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Hepatitis C virus proteins exhibit conflicting effects on the interferon system in human hepatocyte cells

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Abstract

We previously found that hepatitis C virus (HCV) core protein (Core) activated the interferon (IFN)-inducible 40/46 kDa *2′-5′-oligoadenylate synthetase* (*2′-5′-OAS*) gene through an IFN-stimulated response element (ISRE) in non-neoplastic human hepatocyte PH5CH8 cells. Here, we found that Core and NS5B synergistically enhanced the *2′-5′-OAS* gene promoter activity through ISRE. Further analysis revealed that amino acid positions 12 and/or 13 of Core and RNA-dependent RNA polymerase activity of NS5B were essential for the activation of the *2′-5′-OAS* gene promoter. Interestingly, we observed that the activation by Core or NS5B was still partially enhanced by even the NS5B or Core mutant lacking the activating ability, respectively, suggesting an indirect interaction between Core and NS5B. Furthermore, we showed that the activation by NS5B could be explained by NS5B's induction of IFN-β, however, IFN-β was not induced by Core. Moreover, we showed that the synergistic effect of Core and NS5B was not invalidated by NS3-4A, although NS3-4A significantly inhibited the activation by combination of Core and NS5B. Taken together, our findings reveal that NS5B/Core and NS3-4A exhibit conflicting effects (activation and inhibition) on the IFN system in PH5CH8 cells, and suggest that such effects may promote the distraction of the host defense system to lead to persistent infection.

Keywords: Hepatitis C virus; Interferon system; Core; NS5B; *2′-5′-Oligoadenylate synthetase*; Interferon-β; NS3-4A

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis [1,2], which progresses to liver cirrhosis and hepatocellular carcinoma [3,4]. Since at least 170 million people are currently infected with HCV worldwide, this infection is a global health problem [5]. HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the Flaviviridae [6,7]. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acid (aa) residues, which is cleaved co- and post-translationally into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. These cleavages are mediated by the host and virally encoded serine proteinase located in the amino-terminal domain of NS3. Activity of NS3 requires NS4A, a protein consisting of 54 aa residues, to form a stable complex with the NS3 domain [8–10]. NS5B possessing an RNA-dependent RNA polymerase (RdRp) activity is the central enzyme in replication of the HCV genome [10].

Interferon (IFN), one important effector of the innate immune response, is induced by different viral or bacterial components through Toll-like receptor (TLR)-dependent and -independent mechanisms. The binding of type I IFNs (IFN-α and IFN-β) to specific cell-surface receptors (IFNAR1 and IFNAR2c) triggers activation of the intracellular IFN signaling pathway (JAK–STAT). The activated JAK–STAT pathway induces the expression of a large number of IFN-stimulated genes (ISG), including cellular antiviral molecules such as *2′-5′-oligoadenylate synthetase* (*2′-5′-OAS*), *double stranded RNA* (*dsRNA*)*-activated*

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protein kinase (*PKR*), *dsRNA-specific adenosine deaminase 1* (*ADAR1*), and *Mx* genes through the JAK–STAT signaling transduction pathway [11–14].

We previously found that HCV core protein (Core) activated the IFN-inducible 40/46 kDa *2′-5′-OAS* gene in noncancerous human hepatocyte PH5CH8 cells [15], but not in cancerous human hepatocyte HuH-7 cells (H. Dansako and N. Kato, unpublished). Further analysis revealed that Core, irrespective of HCV genotypes and strains, activated the gene (*2′-5′-OAS*, *PKR*, *ADAR1*, etc.) promoters possessing an IFN-stimulated response element (ISRE) [16], and that the N-terminal 20 aa region of Core was important to the activation of the promoter, although this N-terminal region did not change the perinuclear localization of Core [16]. These findings suggest that the Core's activation of the *2′-5′-OAS* gene contributes to the degradation of HCV RNA. However, it is still difficult to clarify this point, because of the lack of a reproducible and efficient HCV proliferation system using PH5CH8 cells [17].

On the other hand, Foy et al. [18] recently found that an HCV serine protease, NS3-4A, blocks virus-induced activation of IFN regulatory factor 3 (IRF-3), a transcription factor playing a critical role in the induction of type I IFNs (initially IFN-β and subsequently IFN-α). This finding using HuH-7 cells suggests that NS3-4A mediates proteolysis of a cellular protein within an antiviral signaling pathway upstream of IRF-3, leading to the persistent viral infection. The recently identified Toll-IL1 receptor domain-containing adaptor inducing IFN-β (TRIF) is a possible candidate for this cellular protein [19]. However, the activation by Core and the suppression by NS3-4A on the IFN system seem to be contradictory phenomena, although both findings have been obtained by using different human hepatocyte cell lines [15,16,18].

To clarify the mechanism(s) underlying activation or suppression by HCV proteins on the IFN system, we further characterized the effects of Core and NS proteins, including NS3-4A, on IFN signaling using PH5CH8 cells, which have recently been shown to retain robust IFN responses to dsRNA as well as viral infection, suggesting that they more closely resemble normal hepatocytes in vivo [20].

In the present study using PH5CH8 cells, we report that NS5B synergistically enhanced the gene activation by Core through ISRE, and that the activation by Core or NS5B was suppressed by NS3-4A, but the synergistic effect of Core and NS5B was still observed even in the presence of NS3-4A.

Materials and methods

Cell lines. Non-neoplastic human PH5CH8 hepatocytes, which are susceptible to HCV infection and supportive of HCV replication [21], were maintained as described previously [22].

Construction of expression vectors. pCXbsr/NS3-4A, pCXbsr/NS4A, pCXbsr/NS4B, and pCXbsr/NS5B, which contain the resistance gene for blasticidin and encode NS3-4A, NS4A, NS4B, and NS5B derived from the HCV 1B-1 strain belonging to genotype 1b (Accession No. AB0802999) [23], respectively, were constructed according to the previously described method [15]. pCXpur [24], which contains the resistance gene for puromycin, was also used for the construction of pCXpur/Core and pCXpur/NS3-4A. The DNA fragments encoding Core and NS3-4A derived from HCV 1B-1 strain [23] were also subcloned into the *Eco*RI and *Not*I sites of pCXpur.

pCXbsr/Core Δ (2–6), pCXbsr/Core Δ (2–11), PCXbsr/Core Δ (2–16), and pCXbsr/Core Δ (2–21), which encode 5, 10, 15, and 20 aa N-truncated Core (1b-P) [15], respectively, were constructed according to the previously described method [15]. pCXbsr/Core R9T-K10S-Δ (11–13) was constructed by PCR mutagenesis with primers containing base alterations. pCXbsr/NS5BΔ C21, pCXbsr/NS5BΔ C56, and pCXbsr/NS5BΔ C97, which encode 21, 56, and 97 aa C-truncated NS5B derived from HCV 1B-1 strain [23], respectively, were also constructed according to the previously described method [15]. pCXbsr/NS5B G317V and pCXbsr/NS5B R154T-K155S-Δ (156–158) were constructed by PCR mutagenesis with primers containing base alterations. The nucleotide sequences of these constructed expression vectors were confirmed by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Luciferase reporter assay. For the dual luciferase assay, we used firefly luciferase reporter vectors, p2′-5′-OAS(−159)-Luci [25] containing the −159 to +82 region of the *2′-5′-OAS* gene, pIFNβ(−125)-Luc [25] containing the *IFN-β* gene promoter region (−125 to +19), and pISRE-Luc (Stratagene, La Jolla, CA) containing five repeats of the consensus ISRE sequence (AGTTTCACTTTCCC). The reporter assay was carried out as previously described [15,16]. Briefly, a total of 1.5×10^5 cells were seeded in a six-well plate 24 h before transfection. Then, 0.5 μg firefly luciferase reporter plasmid (p2′-5′-OAS(−159)-Luci, pIFNβ(−125)-Luc, or pISRE-Luc), 1–2 μg HCV protein expression effector plasmid (pCXbsr series), and 1 ng pRL-CMV (Promega, Madison, WI) as an internal control reporter were transfected into PH5CH8 cells. To maintain the efficiency of transfection, up to 2 μg (4 μg in some cases) of pCXbsr instead of HCV protein expression vectors was used as the effector plasmid DNA. The cells were cultured for 48 h, and then a dual luciferase assay was performed according to the manufacturer's protocol (Promega). In some cases, the cells were cultured for 42 h and then treated with IFN-β (500 IU/ml) for 6 h before the reporter assay. Three independent triplicate transfection experiments were conduced in order to verify the reproducibility of the results. Relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used to detect luciferase activity.

Western blot analysis. Preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were performed as described previously [26]. The antibodies used in this study were those against Core (Institute of Immunology, Tokyo), anti-NS3 (Novocastra Laboratories, Newcastle, UK), anti-NS4A (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), anti-NS4B [9], anti-NS5A [9], anti-NS5B (a generous gift from Dr. M. Kohara), and β-actin (Sigma, St. Louis, MO). Immunocomplexes were detected by a Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

Reverse transcription-PCR. Total cellular RNA was extracted using an Isogen extraction kit (Nippon Gene, Toyama, Japan). Before reverse transcription (RT), the RNA was treated with RNase-free DNase I (TaKaRa Bio, Ohtsu, Japan) to completely remove the genomic DNA as described previously [16]. RT-PCR was performed by a method described previously [16]. The sequences of IFN-β (Accession No. V00547), IRF-1 (Accession No. NM_002198), IRF-3 (Accession No. NM_001571), and IRF-7 (Accession No. U73036) were used to design specific primers. The sequences of the sense and antisense primers for IFN-β were 5′-CCCTG AGGAGATTAAGCAGCTGC-3′ and 5′-AGTTCCTTAGGATTTC CACTCTGAC-3′. The sequences of the sense and antisense primers for IRF-1 were 5′-GCCCTGACTCCAGCACTGTCG-3′ and 5′-ATTG AGTAGGTACCCCTTCCC-3′. The sequences of the sense and antisense primers for IRF-3 were 5′-ACACATACTGGGCAGTGAGC-3′ and 5′-G CAGGTCCACAGTATTCTCC-3′. The sequences of the sense and

antisense primers for IRF-7 were 5′-GTACGGGTGGGCAGTAGAGAC-3′ and 5′-CAGCAGTTCCTCCGTGTAGC-3′. RT-PCR was performed using primer sets for IFNAR1 [16], IFNAR2c [16], STAT1 [16], STAT2 [16], and GAPDH [27] used in the previous reports [16]. Real-time LightCycler PCR was performed by a method described previously [27].

Preparation of PH5CH8 cells stably expressing HCV proteins. PH5CH8 cells were infected with retrovirus pCXbsr [24] encoding various HCV proteins, as described previously [28]. pCXbsr/Core (1b-P) [15], pCXbsr/NS3-4A, pCXbsr/NS5A (1b-P) [15], and pCXbsr/NS5B were used to obtain the PH5CH8 cells stably expressing Core, NS3-4A, NS5A, and NS5B, respectively. At 2 days postinfection, the PH5CH8 cells were changed with fresh medium containing blasticidin (20 μg/ml), and the culture was continued for 7 days to select the cells expressing HCV proteins.

Results

Core and NS5B synergistically enhance 2′-5′-OAS gene promoter activity

We previously found that Core activated the IFN-inducible 40/46 kDa *2′-5′-OAS* gene through an ISRE in human immortalized hepatocyte PH5CH8 cells [15]. However, in that study, the effect of Core in the presence of other HCV proteins, especially NS proteins, was not examined. Since NS proteins coexist with Core when HCV replicates and proliferates in the infected cells, we examined the effects of the combination of Core and NS proteins (NS3, NS4A, NS4B, NS5A, and NS5B) on the *2′-5′-OAS* gene promoter in PH5CH8 cells using a dual luciferase reporter assay. As shown in Fig. 1A, we found that the combination of Core and NS5B exhibited more effective enhancement (approximately 20-fold) than the core protein alone (approximately 7-fold), whereas NS3, NS4A, NS4B, and NS5A had no effects when used in combination with Core. Since this finding suggested that NS5B per se might be able to activate the *2′-5′-OAS* gene promoter, we next examined the effect of NS5B alone on the *2′-5′-OAS* gene promoter. The results revealed that NS5B, but not NS3, NS4A, NS4B, or NS5A, could enhance luciferase activity as well as Core—i.e., by approximately 7-fold (Fig. 1B). The effect of NS5B was not further enhanced by the combination with other NS proteins (data not shown). In addition, we confirmed the transient expression of Core and NS proteins from the expression vectors used in these experiments (Fig. 1C). In summary, these results indicated that the combination of Core and NS5B synergistically (approximately 1.5-fold) enhanced the *2′-5′-OAS* gene promoter activity in PH5CH8 cells.

Deletion analysis of Core and NS5B to identify the critical region for activation of the 2′-5′-OAS gene promoter

Since we previously showed that the N-terminal 20 aa region of Core was important for activation of the *2′-5′-OAS* gene promoter [16], we here speculated that NS5B may also possess an aa sequence similar to the N-terminal 20 aa region of Core. In confirmation of this hypoth-

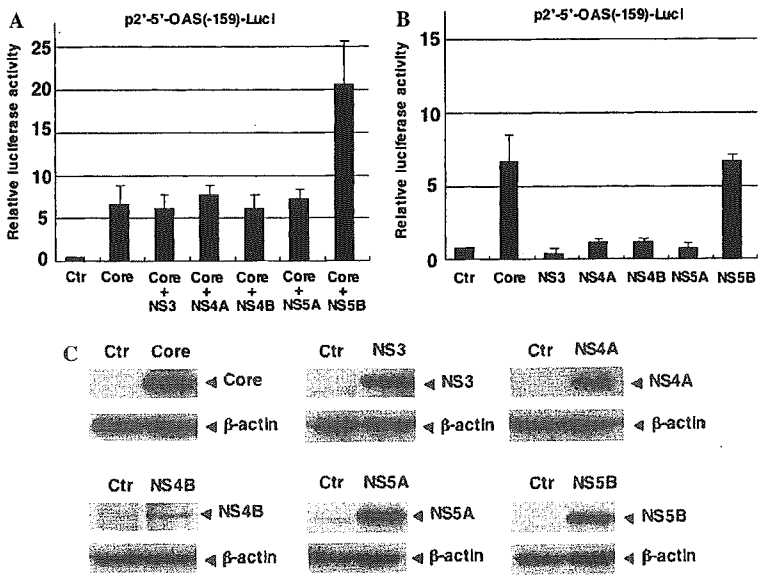

Fig. 1. NS5B synergistically enhanced Core's activation of the human *2′-5′-OAS* gene promoter in PH5CH8 cells. (A) NS5B enhanced Core's activation of the *2′-5′-OAS* gene promoter. The dual luciferase reporter assay was performed as described in Materials and methods. The relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). The lysate of cells transfected with expression vector pCXbsr was used as a control (Ctr). One microgram of HCV protein expression effector plasmid was used. (B) NS5B per se activated the *2′-5′-OAS* gene promoter. The dual luciferase reporter assay was performed as described in (A). (C) Western blot analysis of HCV proteins. The production of Core, NS3, NS4A, NS4B, NS5A, and NS5B in PH5CH8 cells transfected with HCV protein expression plasmids was analyzed by immunoblotting using anti-Core, anti-NS3, anti-NS4A, anti-NS4B, anti-NS5A, and anti-NS5B antibodies, respectively. PH5CH8 cells transfected with pCXbsr plasmid were used as a control (Ctr). β-Actin was used as a control for the amount of protein loaded per lane.

esis, our analysis revealed a KxxRKxxR motif in both Core (KPQRKTKR at aa 6–13) and NS5B (KGGRKPAR at 151–158) (Figs. 2A and B). In NS5B, this motif is located in the priming and interrogation sites, which are essential for the RdRp activity of NS5B [29]. Therefore, we examined whether or not this motif is critical for the activation of the *2′-5′-OAS* gene promoter by using the Core and NS5B mutants lacking this motif. Several N-truncated forms of Core were also used in order to narrow down the critical region for the promoter activation (Fig. 2A). In addition, one NS5B mutant (G317V in the GDD motif, aa 317–319, located in the catalytic site) and three C-truncated forms (ΔC21, ΔC56, and ΔC97, lacking 21, 56, and 97 aa, respectively) of NS5B were used in order to clarify whether or not RdRp activity and endoplasmic reticulum (ER) membrane anchorage of NS5B are required for the promoter activation (Fig. 2B). It has been known that the last 21 aa are necessary and sufficient to target NS5B to the cytosolic side of the ER membrane [30]. Although ΔC21 and ΔC56, but not ΔC97, possess RdRp activity in vitro, ΔC56 shows higher RdRp activity than ΔC21 [31].

The results of the reporter assay regarding the Core mutants revealed that aa 12 and 13 were critical aa residues for the activation of the *2′-5′-OAS* gene promoter, because the activity of Core R9T-K10S-Δ (11–13) was remarkably decreased, whereas core Δ (2–11) lacking aa 2–11 still maintained the activity for the promoter activation (Fig. 2C). It is noteworthy that aa 12 and 13 are located within the KxxRKxxR motif (aa 12 and 13 are underlined). In addition, the results revealed that aa 17–21 was also involved in the promoter activation, because the enhancing activity of core Δ (2–16) (approximately 5-fold) was completely abolished in core Δ (2–21) (Fig. 2C).

Regarding the NS5B mutant forms, the results revealed that the enhancing activities of NS5B R154T-K155S-ΔC (156–158) lacking a KxxRKxxR motif, NS5BΔC97, and NS5B G317V were almost impaired (Fig. 2D), and that NS5BΔC56 and NS5BΔC21 still possessed weak enhancing activities (approximately 6- and 3-fold, respectively). These results suggest that NS5B's activation of the *2′-5′-OAS* gene promoter is dependent on the RdRp activity of NS5B.

Characterization of the synergistic effect of Core and NS5B on the 2′-5′-OAS gene promoter

To clarify the mechanism underlying the synergistic effect of Core and NS5B, we further examined the effects of the combinations of Core and NS5B mutants lacking the enhancing activity, or NS5B and Core mutants lacking the enhancing activity. The results showed that the Core's activation of the *2′-5′-OAS* gene promoter was no longer enhanced in the combination with NS5BΔC97 or NS5B R154T-K155S-Δ (156–158) (Fig. 3). Interestingly, however, NS5B G317V lacking the enhancing activity could partially enhance the Core's activation of the *2′-5′-OAS* gene promoter (Fig. 3). Similarly, core Δ (2–21) lacking the enhancing activity also could partially enhance the activation by NS5B, whereas the combination of core Δ (2–21) and NS5B mutants such as NS5BΔC97 exhibited no effect on the promoter activity (Fig. 3). In addition, co-expression of NS5B and NS5B mutants lacking the enhancing activity also had no effect on the promoter activity (Fig. 3), suggesting that these NS5B mutants are not a competitive or dominant-negative inhibitor for the *2′-5′-OAS* gene promoter activity. These results also suggest that direct or indirect interaction between Core and NS5B is involved in the

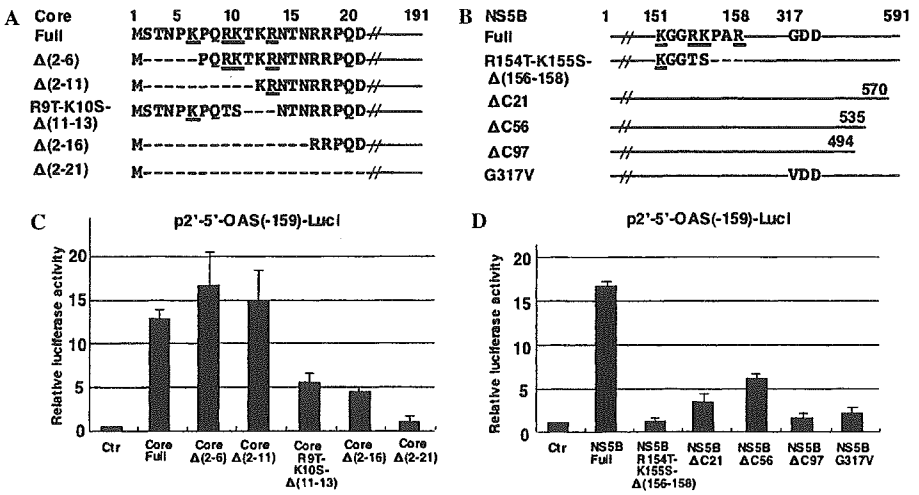

Fig. 2. Deletion analysis of the Core and NS5B. (A) Schematic presentation of the Core mutants used. The aa sequences of aa 1–21 in the Core (1b-P) [15] are indicated. The bars indicate the deleted aa residues, and K and R in the KxxRKxxR motif are underlined. (B) Schematic presentation of the NS5B mutants used. Only the aa sequences in the mutated regions of NS5B are indicated. The bars indicate the deleted aa residues, and K and R in the KxxRKxxR motif are underlined. (C) Effects of the Core mutants on the *2′-5′-OAS* gene promoter activity in PH5CH8 cells. The dual luciferase reporter assay was performed as described in Fig. 1A. Two micrograms of the HCV protein expression effector plasmid was used. (D) Effects of the NS5B mutants on the *2′-5′-OAS* gene promoter activity in PH5CH8 cells. The dual luciferase reporter assay was performed as described in (C).

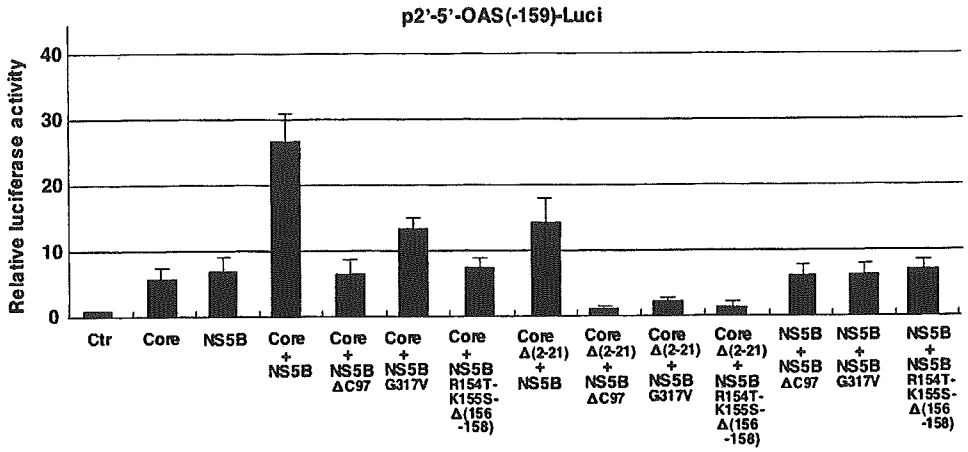

Fig. 3. Characterization of the synergistic effect of Core and NS5B on the *2'-5'-OAS* gene promoter. The dual luciferase reporter assay was performed as described in Fig. 1A. One microgram of each HCV protein expression effector plasmid was used.

synergistic effect of both proteins. Although Uchida et al. [32] reported the formation of a complex between Core and NS5B in mammalian cells, we failed to obtain evidence that Core and NS5B could form a complex in PH5CH8 cells using an immunoprecipitation method (data not shown).

The promoter activations by Core and NS5B are differentially suppressed by NS3-4A

Although we showed that Core and NS5B synergistically enhanced the *2'-5'-OAS* gene promoter in PH5CH8 cells (Fig. 1), it has recently been reported that the NS3-4A serine protease prevented virus-induced activation of IRF-3, which is a critical factor for the induction of IFN-β, using human hepatoma cell lines including HuH-7 [18]. According to this information, we examined the effect of NS3-4A on the activation of the *2'-5'-OAS* gene promoter by Core or NS5B in PH5CH8 cells. We first prepared the NS3-4A expression vector using NS3-4A derived from the 50-1 HCV replicon [33], which could efficiently replicate in HuH-7 cells, suggesting that NS3-4A possessed a powerful serine protease activity. In order to evaluate the effect of NS3-4A, the dose of expression vector for NS3-4A was changed from 0.1 to 1 μg under the condition of a fixed dose (1 μg) of expression vector for Core or NS5B. NS3-4A and Core or NS5B were transiently co-expressed in PH5CH8 cells, and a luciferase reporter assay was performed. The results revealed that the NS5B's activation of the *2'-5'-OAS* gene promoter was drastically suppressed even when 0.1 μg NS3-4A expression vector was used (Fig. 4A); however, the activation by Core was only partially suppressed even when 1 μg NS3-4A expression vector was used (Fig. 4B). These results indicated that NS3-4A had differential suppressive effects toward the activations by Core and NS5B, although the suppressive effect of NS3-4A was consistent with the results reported by Foy et al. [18]. This finding also suggests that the mechanism underlying the activation by Core is different from that of the activation by NS5B. Additional similar results were obtained by a luciferase reporter assay using a synthetic promoter possessing five repeats of the consensus ISRE (Figs. 4C and D). It was noteworthy that the enhancement of luciferase activity by NS5B (1 μg) was impaired when 0.1 μg NS3-4A expression vector was used for the assay (Fig. 4C). Furthermore, we observed that this suppressive effect of NS3-4A toward the promoter activation by Core or NS5B was clearly impaired when a NS3-4A/S1165A mutant lacking serine protease activity [34] was co-expressed with NS5B or Core (Figs. 4C and D), suggesting that the suppressive effect of NS3-4A is dependent on its serine protease activity. Moreover, we confirmed that NS3 alone (Figs. 5A and B) or NS4A alone (Figs. 5C and D) was not able to suppress the promoter activation by Core or NS5B (each 1 μg as effector plasmid), even when 1 μg NS3 or NS4A expression vector was used for the assay. In addition, we confirmed that co-transfection of the NS3 and NS4A expression vectors also showed a similar suppressive effect toward the activation by NS5B (Fig. 5E), indicating that the NS3/4A complex in *trans* [34] is also able to suppress the activation by NS5B. These results suggest that the full protease activity occurring by complex formation between NS3 and NS4A is required for the suppressive effect toward the activation by Core or NS5B. In addition, we observed that NS3/4A complexes in *cis* and in *trans* were no longer able to suppress the signaling occurring after IFN-β treatment (Fig. 5F), suggesting that the target site(s) of NS3-4A is some upstream molecule(s) involved in IFN-β production.

IFN-β is induced by NS5B, but not by Core

As described above, we suggested that NS5B's activation of the *2'-5'-OAS* gene promoter was dependent on the RdRp activity of NS5B. Although the *2'-5'-OAS* gene

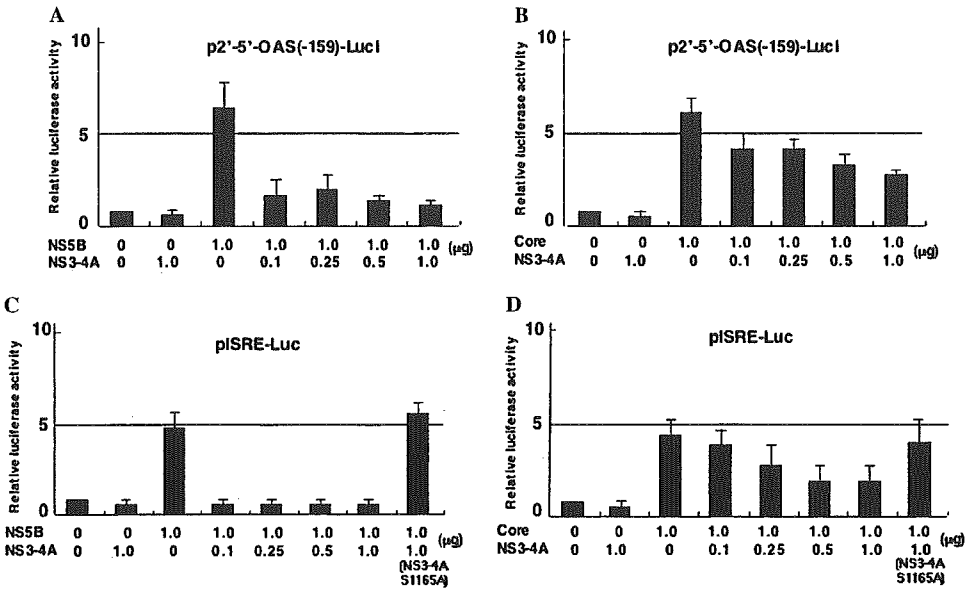

Fig. 4. NS3-4A differentially suppressed the promoter activations by Core and NS5B. The dual luciferase reporter assay was performed as described in Fig. 1A. (A) Effect of NS3-4A on NS5B's activation of the *2′-5′-OAS* gene promoter. (B) Effect of NS3-4A on Core's activation of the *2′-5′-OAS* gene promoter. (C) Effect of NS3-4A on NS5B's activation of the synthetic ISRE promoter. The expression vector of the NS3-4A/S1165A mutant lacking serine protease activity was also used. (D) Effect of NS3-4A on Core's activation of the synthetic ISRE promoter. The expression vector of the NS3-4A/S1165A mutant lacking serine protease activity was also used.

promoter possesses the ISRE sequence of a variant type (GGTTTCGTTTCCTC), this suggestion is consistent with our recent finding (Naka et al., submitted) that NS5B full form activates the *IFN-β* gene promoter possessing the IRF3 target sequence, which is the same as the consensus ISRE sequence (AGTTTCACTTTCCC). Furthermore, since NS5B could induce the expression of IFN-β through the TLR3 signaling pathway in PH5CH8 cells (Naka et al., submitted), we speculated that the activation of the *2′-5′-OAS* gene promoter was caused by IFN-β induced not only by NS5B but also Core.

To clarify whether or not Core is able to induce the expression of IFN-β, RT-PCR analysis of IFN-β was performed using PH5CH8 cells stably expressing Core, NS5B, or NS5A (as a control). The expression levels of IRF1, IRF3, IRF7, type I IFN receptors (IFNAR1 and IFNAR2c), STAT1, and STAT2, all of which are involved in IFN system, were also examined. The results revealed that Core did not induce IFN-β, whereas NS5B induced IFN-β and the downstream effector gene IRF7 (Fig. 6). Neither Core nor NS5A had any effect on the expression levels of the components examined (Fig. 6). We previously showed that Core did not enhance the expression levels or phosphorylation status of the components (STAT1, STAT2, Jak1, and Tyk2) of the JAK–STAT signaling pathway [16]. This previous finding, taken together with the present results, suggests that the mechanism of activation of the *2′-5′-OAS* gene by Core differs from the mechanism of NS5B's induction of IFN-β.

The synergistic effect of Core and NS5B toward IFN-β gene activation is not invalidated by NS3-4A

Since the synergistic effect of Core and NS5B on the *2′-5′-OAS* gene promoter was found in PH5CH8 cells and the activation of the *2′-5′-OAS* gene promoter by NS5B could be explained by NS5B's induction of IFN-β, we next examined whether or not such a synergistic effect on the *IFN-β* gene promoter is observed in PH5CH8 cells. The effect of NS3-4A was also examined in this experiment. The results revealed that the activity of the *IFN-β* gene promoter was also synergistically (approximately 1.6-fold) enhanced by Core and NS5B, and that NS3-4A drastically suppressed the enhancement by NS5B and partially suppressed the enhancement by Core (Fig. 7A), as observed when the *2′-5′-OAS* gene promoter was used (Figs. 1 and 5). In addition, when 0.01, 0.025, and 0.1 μg NS3-4A expression vector were used, the synergistic effects (approximately 3.5-, 3-, and 1.8-fold, respectively) of Core and NS5B were not invalidated (Fig. 7A), although the suppressive effect by NS3-4A was observed. In the assay in which the *IFN-β* gene promoter was also used, the suppressive effect of NS3-4A was clearly impaired when the NS3-4A/S1165A mutant lacking the serine protease activity was expressed, suggesting that the suppressive effect of NS3-4A is dependent on its serine protease activity (Fig. 7A). The expression of Core, NS3, NS3-4A/S1165A, or NS5B in the cells examined was confirmed by Western blot analysis (data not shown).

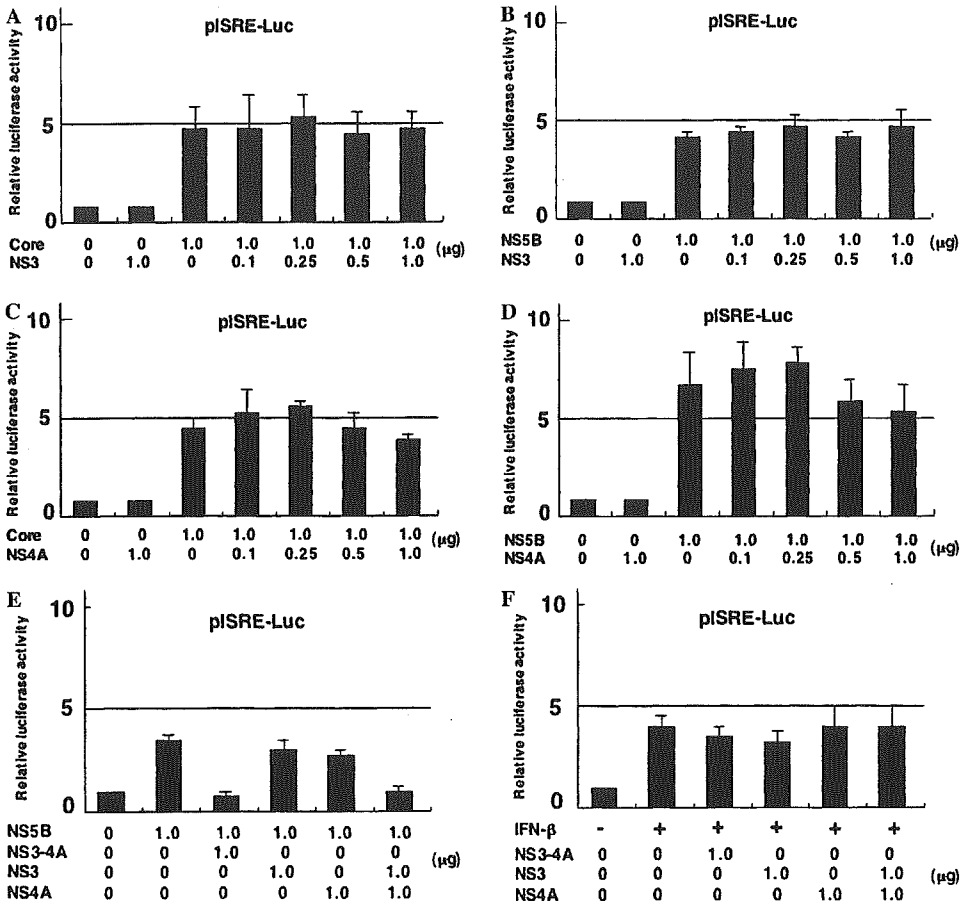

Fig. 5. The synthetic ISRE promoter activation by Core or NS5B is not suppressed by only NS3 or NS4A. Dual luciferase reporter assay was performed as described in Fig. 1A. (A) Effect of NS3 on Core's activation of the synthetic ISRE promoter. (B) Effect of NS3 on NS5B's activation of the synthetic ISRE promoter. (C) Effect of NS4A on Core's activation of the synthetic ISRE promoter. (D) Effect of NS4A on NS5B's activation of the synthetic ISRE promoter. (E) The NS3/4A complex in *trans* as well as in *cis* can suppress NS5B's activation of the synthetic ISRE promoter. (F) The NS3/4A complex in *trans* and in *cis* is not able to suppress the signaling after IFN-β treatment. PH5CH8 cells were treated with IFN-β (500 IU/ml) for 6 h before the reporter assay.

Next, we examined the effect of NS3-4A toward the expression level of IFN-β mRNA in PH5CH8 cells stably expressing Core and/or NS5B. In order to obtain the actual ratios of IFN-β mRNA expression, real-time Light-Cycler PCR was performed. The results revealed that the expression level of IFN-β mRNA in the cells co-expressing Core and NS5B became approximately 8-fold higher than that in the cells expressing NS5B alone (Fig. 7B). Furthermore, we observed that the elevation of IFN-β mRNA in the cells co-expressing Core and NS5B was partially suppressed by NS3-4A expression, although NS3-4A expression in the cells expressing NS5B alone led to complete impairment of the expression of IFN-β mRNA (Fig. 7B). These results are consistent with the results of the reporter assay using the *IFN-β* gene promoter, as described above. The expression of Core, NS3, or NS5B in the cell lines examined was also confirmed by Western blot analysis (Fig. 7C).

Discussion

In the present study, we found that NS5B as well as Core activated the *2′-5′-OAS* gene promoter in PH5CH8 cells, that the activity of NS5B was synergistically enhanced in combination with Core, and that this gene activation was dependent on the RdRp activity of NS5B and on aa 12 and 13 of Core. We obtained some data, suggesting that an indirect interaction between Core and NS5B was involved in the synergistic effect for activation of the *2′-5′-OAS* gene promoter. The activation of the *2′-5′-OAS* gene promoter by NS5B could be explained by our recent finding that NS5B induces IFN-β (Naka et al., submitted). On the other hand, we observed that NS3-4A extensively suppressed NS5B's activation of the *2′-5′-OAS* and *IFN-β* gene promoters in a manner that was dependent on its own protease activity. However, the activation of these gene promoters by Core was only partially

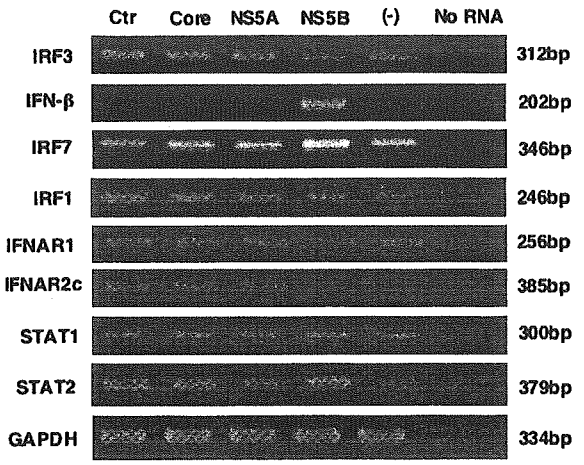

Fig. 6. RT-PCR analysis of IFN-β, interferon regulatory factors (IRF1, IRF3, and IRF7), type I IFN receptors (IFNAR1 and IFNAR2c), transcriptional factors (STAT1 and STAT2), and GAPDH in PH5CH8 cells infected with pCXbsr retrovirus encoding Core, NS5A, or NS5B. The pCXbsr retrovirus was used as a control infection (Ctr). At 48 h postinfection, total RNA was extracted and subjected to RT-PCR analysis using the primer set for IRF3 (312 bp), IFN-β (202 bp), IRF7 (346 bp), IRF1 (246 bp), IFNAR1 (256 bp), IFNAR2c (385 bp), STAT1 (300 bp), STAT2 (379 bp), or GAPDH (334 bp). The PCR products were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. Lane (−), PH5CH8 cells without retrovirus infection.

suppressed by NS3-4A, and the synergistic effect of Core and NS5B was not invalidated by NS3-4A.

Since Core and NS5B equivalently activate the *2′-5′-OAS* and *IFN-β* gene promoters and a synthetic ISRE promoter, it is of interest that IFN-β was induced by NS5B, but not by Core. Furthermore, the synergistic effect of Core on the activation of these gene promoters by NS5B is also interesting. Recently, we found that NS5B, but not Core, delayed the cell cycle progression through the S phase in PH5CH8 cells, and we considered that this phenomenon may have been caused by IFN-β induced by NS5B through the activation of TLR3 (Naka et al., submitted). In the present study, we observed that Core enhanced NS5B's induction of IFN-β mRNA and that Core might indirectly interact with NS5B. Therefore, our findings suggest not only that Core's activation of gene promoters through ISRE is synergistically enhanced by NS5B, but also that Core positively modifies the activation of the TLR3 signaling pathway by NS5B, although Core does not activate this pathway directly.

In the course of studies on the mechanism of Core's activation of gene promoters containing ISRE, we previously showed that Core had no effects on the gene expression levels or phosphorylation status of the major components involved in the JAK–STAT signaling pathway, such as STAT1 and STAT2 [16]. Recently, Imanaka et al. [35] reported that IFN-α-induced STAT-1 phosphorylation and the expression of antiviral genes were inhibited in the suppressor of cytokine signaling (SOCS)-1-expressing cells. Since SOCS-1 is referred to as the STAT-induced STAT inhibitor or the JAK-binding protein, we speculated that Core may be able to suppress the SOCS-1 expression in PH5CH8 cells, resulting in activation of the JAK–STAT signaling pathway. However, RT-PCR analysis revealed that the level of SOCS-1 mRNA was not changed regardless of the expression of Core in PH5CH8 cells. The other possibility is that Core may modify the affinity of IFN-stimulated gene factor 3 (ISGF3) to the ISRE sequence, since it has recently been reported that Core expression is associated with increased ISGF3-binding activity to the ISRE sequence [36]. Further analysis will be necessary to clarify whether or not the same phenomenon is observed in PH5CH8 cells.

Our observations that NS3-4A drastically suppresses NS5B's activation of the IFN system, especially induction of IFN-β, are consistent with the recent findings [18] that NS3-4A effectively blocks the phosphorylation of IRF3 (a key molecule in innate immunity) that normally occurs in response to virus infection. Recent studies [19,37,38] have indicated that NS3-4A inhibits both the TLR3 signaling pathway (TRIF-dependent pathway) and RIG-I signaling pathway (TRIF-independent pathway). The present study showed that NS3-4A in a serine protease activity-dependent manner inhibited the TLR3 signaling pathway in PH5CH8 cells, based on our recent finding that the induction of IFN-β by NS5B was mediated through the TLR3 but not the RIG-I signaling pathway (Naka et al., submitted). It is likely that TRIF, which was identified recently [19] as a target molecule of cellular components upstream of IRF3, is cleaved by NS3-4A in PH5CH8 cells. However, the induction of IFN-β in PH5CH8 cells co-expressing Core and NS5B was only partially suppressed by NS3-4A, whereas the induction of IFN-β by NS5B only was completely suppressed by NS3-4A. As one of the biological implications of this phenomenon, we speculate that HCV proteins contribute to the maintenance of a low steady state of virus by controlling the expression level of IFN-β in the infected cells, thereby enabling HCV to escape from the host immuno-surveillance system, and facilitating persistent viral infection.

To evaluate this hypothesis, it is important to clarify whether or not the activation of the IFN system by Core and/or NS5B or suppression of the IFN system by NS3-4A occur during the HCV life cycle. Although four kinds of genome-length HCV RNA-replicating cells [39–42] and a reproducible HCV proliferation system in cell culture [43] have been established to date using HuH-7 cells, these HuH-7-based cells would not be suitable to prove our hypothesis, because the TLR3 and/or RIG-I signaling pathway does not function in these cells [38,44]. Therefore, a new HCV RNA-replicating or HCV proliferation cell system needs to be developed using other hepatocyte cell lines possessing intact TLR3 and RIG-I signaling pathways, such as PH5CH8 cells [20]. We are currently conducting a trial to establish genome-length HCV RNA-replicating PH5CH8 cells.

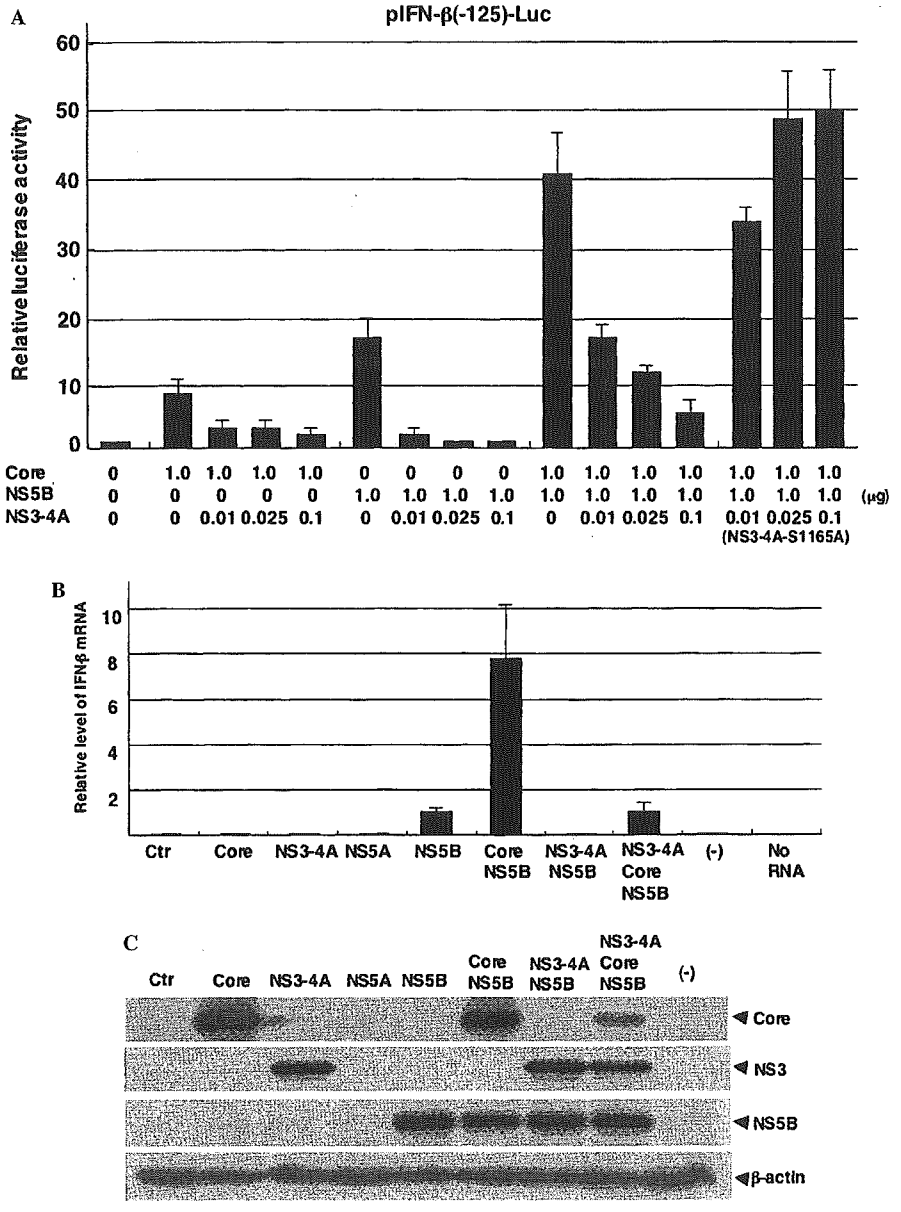

Fig. 7. NS3-4A did not suppress the synergistic effect of Core and NS5B on IFN-β gene expression. (A) Effect of NS3-4A on the activation of the *IFN-β* gene promoter by Core, by NS5B, and by the synergistic effect of Core and NS5B. The dual luciferase reporter assay was performed as described in Fig. 1A. The expression vector of the NS3-4A/S1165A mutant lacking serine protease activity was also used. (B) Real-time LightCycler PCR analysis of IFN-β in PH5CH8 cells stably expressing HCV protein(s). PH5CH8 cells were infected with retrovirus pCXbsr encoding various HCV proteins, and PH5CH8 cells stably expressing HCV protein(s) were obtained as described in Materials and methods. The pCXbsr retrovirus was used as a control infection (Ctr). Total RNA was extracted from the cells and was subjected to real-time LightCycler PCR analysis using the primer set for IFN-β (202 bp). The experiments were performed in at least triplicate. To correct the differences in RNA quality and quantity between the samples, data were normalized using the ratio of IFN-β mRNA concentration to that of GAPDH. The relative level of IFN-β mRNA calculated, when the level of IFN-β mRNA of PH5CH8 cells expressing NS5B alone was assigned to be 1.0, is presented here. (C) Western blot analysis of HCV proteins. Production of Core, NS3, and NS5B in PH5CH8 cells indicated in (B) was analyzed by immunoblotting using anti-Core, anti-NS3, and anti-NS5B antibodies, respectively. The PH5CH8 cells transfected with pCXbsr plasmid were used as control (Ctr). β-Actin was used as a control for the amount of protein loaded per lane.

Acknowledgments

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