximately five-fold (Fig. 10A) and eight-fold (Fig. 10B) only in PH/NS5B cells,

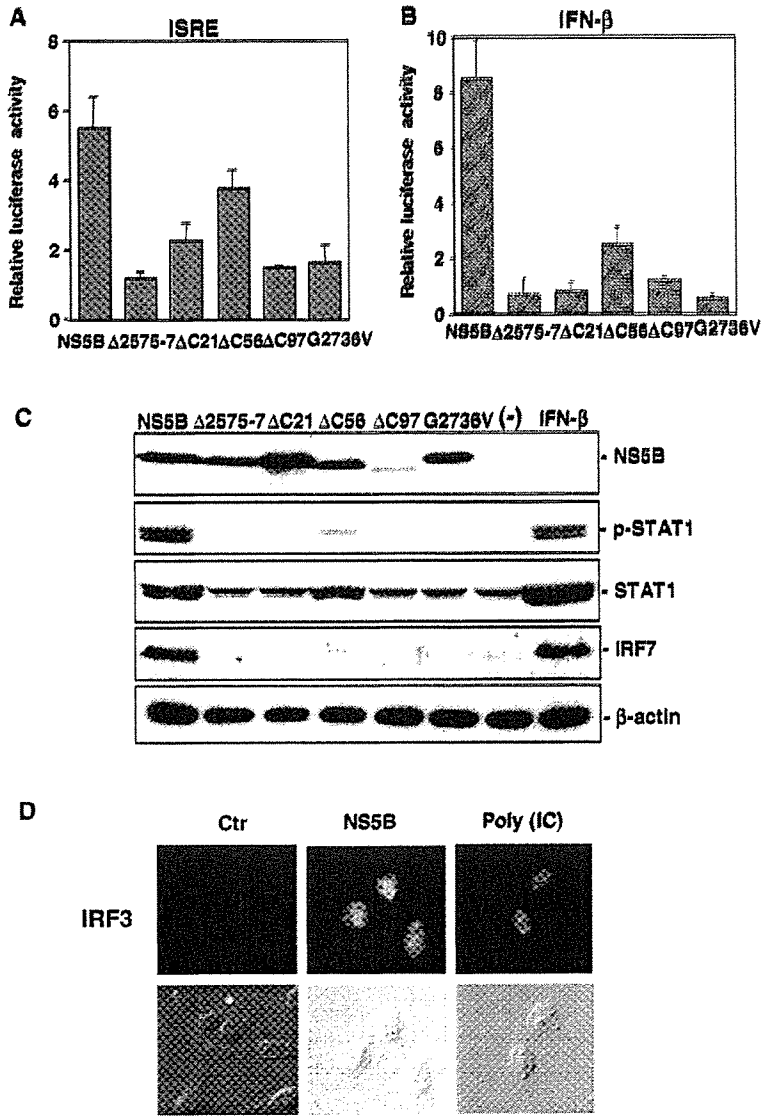

Fig. 10. NS5B full form is required for activation of IRF3 target sequences and IFN-β signaling pathway. (A) Dual luciferase reporter assay toward IRF3 target sequences. PH5CH8 cells were transfected with the pISRE-Luc (Stratagene) and pCXbsr encoding NS5B or its mutant, and the dual luciferase assay was performed as indicated in Fig. 6B. The lysates of cells transfected with pCXbsr were used as a control. (B) Dual luciferase reporter assay of the IFN-β gene promoter. Dual luciferase assay was performed as described in panel A except using pIFN-β(-125)-Luc instead of pISRE-Luc. (C) Western blot analysis of the components involved in the IFN-β signaling pathway. The lysates of PH/NS5B and PH5CH8 cells expressing NS5B mutants were subjected to immunoblotting using anti-NS5B, anti-p-STAT1(Y701), anti-STAT1, anti-IRF7, and anti-β-actin antibodies. PH5CH8 cells treated with or without IFN-β (500 IU/ml for 24 h) were also analyzed as a control. (D) Subcellular distribution of endogenous IRF3. PH/Ctr and PH/NS5B cells were processed and stained with anti-IRF3 antibody and an FITC-conjugated secondary antibody. PH5CH8 cells treated with poly (IC) were also used as a positive control.

suggesting that IRF3 is activated by the NS5B full form. Interestingly, however, luciferase activity was enhanced approximately four-fold (Fig. 10A) and three-fold (Fig. 10B) in PH5CH8 cells expressing ΔC56, although the enhancement was not as great as the five-fold (Fig. 10A) and eight-fold (Fig. 10B) in PH/NS5B cells, respectively. We then examined the phosphorylation status of STAT1 on Y701 and the level of IRF7, one of the downstream targets of the IFN-β signaling pathway (Katze et al., 2002). Western blot analysis revealed marked phosphorylation of STAT1 and IRF7 expression in PH/NS5B cells as well as in PH5CH8 cells treated with IFN-β (Fig. 10C). Although slight phosphorylation of STAT1 was observed in the PH5CH8 cells expressing ΔC56, IRF7 expression was not observed (Fig. 10C). Unlike PH/NS5B cells and PH5CH8 cells expressing ΔC56, neither the phosphorylation of STAT1 nor the expression of IRF7 was detected in PH5CH8 cells expressing other NS5B mutants. These results indicated that ΔC56 had an extremely low ability to induce IFN-β after activation of TLR3, although ΔC56 was still able to enhance the IRF3 target promoter. To obtain further evidence of the activation of IRF3, we examined the subcellular distribution of endogenous IRF3 in PH/Ctr and PH/NS5B

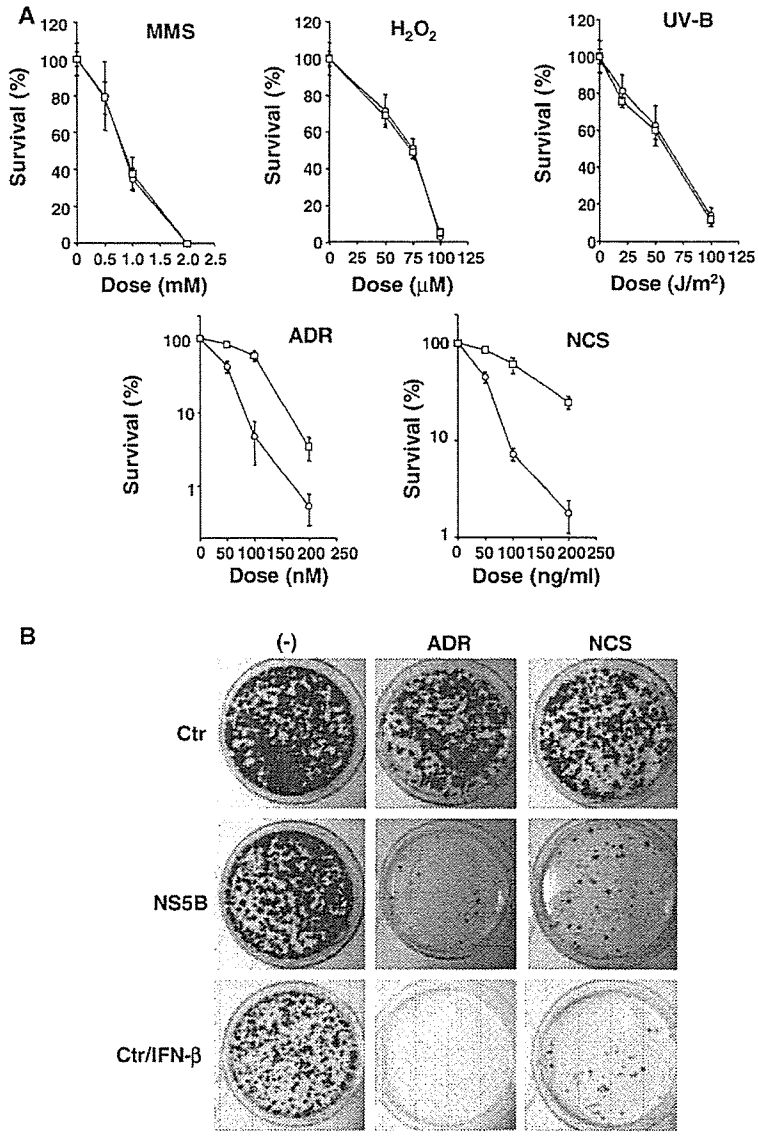

Fig. 11. Sensitivity of PH/NS5B cells against DNA-damaging reagents. (A) Clonogenic assays for PH/Ctr (square) and PH/NS5B (circle) cells after treatment with increasing doses of DNA-damaging reagents. Cells were treated with MMS, H_2O_2, UV-B, ADR, and NCS. Ten days after the treatment, cells were fixed and stained with Coomassie brilliant blue. Only colonies containing >50 cells were scored as being derived from viable clonogenic cells. Data are means ± SD from two independent triplicate experiments. (B) PH/Ctr, PH/NS5B, and IFN-β-treated (20 IU/ml) PH/Ctr cells were treated with ADR (100 nM) or NCS (100 ng/ml). The panels show survived colonies that are stained with Coomassie brilliant blue at 10 days after the treatment.

cells. In PH/Ctr cells, IRF3 was distributed in a perinuclear and/or cytoplasmic context. However, in PH/NS5B cells as well as PH5CH8 cells treated with poly (IC), IRF3 was distributed to the nucleus, a finding consistent with its activated state (Fig. 10D). Taken together, our findings indicate that the RdRp activity of HCV NS5B anchoring on ER membrane is necessary and sufficient to activate the TLR3 signaling pathway.

PH/NS5B cells are more susceptible than PH/Ctr cells to DNA-damaging reagents

To better understand the effect of IFN-β induction in PH/NS5B cells, we next examined the susceptibilities of PH/NS5B and PH/Ctr cells against various types of DNA-damaging reagents. A clonogenic assay using PH/NS5B and PH/Ctr cells was performed by treatment with MMS (a DNA alkylating reagent) and H_2O_2 (a DNA oxidative reagent) and by UV-B irradiation, which induces DNA single-strand breaks and/or thymidine dimer formation in DNA. ADR and NCS, which induce DNA double-strand breaks, were also used for the clonogenic assay. As shown in Fig. 11A, PH/NS5B and PH/Ctr cells were susceptible to the MMS treatment, the H_2O_2 treatment, and the UV-B irradiation, and no differences were observed between their susceptibilities. Interestingly, however, PH/NS5B cells were more susceptible than PH/Ctr cells against ADR or NCS treatment (Fig. 11A). These results suggest that PH/NS5B cells are more sensitive than PH/Ctr cells to damage in the form of DNA double-strand breaks. To clarify whether or not IFN-β induction increases the susceptibility against ADR or NCS treatment, we examined the effect of ADR or NCS in PH/Ctr cells treated with IFN-β. In this treatment, the cells changed to susceptible phenotype against ADR or NCS treatment, as observed in PH/NS5B cells (Fig. 11B). These results suggest that IFN-β induced by NS5B in PH5CH8 cells changes the cells into the hypersensitive phenotype, making them susceptible to DNA damage in the form of double-strand breaks.

Discussion

In the present study, we found that HCV NS5B induced IFN-β in two kinds of immortalized human hepatocyte cell lines, PH5CH8 and NKNT-3. We showed that NS5B's induction of IFN-β was mediated through the TLR3 but not the RIG-I signaling pathway. The induction of IFN-β caused the delay of cell cycle progression through the S phase in these cells. Since it has been generally known that the activation of the TLR3 signaling pathway is caused by dsRNA, a molecular pattern associated with replicating viral genomes, we first obtained data suggesting that dsRNA is generated by NS5B even without replication of the viral genome.

TLRs belong to a family of evolutionarily conserved innate immune recognition molecules, and ten members of the TLR family have been identified in human (Medzhitov, 2001; Takeda et al., 2003). TLR3 recognizes dsRNA and induces the antiviral immune responses (Alexopoulou et al., 2001; Matsumoto et al., 2002). TLR3 activates transcription factor IRF3 through TRIF, leading to IFN-β production (Oshiumi et al., 2003; Yamamoto et al., 2002, 2003). We speculated on two possible mechanisms underlying the activation of the TLR3 signaling pathway by NS5B. The first possibility is that protein–protein interaction between NS5B and TRIF is involved in the activation of the TLR3 signaling pathway. However, we failed to obtain evidence of direct interaction between NS5B and TRIF. The second possibility is that the RdRp activity of NS5B contributes to the activation of TLR3. To evaluate this hypothesis, we examined whether or not several NS5B mutants, including carboxyl-truncated mutants or an RdRp activity-defective mutant (G2736V), could induce IFN-β. The experimental data clearly showed that neither the G2736V mutant nor the carboxyl-truncated mutants could induce IFN-β. Therefore, we suggested that NS5B RdRp activity anchoring the ER membrane is critical for the activation of the TLR3 signaling pathway.

The finding that NS5B RdRp activity on the ER membrane was a critical factor for the induction of IFN-β surprised us because we expected that dsRNA, a natural ligand for TLR3, was produced in NS5B-expressing hepatocyte cells without replication of the viral RNA genome. Therefore, we now presume a daring hypothesis: that NS5B can produce dsRNA using cellular RNA as a template on the ER membrane. Since no direct evidence has been found to support this hypothesis at this stage, further experiments are necessary to evaluate this hypothesis. For instance, if possible, the detection of newly synthesized dsRNA in NS5B-expressing cells or the detection of newly synthesized dsRNA by recombinant NS5B using cellular RNA in vitro may become positive evidence. Furthermore, since the formation of a membrane-associated replication complex is a characteristic of positive-stranded RNA viruses, including HCV (Shi et al., 2003), it will also be interesting to examine whether or not the RdRps of the other RNA viruses possess novel activity similar to that observed in this study. At least we recently detected that NS5B derived from an HCV-2a genome designated JFH-1, which produces virus particles infectious for HuH-7 cells (Wakita et al., 2005), also strongly induced IFN-β in PH5CH8 cells (Ikeda et al., unpublished data). In addition, we are not able to completely exclude the possibility that NS5B-encoding RNA, but not NS5B, specifically activates the TLR3 signaling pathway. However, this possibility is unlikely because the G2736V mutant with only one nucleotide substitution could not activate the TLR3 signaling pathway.

Since the activation of the TLR3 signaling pathway in NS5B-expressing PH5CH8 or NKNT-3 cells is considered to be due to a novel function of NS5B, it is important to clarify whether or not this function occurs in the HCV life cycle. Although HCV replicon systems carrying autonomously replicating HCV RNA genomes developed using HuH-7 (Blight et al., 2000; Ikeda et al., 2002; Lohmann et al., 1999) and HeLa (Zhu et al., 2003) cells have become powerful tools for basic studies of HCV, these systems would not be suitable to prove our hypothesis because the induction of IFN-β by NS5B was not observed in HuH-7 or HeLa cells, in which the TLR3 signaling pathway was suggested to be defective. In fact, it has been recently reported that HuH-7 cells lack a TLR3

response to external dsRNA (Lanford et al., 2003). Therefore, a new HCV replicon system needs to be developed using other human cell lines possessing intact TLR3 signaling pathways. We are currently making a trial to establish an HCV replicon system using PH5CH8 or NKNT-3 cells.

On the other hand, it has been recently found that an HCV serine protease, NS3-4A, can block the phosphorylation and effector action of IRF3 (Foy et al., 2003). This finding using HuH-7 cells suggests that NS3-4A mediates the proteolysis of cellular proteins within an antiviral signaling pathway upstream of IRF3, leading to persistent viral infection. The recently identified TRIF (Li et al., 2005a, 2005b) and RIG-I (Foy et al., 2005) are possible candidates for these cellular proteins. Therefore, it was thought that IFN-β induction by NS5B through the activation of TLR3 might be suppressed by NS3-4A in PH5CH8 cells. In fact, our recent study showed that NS3-4A, in a serine protease activity-dependent manner, suppressed NS5B's activation of the IFN system (Dansako et al., 2005). However, the synergistic induction of IFN-β in PH5CH8 cells co-expressing Core and NS5B was only partially suppressed by NS3-4A, whereas the induction of IFN-β by NS5B only was drastically suppressed by NS3-4A (Dansako et al., 2005). We speculate that a biological implication of this phenomenon is that HCV proteins contribute to the maintenance of a low steady state of the virus by controlling the expression level of IFN-β in the infected cells.

In addition to the delay of cell cycle progression through the S phase, enhanced susceptibility to DNA-damaging reagents was found in NS5B-expressing PH5CH8 cells. This phenomenon was attributed to IFN-β induced by NS5B. Further characterization of this phenomenon may contribute to the understanding of IFN-β's biological effects on hepatocytes and effects on the pathogenesis of hepatocellular carcinoma caused by HCV. Furthermore, the findings of the present study may contribute to an understanding of the mechanisms underlying the TLR3 activation involved in innate immunity against viral infection. In addition, our findings suggest that an antiviral state in uninfected cells may be induced by the expression of a viral protein, NS5B.

Materials and methods

Cell culture and cell cycle analysis

The non-neoplastic immortalized human hepatocyte cell lines, PH5CH8 and NKNT-3 cells, were maintained as described previously (Ikeda et al., 1998; Kobayashi et al., 2000). Human hepatoma cell line HuH-7 cells, human cervical carcinoma HeLa cells, and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

To synchronize the cells at the G1/S transition, growing cells were treated with thymidine (Sigma, St. Louis, MO) (2.5 mM) for 19 h, washed in PBS, and released into fresh medium for 11 h. The cells were then treated with aphidicolin (Sigma) (5 μM) for 13 h, washed in PBS, and released into fresh medium. The cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdUrd; Sigma) for 1 h, fixed with 70% ethanol at indicated time points, stained with fluorescein-isothiocyanate (FITC)-conjugated mouse monoclonal antibody to BrdUrd (BD Pharmingen, San Diego, CA), and counterstained with propidium iodide (PI) (Sigma). The cellular content of DNA was determined by flow cytometry with FACScalibur instrument, and data were analyzed with CELL Quest software (BD Biosciences, San Jose, CA) (Naka et al., 2004). To determine the population of G2-M phase reached cells, the cells were treated with Nocodazole (Noc; Sigma) (200 ng/ml) at 5 h after release into the S phase. Then, after 7 h (post release from 12 h), the cell population that had accumulated in the G2-M phase was analyzed by flow cytometry. To examine the effects of IFN-β, PH5CH8 and NKNT-3 cells were treated with or without IFN-β (500 IU/ml) at 12 h prior to release, and cell cycle progression was analyzed. To assess the effect of anti-IFN-β neutralizing antibody, NS5B-expressing cells were treated with anti-IFN-β antibody (70 U/ml, OBT0377, Oxford Biotechnology, Oxfordshire, UK) during cell cycle synchronization and after release from the G1/S boundary.

Vector construction and retrovirus infection

Retroviral vectors pCXbsr (Akagi et al., 2000) and pCX4bsr (Akagi et al., 2003), which contain the resistance gene for blasticidin, were used in this study. The DNA fragments encoding the influenza hemagglutinin tagged (HA)-core, NS3, HA-NS4B, HA-NS5A, HA-NS5B, and NS5B were amplified from pMILE (HCV 1B-1 strain belonging to genotype 1b; accession no. AB080299) by PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The obtained DNA fragments were subcloned into the *Eco*RI (*Bam*HI for NS5B) and *Not*I sites of pCXbsr or pCX4bsr. The DNA fragment encoding myc-tagged NS5A was also amplified from pMILE by PCR. The obtained DNA fragment was subcloned into the *Eco*RI and *Not*I sites of pCXpur (Akagi et al., 2000), which contains the resistance gene for puromycin. The DNA fragments encoding TLR3 (accession no. NM_003265), Toll-IL-1 receptor (TIR) domain-containing adaptor-inducing IFN-β (TRIF or TICAM-1, accession no. NM_182919), and RIG-IC, a dominant negative inhibitor of retinoic acid-inducible gene-I (RIG-I) (Yoneyama et al., 2004), were amplified from cDNAs obtained from PH5CH8 cells. The primer sequences containing the *Sph*I (for forward) or *Not*I (for reverse) recognition sites for TLR3, TRIF, and RIG-IC were designed to enable expression of the TLR3, TRIF, and RIG-IC open-reading frames, respectively. The obtained DNA fragments were subcloned into the *Sph*I and *Not*I sites of pCXpur/myc, which can express myc-tagged protein. The *IFN-β* gene promoter region (−125 to +19) described previously (Fujita et al., 1988) was amplified using genomic DNA derived from PH5CH8 cells and a primer set of 5′-ACGGGGTACC-GAGTTTTAGAAACTACTAAAATG-3′ containing the *Kpn*I recognition site (underlined) and 5′-AGGAAGATCTTC-GAAAGGTTGCAGTTAGAATG-3′ containing the *Bgl*II recognition site (underlined). The obtained DNA fragment was

subcloned into the *Kpn*I and *Bgl*II sites of pGL3-Basic (Promega) and was termed pIFN-β(-125)-Luc. Retrovirus infections were performed as described previously (Naganuma et al., 2004).

RT-PCR and RNA interference

RT-PCR was carried out as described previously (Dansako et al., 2003). The sequences of sense and antisense primers for IFN-β (accession no. V00547) were 5′-CCCTGAGGAGATTAAGCAGCTGC-3′ and 5′-AGTTCCTTAGGATTTCCACTCTGAC-3′. The sequences of primer set for ISG56 (accession no. X03557) were 5′-AGAAGCAGGCAATCACAGAAAAGCTG-3′ and 5′-CCAGGGCTTCATTCATATTTCCTTCC-3′. Small-interference RNA (siRNA) duplexes targeting the coding regions of human TLR3, TLR4, and luciferase GL2 (Elbashir et al., 2001) as a control were chemically synthesized (Greiner, Tokyo, Japan). The sequences of the human TLR3 oligonucleotides were: 5′-CCUCCAGCACAAUGAGCUATT-3′ and 5′-UAGCUCAUUGUGCUGGAGGTT-3′. The sequences of the human TLR4 oligonucleotides were: 5′-CCUCCCCUUCUCAACCAAGTT-3′ and 5′-CUUGGUUGAGAAGGGGAGGTT-3′. The cells were transfected with the indicated siRNA duplex using OligofectAMINE (Invitrogen, Carlsbad, CA). Total RNAs were extracted after 3 days, and RT-PCR was performed using primer sets for TLR3 (Kadowaki et al., 2001), TLR4 (Kadowaki et al., 2001), and GAPDH (Dansako et al., 2003).

Western blot analysis

The preparation of cell lysates, SDS-PAGE, and immunoblotting analysis were performed according to standard procedures using primary antibodies, rat monoclonal anti-HA (3F10; Roche Molecular Biochemicals, Mannheim, Germany), mouse monoclonal anti-NS3 (clone MMM33; Novacastra Laboratories, Newcastle upon Tyne, UK), anti-NS5B (a gift from Dr. M. Kohara), anti-myc (PL14; Medical and biological laboratories, Nagoya, Japan), anti-β-actin (AC-15, Sigma), anti-STAT1 (clone 42; BD Transduction Laboratories, San Diego, CA), rabbit polyclonal anti-phospho-STAT1(Y701) (Cell Signaling Technology, Beverly, MA), and anti-IRF7 (H-246, Santa Cruz Biotechnology, Santa Cruz, CA), and horseradish-peroxidase-conjugated secondary antibodies. The immune complex was visualized using the ECL Western blot detection system (Amersham Bioscience, Piscataway, NJ).

Reporter assay

The luciferase activity was measured by dual-luciferase assay system (Promega, Madison, WI) as previously described (Dansako et al., 2003). Briefly, cells were transfected with pISRE-Luc (Stratagene, LaJolla, CA) or pIFN-β(-125)-Luc reporter plasmid together with phRL-CMV (Promega) as an internal control reporter plasmid by FuGENE6 (Roche). After 48 h of transfection, cell lysates were then prepared and assayed for luciferase activities; transfection efficiency was normalized by renilla luciferase activity (internal control) derived from phRL-CMV. Three independent triplicate transfection experiments were conducted in order to verify the reproducibly of the results.

Immunoprecipitation

Cells were lysed in a buffer containing 50 mM Tris (pH 7.4), 125 mM NaCl, 0.1%(v/v) Nonidet P-40 (NP-40; Sigma), 5 mM EDTA, 0.1 M NaF, and a mixture of protease inhibitors (Complete; Roche). Pre-cleared cell lysates were subjected to immunoprecipitation using agarose-conjugated anti-myc antibody (PL14, MBL). Bound proteins were eluted from beads by boiling in SDS sample buffer, and immunoblotting analysis was performed using anti-myc or anti-NS5B antibody, and HRP-conjugated anti-mouse IgG TrueBlot (eBioscience, San Diego, CA).

Immunofluorescence analysis

To examine the intracellular protein localization, 2×10^4 cells were cultured and treated on chamber slides then fixed and probed with polyclonal rabbit anti-IRF3 antibody (FL-425, Santa Cruz Biotechnology) and FITC-conjugated donkey anti-rabbit secondary antibody according to a method described previously (Foy et al., 2003). PH5CH8 cells treated with poly (IC) (2.5 μg/ml for 6 h; Amersham Biosciences) were used as a positive control for the activation of IRF3.

Evaluation of sensitivity to DNA damage

Cells in an exponential growth phase were plated onto 10-cm plates (5×10^3 cells/plate) and cultured for 4 days. The cells were treated with hydrogen peroxide (H_2O_2; Wako Pure Chemical, Osaka, Japan), methylmethane sulfonate (MMS; Sigma), Adriamycin (ADR; doxorubicin; Sigma), and neocarzinostatin chromophore (NCS; generously provided by Kayaku, Tokyo, Japan) for 2 h at 37 °C. For UV-B treatment (UV-B radiation at 302 nm), the medium was aspirated prior to exposure, the cells were washed twice with PBS, and then a fresh culture medium was added. Ten days later, the cells were fixed and stained with Coomassie brilliant blue as described previously (Naganuma et al., 2004). Only colonies containing >50 cells were scored as being derived from viable clonogenic cells.

Acknowledgments

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Epigenetic silencing of interferon-inducible genes is implicated in interferon resistance of hepatitis C virus replicon-harboring cells

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***Background/Aims*: We previously established hepatitis C virus (HCV) replicon-harboring cell lines possessing two interferon (IFN)-resistant phenotypes: a partially resistant phenotype (αR series) and a severely resistant phenotype (βR series). We recently found that the severe IFN resistance of the βR-series cells is caused by the functional disruption of type I IFN receptors. Here, we aimed to clarify the mechanism(s) underlying the partial IFN resistance of the αR-series cells.**

***Methods*: αR-series cells were pre-treated with 5-azacytidine to evaluate the effects of DNA demethylation on IFN resistance. cDNA microarray analysis was carried out in order to compare 1αR cells, which belong to the αR series, treated with both 5-azacytidine and IFN-α with cells treated with 5-azacytidine or IFN-α alone.**

***Results*: We found that the IFN-resistant phenotype of αR-series cells was impaired by treatment with 5-azacytidine. cDNA microarray analysis identified seven IFN-stimulated genes, which were up-regulated by 5-azacytidine treatment. We demonstrated here that the ectopic expression of each of these seven genes in 1αR cells frequently weakened the IFN resistance of these cells.**

***Conclusions*: The present results suggest that the epigenetic silencing of IFN-stimulated genes is implicated in the acquisition of a partially IFN-resistant phenotype of HCV replicon-harboring cells.**

***Keywords*: IFN resistance; HCV replicon; DNA methylation; cDNA microarray; Epigenetic silencing**

1. Introduction

Persistent infection by the hepatitis C virus (HCV) is a major cause of chronic hepatitis (CH) [1,2], which can progress to liver cirrhosis and hepatocellular carcinoma [3]. HCV is an enveloped RNA virus belonging to the family Flaviviridae, the genome of which consists of a positive-stranded 9.6-kb RNA encoding at least 10 structural and non-structural proteins [4]. Since, at least 170 million people are currently infected with HCV worldwide, this type of infection constitutes a global health problem [5]. Interferon (IFN)-α/ribavirin combination therapy is currently the standard clinical therapy for patients with CH C; however, the effectiveness of IFN is limited to approximately half of these patients [6]. This clinical finding suggests that HCV is resistant to the antiviral effects of IFN, and that HCV proteins directly or indirectly attenuate those effects [7].

Although HCV replicon system harboring autonomously replicating HCV subgenomic RNA containing the non-structural region [8] provides a powerful tool for various HCV studies, all of the HCV replicons established to date

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Abbreviations: aa, amino acid; 5-azaC, 5-azacytidine; CH, chronic hepatitis; HCV, hepatitis C virus; IFN, interferon; IFNAR, IFN receptor; IRF-1, IFN regulatory factor 1; ISG, IFN-stimulated gene; RT-PCR, reverse transcription-polymerase chain reaction.

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have been highly sensitive to IFN [9–12]. Based on our assumption that prolonged IFN treatment might change HCV replicons from the IFN-sensitive phenotype into an IFN-resistant phenotype, we established nine HCV replicon-harboring cell lines possessing two IFN-resistant phenotypes: a partially resistant phenotype (αR series; 1αR, 3αR, 4αR, 5αR, and αRmix) and a severely resistant phenotype (βR series; 1βR, 3βR, 4βR, and 5βR) obtained by IFN-α and IFN-β treatment, respectively [13]. αR- and βR-series cells were derived from clones 1, 3, 4, and 5, and 1αR, 3αR, 4αR, and 5αR cells were counterparts of 1βR, 3βR, 4βR, and 5βR cells, respectively [13]. Although genetic analysis of these replicons identified one common amino acid (aa) substitution and several cell line-specific aa substitutions, we failed to obtain the evidence of the direct involvement of these aa substitutions to IFN resistance [14]. However, we found frequent non-sense mutations and deletions in type I IFN receptor (IFNAR) genes (IFNAR1 and IFNAR2c) in the βR-series cells, but such mutations were rarely observed in the αR-series cells [14]. Since we demonstrated that the ectopic expression of wild-type IFNAR in the βR-series cells restored IFN signaling, we determined that the functional disruption of type I IFNAR was responsible for this type of resistance [14]. However, the mechanism underlying the partial IFN-resistance of αR-series cells remains unclear. Since, the expression levels of IFNARs, Tyk2, and Jak1 were not lower in the αR-series cells [13,14], the functional degeneration of other cellular factor(s) involved in IFN signaling or IFN-stimulated gene(s) (ISG) was thought to contribute to the acquisition of IFN resistance.

As one potential mechanism for the partial resistance, we assumed that the epigenetic silencing of some ISGs, which are known to be involved in the anti-HCV activity of IFN, by DNA methylation around the promoter region might convert HCV replicon-harboring cells from the IFN-sensitive phenotype to the IFN-resistant phenotype. To evaluate our hypothesis, we examined whether or not pre-treatment of αR-series cells with 5-azacytidine (5-azaC), an inhibitor of DNA methyltransferase and an inducer of gene suppressed by DNA methylation, could alter the IFN sensitivity of the cells.

Here, we report that the epigenetic silencing of ISGs is implicated in the IFN-resistance of αR-series cells. We have also identified several ISGs that are up-regulated by 5-azaC treatment and weaken the IFN resistance of αR-series cells.

2. Materials and methods

2.1. Cell cultures

HCV replicon-harboring cells and cured 6Mc cells [14], from which the HCV replicons had been eliminated by IFN-γ treatment, were maintained as described previously [13].

2.2. Analysis of IFN sensitivity

HCV replicon-harboring cells were treated with 5-azaC (2–10 μM) (A-2385, Sigma, St Louis, MO) for 2 weeks. Then, human IFN-α (I-2396; Sigma) was added to the cells (with or without pre-treatment with 5-azaC) as described previously [12,13]. After 3 weeks in culture, the colonies obtained on the culture dishes were stained with Coomassie brilliant blue (CBB) as described previously [15].

2.3. Quantitative analysis of HCV replicon RNA

The quantification of HCV RNA was carried out to monitor the antiviral effects of IFN-α, and was performed by real-time LightCycler polymerase chain reaction (PCR) as described previously [16,17].

2.4. Construction of replicon plasmid and RNA synthesis

The non-structural region (6.1 kb) fragment of a 1βR1 clone obtained from 1βR cells [13] was digested with SpeI and BsiWI, and the digested

Table 1
The primers used for RT-PCR analysis of mRNA expression

Gene	Direction	Nucleotide sequence	Products (bp)	Cycles
IFI27	Forward	gttttgcccctggccaggattgct	252	27
	Reverse	aatggagcccaggatgaacttggt		
9-27	Forward	tcttcttgaactggtgctgtctggg	191	28
	Reverse	agagccgaataccagtgacaggat		
LMP2	Forward	atggaaccctgggaggaatgctg	145	27
	Reverse	gcaatagcgtctgtggtgaagcg		
LMP7	Forward	ctgggataagaagggtcctggac	293	27
	Reverse	tactggtgcagcaggtcactggac		
Viperin	Forward	tggagcgccacaaagaagtgtcct	240	27
	Reverse	ccagcttcagatcagccttactcc		
IFI44	Forward	tgtggctttgctcactcatgtgga	227	31
	Reverse	cagcccatagcattcgtctcagag		
IFIT2	Forward	aggccatccaccactttatagagg	272	28
	Reverse	tgggcaccacatctctattctcca		
ISG56	Forward	tagccaacatgtcctcacagac	396	32
	Reverse	tcttctaccactggtttcatgc		
GAPDH	Forward	gactcatgaccacagtccatgc	334	26
	Reverse	gaggagaccacctggtgctcag		

fragment (5.7 kb) was ligated into the plasmid pNSS1RZ2RU [12], which was predigested with SpeI and BsiWI. The obtained plasmid was linearized by XbaI and was used for RNA synthesis with T7 MEGAscript (Ambion) as previously described [12].

2.5. RNA transfection and selection of G418-resistant cells

The transfection of HCV replicon RNA synthesized in vitro into 6Mc cells was performed by electroporation, and the cells were selected in the presence of G418 (300 μg/ml; Invitrogen) for 3 weeks as described previously [14].

2.6. cDNA microarray analysis

The 1αR cells (5×10^5 cells), which were cultured for 2 weeks in the absence or presence of 5-azaC (10 μM), were plated onto a 10-cm diameter dish, and were cultured for 2 days in the absence of G418. Then the cells were treated with or without IFN-α (500 IU/ml) for 8 h. Total RNAs prepared from 80% confluent cells were subjected to cDNA microarray analysis (CodeLink™, Uniset human I containing 54840 spots of 30-mer oligonucleotides; Amersham Biosciences) as described previously [18].

2.7. Reverse transcription (RT)-PCR

RT-PCR and real-time LightCycler PCR were performed as described previously [18,19] using the primer sets in Table 1.

2.8. Expression vectors and retroviral infection

Retroviral vectors pCXbsr [20] and pC4bsr(IRES) (kindly provided by Dr T. Akagi), which contain the resistance gene for blasticidin, were used in this study. Retroviral infection and selection by blasticidin were performed as described previously [15].

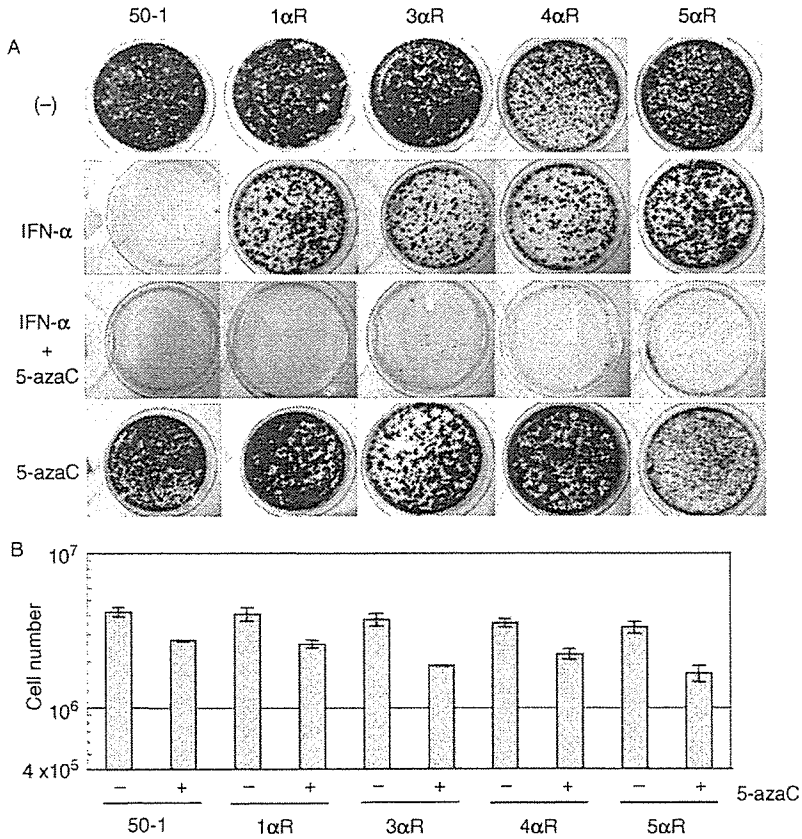

Fig. 1. 5-AzaC treatment converts αR-series cells from an IFN-resistant phenotype into an IFN-sensitive phenotype. (A) IFN sensitivities of various HCV replicon-harboring cells. αR-series cells (1αR, 3αR, 4αR, and 5αR) showing a partially IFN-resistant phenotype and the parent 50-1 cells with an IFN-sensitive phenotype were used for the analysis. These cells were plated onto 10-cm diameter dishes (2×10^4 cells/dish) and were cultured for 1 day before treatment with 5-azaC. 5-AzaC was added to the cultures at a final concentration of 10 μM (4 μM for 4αR cells, and 2 μM for 5αR cells) and the cells were then cultured for 2 weeks, and were subsequently treated with or without IFN-α (400 IU/ml) for 3 weeks in the presence of G418 (300 μg/ml). The HCV replicon-harboring cells were known to possess the G418-resistant phenotype, because neomycin phosphotransferase was produced by the efficient replication of the HCV replicon in the cells. Therefore, when an HCV replicon was excluded from the cells or levels of the replicon were low, the cells did not survive in the presence of G418. The panels show G418-resistant colonies stained with CBB as described previously [15]. (B) Effect of 5-azaC on the growth of HCV replicon-harboring cells. 50-1 and αR-series cells were plated onto 10-cm diameter dishes (4×10^5 cells/dish) and were cultured for 1 day before treatment with 5-azaC. These cells were cultured in the absence or presence of 5-azaC (10 μM for 50-1, 1αR, and 3αR cells, 4 μM for 4αR cells, and 2 μM for 5αR cells) for 3 days, and then the cell number was determined by a method described previously [27]. The data indicate means ± SD of triplicates from two independent experiments. [This figure appears in colour on the web.]

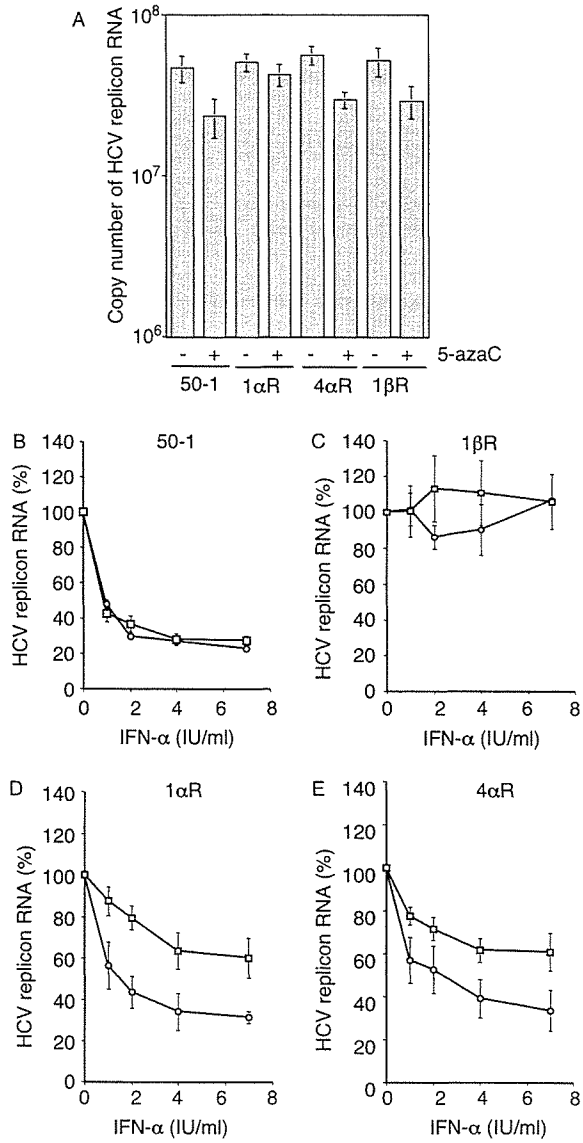

Fig. 2. IFN sensitivities of HCV replicons. (A) HCV replicon RNA levels of 50-1, 1βR, 1αR, and 4αR cells cultured in the presence or absence of 5-azaC (10 μM for 50-1, 1βR, and 1αR cells, 4 μM for 4αR cells) for 2 weeks. Real-time LightCycler PCR was carried out in order to quantitatively monitor the levels of replicon RNAs in the cells, as described previously [16,17]. The copy numbers of HCV replicon RNA per μg of total RNA determined by quantitative RT-PCR are shown. (B) 50-1, (C) 1βR, (D) 1αR, and (E) 4αR cells were cultured in the presence (open circles) or absence (open squares) of 5-azaC as described in (A), and then the cells were treated with IFN-α (0, 1, 2, 4, and 7 IU/ml each) for 3 days. Quantitative RT-PCR was carried out as described in (A). The relative level of HCV replicon RNA (%) calculated at each point, when the HCV replicon RNA level of IFN non-treated cells was assigned to be 100%, is shown here. The data indicate means ± SD of triplicates from two independent experiments.

2.9. Western blot analysis and immunoprecipitation

The preparation of cell lysates, SDS-PAGE, and immunoblotting analysis were performed as previously described [21].

Table 2
Genes whose elevated levels in the C vs. IFN + 5-azaC experiment were more than 2.5-fold those in the C vs. IFN or C vs. 5-azaC experiment

Gene	C vs. IFN + 5-azaC	C vs. IFN	C vs. 5azaC	Accession no.
IFI27	25.21[a]	1.56	1.72	NM_005532
9-27	2703.30	600.65	−0.04	NM_003641
LMP2	23.60	6.20	1.57	NM_002800
LMP7	22.27	8.79	3.72	NM_004159
Viperin	25.26	9.07	−2.26	NM_080657
IFI44	29.60	7.05	0.03	NM_006417
IFIT2	10.67	2.96	−3.02	NM_001547

The manufacturer's protocol (Amersham Biosciences) recommended the use of a ratio of at least 2:1 as a standard for the selection of genes showing significant differences in expression.
[a] Fold.

3. Results

3.1. IFN-resistant phenotype of HCV replicon-harboring cells is impaired by co-treatment with 5-azaC and IFN-α

Based on our assumption, we examined whether or not pre-treatment of αR-series cells with 5-azaC could convert these cells from the IFN-resistant phenotype to the IFN-sensitive phenotype. Parent HCV replicon-harboring cells (50-1) [22], and αR-series cells were treated with IFN-α and/or 5-azaC, and their IFN sensitivities were compared. After IFN-α treatment, many 1αR, 3αR, 4αR, and 5αR cell colonies survived, although only a few small colonies of 50-1 cells survived. However, when 5-azaC pre-treatment following IFN-α treatment was applied, almost none of the 1αR, 3αR, 4αR, or 5αR cell colonies survived, nor did the colonies of 50-1 cells (Fig. 1(A)). Although, we observed that 5-azaC treatment slightly lowered the growth rates of these cells (Fig. 1(B)), the cell viabilities exceeded 99%, and a number of colonies were finally obtained after treatment with 5-azaC alone (Fig. 1(A)). These results suggest that 5-azaC treatment converts αR-series cells from an IFN-resistant phenotype into an IFN-sensitive phenotype.

Since, we previously observed that none of the non-sense mutations in IFNAR genes found in all clones derived from 1βR and 4βR cells were detected in 1αR and 4αR cells, which were the counterparts of 1βR and 4βR cells, respectively [14], we focused on 1αR and 4αR cells to assess the effect of 5-azaC treatment on IFN sensitivity. Quantitative RT-PCR analysis of replicon RNA in cells treated with IFN-α was performed using 1αR, 4αR, 1βR (a counterpart of 1αR), and 50-1 cells pre-treated with or without 5-azaC. We confirmed that HCV replicon RNA levels in the cells pre-treated with 5-azaC were sufficient for analysis, although HCV replicon RNA levels were a little lower by 5-azaC treatment (Fig. 2(A)). The level of replicon RNA in 50-1 cells drastically decreased after IFN-α treatment, regardless of 5-azaC pre-treatment (Fig. 2(B)). Contrary to the level observed in 50-1 cells, that of replicon

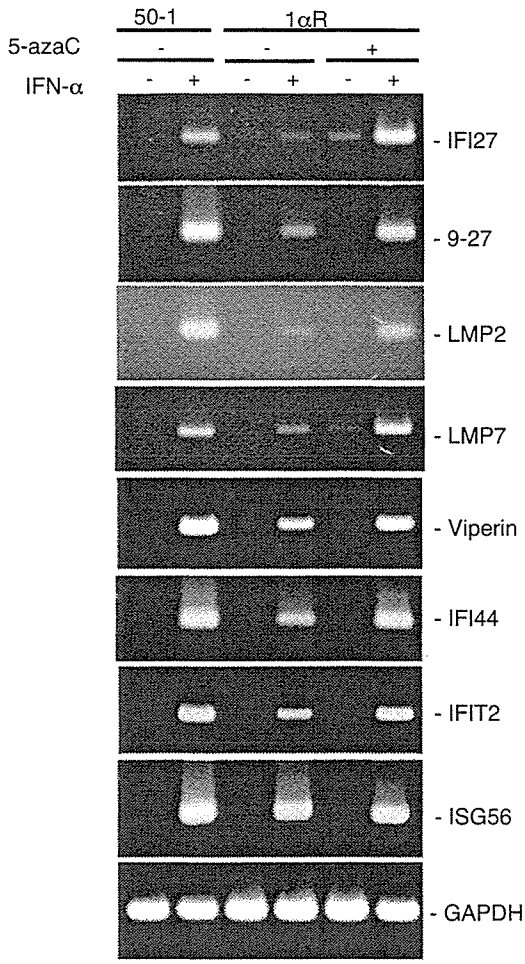

Fig. 3. RT-PCR analysis of mRNA expression of selected ISGs based on the microarray results. 1αR cells were cultured in the presence or absence of 5-azaC (10 μM) for 2 weeks. The 1αR cells treated with 5-azaC and the 50-1 cells were treated with or without IFN-α (500 IU/ml) for 8 h. The total RNAs extracted from these cells were subjected to RT-PCR using the primer sets listed in Table 1. ISG56 was used as the representative ISG, which was not selected by the microarray analysis. GAPDH was used as an internal control. RT-PCR products were detected by staining the samples with ethidium bromide after 3% agarose gel electrophoresis.

RNA in 1βR cells did not decrease after IFN-α treatment, regardless of 5-azaC pre-treatment (Fig. 2(C)). However, we observed that the level of replicon RNA in 1αR cells (Fig. 2(D)) and that in 4αR cells (Fig. 2(E)) pre-treated with 5-azaC had decreased more substantially after IFN-α treatment than that in the cells lacking 5-azaC pre-treatment. In general, these results indicate that 5-azaC treatment of αR-series cells (at least 1αR and 4αR cells) may convert the cells from a partially IFN-resistant phenotype to an IFN-sensitive phenotype; moreover, the present findings suggest that some ISGs known to contribute to the anti-HCV activity of IFN are suppressed by DNA methylation in αR-series cells.

3.2. cDNA microarray analysis using 1αR cells

Based on the results presented above, we attempted to identify those ISGs that are determinative of IFN sensitivity; to this end, cDNA microarray analyses were performed by comparing the following: 1αR cells with 1αR cells treated with 5-azaC (C vs. 5-azaC), 1αR cells with 1αR cells treated with IFN-α (C vs. IFN), and 1αR cells with 1αR cells treated with IFN-α after pre-treatment with 5-azaC (C vs. IFN+5-azaC). As the first step in this analysis, we selected the only already-known genes whose expression levels were up-regulated at a ratio of more than 10 in C vs. IFN+5-azaC. Then, from among those that were selected in the first step, we performed an additional selection of genes whose elevated levels in the C vs. IFN+5-azaC experiment were more than 2.5-fold, as compared with those in the C vs. IFN or C vs. 5-azaC experiment. Finally, seven genes (IFI27, IFI44, LMP2, LMP7, 9-27, Viperin, and IFIT2) were identified as genes that were highly and selectively induced by co-treatment with IFN-α and 5-azaC (Table 2).

In order to confirm the results of our microarray selection, we conducted RT-PCR analysis and real-time LightCycler PCR to examine the mRNA levels of the identified seven genes in the 1αR cells treated with or without IFN-α after pre-treatment with or without 5-azaC. As a control, parent 50-1 cells treated with or without IFN-α were used. The results (Fig. 3 and Table 3) confirmed the results of the microarray analysis (Table 2). The findings revealed that the levels of induction of these seven genes in 1αR cells treated with IFN-α were clearly lower than those in 50-1 cells treated with IFN-α. It is noteworthy that the expression of these seven genes in 1αR cells treated with IFN-α after 5-azaC pre-treatment was remarkably elevated, whereas the IFN-α-induced level of expression of the ISG56 gene, an immediate early antiviral ISG, was slightly enhanced after 5-azaC pre-treatment (Fig. 3 and Table 3). These results suggest that the epigenetic silencing of these ISGs is involved in the acquisition of the IFN-resistant phenotype, at least in 1αR cells, and this is also likely to be the case in other αR-series cells.

3.3. Characterization of additional HCV replicon-harboring cells possessing an IFN-resistant phenotype

In order to evaluate the reproducibility of the phenomenon observed in 1αR cells, additional HCV replicon-harboring 1βR1/6Mc cells, which were recently established independently [14], were used for IFN-α treatment (Fig. 4(A)). IFN-α-treated 1βR1/6Mc cells yielded several distinct IFN-resistant colonies, which were designated as 1βR1/6McαR mixed colonies.

To assess the effects of 5-azaC treatment on IFN-sensitivity, quantitative RT-PCR analysis of replicon RNA in the cells treated with IFN-α was performed using 1βR1/6Mc cells, 1βR1/6McαR cells, and 1βR1/6McαR cells pre-treated with 5-azaC (designated as 1βR1/6McαR+AZ cells). The level of replicon RNA in 1βR1/6Mc cells

Table 3
Real-time RT-PCR analysis of mRNA expression of selected ISGs based on the microarray results

Gene	50-1		1αR				5-azaC
	−		−		+		
	−	+	−	+	−	+	IFN-α
IFI27	4.8±1.9	100	6.4±3.2	11.9±1.9	22.9±5.4	380.4±18.9	
9-27	<1.0	100	<1.0	6.8±4.6	<1.0	46.0±6.7	
LMP2	<1.0	100	<1.0	11.7±0.9	2.7±0.3	60.5±4.5	
LMP7	2.3±0.5	100	2.1±0.3	19.8±2.0	8.8±1.3	92.6±18.7	
Viperin	n.d.	100	n.d.	7.4±2.0	<1.0	35.2±5.2	
IFI44	n.d.	100	n.d.	5.9±0.5	n.d.	56.0±7.1	
IFIT2	<1.0	100	<1.0	16.8±9.1	<1.0	48.4±13.3	
ISG56	<1.0	100	<1.0	76.2±4.1	<1.0	117.1±8.7	

The experiments were performed in at least triplicate. n.d., not detected. To correct the differences in RNA quality and quantity between the samples, data were normalized using the ratio of each mRNA concentration to that of GAPDH. The relative level (mean±SD) of each mRNA calculated, when the level of each mRNA of 50-1 cells treated with IFN-α was assigned to be 100, is shown here.

decreased markedly after IFN-α treatment (Fig. 4(B)), as had also been observed in the 50-1 cells (Fig. 2(B)). In contrast to the 1βR1/6Mc cells, 1βR1/6McαR cells exhibited a partially IFN-resistant phenotype (Fig. 4(B)), as had been observed in the 1αR cells (Fig. 2(D)) and 4αR cells (Fig. 2(E)). However, after IFN-α treatment, the level of replicon RNA in the 1βR1/6McαR+AZ cells was lower than that in 1βR1/6McαR cells (Fig. 4(B)). This result suggests that the treatment of 1βR1/6McαR cells with 5-azaC also contributed to a weakening of the IFN-resistant phenotype in these cells.

We next considered the expression levels of the seven genes that had been identified as epigenetically suppressed genes in 1αR cells; here, IFN-α-treated 1βR1/6Mc and 1βR1/6McαR cells were compared. The results obtained by RT-PCR analysis revealed that the induction of these seven genes by IFN-α in the 1βR1/6McαR cells was weaker than

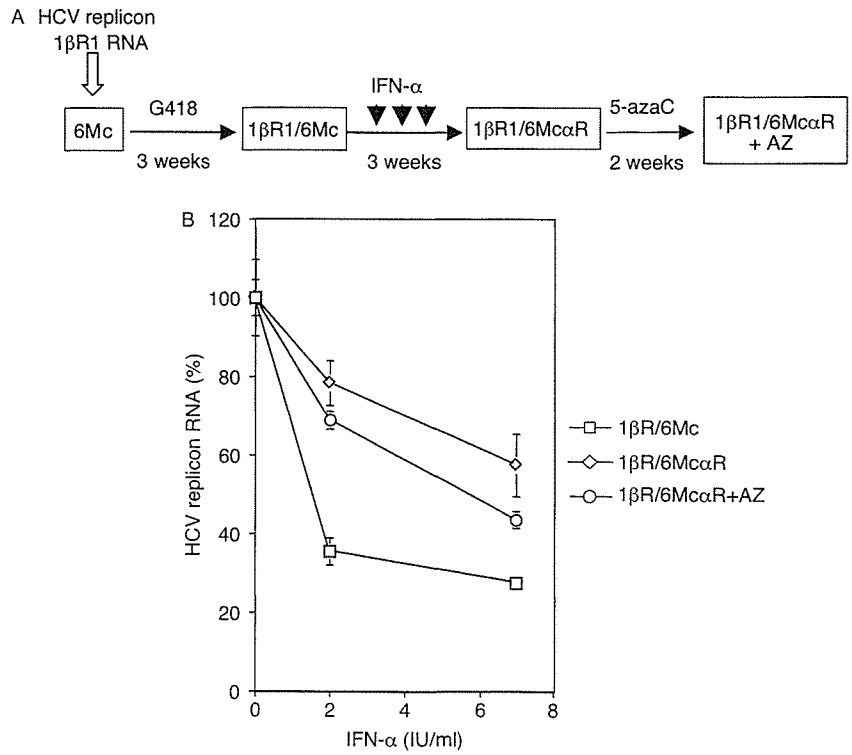

Fig. 4. Effect of 5-azaC treatment on IFN resistance in newly isolated HCV replicon-harboring cells showing a partially IFN-resistant phenotype. (A) Outline of the isolation of HCV replicon-harboring cells showing partial IFN resistance. HCV replicon-harboring cells (designated as 1βR1/6Mc cells) [14], established by the transfection of in vitro-synthesized replicon RNA (1βR1 obtained from 1βR cells) [13] into 6Mc cells, were treated with 400 IU/ml of IFN-α for 3 weeks in the presence of G418, and several colonies survived as cells with an IFN-resistant phenotype (1βR1/6McαR cells). The 1βR1/6McαR cells were treated with 5-azaC (4μM) for 2 weeks. (B) IFN sensitivities of HCV replicons. The HCV replicon-harboring cells treated with IFN-α (0, 2, and 7 IU/ml each) for 3 days were subjected to quantification of HCV replicon RNA, as described in Fig. 2.

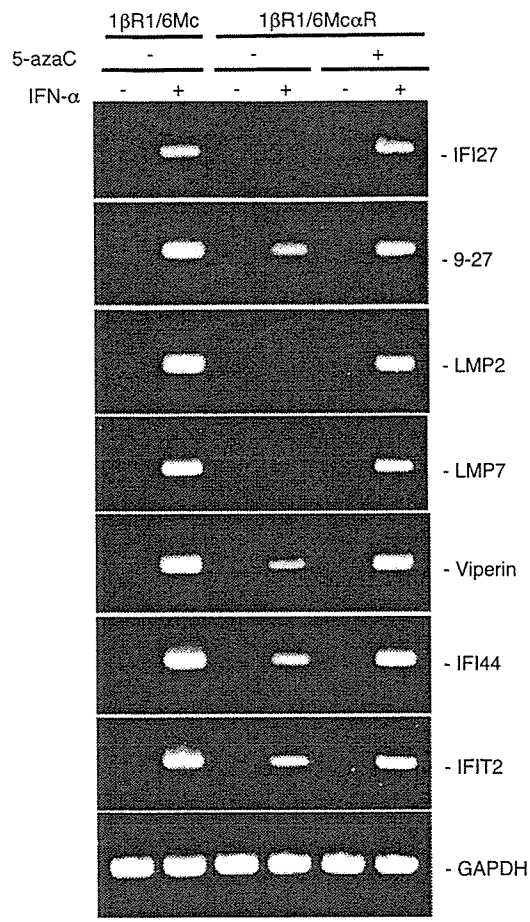

Fig. 5. RT-PCR analysis of mRNA expression of seven ISGs selected by microarray analysis. 1βR1/6McαR cells were cultured in the presence or absence of 5-azaC (4 μM) for 2 weeks. The 1βR1/6McαR cells were treated with 5-azaC and the 1βR/6Mc cells were treated with or without IFN-α (500 IU/ml) for 8 h. The total RNAs extracted from the cells were subjected to RT-PCR using the primer sets listed in Table 1. GAPDH was used as an internal control. RT-PCR products were detected by staining the samples with ethidium bromide after 3% agarose gel electrophoresis.

that in the parent 1βR1/6Mc cells (Fig. 5). However, these seven genes were induced by IFN-α in 1βR1/6McαR cells pre-treated with 5-azaC (Fig. 5). These results were confirmed by real-time LightCycler PCR (Table 4), and were similar to those obtained in the study of 1αR cells (Fig. 3 and Table 3), thus demonstrating that the appearance of the IFN-resistant phenotype is reproducible. Furthermore, these results suggest that some form of epigenetic silencing such as DNA methylation is frequently involved in the acquisition of the IFN-resistant phenotype.

3.4. Ectopic expression of epigenetically suppressed genes weakens the IFN resistance of 1αR cells

To evaluate the effects of epigenetically suppressed genes on the IFN sensitivity of HCV replicons, we prepared 1αR cells stably expressing IFI27, IFI44, LMP2, LMP7, 9-27, Viperin, or IFIT2 using retroviral gene transfer system [15]. The ectopic expression of each ISG protein was confirmed by Western blot analysis or immunoprecipitation following Western blot analysis (Fig. 6(A)). The results obtained by quantitative RT-PCR analysis revealed that the level of HCV replicon RNA decreased to about half that in the cells expressing Viperin. Also, in LMP2-, LMP7-, and IFIT2-expressing cells, significant reductions of HCV replicon RNA were detected (Fig. 6(B)). These results indicate that the simple expression of these genes into the cells interferes with the level of HCV replicon RNA, and that the combination of ISGs contributes to the IFN resistance of the HCV replicon. Therefore, 1αR cells expressing each ISG protein were initially treated with IFN-α, and then the level of HCV replicon RNA was monitored by quantitative RT-PCR analysis. Interestingly, the results revealed that the level of HCV replicon RNA in the 1αR cells expressing Viperin or LMP7 was largely decreased to the level in parent 50-1 cells (Fig. 6(C)). Furthermore, we observed that the 1αR cells expressing IFIT2, 9-27, LMP2, or IFI44 also partially changed to the IFN-sensitive phenotype (Fig. 6(C)). These results suggest that the transcriptional suppression of ISGs such as LMP-7

Table 4
Real-time RT-PCR analysis of mRNA expression of seven ISGs selected by microarray analysis

Gene	1βR1/6Mc		1βR1/6McαR				5-azaC
	−		−		+		
	−	+	−	+	−	+	IFN-α
IFI27	4.0±0.8	100	13.7±2.0	18.4±3.7	60.9±10.7	383±16.4	
9-27	<1.0	100	<1.0	14.8±0.2	1.0±0.2	101.7±26.3	
LMP2	1.1±0.1	100	<1.0	6.9±0.6	3.0±0.3	112.2±38.1	
LMP7	3.5±0.4	100	<1.0	9.9±0.7	8.7±0.9	96.5±11.2	
Viperin	n.d.	100	n.d.	23.2±9.4	<1.0	104.8±14.0	
IFI44	n.d.	100	n.d.	4.5±0.9	n.d.	57.3±1.9	
IFIT2	<1.0	100	<1.0	10.2±16	<1.0	67.9±9.2	

The experiments were performed in at least triplicate. n.d., not detected. To correct the differences in RNA quality and quantity between the samples, data were normalized using the ratio of each mRNA concentration to that of GAPDH. The relative level (mean±SD) of each mRNA calculated, when the level of each mRNA of 1βR1/6Mc cells treated with IFN-α was assigned to be 100, is shown here.

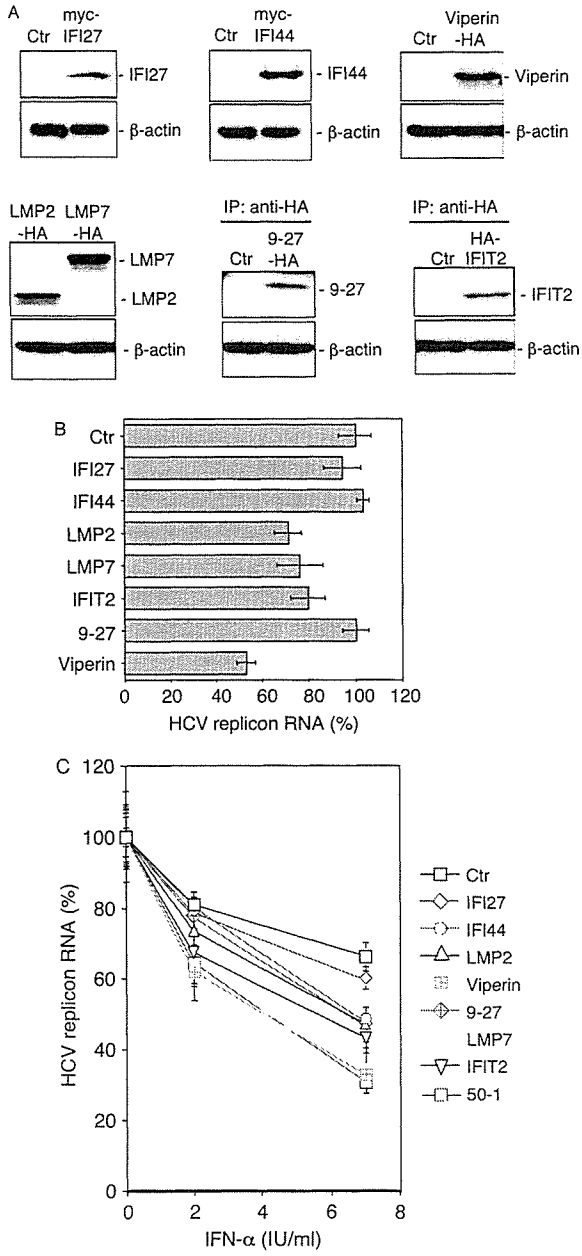

Fig. 6. IFN sensitivities of HCV replicons in 1αR cells expressing epigenetically silenced ISG. (A) Ectopic expression of epigenetically silenced ISG in 1αR cells introduced by retrovirus-mediated gene transfer. The DNA fragments encoding myc-tagged IFI27 and IFI44, and influenza hemagglutinin-tagged (HA)-LMP2, LMP7, 9-27, and IFIT2 were obtained by the PCR amplification of cDNAs derived from HCV replicon-harboring cells using primer sets designed from the nucleotide sequences (see the Accession no. in Table 2). The obtained DNA fragments were cloned into the EcoRI and NotI sites of pCXbsr, as described previously [28]. The DNA fragment encoding the HA-IFIT2 was cloned into the EcoRI and NotI sites of pC4bsr(IRES). The sequences of the plasmid inserts were confirmed by Big Dye terminator cycle sequencing on an ABI PRISM 310 genetic analyzer (Applied Biosystems). Western blot analysis or immunoprecipitation (IP) following Western blot analysis of 1αR cells infected with pCXbsr retroviruses encoding myc-IFI27, myc-IFI44, Viperin-HA, LMP2-HA, LMP7-HA, 9-27-HA, and HA-IFIT2 was performed by anti-HA (rat monoclonal 3F10; Roche, Mannheim, Germany) or anti-myc (mouse monoclonal PL14; Medical and Biological Laboratories, Nagoya, Japan) antibodies. In order to detect 9-27-HA and HA-IFIT2, IP was performed by using anti-HA affinity matrix (3F10, Roche). pCXbsr or pC4bsr (IRES) retrovirus-infected 1αR cells were used as a control (Ctr). β-actin was detected by anti-β-actin antibody (AC-15, Sigma) as a control for the amount of protein loaded per lane. (B) The quantification of HCV replicon RNA in 1αR cells expressing epigenetically silenced ISG was performed as described in Fig. 2. pCXbsr retrovirus-infected 1αR cells were used as a control (Ctr). (C) IFN sensitivities of HCV replicon RNA in 1αR cells expressing epigenetically silenced ISG. ISG-expressed 1αR cells treated with IFN-α (0, 2, and 7 IU/ml each) for 3 days were subjected to quantification of HCV replicon RNA, as described in Fig. 2. pCXbsr retrovirus-infected 1αR cells were used as a control (Ctr). The relative level of HCV replicon RNA (%) calculated at each point, when the HCV replicon RNA level of IFN non-treated cells expressing each ISG was assigned to be 100%, is shown here. [This figure appears in colour on the web.]

and Viperin, among other ISGs, is involved in the partial IFN resistance of HCV replicon-harboring cells.

4. Discussion

Although, we identified several ISGs, which are expected to contribute to the acquisition of the IFN-resistant phenotype, the relationships among these ISGs remain unclear, in particular as regards issues such as anti-HCV activity and the mechanisms of induction of these ISGs after 5-azaC treatment. In this context, several plausible explanations can be considered: (1) all seven ISGs are independently induced by 5-azaC treatment; (2) one of the seven ISGs is induced by 5-azaC treatment, and then this ISG activates the other ISGs; (3) a certain transcriptional factor (e.g. IFN regulatory factor 1 (IRF-1), IRF-7, etc.) is induced by 5-azaC treatment, and then this factor activates the seven ISGs; (4) a certain combination of a identified ISG and an unidentified ISG in the present study exhibits anti-HCV activity; and/or (5) a certain combination of a number of ISGs identified in the present study exhibits anti-HCV activity. As regards possibilities (1) and (2), we examined the expression levels of seven ISGs in 1αR cells ectopically expressing one of these seven ISGs. We did not obtain evidence demonstrating that one of the seven ISGs activates the other ISGs (data not shown); therefore, possibility (1) was supported and possibility (2) was excluded (data not shown). As regards option (3), it was noted that ectopic IRF-1 expression suppresses the replication of the HCV replicon via the activation of ISGs [23]. However, we did not observe any significant induction of the mRNA for the IRF-1 gene in 1αR cells co-treated with IFN-α and 5-azaC (data not shown). Moreover, no induction of the IRF-7 gene was observed in 1αR cells co-treated with IFN-α and 5-azaC (data not shown). Therefore, possibility (3) also appears unlikely, although the possibility of the induction of another transcriptional factor(s) cannot be excluded. As regards

option (4), a number of ISGs that were not selected by the present microarray analysis could be considered as candidates for contributing to anti-HCV activity. However, at least with respect to the representative 2′-5′-oligoadenylate synthetase (2′-5′-OAS) and double-stranded RNA-specific adenosine deaminase (ADAR1), no significant differences in mRNA induction of 2′-5′-OAS or ADAR1 genes by IFN-α treatment were observed between 50-1 and 1αR cells, regardless of 5-azaC pre-treatment (data not shown). As regards option (5), it is likely that a combination of several ISGs is involved in the conversion to the IFN-sensitive phenotype of these cells, because the expression of LMP-2, LMP-7, or Viperin alone was associated with reductions in the level of HCV replicon RNA (Fig. 6(B)). Therefore, in order to clarify this issue, future studies involving the co-expression of these ISGs will also be necessary.

Among the genes selected in this study, LMP2 and LMP7 are of interest; these genes are known as catalytic subunits of immunoproteasome, which is induced by IFN-γ [24]. These genes have recently been identified as being suppressed in HCV replicon-harboring cells [18]. The down-regulation of these genes may contribute to persistent viral infection due to the acquisition of the IFN-resistant phenotype.

Viperin is a third gene of interest identified in this study. Viperin is induced by IFN-α/β, IFN-γ, as well as by human cytomegalovirus (HCMV) infection [25]. Stable expression of Viperin in fibroblasts inhibited HCMV infection and downregulated several HCMV proteins, although its molecular mechanism remains unclear at present [25]. Since, HCV replication complexes have been associated with inner cellular lipid membrane structures [26], viperin may contribute to the inhibition of HCV replication.

The present study using HCV replicon-harboring cell lines to investigate the mechanisms of IFN resistance may contribute to further diagnostic study of IFN sensitivity among patients with CH C. However, additional comparisons of ISG expression levels in IFN responders and non-responders with CH C will be required to understand the mechanisms underlying IFN resistance associated with HCV.

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Oligomerization of Hepatitis C Virus Core Protein Is Crucial for Interaction with the Cytoplasmic Domain of E1 Envelope Protein▽

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Hepatitis C virus (HCV) contains two membrane-associated envelope glycoproteins, E1 and E2, which assemble as a heterodimer in the endoplasmic reticulum (ER). In this study, predictive algorithms and genetic analyses of deletion mutants and glycosylation site variants of the E1 glycoprotein were used to suggest that the glycoprotein can adopt two topologies in the ER membrane: the conventional type I membrane topology and a polytopic topology in which the protein spans the ER membrane twice with an intervening cytoplasmic loop (amino acid residues 288 to 360). We also demonstrate that the E1 glycoprotein is able to associate with the HCV core protein, but only upon oligomerization of the core protein in the presence of tRNA to form capsid-like structures. Yeast two-hybrid and immunoprecipitation analyses reveal that oligomerization of the core protein is promoted by amino acid residues 72 to 91 in the core. Furthermore, the association between the E1 glycoprotein and the assembled core can be recapitulated using a fusion protein containing the putative cytoplasmic loop of the E1 glycoprotein. This fusion protein is also able to compete with the intact E1 glycoprotein for binding to the core. Mutagenesis of the cytoplasmic loop of E1 was used to define a region of four amino acids (residues 312 to 315) that is important for interaction with the assembled HCV core. Taken together, our studies suggest that interaction between the self-oligomerized HCV core and the E1 glycoprotein is mediated through the cytoplasmic loop present in a polytopic form of the E1 glycoprotein.

Hepatitis C virus (HCV) is the causative agent of chronic hepatitis C, leading to steatosis, cirrhosis, and hepatocellular carcinoma. It is estimated that over 170 million people are infected with HCV worldwide (5, 18, 37). HCV is an enveloped single-stranded plus-sense RNA virus in the *Hepacivirus* genus of the family *Flaviviridae*, which also includes the flaviviruses and pestiviruses (36). The genome of HCV encodes a polyprotein of approximately 3,000 amino acids which is cotranslationally and posttranslationally processed to generate at least 10 viral proteins (12). The structural proteins, the core and E1 and E2 envelope glycoproteins, are encoded in the N-terminal portion of the polyprotein, and the nonstructural proteins, thought to be required for replication of the viral genome, are encoded in the C-terminal region (11). The core protein, which interacts with viral RNA (47) to form the nucleocapsid, is liberated from the N terminus of the polyprotein by signal peptidase cleavage in the downstream E1 protein (at position 191), and the C-terminal transmembrane region of the core protein (residue 164 to 191) is further cleaved at residues 177 or 179 by the signal peptide peptidase (16, 43). The remaining hydrophobic region of the core protein (domain II; residues 119 to 174) has been shown to affect the efficiency of signal peptide peptidase cleavage and the intracellular localization of core protein (14, 44). Although the C-terminal transmembrane region of core protein and E1 were reported to interact with each other within the intramembrane space (25), the central hydrophobic region from residues 119 to 152 within domain II was also suggested to be responsible for the interaction between core and E1 (27).

Recently, in vitro replication of a JFH1 clone of HCV genotype 2a derived from a patient with fulminant hepatitis C was reported in a cell line that had been cured of its HCV replicon by treatment with interferon (23, 50, 51). However, this reverse genetics system is limited to the JFH-1 clone of genotype 2a and specific cell lines. Robust and reliable in vitro replication of other major genotypes of HCV such as genotypes 1a and 1b has yet to be developed. So far, biological functions of HCV envelope proteins have been characterized by using recombinant envelope proteins expressed in vitro, HCV-like particles produced in insect cells, and the pseudotyped virions based on vesicular stomatitis virus and retroviruses (8). The HCV polyprotein precursor must be specifically threaded through the membrane of the endoplasmic reticulum (ER) to undergo maturation to form the mature envelope glycoproteins (7). In the polyprotein, the C-terminal regions of E1 and E2 each contain a membrane-spanning domain as well as the hydrophobic signal peptide of the downstream viral protein (E2 and p7, respectively). These domains form hairpin structures that pass through the membrane twice, to allow processing by signal peptidase in the ER lumen. Upon signal peptidase cleavage, the C termini are thought to translocate into the cytoplasm to generate the type I membrane topology of the mature glycoproteins. The mature E1 and E2 glycoproteins

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