

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

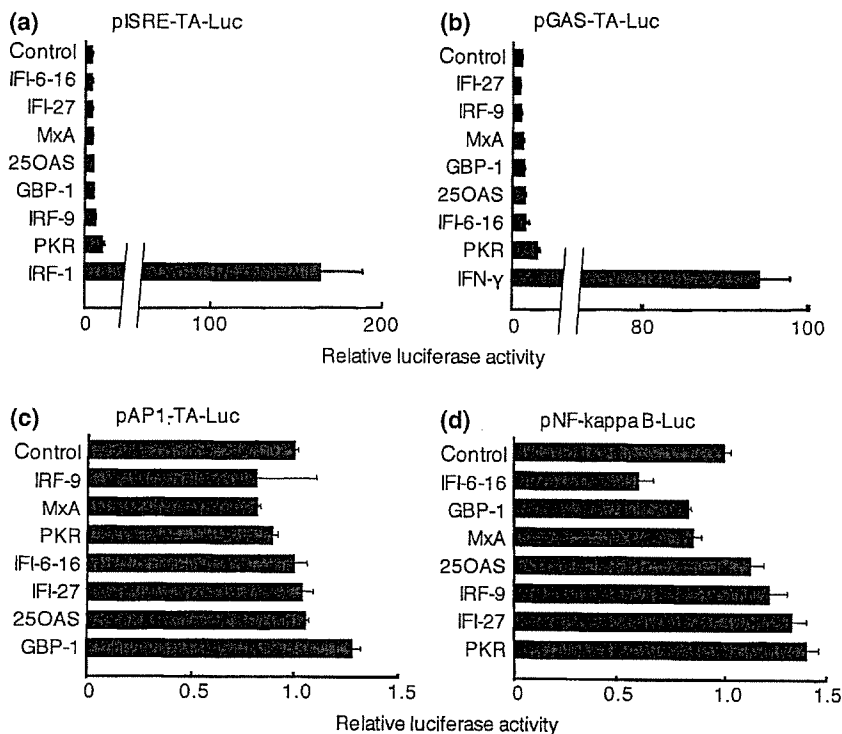


Fig. 4 The effects of interferon-stimulated genes (ISGs) on cellular signal transduction pathways. Reporter plasmids, pISRE-TA-Luc (panel A), pGAS-TA-Luc (panel B), pAP1-TA-luc (panel C), or pNF-kappa B-TA-luc (panel D) were cotransfected into Huh7 cells with the indicated ISG-expression plasmid vectors.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

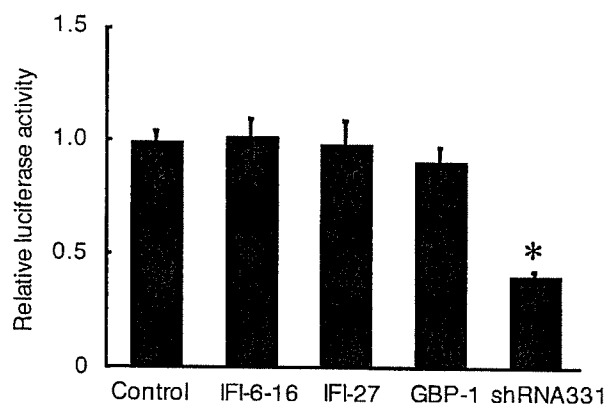


Fig. 5 The effect of guanylate binding protein (GBP)-1, interferon-induced protein (IFI)-6-16 and IFI-27 expression on hepatitis C virus (HCV)-internal ribosome entry site (IRES). HCV-IRES-luciferase construct, pCneo.Rluc-IRES-Fluc was cotransfected with the indicated ISG-expression plasmid, pcDNA-ISG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

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

DISCUSSION

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

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

the nonhepatocyte HeLa cell lines which support replication of HCV replicon, ISRE activity was significantly lower in the replicon-expressing cells than in the naive cells. These findings suggest that the decreased baseline ISRE activities may contribute to the high permissiveness of HCV replicon.

The actions of IFN are mediated by expressional induction of various ISGs which have a wide spectrum of activities. The decreased ISG expression levels in the replicon-expressing cells and the enhanced HCV replication in the cured cells, in which ISG expression had been decreased, have led us to speculate that certain ISGs which were down-regulated in the replicon-expressing cells may have direct effects on viral replication. Thus we have investigated the IFN-induced proteins through [1] the generation of cells overexpressing IFN-induced gene products and [31] the generation of cells in which the expression of IFN-inducible gene products have been knocked down. Our overexpression analyses using the cell lines expressing HCV replicon (Huh7/Rep-Feo) identified eight genes that inhibited HCV replication and expression of the virus proteins; PKR, 25OAS, IRF-1, IRF-9, MxA, GBP-1, IFI-6-16 and IFI-27 (Fig. 3a,c). These genes did not activate ISRE, GAS, NF-kappa B, or AP1 promoter activities (Fig. 4a-d), suggesting that the genes directly mediate antiviral effects without activating aberrant signal transduction pathways which may induce aberrant antiviral actions. Among the genes, the antiviral activities of PKR, 25OAS, IFI-56K and MxA have been well documented [10,11,13,14], while the effects of GBP-1, IFI-6-16 and IFI-27 on virus replication have not been reported, with the exception of one study on IFI-6-16 [27]. Overexpression of GBP-1, IFI-6-16 and IFI-27 did not influence HCV-IRES-mediated translation efficiency (Fig. 5); shRNA-directed knock down of the genes caused a significant increase in the HCV replication level (Fig. 6). These results suggest that GBP-1, IFI-6-16 and IFI-27, which we have identified as having antiviral activities, may contribute to the IFN-induced cellular antiviral responses.

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

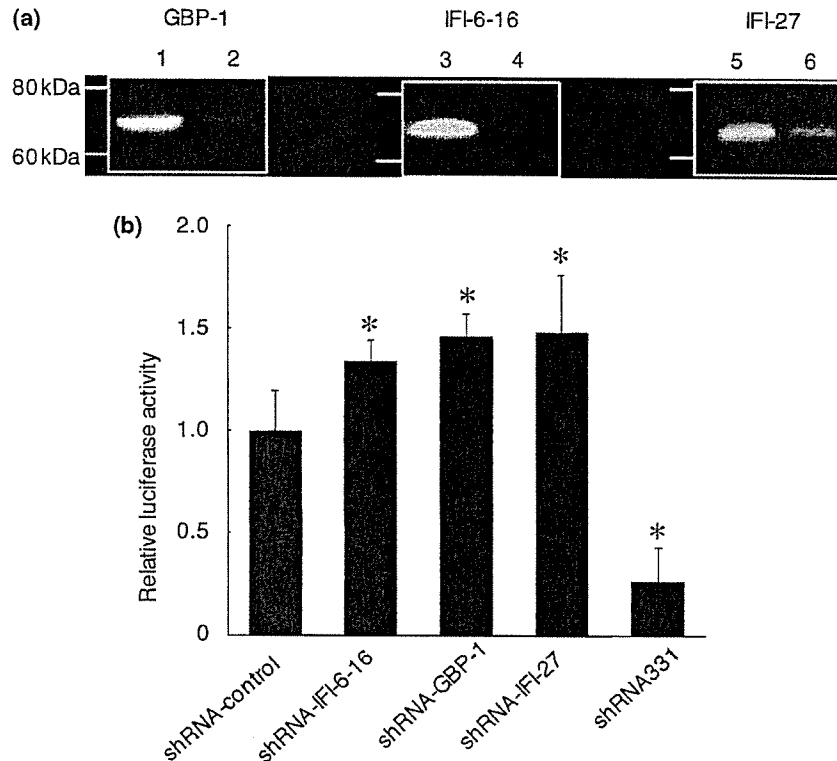


Fig. 6 Effects of shRNA-directed suppression of guanylate binding protein (GBP)-1, interferon-induced protein (IFI)-6-16 and IFI-27 expression on hepatitis C virus (HCV) replication. (a) The indicated interferon-stimulated gene (ISG)-expression plasmids, pcDNA-GBP-1 (Lanes 1 and 2), pcDNA-IFI-6-16 (Lanes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

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

A group of small ISGs including IFI-27 and IFI-6-16 remains uncharacterized, and their functions are still basically unknown [37–42]. IFI-6-16 is a hydrophobic protein and thought to be present in the membrane. It is postulated to be a precursor protein but its signal peptide region has not been confirmed. Little is known about molecular functions of IFI-6-16 except that its promoter region contains one ISRE [43]. One study has reported on the antiviral effect of IFI-6-16 on HCV replication, however, the mechanism of action remained uncharacterized [27]. The study reported that the expression of IFI-6-16 protein enhanced IFN-mediated antiviral activity, while transfection of IFI-6-16 alone did not

cause significant inhibition of HCV replication. IFI-27 (also referred to as ISG12) is a putative highly hydrophobic protein of 122 amino acids which has a 33% overall sequence similarity to IFI-6-16. IFI-27 is unique in that it is the only protein found thus far to localize to the nuclear envelope, although its function in the nuclear envelope has not yet been explored [44]. It has also been reported that ISG12 exerts protective effects during lethal Sindbis virus infection and that ISG12 is the only ISG with greater expression in the disease recovery phase than the acute phase of the viral infection [45]. In our present study, overexpression of IFI-6-16 or IFI-27 inhibited replication of HCV replicon (Fig. 3), and the shRNA-knock down of each gene caused HCV replication to increase (Fig. 6). These findings also suggest that the expression of IFI-6-16 or IFI-27 without IFN treatment inhibits HCV-replication. In a previous DNA microarray study in an HCV infection model, IFI-6-16 gene expression was highly induced at the early stage of infection, while IFI-27 was induced in both the acute and recovery phases of infection [6]. Because neither of the proteins suppressed HCV-

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

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

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

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SUMMARY. The number of amino acid substitutions in the interferon sensitivity-determining region (ISDR) in the non-structural 5A (NS5A) gene of hepatitis C virus (HCV) is closely associated with the interferon (IFN) response and viral load. Several HCV replicon-based studies have reported that ISDR sequences had an influence on viral replication *in vitro*. However, it is unclear as to how different ISDR sequences affect HCV replication. Various clinically observed ISDR sequences were introduced into HCV replicons and their contribution to viral replication was investigated using a colony formation assay and/or a transient replication assay. A mapping study of the ISDR was performed to identify the amino acid positions that critically affect replication. While no colonies were formed in the colony for-

mation assay using HCV replicons with few mutations (0, 1 and 3) in the ISDR, numerous colonies (>200) appeared when using constructs with six mutations. Introduction of various distinct ISDR sequences with multiple mutations resulted in replication enhancement in transient assays. A mapping study identified several specific sites in the ISDR that critically affected replication, including codon 2209 which, in patients, was closely associated with a strong response to IFN. ISDR sequences associated with a clinical IFN response and viral load modulated the replication of HCV replicons, suggesting the importance of the ISDR sequence in HCV infection.

Keywords: HCV replicon, NS5A, ISDR.

INTRODUCTION

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

The interferon sensitivity-determining region (ISDR) at position 2209–2248 of NS5A in the HCV genome was originally identified as the viral genomic element in which

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

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

*Both these authors contributed equally to this study.

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

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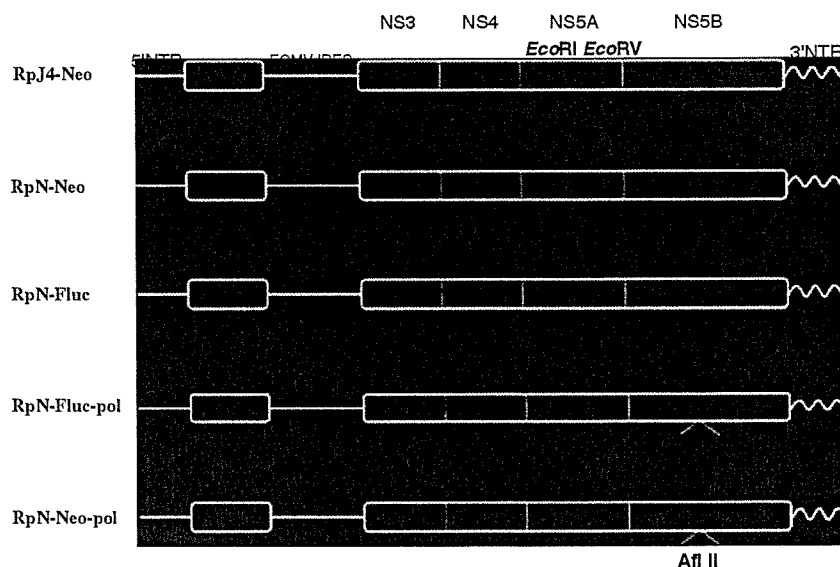


Fig. 1 Construction of HCV replicons. HCV replicons derived from HCV-N and HC-J4 isolates, and with the reporter genes *Neo* or *Fluc*, were used in this study. Using *EcoRI* and *EcoRV* digestion sites for HC-J4, and *BamHI* and *EcoRV* sites for HCV-N, ISDR sequences could be substituted by mutated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

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

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

On the other hand, we have recently conducted a clinical study of 334 patients with HCV-1b who were treated with IFN and found a close correlation between the IFN response and several individual mutations in the ISDR, suggesting that the effect of mutations in the ISDR varies according to the site [34]. This correlation also was reported in a meta-analysis study [10]. Because the tertiary structures of the NS5A protein determine their functions and affinity with

host or other viral proteins, it may be speculated that the ISDR function is not simply regulated by the number of mutations, but by the resultant NS5A protein conformation as determined by the respective ISDR amino acid sequences.

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

MATERIALS AND METHODS

Cell culture

Human hepatoma Huh7 cells were grown in Dulbecco's modified minimal essential medium (Sigma, St Louis, MO, USA) supplemented with 2 mM L-glutamine, non-essential amino acids, 100 IU of penicillin, 100 mg/L of streptomycin, and 10% foetal bovine serum at 37 °C under 5.0% CO₂. G418 (Wako, Osaka, Japan) was added to the culture medium at a final concentration of 200 µg/mL for cells carrying HCV replicons. The cells were split twice weekly at 1 : 5 to 1 : 6, depending on their confluence.

Plasmid constructions

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

of replication. Next, in order to introduce various ISDR sequences into 1bneo/delS, a *Bam*HI site [nucleotide (nt.) 5302 to 5307 of 1bneo/delS] and an *Eco*RV site (nt. 5335 to 5340 of 1bneo/delS) were introduced into the NS5A region by site-directed mutagenesis (Quick-Change Multi Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA). Mutagenesis primers were as follows: ISDR *Bam*HI-mutagenesis, 5'-CGTAGGTTGGCCAGGGGATCCCCCCTCCTTGCC-3', and ISDR *Eco*RV-mutagenesis, 5'-GAGGGGGATGA-GAATGAGATATCCATTGCGGCGGAG-3'. Digestion with *Bam*HI and *Eco*RV excised a DNA fragment encompassing nt. 5304 to 5537 of 1bneo/delS, including the ISDR. The DNA fragment was subcloned into the pLITMUS 28i vector (New England Biolabs, Beverly, MA, USA) and various ISDR mutations were introduced by site-directed mutagenesis of the pLITMUS 28i vector. Finally, these ISDR-mutated *Bam*HI-*Eco*RV fragments were subcloned back into the parental plasmid. From pRpJ4, digestion with *Eco*RI and *Eco*RV excised a DNA fragment encompassing nt. 5084 to 5528, including the ISDR, without mutagenesis. Likewise, various mutations were introduced into this DNA fragment in the subcloning vector pLITMUS28i. As replication-deficient controls, HCV-N derived replication-deficient replicons were constructed by introducing an NS5B frameshift. In order to construct this NS5B frameshift, plasmid 1bneo/delS was digested with *Afl*II, then subjected to blunting using a DNA Blunting Kit (Takara, Tokyo, Japan) and finally self-ligated (RpN-Fluc-polΔ as a replication-deficient control for RpN-Fluc and RpN-Neo-pol $\frac{1}{2}$ as a replication-deficient control for RpN-Neo).

In vitro transcription and transfection

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

Luciferase assays

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

Colony formation assay

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

Analyses of clinical data of IFN-treated patients

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

RESULTS

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

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

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

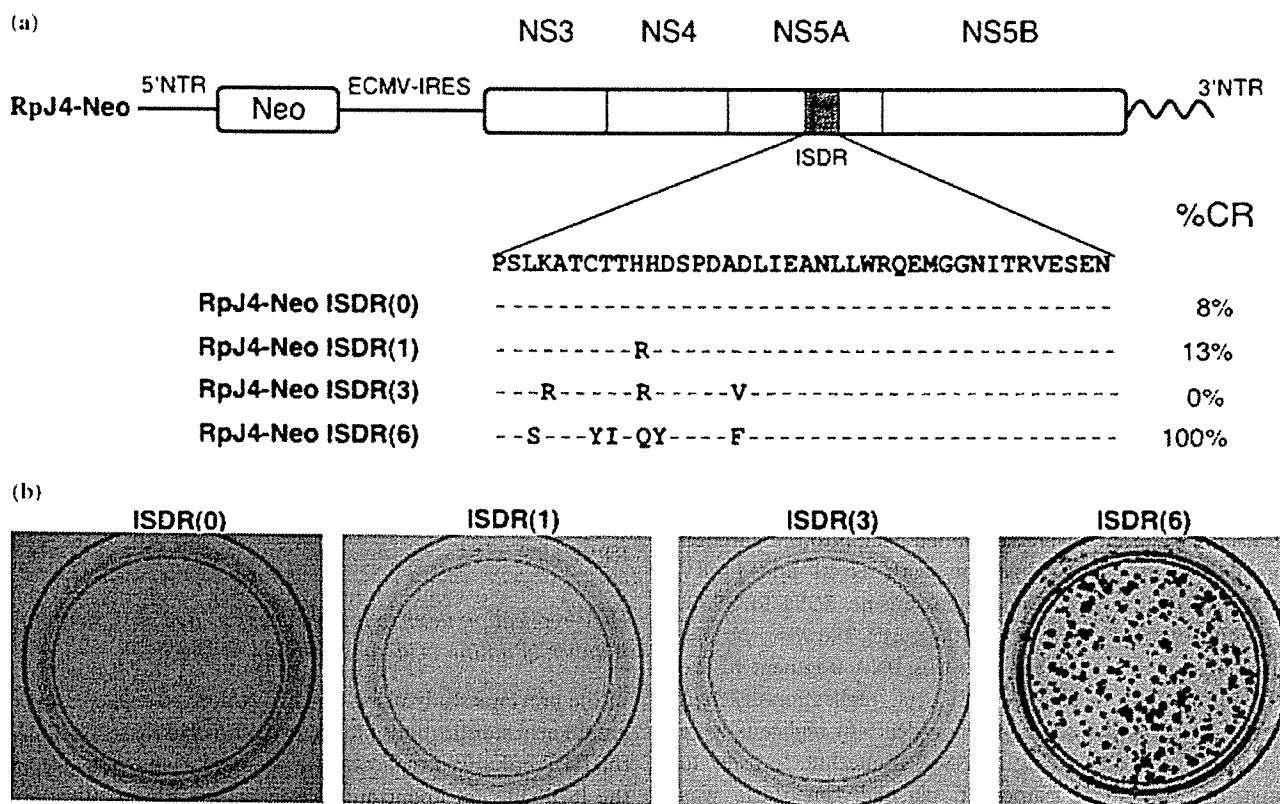


Fig. 2 HCV replication *in vitro* is regulated by the total number of amino acid substitutions in the product of the ISDR. (a) Alignment of ISDR sequences in HC-J4 replicons with different numbers of mutations in the ISDR. These ISDR sequences were obtained from patients with various IFN responses. As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

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

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

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

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

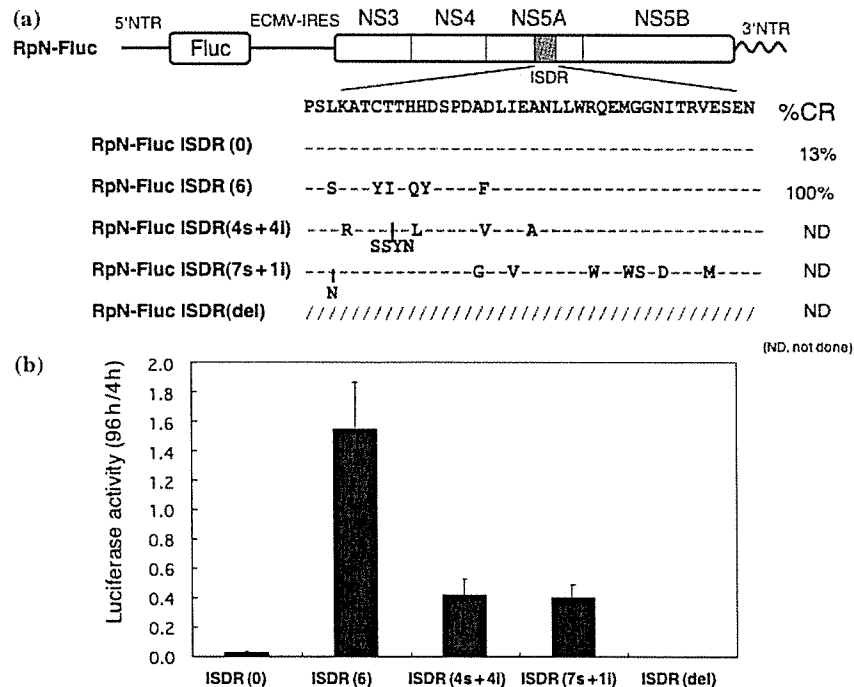


Fig. 3 Various patterns of ISDR sequences with multiple mutations all result in enhancement of HCV replication *in vitro*. (a) Alignment of ISDR sequences in HCV-N replicons with diverse ISDR sequences with multiple mutations observed in patients. In order to demonstrate the effect of deletion of the ISDR, an HCV-N replicon with complete deletion of the ISDR was engineered. (b) Replication capacity of three replicons with multiple ISDR mutations: all were greatly enhanced. In contrast, the replication capacity of replicons with deletion of the entire ISDR was almost completely abrogated. Values correspond to the ratio of the luciferase activity measured 96 and 4 h after transfection. The 4-h value was used to correct for different transfection efficiencies because at this time only luciferase translated from the input RNA was measurable.

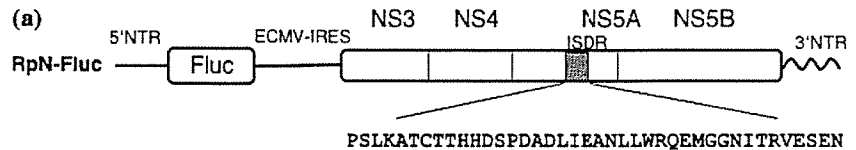
introduced into the ISDR of RpN-Fluc (Fig. 4a) [34]. As shown in Fig. 4b, although the replication capacity of most of the replicons was quite low even after the introduction of ISDR mutations, the replication capacity of RpN-Fluc-P2209L was strongly enhanced to almost 20-fold more than that of RpN-Fluc-ISDR(0) (RpN-Fluc-ISDR(0): 387 ± 60 , RpN-Fluc-ISDR(1)-P2209L: 6111 ± 500 , RpN-Fluc-ISDR(1)-T2216A: 263 ± 20 , RpN-Fluc-ISDR(1)-I2227V: 160 ± 50 , RpN-Fluc ISDR(1)-H2218R: 703 ± 270 , RpN-Fluc-ISDR(1)-H2218C: 241 ± 80 , RpN-Fluc-ISDR(1)-A2224V: 580 ± 90).

Finally, using the ISDR-6aa mutant replicon [RpN-Fluc-ISDR(6)] with high replication capacity as the starting material, we determined the specific sites that critically reduced replication by systematically reversing each mutant sequence (Fig. 5a). As demonstrated in Fig. 5b, each back mutation lowered replication capacity to a different extent. Among them, the Q2218H mutation greatly weakened replication capacity (RpN-Fluc-ISDR(0): 101 ± 40 , RpN-Fluc-ISDR(6): 11050 ± 690 , RpN-Fluc-ISDR(5)-S2211L: 5123 ± 460 , RpN-Fluc-ISDR(5)-Y2215C: 3157 ± 480 , RpN-Fluc ISDR(5)-I2216T: 5501 ± 1200 , RpN-Fluc-ISDR(5)-Q2218H: 404 ± 80 , RpN-Fluc-ISDR(5)-Y2219H: 2094 ± 250 , RpN-Fluc-ISDR(5)-F2224A: 2795 ± 550).

DISCUSSION

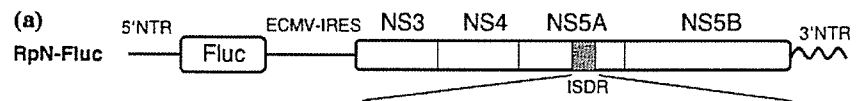
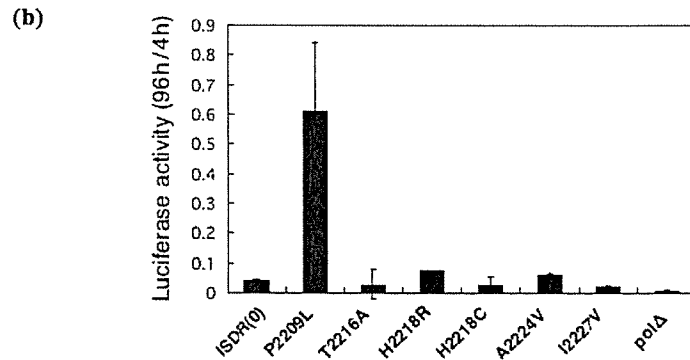
In this study, we demonstrated that the ISDR sequences regulate the replication capacity of HCV replicons, depending on IFN response-related structures. Furthermore, a mapping study revealed that the influence of each ISDR mutation on replication was site-specific, and the critical sites modulating replication *in vitro* coincided with those strongly associated with the IFN response *in vivo*.

As demonstrated in Figs 2 and 3, ISDR sequences regulated replication of HCV replicons, depending on the number of mutations in the region. Even though few mutations (0 to 3 codon changes) in the ISDR did not enhance replication, multiple mutations (resulting in six substitutions, seven substitutions plus one insertion, and four substitutions plus four insertions) all resulted in replication enhancement, excepting artificially engineered total ISDR deletion that abrogated replication capacity. These results were consistent between two replicon clones, HCV-N and HC-J4, indicating that a relationship between the number of ISDR mutations and virus replication *in vitro* may be found widely, beyond specific HCV isolates. Meanwhile, the finding that complete deletion of the ISDR abrogated replication capacity may indicate that maintenance of the conformation of the NS5A



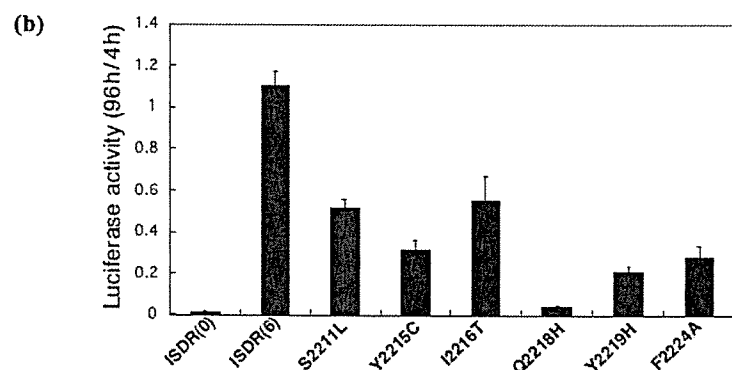
RpN-Fluc ISDR(0)	-----
RpN-Fluc ISDR(1) P2209L	L-----
RpN-Fluc ISDR(1) T2216A	-----A-----
RpN-Fluc ISDR(1) H2218R	-----R-----
RpN-Fluc ISDR(1) H2218C	-----C-----
RpN-Fluc ISDR(1) A2224V	-----V-----
RpN-Fluc ISDR(1) I2227V	-----V-----

Fig. 4 Determination of mutational points in the ISDR that critically upregulates HCV replication *in vitro*. Alignment of ISDR sequences in the HCV-N replicon with various single amino acid substitutions. These individual mutations were selected for this study because of their frequent appearance in clinical infections. Transient replication capacity of replicons with various single codon changes in the ISDR was analysed by luciferase assay. Although a parental replicon possessing no amino acid mutations in the ISDR had low luciferase activity, the replication capacity increased 20-fold upon introduction of P2209L. In contrast, no other mutation significantly altered replication.



RpN-Fluc ISDR(0)	-----
RpN-Fluc ISDR(6)	--S--YI-QY--F-----
RpN-Fluc ISDR(5) S2211L	-----YI-QY--F-----
RpN-Fluc ISDR(5) Y2215C	--S--I-QY--F-----
RpN-Fluc ISDR(5) I2216T	--S--Y--QY--F-----
RpN-Fluc ISDR(5) Q2218H	--S--YI--Y--F-----
RpN-Fluc ISDR(5) Y2219H	--S--YI-Q--F-----
RpN-Fluc ISDR(5) F2224A	--S--YI-QY-----

Fig. 5 Determination of mutational points in the ISDR that critically downregulate HCV replication *in vitro*. (a) Alignment of ISDR sequences in the HCV-N replicon with five amino acid substitutions in ISDR. In order to determine the position that critically downregulates replication of a highly replication-competent replicon with six codon changes in the ISDR, we systematically introduced back mutations. (b) Transient replication capacities of replicons with five codon changes in the ISDR were analysed by luciferase assay. Each back mutation lowered replication capacity to a different degree and, in particular, the Q2218H substitution reduced replication capacity greatly.



protein, with inclusion of the ISDR, is required for the replication of the HCV genome. Nevertheless, the need for ISDR for HCV replication should be confirmed in further studies, because a previous study reported that a partial deletion in NS5A, including the ISDR, rather supported replication [26].

This paradox, however, indicates that NS5A conformation has a key role in HCV replication.

At the same time, the effect of mutations in the ISDR on replication was site-specific. The introduction of single codon changes into the wild-type ISDR revealed that substitution of

codon 2209 greatly enhanced replication, almost 20-fold, indicating this site had a great influence on the regulation of replication (Fig. 4). On the other hand, in the experiments using the highly replication-competent replicon with six codon changes in the ISDR as starting material, the influence of reversion of each change on replication also was site-specific, and reversion of the change at codon 2218 decreased replication capacity most effectively. Importantly, codon 2209 was revealed in the clinical analysis to be one of the most important sites determining the IFN response, and codon 2218 was the site most frequently mutated among all codons in the ISDR [10,34]. Collectively, it is considered that the replication of HCV replicons reflects the ISDR sequences related to IFN response and virus replication. From these results, the HCV replicon system reflecting clinical findings could be a powerful tool to analyse the actual NS5A-ISDR function in HCV replication. For further confirmation of the relationship between HCV replication *in vitro* and that *in vivo*, we are now investigating how various NS5A-ISDR-mutant replicons respond to IFN.

Even though the HCV replicon reflects the ISDR sequences related to IFN response and virus replication, why does the influence of ISDR mutations on virus replication *in vitro* and *in vivo* seem to be contrary? In order to answer the question, the mechanism(s) by which adaptive mutations enhance replication must be clarified. Whether adaptive mutations scattered throughout the viral genome all have the same function is as yet unknown, but the functional role of the NS5A-serine cluster region recently has been investigated intensively, and mutations in the region were demonstrated to modify the phosphorylated state of NS5A proteins [26,29–31,37]. NS5A is a phosphoprotein, and has two phosphorylated isoforms: the basal phosphorylated form (p56) and the hyperphosphorylated form (p58). The NS5A protein is part of the HCV replication complex, and p56 must bind directly to a SNARE-like protein, hVAP-A, expressed in the ER membrane, in order to form the HCV replication complex [29,30,38]. On the other hand, p58 dissociates the NS5A protein from hVAP-A, preventing the HCV replication complex from forming on the ER membrane, and has been implicated in playing a certain role in virus particle assembly or excretion of virus from the cells. Thus, adaptive mutations have been proposed to prevent disassociation of the replication complex, freezing the viral life cycle at the RNA replication stage and inhibiting virion production and viral spread [39]. Although it is not clear whether the ISDR has the same function as the serine cluster region, it is possible that the ISDR has some influence on this function, because it is located adjacent to the serine cluster region and ISDR mutations enhance HCV replication synergistically with serine cluster mutations. If this is the mechanism, the inverse relationship of the *in vitro* and *in vivo* effects can be explained, because the HCV-RNA titre might reflect virion production and their secretion into the serum, while replicon

replication reflects intracellular viral replication. However, further studies are needed to test this hypothesis.

On the other hand, there is a big difference in the environment of cultured cells and the human liver. The human liver contains many immune cells, such as cytotoxic T lymphocytes, natural killer cells and dendritic cells, and a variety of cytokines, including endogenous IFN-alpha/beta produced by these cells [40]. Consequently, immunological signal pathways such as the JAK-STAT pathway and downstream interferon-inducible genes are thought to be always active to some extent in the human liver [11], in contrast to cultured cells that are not affected by immune cells or cytokines. In the innate immune system, there are two major pathways: the primary anti-viral pathway not requiring type I IFN, and the second anti-viral pathway requiring IFN for its activation [41]. It is considered that the JAK-STAT pathway is involved in the second pathway, while IRFs are involved in the primary pathway. Because of the differences in these environments, it has been suggested that the primary pathway should have a dominant role as an innate immunity in cultured cells, in contrast to the human liver where the second pathway might have an important role. We have shown recently that expression of IRF-1 was greatly suppressed in replicon-harboring cells, or even in replicon-cured cells, from which replicons had been eliminated by exogenous IFN treatment, compared with naïve Huh-7 cells [42]. When IRF-1 was induced by plasmid expression, replicon replication was greatly attenuated, indicating that downregulation of the IRF-1 pathway was necessary and was a key pathway regulating replicon replication. On the other hand, we also have shown in the previous study that the need for NS5A-adaptive mutations for replication decreases when IRF-1-suppressed cured cells are used [33], indicating that NS5A-adaptive mutations and IRF-1 suppression can effectively complement one another to permit replication. Thus, the IRF-1 pathway is also considered as a candidate target of NS5A adaptive mutations, including the serine cluster region and ISDR.

In conclusion, we have demonstrated that the numbers of mutations in the ISDR regulate the replication of replicons, suggesting that ISDR sequences associated with IFN sensitivity influence the replication of replicons *in vitro*. Moreover, specific sites in the ISDR critically regulate replication, showing that the effects of mutations in the ISDR vary according to the site. Although the numbers of analysed ISDR sequences were still small, our results indicate that the replicon system might be used as a tool to clarify the molecular mechanism of NS5A-ISDR to modulate HCV replication.

CONFLICT OF INTEREST

We do not have a commercial or other association that might pose a conflict of interest.

ACKNOWLEDGEMENTS

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Divergent activities of interferon-alpha subtypes against intracellular hepatitis C virus replication

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Abstract

Backgrounds: Interferon (IFN)-alpha is represented by several structurally related subtypes that show different antiviral and anti-tumor effects. Here, we analyzed differential effects of IFN-alpha subtypes on intracellular hepatitis C virus (HCV) replication using HCV subgenomic replicon system as a model.

Methods: Huh7 and HeLa cells supporting expression of HCV replicon were treated with various concentrations of five recombinant human IFN-alpha subtypes 1, 2, 5, 8, and 10, and with IFN-alpha con1. The effects of IFNs on various cell-signaling pathways were assayed by using ISRE-, GAS-, AP1-, NF-kappa B-, CRE-, and SRE-luciferase reporter plasmids.

Results: Each IFN-alpha subtype suppressed HCV replication in a dose-dependent manner. Among them, IFN-alpha8 was the most effective, while IFN-alpha1 was the least effective with 50% inhibitory concentrations of 0.123 IU/ml versus 0.375 IU/ml, respectively. These differential effects against HCV replication did not correlate with levels of the IFN-responsive ISRE or GAS reporter activities, nor they did activate the other reporters, AP1, NF-kappa B, CRE and SRE.

Conclusion: There were divergent effects of IFN-alpha subtypes against HCV replication that may be through JAK-STAT-independent pathways. Exploring further mechanisms of action may elucidate IFN-mediated cellular antiviral mechanisms.

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Keywords: Interferon subtype; Consensus interferon; Hepatitis C virus replicon; ISRE; GAS; Drug synergy

1. Introduction

Hepatitis C virus (HCV) is a world-wide health care problem causing a spectrum of liver disease ranging from

asymptomatic carrier state to liver cirrhosis and hepatocellular carcinomas [1]. Currently available anti-HCV treatments are based on administration of a major antiviral cytokine, type I interferons (IFN-alpha or -beta). Among them, therapies with recombinant interferon-alpha subtype 2a or 2b and their chemically modified derivatives, PEG-IFNs, are the de facto standard in the current clinical therapeutics. Their effectiveness, however, remains around 50% of patients treated [2].

IFN plays a central role in eliminating viruses not only as therapeutic applications but also as natural cellular antiviral defense mechanisms [3,4]. Also in HCV, a DNA microarray analysis of chimpanzee liver experimentally inoculated with

Abbreviations: AP1, activator protein 1; CRE, cAMP response element; GAS, interferon-gamma activation sequence; HCV, hepatitis C virus; IFN, interferon; ISRE, interferon stimulation response element; JAK, janus kinase; SRE, serum response element; STAT, signal transducers and activators of transcription

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HCV revealed that expression of the interferon-stimulated genes (ISGs) was the principal reaction during the course of the viral infection and its clearance [5].

Type I IFNs are represented by the leukocyte and fibroblast types [6]. IFN-beta is encoded by a single gene, whereas IFN-alpha is represented by a large family of structurally related genes expressing 13 subtypes [7–10]. Among 13 proteins produced from these genes, IFN-alpha13 is identical to that produced from IFN-alpha1 gene. Thus, there are 12 different human IFN-alpha proteins. Several studies have shown that IFN-alpha subtypes have different properties in their mode of expression and the effects on the target cells depending on the cell types and on the IFN inducer used. In response to virus infection to murine cells, expression of IFN-alpha1, -alpha4, and -alpha6 mRNA are induced differently between cell types [11]. An analysis of mRNA expression in liver of chronic hepatitis C patients has shown that the expression patterns of each IFN subtype are different from those of normal controls [12].

In addition to the difference of the expression profile, IFN-alpha subtypes show different biological activities on their target cells. Each IFN-alpha subtype shows distinct patterns of antiviral, growth inhibitory, and other biologic activities [13–16]. Regarding antiviral activity, studies using murine encephalomyelitis virus and various human cell lines have shown that IFN-alpha8 was the most potent, while IFN-alpha1 was the least potent [17]. These reports have made us speculate that there might be subtype-specific IFN-signaling pathways.

Actions of type I IFN are initiated by binding of the IFN-alpha receptors (IFNARs) expressed on cell surface. The binding of IFN to their receptor activates two kinases; janus kinase 1 (JAK1) and the tyrosine kinase 2 (TyK2), which associate with the intracellular domain of IFNARs). These receptor-kinases activate the SH2 domain of the signal transducer and activator transcription factors (STATs). The phosphorylated STAT1 and STAT2 recruit IFN regulatory factor (IRF)-9 to form the IFN-stimulated gene factor-3 (ISGF-3) and activates expression of the ISGs by binding the interferon stimulation response element (ISRE) in their promoter/enhancer domain [18,19].

Studies on HCV replication have been hampered by the lack of efficient cell culture models. The problem was partly overcome by the development of an HCV subgenomic replicon system, which simulates cellular autonomous replication of HCV genomic RNA [20]. More recently, an efficient HCV cell culture system has been reported [21]. Replication of HCV replicon can be abolished by treatment with type I and type II IFNs [22–24]. These findings indicate that the IFN receptor-mediated cellular responses are intact in the HCV replicon system. In the present study, we have analyzed effects of five IFN-alpha subtypes on intracellular HCV replication using HCV subgenomic replicon system, and investigated on the difference of mechanisms of action.

2. Materials and methods

2.1. Cell culture

Huh7 and HeLa cells were maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37 °C under 5% CO₂. Huh7 cells containing HCV replicon were cultured in medium containing 500 µg/ml G418 (Nakalai, Kyoto, Japan).

2.2. HCV replicon constructs and transfected cell lines

An HCV subgenomic replicon plasmid, pHCVIbneo-delS was derived from an infectious HCV clone, HCV-N, genotype 1b [24]. The replicon was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotransferase (pRep-Feo) [25,26]. Another replicon construct, pSGR-JFH1 was derived from an infectious HCV clone, JFH-1, genotype 2a [27]. The replicon was reconstructed by substituting neomycin phosphotransferase gene with a fusion gene comprising of renilla luciferase and neomycin phosphotransferase (pRep-Reo). RNA was synthesized from linearized replicon plasmids using T7-polymerase (Promega, Madison, WI) and transfected into HeLa cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/Rep-Feo and HeLa/Rep-Reo). The expression levels of the replicon in the cell lines were determined by measuring luciferase activities of the cell lysates.

2.3. Reporter plasmids

A plasmid, pISRE-TA-Luc (BD Biosciences Clontech, Palo Alto, CA), expressed the Fluc gene under the control of the ISRE [28]. pGAS-TA-Luc, pAPI1-TA-Luc, NF-kappa B-TA-Luc pSRE-TAL-luc, and pCRE-TAL-luc (BD Biosciences Clontech) are also used to see the expressed Fluc gene under GAS, AP1, NF-kappa B, SRE and CRE. A plasmid, pRL-CMV (Promega), which expresses the renilla luciferase gene under the control of the cytomegalovirus early promoter/enhancer, was used to normalize transfection efficiency.

2.4. Recombinant IFN-alpha subtypes

Recombinant human IFN-alpha1, -alpha2, -alpha5, -alpha8, and -alpha10 were prepared via the pET-3a-BL21 system (Novagen, Madison, WI) as described previously [29]. IFN-alpha activities were measured in a cytopathic effect (CPE) reduction assay using Sindbis virus-infected FL cells, and the IFN titers were standardized in international units (IU/ml) by comparison with the WHO standard [29]. Cells were seeded in 96-well plates and incubated with two-fold dilutions of each IFN-alpha subtype. After incubation for 72 h, The CPE in each well was determined, and the specific

antiviral activity was expressed in terms of the mean 50% inhibitory concentration (IC50) of each IFN- α subtype on a weight basis. The activities were 3.0×10^6 IU/mg for subtype $\alpha 1$, 7.2×10^7 IU/mg for $\alpha 2$, 4.0×10^7 IU/mg for $\alpha 5$, 2.9×10^8 IU/mg for $\alpha 8$, and 4.9×10^7 IU/mg for $\alpha 10$. As regards IFN- $\alpha 2$, because of glycosylation of the naturally occurring subtype that may influence its biologic activity, we also purified natural IFN- $\alpha 2$ from cultures of BALL-1 cells stimulated with Sendai virus instead of producing a recombinant product. Recombinant interferon- α con1 was kindly provided by Yamanouchi Co. Ltd. (Tokyo, Japan). Specific activity of IFN- α con1 was 2.05×10^9 IU/mg [30].

2.5. Luciferase assays

Luciferase assays were done as described previously [28,31]. Typically, the replicon-expressing cells were seeded on a 24-well plate at a density of 1×10^4 cells per well. IFNs were applied to the culture medium at various concentrations (0.01–100 IU/ml). After 48 h of culture, expression levels of HCV replicon were measured by luciferase assay using the Bright-Glo Luciferase Assay System (Promega) or the Renilla Luciferase Assay System (Promega) and a luminometer, TD-20/20 (Turner Designs, Sunnyvale, CA). Assays were done in triplicate and the results were expressed as means \pm S.D. To perform dual reporter assays, cells were seeded onto 24-well plate at 5×10^4 cells per well, and transfected with 400 ng of pISRE-TA-Luc and pRL-CMV (1 ng for Huh7, 10 ng for HeLa, respectively) using 1.2 μ l of FuGene6 (Roche, Indianapolis, IN) per well. IFN- α subtypes were applied at 24 h after the transfection. Cells were harvested at 24 h after the addition of IFNs. The dual reporter assays were done using the Dual-Luciferase Reporter Assay System (Promega).

2.6. Western blot analysis

Ten micrograms of nuclear extract lysate was separated in a NuPAGE 10% Bis-Tris Gel (Invitrogen) and blotted onto the PVDF Western Blotting Membrane (Roche). The membranes were incubated with mouse monoclonal anti-NS5A antibody followed by POD-labeled anti-mouse IgG antibody. Chemiluminescence was detected using the ECL Western blotting Analysis System (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's protocol.

2.7. Analyses of drug synergism

The effects of treatment of Huh7/Rep-Feo cells with the IFN subtypes, alone and in combination, were analyzed according to classical isobologram analysis [32,33]. Dose-inhibition curves of IFN subtypes A and B were drawn, with the two drugs used alone or in combination. In each drug combination, the concentrations of IFN subtypes A and B that inhibited HCV replication to 50% (IC50) were plotted against

the fractional concentration of each IFN subtype on the X- and Y-axes, respectively. The combination index, CI, for each combination was calculated using the following formula:

$$CI = \frac{IC50(\text{IFN subtypes A and B combined})}{IC50(\text{IFN subtype A alone})} + \frac{IC50(\text{IFN subtypes A and B combined})}{IC50(\text{IFN subtype B alone})}$$

For such plots, the combined effects of two drugs can be assessed as an either additive effect, indicated by CI = 1, synergy, indicated by CI < 1, or antagonism, indicated as CI > 1.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR) assay

Total cellular RNA was extracted from cells using ISOGEN (Wako, Osaka, Japan). Two micrograms of total cellular RNA was used to synthesize cDNA from each sample using the SuperScript II (Invitrogen) reverse transcriptase. PCR was done in a mixture containing 1 μ l cDNA sample; 1.25 mM MgCl₂; 0.5 μ M of each primer; 0.4 mM of dNTP and BD advantage2 PCR Kit (BD Biosciences Clontech, Palo Alto, CA). Primers used were as follows: IFNAR-1, sense, 5'-AGT GTT ATG TGG GCT TTG GAT GGT TTA AGC-3', IFNAR-1, antisense, 5'-TCT GGC TTT CAC ACA ATA TAC AGT CAG TGG-3', IFNAR-2, sense, 5'-ATT TCC ATC TAT TGT TGA GG-3', IFNAR-2, antisense, 5'-CAC TTT CTT CTT TCT GTT GA-3', beta-actin, sense, 5'-CAC CAT GGA TGA TGA TAT CGC CGC GCT CGT-3', and beta-actin, antisense, 5'-GAA GCA TTT GCG GTG GAC GAT GGA GGG GCC-3'. The PCR products were separated on 1% agarose gel, stained with ethidium bromide, and visualized by UV transilluminator.

2.9. Statistical analyses

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

3. Results

3.1. Analysis of suppressive effects of the IFN- α subtypes on expression of HCV replicon in Huh7 cells

Huh7/Rep-Feo cells were treated with various concentrations of IFN subtypes (0.01–100 IU/ml). After 48 h of the treatment, the levels of HCV replication were analyzed by luciferase assay. Replication level of HCV replicon was significantly suppressed by each IFN subtype in dose-dependent manner (Fig. 1A). Fifty percent inhibitory concentrations (IC50) of the IFN subtypes were calculated as 0.375 IU/ml (125 pg/ml) for $\alpha 1$, 0.220 IU/ml (3.05 pg/ml) for $\alpha 2$, 0.238 IU/ml (5.94 pg/ml) for $\alpha 5$, 0.124 IU/ml

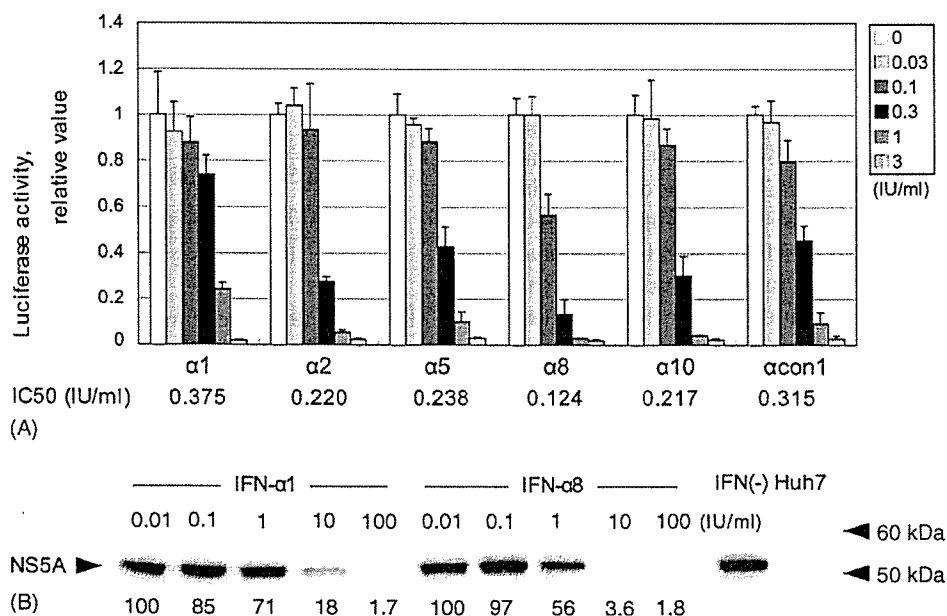


Fig. 1. Analysis of antiviral effect of the IFN- α subtypes using HCV replicon Huh7 cells: (A) luciferase activities of Huh/Rep-Feo were cultured in the presence of indicated concentration of IFN subtypes. Luciferase activities were measured at 48 h after culture. Values indicate relative luciferase activities as percents of IFN-negative controls. Error bars indicate mean \pm S.D. (B) Western blotting. Huh7/Rep-Feo cells were treated with indicated concentrations (numbers on the top) of IFN- α 1 and - α 8. Total cell lysate was harvested after 48 h, and Western blotting was done using primary antibody directed against NS5A. Numbers on the bottom indicate densitometric values displayed as percents of an IFN-negative control.

(0.429 pg/ml) for α 8, 0.217 IU/ml (4.43 pg/ml) for α 10, and 0.315 IU/ml (0.153 pg/ml) for α con1, respectively. As indicated, expression of HCV replicon in Huh7 cells was the most sensitive to IFN- α 8, and the least to IFN- α 1 by titers standardized in IU/ml and in pg/ml. IFN- α 8 was 1.8 times more sensitive than IFN- α 2 to suppress HCV replication. Treatment of Huh7/Rep-Feo with a solvent of IFN proteins showed no effects on expression levels of HCV replicon. To confirm the result of IFN- α 1 and - α 8, we next carried out Western blotting. Cells were treated with IFN- α 1 and - α 8, and total cell lysate was harvested after 48 h. The expression of replicon-derived NS5A protein was dose-dependently suppressed by treatment with IFN- α 1 and - α 8, and the velocities of suppression were comparable to those of luciferase activities (Fig. 1B).

3.2. Effects of IFN subtypes on cellular signal transduction pathways

We next investigated effects of IFN subtypes on the ISRE and GAS promoter activities by luciferase reporter assay. Plasmids, pISRE-luc and pGAS-luc were transfected into Huh7 cells. Twenty-four hours after transfection, various concentrations of IFN subtypes were added into the medium. Luciferase assay was done at 6 h after addition of IFNs. The ISRE- and GAS-mediated luciferase activities were elevated by dose-dependent manner of each IFNs. However, there was no significant difference in their induction velocities between each IFN subtypes (Fig. 2A). Furthermore, a discrepancy was

observed between the suppressive effects of HCV replication and the ISRE and GAS activities; IFN- α 1 and - α 8 apparently showed similar activations of ISRE and GAS promoter, while IFN- α 8 was more effective to inhibit the HCV replication than IFN- α 1. Therefore, we investigate alternate pathway that may be activated by IFN. Reporter assays were performed by using plasmids, pAP1-luc, pNF- κ B-luc, pCRE-luc, and pSRE-luc. Luciferase assays after addition of each IFN subtypes showed that neither of AP1, NF- κ B, CRE, and SRE reporters were activated by IFN treatment (Fig. 2B).

3.3. Analyses of synergism between IFN subtypes

It has been reported that interactions between IFN subtypes may influence cellular response to IFN [16]. Our previous study showed that IFN- α 2 and - α 8 had synergistic antiviral effect against vesicular stomatitis virus (VSV) infection to HepG2 cells. [29]. To investigate if the IFN subtypes synergistic effects on HCV replication, a classical isobologram analysis was performed [32,33]. Huh7/Rep-Feo cells were treated with IFN- α 2 or - α 8 alone, or with IFN- α 2 and - α 8 that were mixed with equivalent IC50 ratio, and the dose-effect plots were drawn (Fig. 3A). Each concentration of IFN- α 2 and - α 8 at 50% inhibition were plotted on the X- and Y-axes, respectively, to generate an isobologram (Fig. 3B). A plot showing the IC50 of IFN- α 2 and - α 8 at 1:1 mixture was located on the line connecting IC50 of IFN- α 2 or - α 8 alone, indicating that the effects of the drug

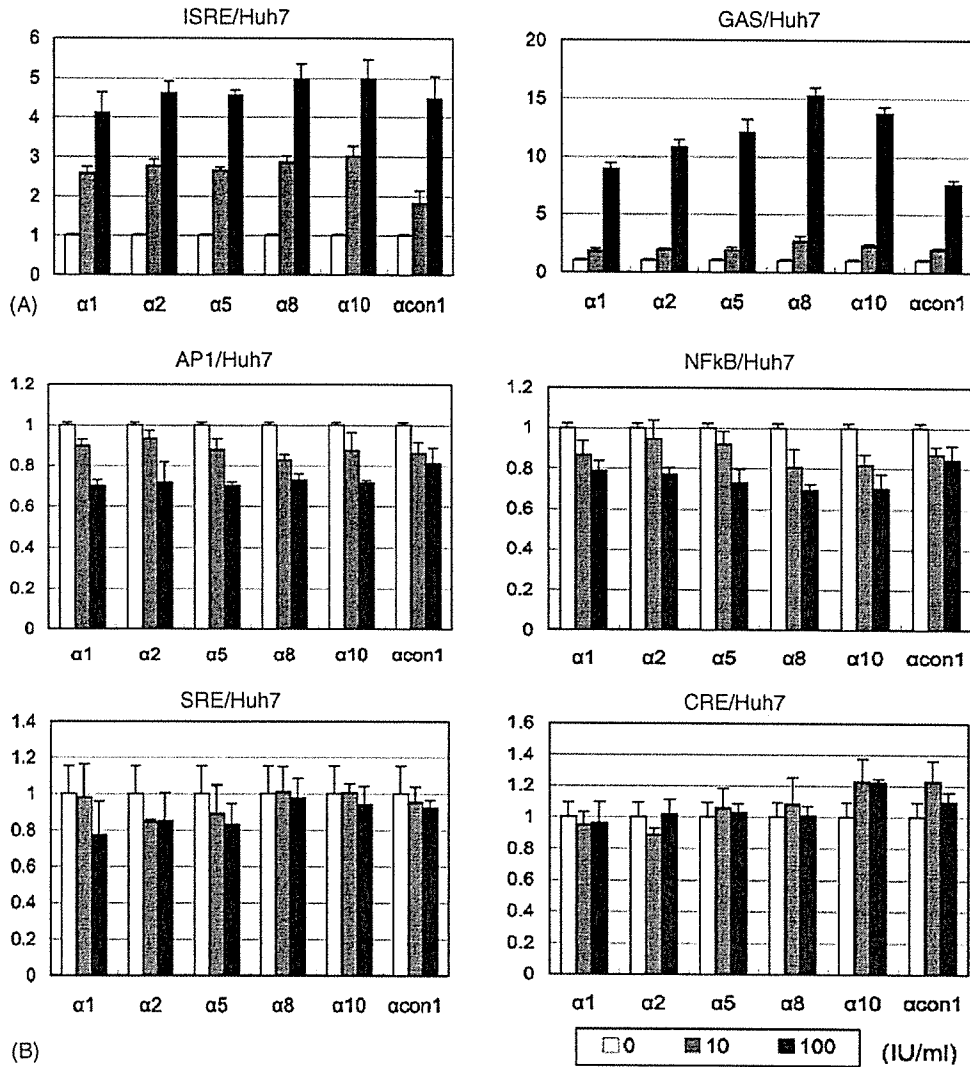


Fig. 2. ISRE, GAS, AP1, NF-kappa B, CRE and SRE reporter assay of Huh7 cells. ISRE-, GAS-, AP1-, NF-kappa B-, CRE-, or SRE-Luc reporter plasmids was transfected into Huh7 with pRL-CMV as a control. Twenty-four hours after transfection, the medium was supplemented with the IFN-alpha subtypes indicated. Dual luciferase assays were done at 6 h after the addition of IFNs. Bars indicate relative reporter activities as percents of IFN-negative controls. Error bars indicate mean \pm S.D.

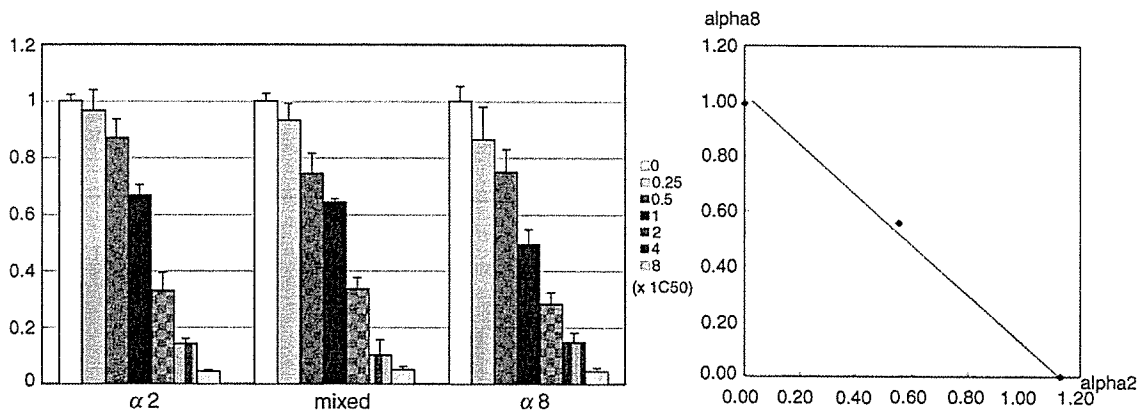


Fig. 3. Analyses of synergism between IFN subtypes 2 and 8: (A) IFNs; IFN-alpha2, -alpha8, mixed, dose modified by IC50 are added to Huh/Rep-Feo. Luciferase activities of Huh/Rep-Feo were measured 48 h after the addition of IFNs. (B) Graphical representation of the isobologram analysis. IC50s were calculated from the values in panel A. Each fractional IC50 for IFN-alpha2 and -alpha8 was plotted on X- and Y-axes, respectively. The FIC plot for the IFN-alpha2 and -alpha8 combinations of 1:1 falls on a theoretical line of additivity that is drawn between the IC50 plot for each IFN alone, indicating the effects of combination of IFN-alpha2 and -alpha8 is additive.

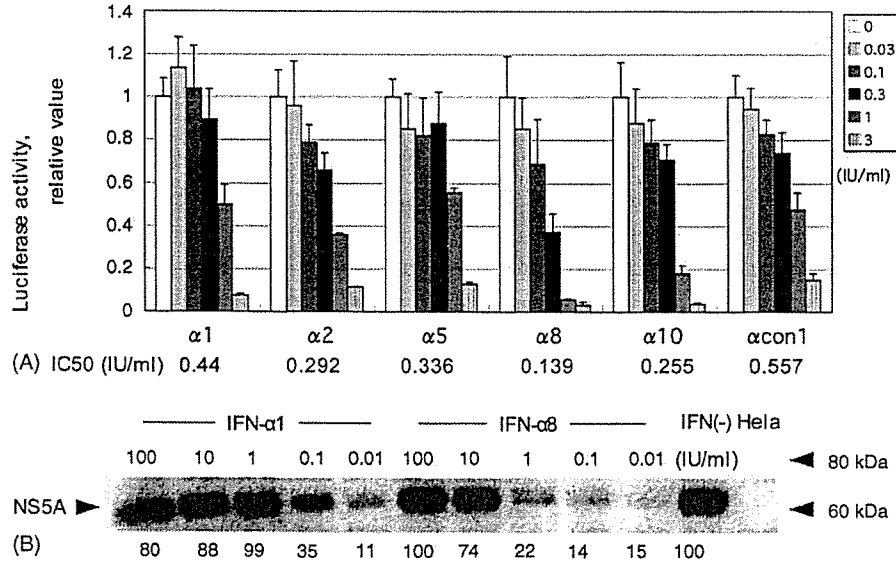


Fig. 4. Analysis of antiviral effect of the IFN-alpha subtypes using HCV replicon HeLa cells: (A) Luciferase activities of HeLa/Rep-Reo were cultured in the presence of indicated concentration of IFN subtypes. Luciferase activities were measured at 48 h after culture. Values indicate relative luciferase activities as percents of IFN-negative controls. Error bars indicate mean ± S.D. (B) Western blotting. HeLa/Rep-Reo cells were treated with indicated concentrations (numbers on the top) of IFN-alpha1 and -alpha8. Total cell lysate was harvested after 48 h, and Western blotting was done using primary antibody directed against NS5A. Numbers on the bottom indicate densitometric values displayed as percents of an IFN-negative control.

combination on intracellular HCV-RNA replication is additive.

3.4. Analysis of antiviral effect of the IFN-alpha subtypes using HCV replicon HeLa cells

Different type of cells may respond to IFN in a different manner possibly depending on the expression profiles of interferon receptors or cellular factors that mediate IFN sig-

nal transduction. To investigate the effects of IFN subtypes in a non-hepatocyte cell line, we used HeLa cells expressing chimeric luciferase reporter HCV replicon, HeLa/Rep-Reo. Treatment of HeLa/Rep-Reo cells with IFN subtypes suppressed expression of HCV replicon in dose-dependent manner (Fig. 4A). The IC50 for each IFN subtypes were 0.44IU/ml (147 pg/ml) for alpha1, 0.292 IU/ml (4.06 pg/ml) for alpha2, 0.336 IU/ml (8.4 pg/ml) for alpha5, 0.139 IU/ml (0.479 pg/ml) for alpha8, 0.255 IU/ml (5.20 pg/ml) for

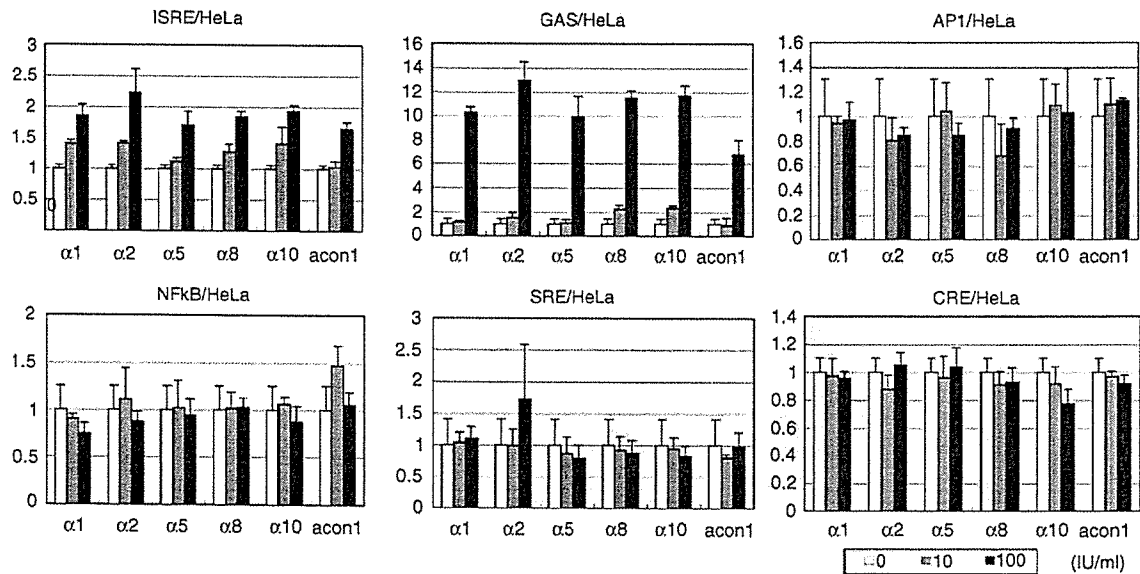


Fig. 5. Reporter assay of HeLa cells. ISRE-, GAS-, AP1-, NF-kappa B-, CRE-, and SRE-Luc reporter plasmids were, respectively, transfected into HeLa cells with pRL-CMV as a control. Twenty-four hours after transfection, IFN subtypes were added onto the medium. Dual luciferase assays were done at 6 h after the addition of IFNs. Bars indicate relative reporter activities as percents of IFN-negative controls. Error bars indicate mean ± S.D.