as protease inhibitors or polymerase inhibitors, which are now undergoing clinical trials, directly target viral proteins and inhibit their enzymatic activity. Because cyclosporins such as CsA and NIM811 target a cellular factor, CyPB, as described above, these compounds could serve as an additional type of anti-HCV agent. Moreover, viruses resistant to cyclosporins are less likely to occur, since antiviral compounds that target cellular factors generally induce less drug resistance than those inhibiting viral proteins; this difference is due to the high mutation rates of RNA viruses [26–30]. Thus, this novel anti-HCV candidate could provide an alternative strategy to combat HCV.

Acknowledgments

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Helper virus-independent *trans*-replication of hepatitis C virus-derived minigenome

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Abstract

We have previously described a synthetic T7-driven cDNA minigenome containing the antisense sequence of luciferase gene and internal ribosome entry site of encephalomyocarditis virus flanked by 5′- and 3′-end sequences of hepatitis C virus (HCV) that contain *cis*-acting replication elements. Synthesis of minus-strand RNA from the artificial minigenome was determined by using Huh-7 cells harboring autonomously replicating HCV subgenome as a helper for provision of functional replication components. To further confirm and extend these studies, we investigated here whether the minigenome replication system could be reconstituted by transfection of naïve Huh-7 cells with plasmid expressing nonstructural (NS) proteins. Reporter assay and Northern blot analysis revealed that *trans*-expression of NS proteins from 3 to 5 resulted in high level of luciferase activity and synthesized minus-strand RNA. The analogous result was also obtained with the minigenome derived from HCV 2a, and both HCV 1b- and 2a-derived NS protein were able to support the chimeric minigenomes whose 5′- or 3′-end was replaced by the respective region of the heterologous virus. These results provide a basis for establishing the reverse genetic system that is helpful to study *cis*- and *trans*-acting factors involved in HCV RNA replication.

Keywords: Hepatitis C virus; Minigenome; *trans*-Replication

Hepatitis C virus (HCV) is an important human pathogen with an estimated 170 million chronic carriers throughout the world, and many of them are at a high risk for developing liver cirrhosis and hepatocellular carcinoma [1]. HCV is a member of the *Flaviviridae* family with a positive-sense RNA genome of ~9600 nucleotides in length. The genome is flanked by highly structured nontranslated regions (NTRs) important for both RNA translation and replication. The viral genome encodes a polyprotein precursor of approximately 3010 amino acids, which is processed by viral and cellular protease to produce the structural proteins (core, E1, and E2) and nonstructural (NS) proteins (p7 and NS2 to NS5B).

Like other plus-stranded RNA viruses, HCV genomic RNA is first transcribed into a minus-strand intermediate, which in turn serves as the template for production of progeny plus-strand RNA. Although the basic steps in replication have been well established, little is known about the detail of these processes. Studies of HCV replication have been hampered by the lack of an efficient tissue culture system. Although the development of subgenomic replicon has facilitated the investigation of viral RNA replication in cell culture [2], culture-adaptive mutations within the NS proteins are required for efficient replication [3,4], and full-length genomes carrying such mutations do not produce infectious virus particles [5,6]. More recently, it was reported that genotype 2a JFH1 genome replicates efficiently independent of the culture-adaptive mutations and supports production of viral particles [7]. This *in vitro* system, together with the later-developed JFH1-based

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chimeras [8,9], are an important progress in HCV research, allowing the study of unknown aspects of HCV life cycle. However, a comparison study showed that JFH1 differs from the earlier-generated HCV 1b replicon in independence of the cellular cofactor (cyclophilin B) for the replication and less sensitivity to antiviral reagent [10], suggesting that the strain- or genotype-specific properties may exist and the observation obtained with JFH1 cannot be simply extrapolated to other isolates.

Synthetic minigenomes have been described in a number of minus- and plus-stranded RNA viruses, which has contributed greatly to the analysis of *cis*-acting sequences and *trans*-acting proteins required for viral replication, maturation, and packaging [11,12]. We previously established a helper virus-dependent expression system utilizing HCV-derived minigenome, and Huh-7 cells harboring autonomously replicating HCV subgenome [13]. In this study, we further investigated whether the minigenome replication system could be reconstituted by transfection of naïve Huh-7 cells with plasmid expressing NS proteins. It was shown that synthesis of minus-strand RNA from HCV minigenome can be supported by *trans*-expressed polyprotein NS3 to NS5B, and the NS proteins were able to replicate not only the homologous minigenome but the heterologous and chimeric minigenome as well.

Materials and methods

Plasmids. HCV 1b-derived minigenome p1b-1b was previously referred to as pT7cRLNS5B1 [13]. For construction of chimeric minigenome p2a-1b, the first 376 nucelotides of HCV 2a cDNA with the T7 promoter directly coupled at the 5′-end were amplified by PCR with primers 5′-tataa gcttTAATACGACTCACTATAACCTGCCCCTAATAGGGGC-3′ and 5′-tgcgcatgcTTTGGTTTTTCTTTGAGGTT-3′. The *Renilla* luciferase gene was amplified from pRL-TK (Promega) using primers 5′-ctctctagaA TGACTTCGAAAGTTTATGA-3′ and 5′- tgcgcatgcTTATTGTTCATT TTTGAGAA-3′. The resulting PCR products were digested with *Hin*dIII–*Sph*I and *Sph*I–*Xba*I, respectively, and inserted into the *Hin*dIII/*Xba*I sites of p1b-1b. The 3′- part of the NS5B coding region fused 3′ UTR of HCV 2a cDNA was amplified by PCR using primers 5′-ataggatccCCTCAGAA AACTTGGGG-3′ and 5′-ataggcgccagcgaggaggctgggaccatgccggccACAT GATCTGCAGAGAGACC-3′, digested with *Bam*HI and *Nar*I, and cloned, along with the annealed oligonucleotides containing partial sequence of the HDV ribozyme [13], into *Bam*HI/*Eco*RI-cut p1b-1b or p2a-1b, creating p1b-2a and p2a-2a, respectively.

To construct plasmid pNS3-51b expressing polyprotein from HCV 1b, a cDNA containing the ORF of NS3 to NS5B was amplified with primers 5′-atatctagaATGGGCCCATCACGGCTTA-3′ and 5′-ataggcgcgccTCA CCGGTTGGGGAGCAGG-3′, digested with *Xba*I and *Asc*I, and cloned, along with *Hin*dIII/*Xba*I-cut HCV sequence (1–341 nt), into pGEMEX-1 vector (Promega) which was modified by deletion of all of the T7 gene 10 and introduction of *Hin*dIII and *Asc*I sites between T7 promoter and terminator [14]. pNS3-52a, which expresses the polyprotein from HCV 2a, was constructed similarly except with the primers of corresponding sequence from genotype 2a. The sequences of these constructs were confirmed by nucleotide sequencing.

Cells. The cell line Huh-7 was purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum and 50 U/ml penicillin and streptomycin in a 5% CO_2 humidified atmosphere. A Huh-7-derived cell line (Huh–NNRZ) stably replicating HCV subgenomic replicon was grown in DMEM containing 300 μg/ml G418 (Geneticin, Invitrogen) [15,16].

Transfection. Huh-7 cells were seeded at 1×10^5 per well of 12-well plates. Twenty-four hours later, 0.5 μg *Eco*RI-linearized minigenome (p1b-1b, p1b-2a, p2a-1b, or p2a-2a), 0.5 μg pGEMEX-1, pNS3-51b, or pNS3-52a, 0.5 μg pAM8-1, and 0.1 μg pGL3-Control vector were cotransfected into cells with Fugene HD Transfection Reagent (Roche). The cells were harvested at the indicated time points, and cell lysates were assayed for luciferase activity as described below.

Luciferase assay. Cell lysates were prepared from transfected cells, centrifuged briefly, and 20 μl of the supernatants was used for luciferase assays with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase activities were measured using a TD-20/20 Luminometer (Promega).

Western blot analysis. Protein was electrophoresed on a sodium dodecyl sulfate–polyacrylamide gel, transferred to Hybond-P PVDV Membrane (Amersham). The blots were probed with Antiserum Product 2871 and 2881 (ViroStat) for detection of NS3 and NS4, rabbit polyclonal antibody (ab2594, Abcam Limited) for NS5A, and goat polyclonal antibody (sc-17532, Santa Cruz Biotechnology, Inc.) for NS5B. Signals were visualized with ECL Plus Western Blotting Detection Reagents (Amersham).

Northern blot analysis. RNAs were isolated from transfected cells with Trizol reagent (Invitrogen) and treated with RNase-free DNase (Promega). The purified RNAs were separated by denaturing agarose gel electrophoresis and analyzed by Northern blot using digoxigenin-labeled antisense *Renilla* luciferase sequence.

Results

Synthetic minigenome derived from HCV

The minigenome construct derived from HCV 1b consists of the antisense sequence of the *Renilla* luciferase gene and internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV) flanked upstream by 5′-end (nucleotides 1–377) and downstream by 3′-end sequence containing NS5B coding region from nucleotides 9067 to 9371 plus 3′-UTR of HCV cDNA. The cassette was positioned precisely at the T7 transcription start site followed by self-cleaving HDV ribozyme to ensure authentic 5′- and 3′-ends (Fig. 1A, p1b-1b). If the minigenome could be accepted as a template by the replication complex provided in *trans*, the luciferase gene, which is encoded by synthesized minus-strand RNA, would express in HCV-infected cells. Fully consistent with this hypothesis, luciferase activity was selectively detected in Huh-7 cells harboring an autonomously replicating HCV subgenome (Huh–NNRZ) [13].

Replication of HCV minigenome in Huh-7 cells expressing polyprotein NS3 to NS5B

The ability of the synthetic minigenome to replicate in replicon cells prompted us to investigate whether the replication of minigenome could be supported by HCV proteins expressed in *trans*. For this purpose, Huh-7 cells were transfected with the minigenomic construct p1b-1b, plasmid encoding a polyprotein encompassing NS3 to NS5B under the control of T7 RNA polymerase promoter, pAM8-1 plasmid expressing T7 RNA polymerase [14], and pGL3-Control vector. The cells were harvested at 3

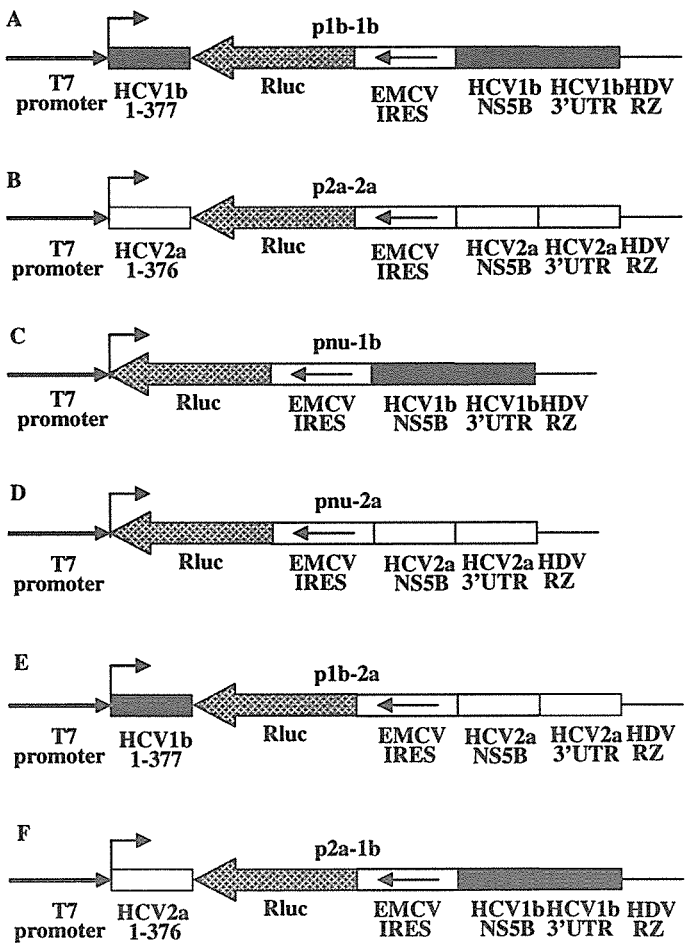

Fig. 1. Schematic diagrams of T7-based minigenomes derived from HCV 1b (A), HCV 2a (B), chimeric minigenomes p1b-2a consisting of 5′-end of HCV 1b and 3′-end of HCV 2a (E) and p2a-1b consisting of 5′-end of HCV 2a and 3′-end of HCV 1b (F). HCV minigenome containing the antisense sequence of the *Renilla* luciferase gene and EMCV IRES flanked by the 5′-end and 3′-partial NS5B coding sequence-connected 3′-UTR was juxtaposed precisely at the T7 transcription start site and followed by the HDV ribozyme sequence. pnu-1b (C) and pnu-2a (D) were identical to p1b-1b and p2a-2a except for the 5′-end sequences deleted.

days posttransfection, protein expression was verified by Western blot analysis (Fig. 2) and the replication of minigenome was determined by luciferase assay and Northern blot analysis. The firefly luciferase activity from cotransfected pGL3-Control vector was simultaneously measured to normalize the transfection efficiency. As shown in Fig. 3A, only background level of *Renilla* luciferase (Rluc) activity was detected in cells transfected with the empty vector. Cotransfection of the plasmid encoding the polyprotein NS3 to NS5B (pNS3-51b) resulted in significant *Renilla* luciferase expression. Omission of pAM8-1 in the transfection mixture completely abrogated *Renilla* luciferase activity, largely ruling out the possibility that the minus-strand RNA used here as the mRNA for reporter gene expression was synthesized as a consequence of the transcription by a cryptic promoter. Consistent with the results reported previously [13], *Renilla* luciferase activity was also detected in Huh–NNRZ cells stably replicating the HCV subgenomic replicon, although it was lower than that in cells *trans*-expressing the polyprotein. The fact that the replicase complex reconstituted by *trans*-expressed polyprotein could support more efficient replication of the minigenome may be attributable to the higher expression level of plasmid-encoded protein on a per-transfected-cell basis. Alternatively, the recruitment of the replication complex to the minigenome may be competed by the subgenomic replicon, because both of these share the replication machinery in replicon cells.

To further confirm the result of reporter assay, RNA was extracted from transfected cells and subjected to Northern blot analysis using digoxigenin-labeled antisense *Renilla* luciferase probes. Also, minus-strand RNA transcripts of the expected size were specifically detected in Huh-7 cells expressing NS 3–5 protein and Huh–NNRZ cells replicating HCV subgenomic replicon (Fig. 3B, lanes 2 and 4). These data demonstrate that *trans*-replication of HCV minigenome does not require replication of the helper viral RNA.

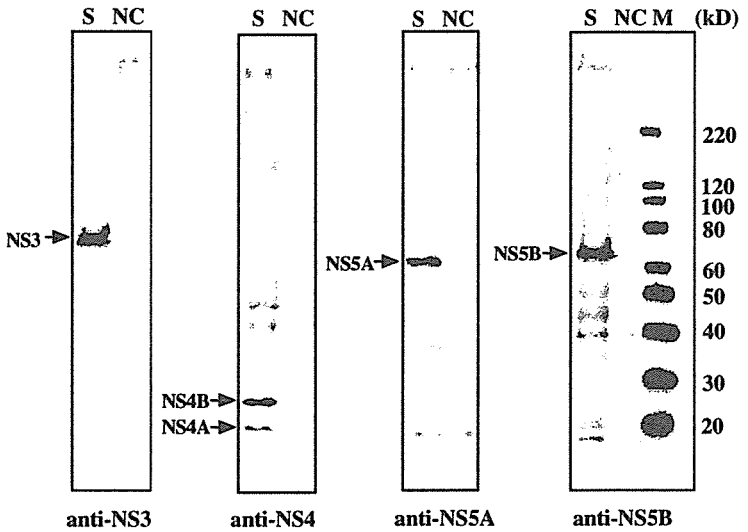

Fig. 2. Expression of NS proteins in Huh-7 cells transfected with plasmid encoding a polyprotein encompassing NS3 to NS5B. Huh-7 cells were transfected with pNS3-51b together with pAM8-1 plasmid expressing T7 RNA polymerase and harvested at day 3 posttransfection. Cell lysates of the transfected cells were analyzed by Western blot using antibodies against each NS protein. Protein standard is shown on the right, and the band corresponding to each NS protein is indicated by an arrowhead. Huh-7 cells transfected with the empty vector served as a negative control (NC).

To document that the reporter gene expression detected above was dependent on HCV replicase reconstituted by *trans*-expressed NS proteins, we employed inactive mutant pNS3-51b/dGDD (in which the GDD motif of NS5B was deleted) and AdsiNS5B expressing siRNA directed against NS5B [13] in the reporter assay. As shown in Fig. 3C, the deletion of GDD motif significantly attenuated the ability of NS proteins to support minigenome replication, and transduction with AdsiNS5B resulted in a substantial and dose-dependent reduction in luciferase expression. These results provide further evidence that the reporter gene was expressed as a result of replication of HCV minigenome by *trans*-supplied NS proteins.

Chimeric minigenomes as templates for HCV replication complex

Next, we were interested in investigating whether the replicase of HCV can recognize the heterologous signals for synthesis of minus-strand RNA. For this purpose, HCV minigenome from distantly related genotype 2a (Fig. 1B, p2a-2a), minigenomes with 5′-end deleted (Fig. 1C and D, pnu-1b and pnu-2a), and chimeric minigenomes whose 5′- or 3′-end was replaced by the respective region of the heterologous virus (Fig. 1E and F, p1b-2a and p2a-1b) were constructed. Huh-7 cells were transfected with these minigenomes together with the plasmid expressing HCV 1b- or 2a-derived NS proteins, pAM8-1, and *Renilla* luciferase activities were measured as fore-mentioned. Consistent with the results described above, replicase of HCV 1b and 2a accepted its respective minigenome as the template for synthesis of minus-strand RNA, and exchange of NS proteins between HCV1b- and 2a-derived minigenome systems also led to reporter gene expression (Fig. 4), implying that the replication complex is not strictly specific for the homologous RNA template. Deletion of the 5′-end region in the minigenome fully abrogated its replication, both NS proteins from HCV 1b and 2a, however, could support the replication of chimeric minigenomes, suggesting that both RNA–protein interaction between replicase and viral genome and long range RNA–RNA interaction between 5′- and 3′-terminal sequence involved in HCV minus-strand RNA synthesis are functionally conserved between genotype 1b and 2a. Additionally, in all tested minigenomes, the NS proteins originated from HCV 1b constantly yielded higher levels of luciferase expression than that from 2a, suggesting that intrinsic differences in the replication capabilities of the replicase complex from different strains may exist. More likely, the superior capability of pNS3-51b in supporting the minigenomes replication may be attributable to the fact that the coding sequence in pNS3-51b was amplified from the replicon which harbors the adaptive mutations due to long-term culture, whereas the coding region in pNS3-52a was directly amplified from HCV 2a-infected serum.

Discussion

Successful establishment of the minigenome system has been described in a number of minus-stranded RNA viruses from different families and plus-stranded RNA viruses belonging to the *Coronaviridae* family, which has contributed greatly to the analysis of *cis*-acting sequences and *trans*-acting proteins essential for viral replication [11,12]. The rescue of synthetic minigenomes was achieved either through helper virus infection of minigenome-transfected

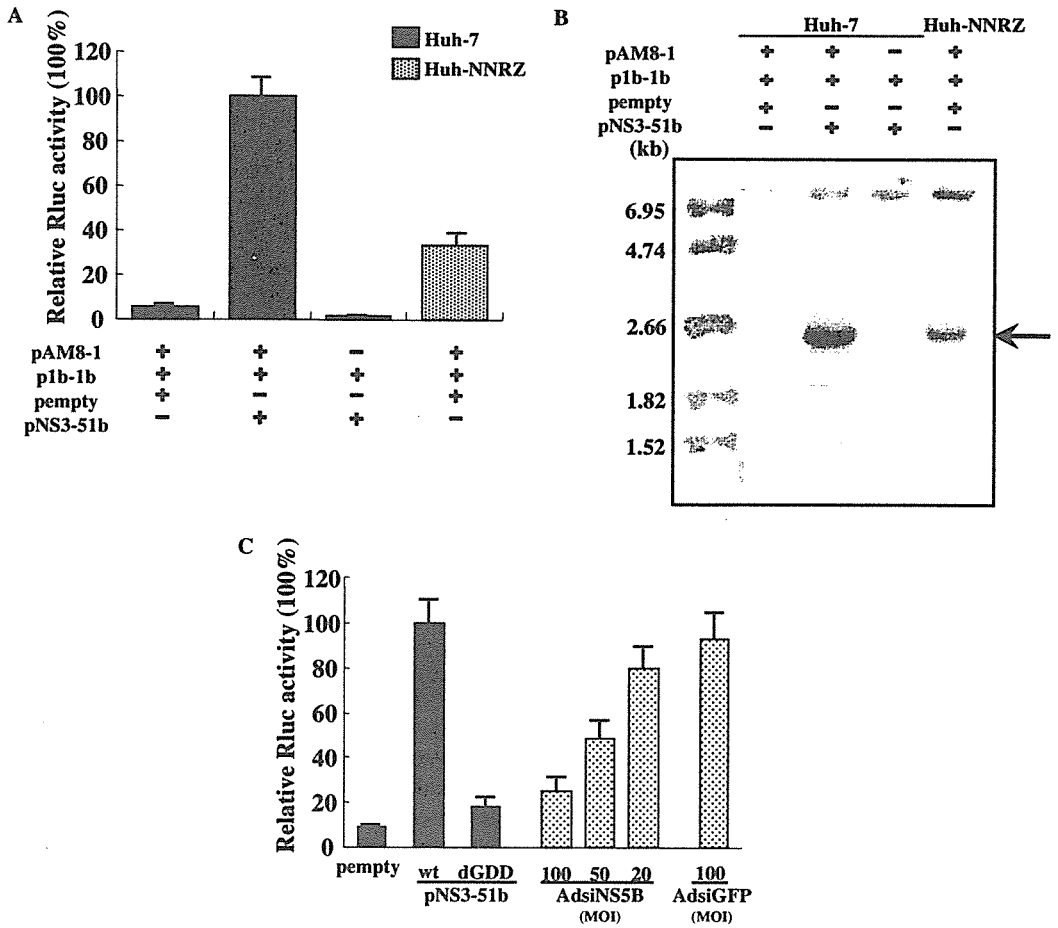

Fig. 3. Replication of HCV minigenome in Huh-7 cells expressing polyprotein NS3 to NS5B. (A) Huh-7 or Huh–NNRZ cells were transfected with p1b-1b, pNS3-51b expressing polyprotein NS3 to NS5B, together with or without pAM8-1. Relative *Renilla* luciferase activities in the lysates were determined at 72 h posttransfection. The columns and bars represent mean and standard deviation of four independent experiments. (B) Northern blot was performed on 8 μg of extracted RNA using digoxigenin-labeled antisense *Renilla* luciferase RNA probe to detect minus-strand transcripts. RNA size markers are shown on the left, and the bands corresponding to minus-strand RNA are indicated on the right. (C) Huh-7 cells were transfected with p1b-1b, pAM8-1, and pempty, pNS3-51b or pNS3-51b/dGDD (column 1–3), or infected with AdsiNS5B at an MOI of 100, 50, and 20 (column 4–6) before transfection, and relative *Renilla* luciferase activities in the lysates were determined as described above.

cells with virus particles or through co-transfection of plasmids expressing viral proteins. For viruses of *Flaviviridae* family, however, a little has been reported in the development of similar approach except an *in vitro* replication system which utilizes cytoplasmic extracts from viral-infected cells and exogenous RNA template containing 5′- and 3′-terminal regions was described for dengue virus [17]. Together with those reported previously [13], the data shown here represent the first example of minigenome system for HCV, indicating that both the replicase complex supplied from replicating subgenomic replicon and that reconstituted by plasmid-encoded NS proteins are capable of supporting the replication of HCV minigenome.

The data shown here further confirm that the viral 5′- and 3′-end sequence together with the 3′-partial NS5B coding region represent sufficient *cis*-acting signals for minus-strand RNA synthesis. These results, however, do not rule out the possibility for the existence of *cis*-acting elements in other coding region, which may act as regulatory elements (either enhancers or silencers) in RNA synthesis. The presence of noncontiguous *cis*-acting signals involved in viral RNA replication has been reported in the viral genome of the brome mosaic virus [18], tobacco mosaic virus [19], and the double-stranded RNA virus of yeast [20].

Similar to that found in dengue virus, it was shown that deletion of the 5′-end region in the minigenome fully abrogated its replication, but substitution of the 5′-end with the respective sequence from heterologous virus (p1b-2a or p2a-1b) did not significantly affect its template ability, suggesting that the long range RNA–RNA interaction between 5′- and 3′-ends essential for RNA replication is functionally conserved between HCV 1b and 2a. In addition to homologous minigenome, both HCV 1b- and 2a-derived replicase were able to accept the heterologous and chimeric minigenomes as the templates for synthesis of minus-strand

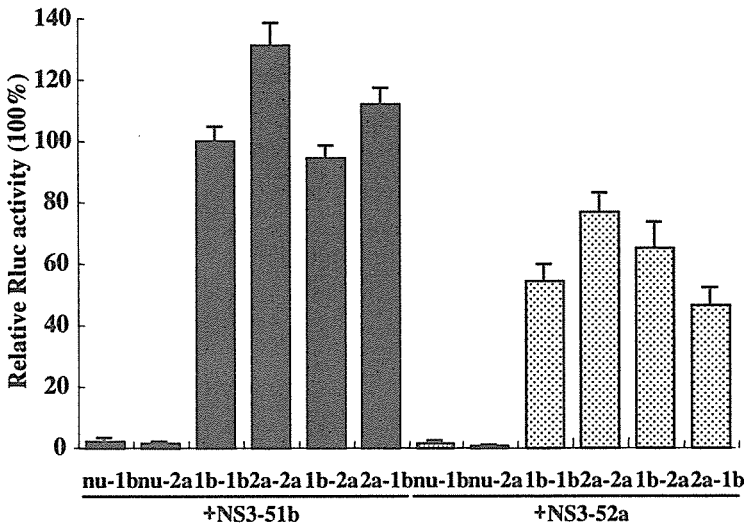

Fig. 4. Replication of chimeric HCV minigenomes. Huh-7 cells were transfected with each indicated minigenome, pAM8-1, and pNS3-51b or pNS3-52a expressing polyprotein NS3 to NS5B derived from genotype 1b or 2a. Relative *Renilla* luciferase activities in the lysates were determined as described for Fig. 3A. The columns and bars represent means and standard deviations of three independent transfections.

RNA, indicating that the replicase-catalyzed RNA synthesis is not strictly strain- or genotype-specific.

Using replicon system, Bartenschlager's group obtained the evidence showing that only mutations in NS5A, but not mutations in NS3, NS4B, and NS5B, could be rescued by *trans*-complementation [21]. Our data presented here indicate that replication of the minigenome can be supported by *trans*-expressed NS proteins. One scenario may make these two different findings compatible: the *cis*-expressed, lethally mutated NS proteins may exert dominant negative effect in reconstituting replication complex, and thus interfere with the incorporation of *trans*-supplied NS proteins into a functional replication complex, which may account for the failure of NS proteins (other than NS5A) to *trans*-complement HCV RNA replication; however, such a dominant negative effect does not exist in the minigenome system described here because there is no NS protein expressed in *cis*, and *trans*-expressed NS proteins might be able to reconstitute the functional replication complex to support minigenome replication. Further experiments are now in progress to substantiate this assumption.

It is generally believed that the HCV replication follows the pathway used by other plus-strand RNA viruses: the input RNA is first transcribed into a minus strand, which in turn serves as the template for production of progeny plus strand. The negative strand intermediates are postulated to exist as a dsRNA form. However, there is no direct evidence demonstrating this postulation in HCV, and whether there is free HCV-specific RNA of negative polarity in infected cells is still an issue to be elucidated. On the other hand, increasing evidence showed that the RNA in native replication intermediates of some positive strand RNA viruses is single-stranded. For example, in polio virus-infected cells, a careful electron microscope analysis using a membrane-permeable cross-linking reagent demonstrated that the native replication intermediate *in vivo* has a predominantly single-stranded backbone attached to several nascent RNA chains with few or no regions of extensive base-pairing, although deproteined (phenol-extracted) replication intermediate has a backbone mostly double-stranded [22]. More recently, Fujimura et al., reported that native replication intermediates of 20 S RNA virus have a single-stranded RNA backbone [23]. After completion of product-strand elongation, both the product and template strands are released from the replication complex as single-stranded RNA. The data presented here indicate that the minus strand RNA could serve as the mRNA for transgene expression, implying a similar scenario may also occur in HCV replication and minus strand RNA may be dissociated and present as a free single-strand form after RNA synthesis is completed.

One issue of concern in using minigenome to study the molecular mechanism of viral replication is whether the elements controlling viral replication in the context of minigenome could authentically reflect those that occurred in the context of full-length genome. Recently, differential effect of a point mutation in the replicase gene on genome and minigenome replication was reported in coronavirus, emphasizing the need to use full-length genome to validate the replication signals obtained from minigenome system [24]. Nonetheless, the HCV minigenome system described here represents a useful tool for identification of *cis*- and *trans*-acting factors involved in viral replication while eliminating biosafety constraints required for work with infectious systems. Additionally, it will be of interest to explore whether the HCV minigenome can be packaged by additional provision of the viral structural protein in *trans*, and its success will not only further broaden the

application of the HCV minigenome, but also facilitate the development of HCV-based gene delivery system.

We describe here a reverse genetic system for HCV that is based on T7-driven minigenome coupled with plasmid-encoded NS proteins. This system opens the possibility of manipulation of *cis*-acting signals and *trans*-acting factors involved in the control of HCV RNA synthesis, which may facilitate future studies aimed at investigation of the mechanisms involved in the replication of viral RNA.

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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].

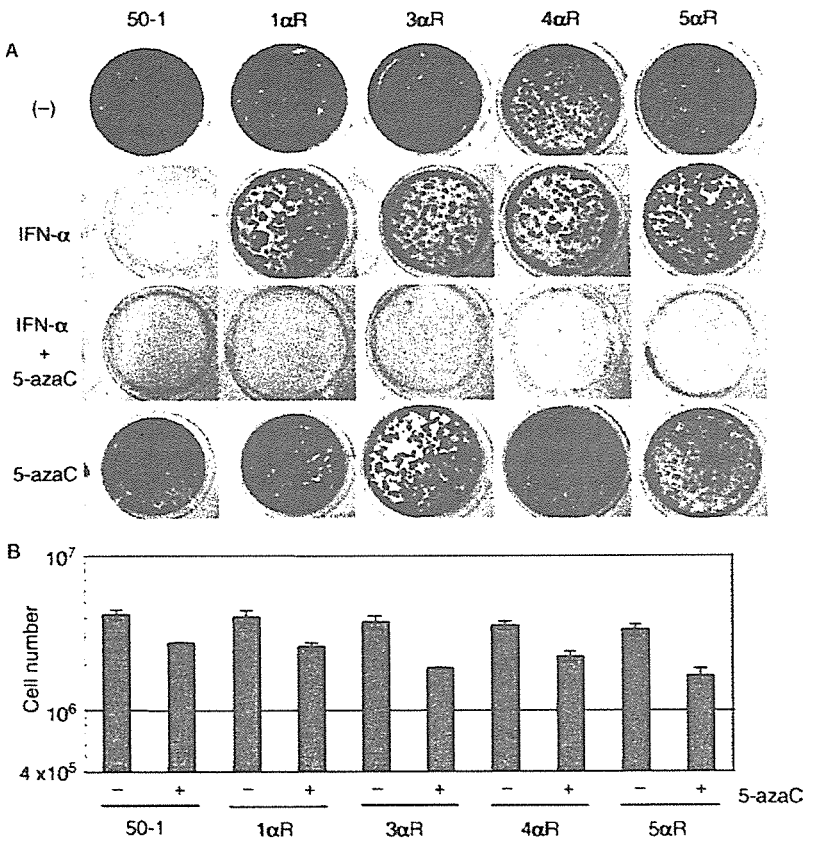

Fig. 1. 5-AzaC treatment converts αR-series cells from an IFN-resistant phenotype into an IFN-sensitive phenotype. (A) IFN sensitivities of various HCV replicon-harboring cells. αR-series cells (1αR, 3αR, 4αR, and 5αR) showing a partially IFN-resistant phenotype and the parent 50-1 cells with an IFN-sensitive 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.]

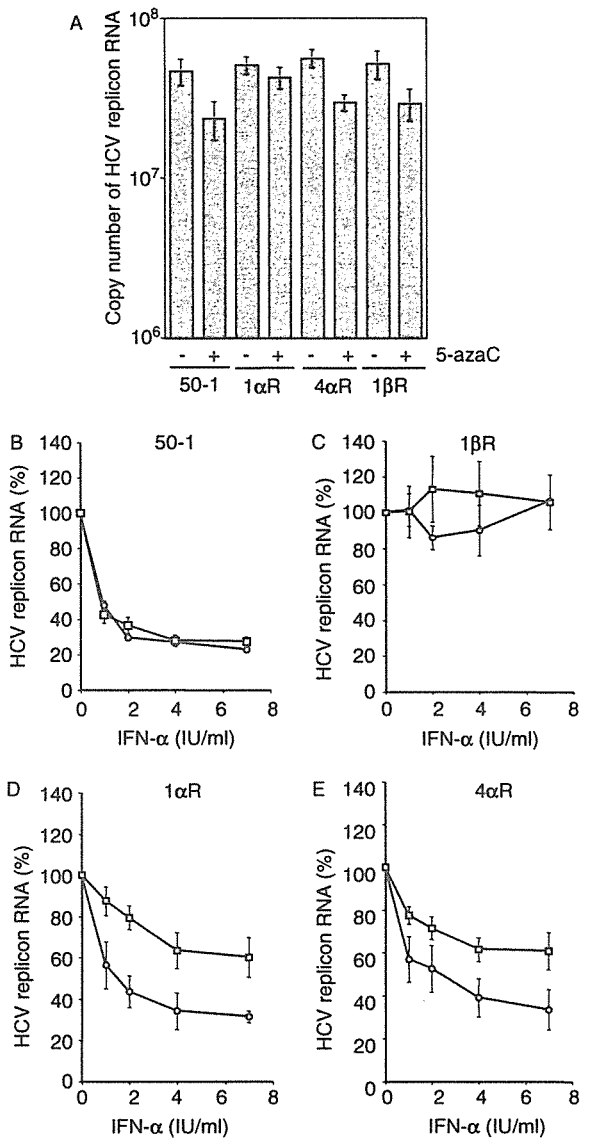

Fig. 2. IFN sensitivities of HCV replicons. (A) HCV replicon RNA levels of 50-1, 1βR, 1αR, and 4αR cells cultured in the presence or absence of 5-azaC (10 μM for 50-1, 1βR, and 1αR cells, 4 μM for 4αR cells) for 2 weeks. Real-time LightCycler PCR was carried out in order to quantitatively monitor the 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

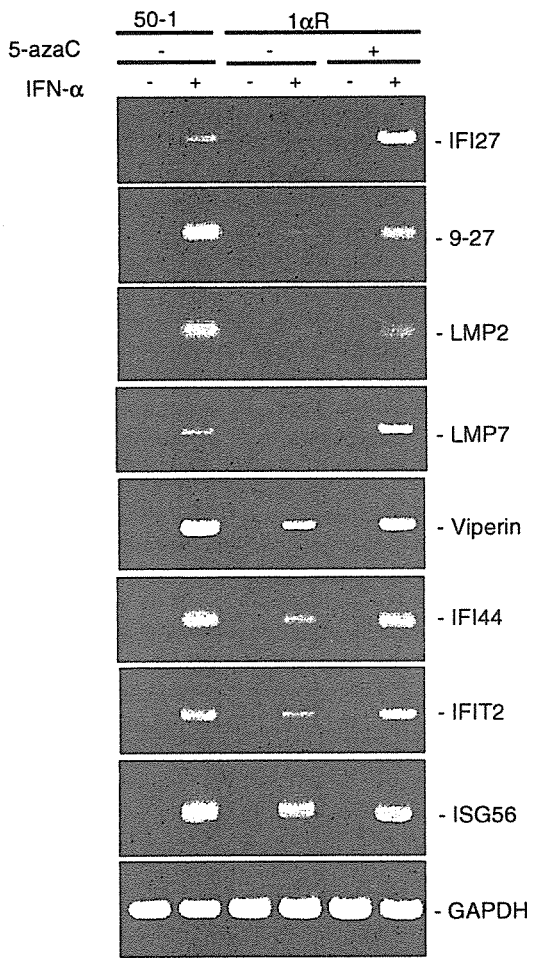

Fig. 3. RT-PCR analysis of mRNA expression of selected ISGs based on the microarray results. 1αR cells were cultured in the presence or absence of 5-azaC (10 μM) for 2 weeks. The 1αR cells treated with 5-azaC and the 50-1 cells were treated with or without IFN-α (500 IU/ml) for 8 h. The total RNAs extracted 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

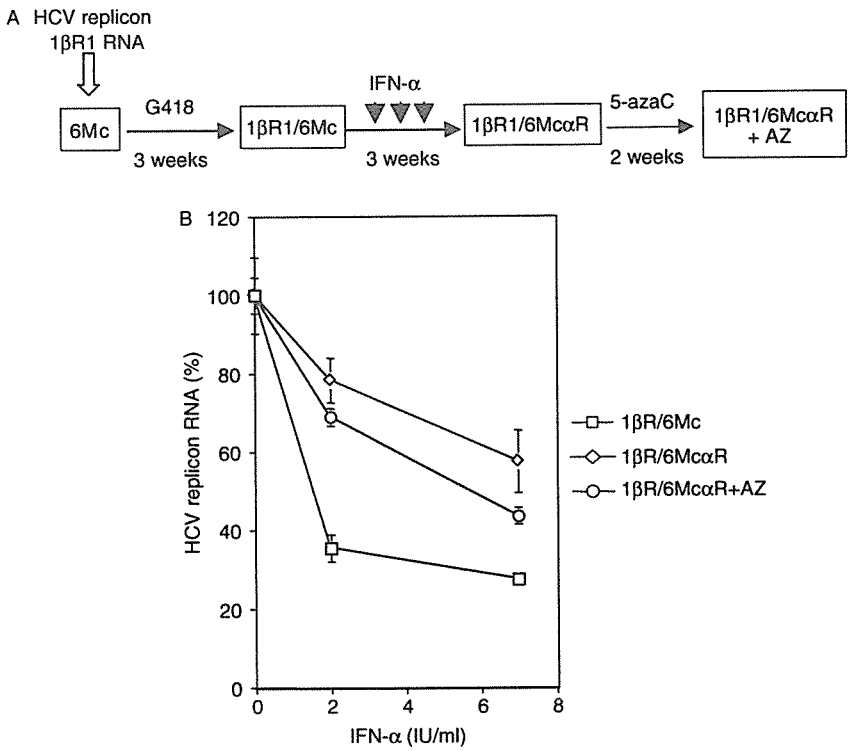

Fig. 4. Effect of 5-azaC treatment on IFN resistance in newly isolated HCV replicon-harboring cells showing a partially IFN-resistant phenotype. (A) Outline of the isolation of HCV replicon-harboring cells showing partial IFN resistance. HCV replicon-harboring cells (designated as 1βR1/6Mc cells) [14], 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.

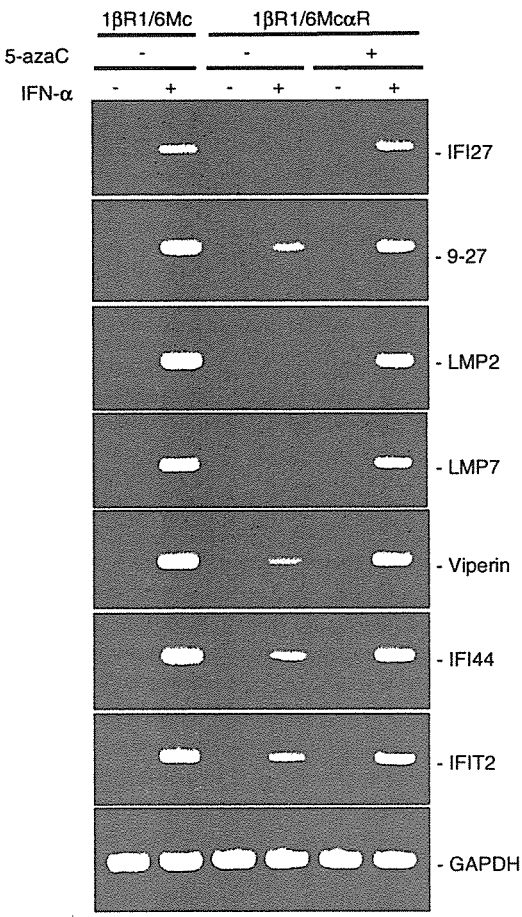

Fig. 5. RT-PCR analysis of mRNA expression of seven ISGs selected by microarray analysis. 1βR1/6McαR cells were cultured in the presence or absence of 5-azaC (4 μM) for 2 weeks. The 1βR1/6McαR cells were treated with 5-azaC and the 1βR/6Mc cells were treated with or without IFN-α (500 IU/ml) for 8 h. 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.

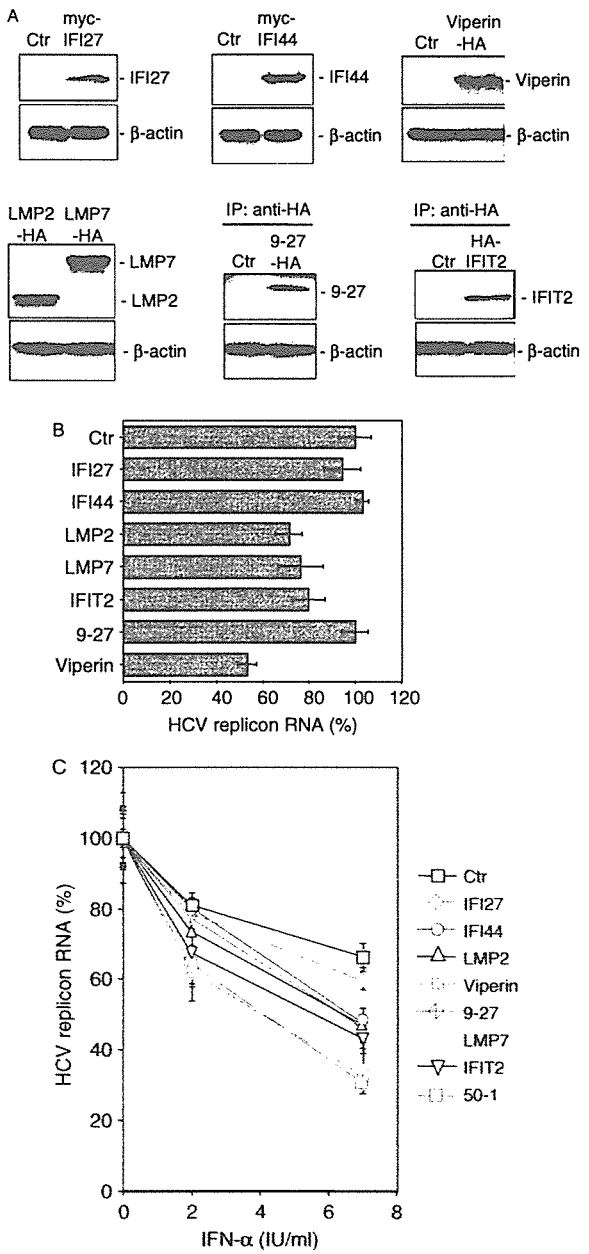

Fig. 6. IFN sensitivities of HCV replicons in 1αR cells expressing epigenetically silenced ISG. (A) Ectopic expression of epigenetically silenced ISG in 1αR cells introduced by retrovirus-mediated gene transfer. The DNA fragments encoding myc-tagged IFI27 and IFI44, and influenza hemagglutinin-tagged (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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Hepatitis C virus NS5B delays cell cycle progression by inducing interferon-β via Toll-like receptor 3 signaling pathway without replicating viral genomes

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Abstract

To clarify the pathogenesis of hepatitis C virus (HCV), we have studied the effects of HCV proteins using human hepatocytes. Here, we found that HCV NS5B, an RNA-dependent RNA polymerase, delayed cell cycle progression through the S phase in PH5CH8 immortalized human hepatocyte cells. Since treatment with anti-interferon (IFN)-β neutralizing antibody restored the cell cycle delay, IFN-β was deemed responsible for the cell cycle delay in NS5B-expressing PH5CH8 cells. The induction of IFN-β and the cell cycle delay were overridden by the down-regulation of Toll-like receptor 3 (TLR3) through RNA interference in NS5B-expressing PH5CH8 cells. Moreover, the NS5B full form was required for the cell cycle delay, the induction of IFN-β, and the activation of the IFN-β signaling pathway. Our findings revealed that NS5B induced IFN-β through the TLR3 signaling pathway in immortalized human hepatocytes even without replicating viral genomes.

Keywords: Hepatitis C virus; NS5B; Interferon-β; TLR3; Hepatocyte cells

Introduction

Since more than 170 million individuals are estimated to be infected with hepatitis C virus (HCV) worldwide, this disease is a global health problem (Thomas, 2000). HCV belongs to the family Flaviviridae, whose positive-stranded RNA genome encodes a large polyprotein precursor of approximately 3000 amino acid residues. This polyprotein is processed by a combination of the host and viral proteases into at least ten proteins in the following order: NH_2-core-envelope 1-envelope 2-p7-nonstructural protein 2 (NS2)-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Kato, 2001; Kato et al., 1990). These viral proteins are not only involved in viral replication but also may affect a variety of cellular functions (Bartenschlager and Lohmann, 2000; Kato, 2001). Although persistent infection with HCV is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (Colombo, 1996; Kato, 2001), the molecular mechanisms leading to liver cell dysplasia and HCC remain elusive.

It has been thought that unregulated cell cycle progression may be a cause of malignant transformation of normal cells. On the other hand, inhibition of cell cycle progression through the S phase may cause replication error during DNA replication, which induces genomic instability and malignant transformation. Therefore, it is important to clarify the effect of HCV proteins on cell cycle progression in order to understand the molecular mechanism underlying the pathogenesis of HCV, including the development of HCC. A number of previous reports suggested that four HCV proteins—the core, NS3, NS4B, and NS5A—are involved in modulating cell cycle progression (Arima et al., 2001; Kato, 2001; Ray and Ray, 2001; Reed and Rice, 2000). For instance, the core protein promotes cell proliferation through the Ras/Raf signaling pathway and the anti-apoptotic function (Mar-

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usawa et al., 1999; Tsuchihara et al., 1999). However, the core has been described to both enhance and repress the function of $p21^{Waf1/Cip1/Sdi1}$, a Cdk inhibitor (Dubourdeau et al., 2002; Jung et al., 2001; Lu et al., 1999; Ray et al., 1998). Recently, Scholle et al. found no significant cell cycle delay in human hepatoma HuH-7-based HCV RNA-replicating cells that were autonomously replicating genome-length HCV RNA, in comparison with cured cells of the same line from which HCV RNA had been eliminated by treatment with interferon (IFN)-α (Scholle et al., 2004). Hence, the effects of cell cycle regulation by HCV proteins are still controversial. Cancerous cell lines, such as the human hepatoma HuH-7 cell line (Hsu et al., 1993), which harbors a mutant *p53* gene, may not be suitable for addressing the effects of HCV proteins on cell cycle progression.

The PH5CH8 cell line was established by immortalization using the SV40 large-T antigen from non-neoplastic liver tissue of an HCV-related HCC patient (Ikeda et al., 1998; Noguchi and Hirohashi, 1996). PH5CH8 cells possess wild-type *p53* and *Rb* tumor suppressor genes. In nude mice, these cells reveal a non-malignant phenotype upon colony formation and tumorigenicity (Noguchi and Hirohashi, 1996), although the SV40 large-T antigen would partially repress the function of p53. Therefore, the PH5CH8 cell line is considered to be more relevant for studying the role of HCV proteins during hepatocarcinogenesis. We have previously reported that the HCV core protein activates the IFN-inducible 2′–5′-oligoadenylate synthetase gene in PH5CH8 cells (Naganuma et al., 2000). Recently, we demonstrated that the core protein's activation of this gene was mediated through the IFN-stimulated response element (ISRE) (Dansako et al., 2003). Furthermore, we found that the core protein promoted microsatellite instability in PH5CH8 cells (Naganuma et al., 2004). In fact, microsatellite instability was detected in approximately 20% of the tumor tissues from HCC patients examined, whereas no microsatellite instability was detected in normal liver tissues from the same patients (Dore et al., 2001; Kondo et al., 2000). In order to clarify the effect of HCV proteins on cell cycle progression in PH5CH8 cells, we examined cell cycle progression after the cells were released from the G1/S boundary in PH5CH8 cells expressing HCV proteins. We found that NS5B delays cell cycle progression by inducing IFN-β through the activation of the Toll-like receptor 3 (TLR3) signaling pathway without replicating viral genomes.

Results

HCV NS5B causes the delay of S phase progression

In a previous study of virus–host interactions, we examined whether or not HCV proteins affect cell cycle progression in PH5CH8 cells that stably expressed core or NS proteins. PH5CH8 cells were infected with retrovirus pCXbsr as a negative control (Ctr) or pCXbsr encoding either an HCV structural protein (HA-core) or NS protein (NS3, HA-NS4B, HA-NS5A, HA-NS5B, or NS5B), and we obtained PH5CH8 cells stably expressing each HCV protein. The expression of each HCV protein was confirmed by Western blot analysis (Fig. 1A). Then, the HCV protein-expressing cells were synchronized at the G1/S boundary, and cell cycle progression (from the S phase to the G2-M phase, then turning back to the G1 phase) was analyzed after the cells were released from synchronization. This cell cycle analysis revealed no significant differences in cell cycle progression between cells (PH/Ctr) infected with a control pCXbsr retrovirus and cells expressing core, NS3, NS4B, or NS5A (Fig. 1B). Unlike the PH/Ctr cells, the apparent delay of S phase progression was found in cells (PH/NS5B) expressing NS5B, regardless of the presence of the HA tag (Fig. 1B). To exclude the possibility that pCXbsr-derived retrovirus proteins synergistically affect the cell cycle together with NS5B, the retrovirus pCX4bsr vector (Akagi et al., 2003), which eliminates the production of any fusion proteins resulting from initiation at upstream AUG codons within the *gag* region of the vector, was used for the cell cycle analysis. As a result, the delay of S phase progression was found again in PH5CH8 cells expressing NS5B (Figs. 1A and C), suggesting that the retrovirus proteins are not involved in the delay of S phase progression. BrdUrd incorporation analysis was also carried out using PH/Ctr and PH/NS5B cells (Fig. 1D). In the PH/Ctr cells, DNA synthesis began early in the S phase (4 h after release). In the late S phase (8 h), more than 61% of the cells indicated final DNA synthesis. Thereafter, the cells either finished DNA replication in the G2-M phase (12 h) or returned to the G1 phase. In contrast, we found that DNA replication in most PH/NS5B cells predominantly remained in the early or middle S phase (8 h), and 49% of the cells were prolonged in the late S phase (12 h). To quantitatively evaluate this delay in S phase progression, the cells that had finished DNA replication were accumulated during the G2 phase by treatment with Nocodazole (Noc), which inhibits the progression of the G2 to M phases, after the cells were released from the G1/S transition. Whereas 77% of PH/Ctr cells reached the G2-M phase, only 37% of PH/NS5B cells did so (Fig. 1D). This level of decrease in cell numbers in the G2-M phase was not observed in PH5CH8 cells expressing core, NS3, NS4B, or NS5A (Fig. 1E), suggesting that NS5B specifically causes the delay of S phase progression in PH5CH8 cells. We further observed that the growth rate of PH/NS5B cells was significantly decreased relative to PH/Ctr cells (Fig. 1F), although the cell cycle distribution in asynchronous PH/NS5B cells was almost the same as that in asynchronous PH/Ctr cells (Fig. 1D). These results indicated that NS5B might delay the cell cycle progression of PH5CH8 cells in the S phase.

Cell cycle delay by NS5B is also found in other immortalized human hepatocytes

To clarify whether or not the delay of S phase progression by NS5B occurs in other human cell lines, we prepared three cell lines (Fig. 2A) that stably express NS5B–non-neoplastic human hepatocyte NKNT-3 (Kobayashi et al., 2000), hepatoma