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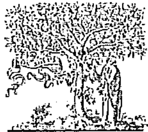

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Hepatitis C virus proteins exhibit conflicting effects on the interferon system in human hepatocyte cells

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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.
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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. AB080299) [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′-CCCTGAGGAGATTAAGCAGCTGC-3′ and 5′-AGTTCCTTAGGATTTCCACTCTGAC-3′. The sequences of the sense and antisense primers for IRF-1 were 5′-GCCCTGACTCCAGCACTGTCG-3′ and 5′-ATTGAGTAGGTACCCCTTCCC-3′. The sequences of the sense and antisense primers for IRF-3 were 5′-ACACATACTGGGCAGTGAGC-3′ and 5′-GCAGGTCCACAGTATTCTCC-3′. The sequences of the sense and

antisense primers for IRF-7 were 5′-GTACGGGTGGGCAGTAGA GAC-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-

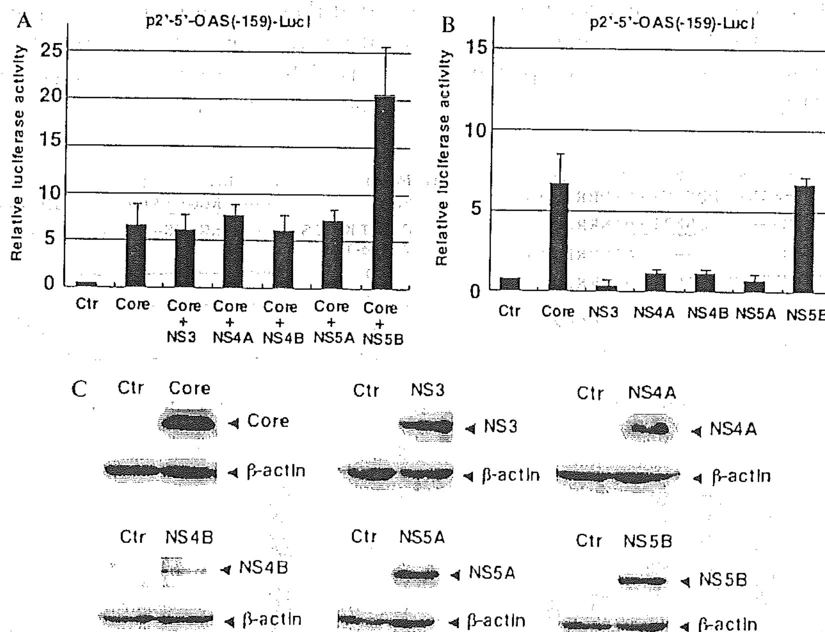

Fig. 1. NS5B synergistically enhanced Core's activation of the human *2′-5′-OAS* gene promoter in PH5CH8 cells. (A) NS5B enhanced Core's activation of the *2′-5′-OAS* gene promoter. The dual luciferase reporter assay was performed as described in Materials and methods. The relative luciferase activity 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

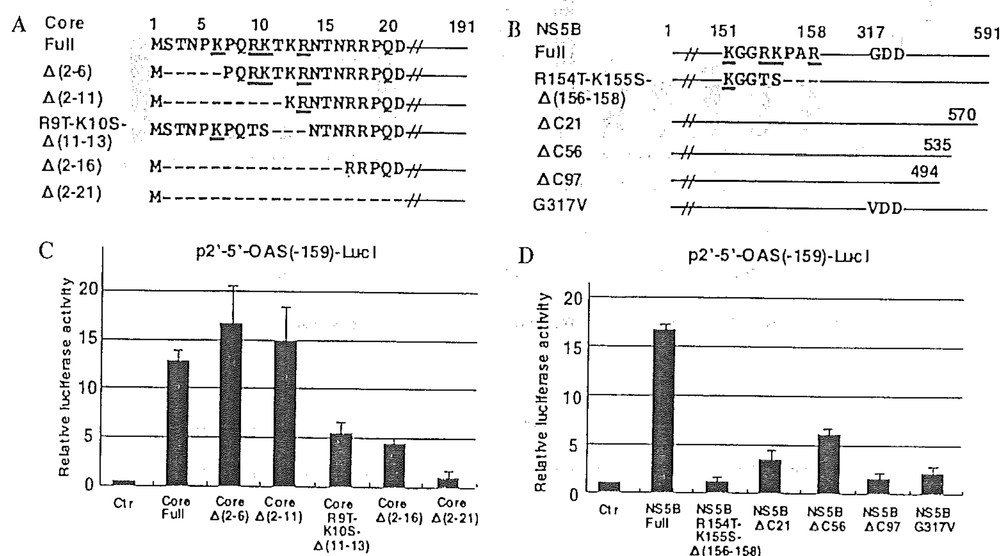

Fig. 2. Deletion analysis of the Core and NS5B. (A) Schematic presentation of the Core mutants used. The aa sequences of aa 1–21 in the Core (1b-P) [15] are indicated. The bars indicate the deleted aa residues, and K and R in the KxxRKxxR motif are underlined. (B) Schematic presentation of the NS5B mutants 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).

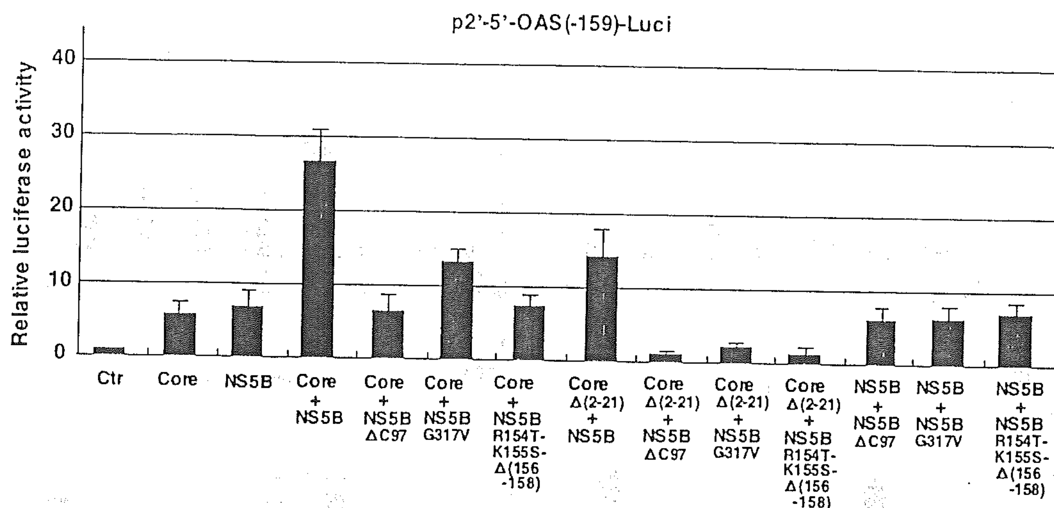

Fig. 3. Characterization of the synergistic effect of Core and NS5B on the *2′-5′-OAS* gene promoter. The dual luciferase reporter assay was performed as described in Fig. 1A. One microgram of each HCV protein expression effector plasmid was used.

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

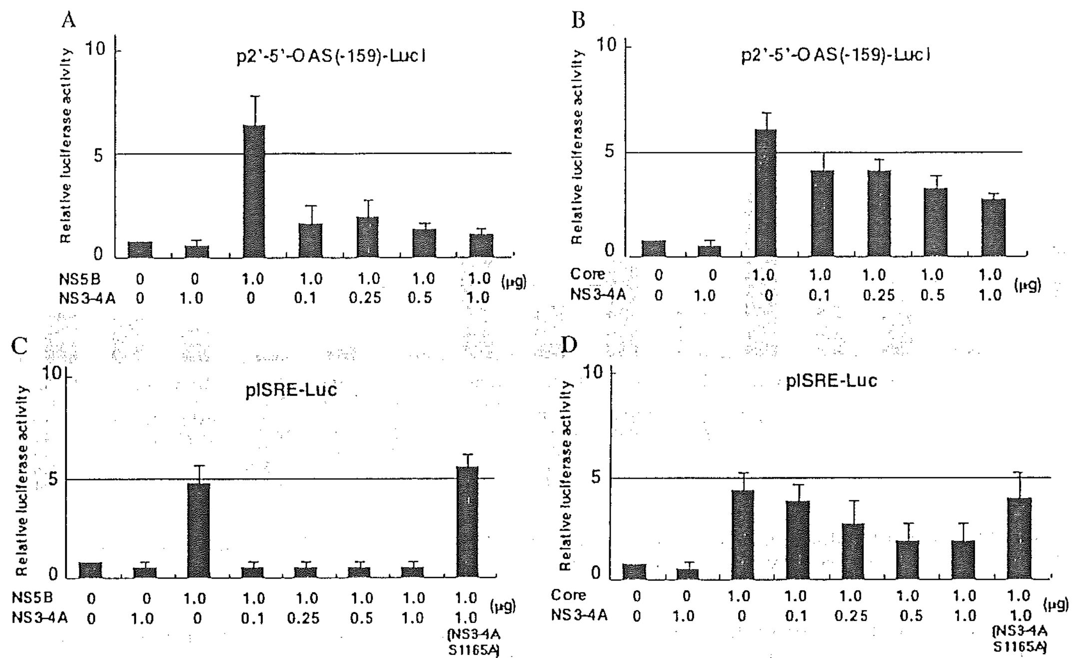

Fig. 4. NS3-4A differentially suppressed the promoter activations by Core and NS5B. The dual luciferase reporter assay was performed as described in Fig. 1A. (A) Effect of NS3-4A on NS5B's activation of the *2'-5'-OAS* gene promoter. (B) Effect of NS3-4A on Core's activation of the *2'-5'-OAS* gene promoter. (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).

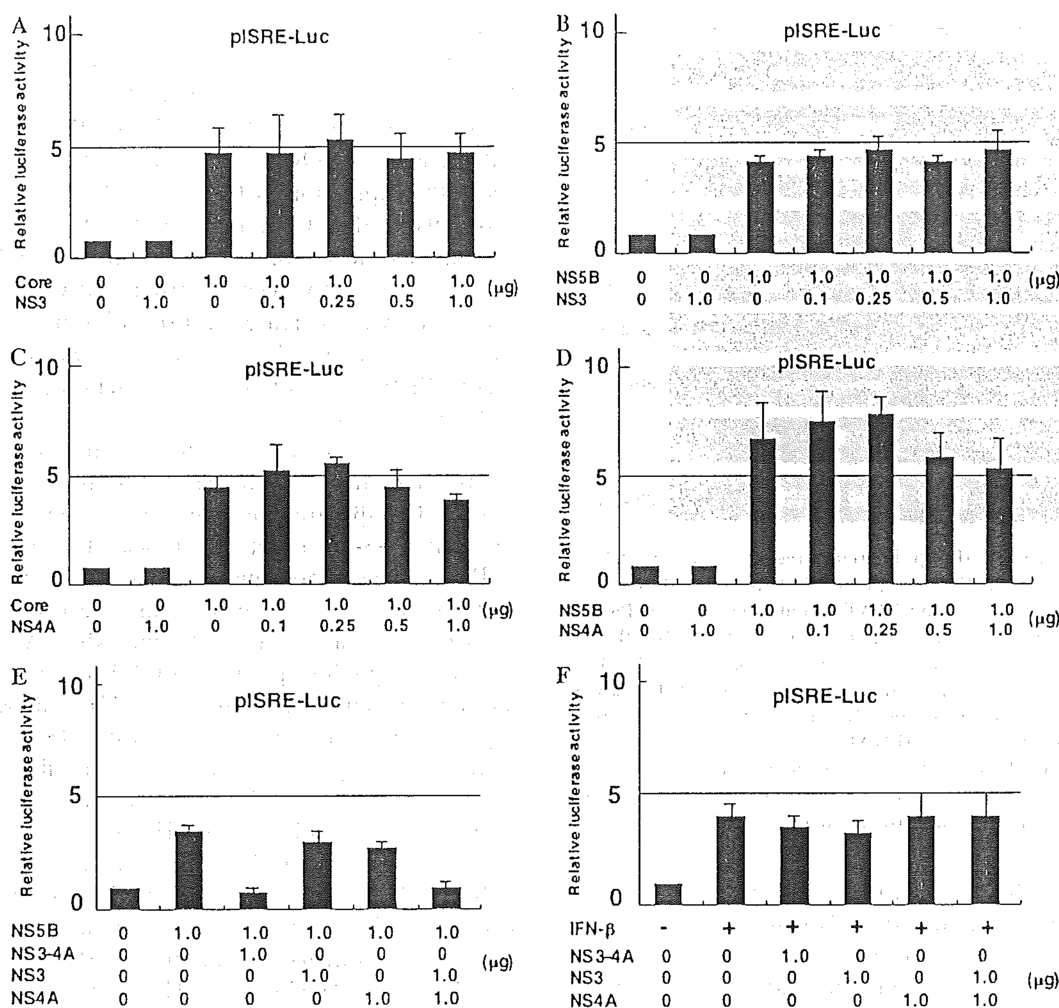

Fig. 5. The synthetic ISRE promoter activation by Core or NS5B is not suppressed by only NS3 or NS4A. Dual luciferase reporter assay was performed as described in Fig. 1A. (A) Effect of NS3 on Core's activation of the synthetic ISRE promoter. (B) Effect of NS3 on NS5B's activation of the synthetic ISRE 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 LightCycler 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

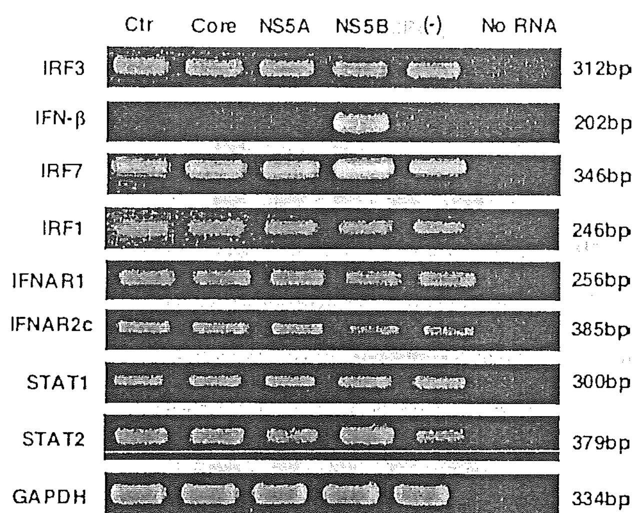

Fig. 6. RT-PCR analysis of IFN-β, interferon regulatory factors (IRF1, IRF3, and IRF7), type I IFN receptors (IFNAR1 and IFNAR2c), transcriptional factors (STAT1 and STAT2), and GAPDH in PH5CH8 cells infected with pCXbsr retrovirus encoding Core, NS5A, or NS5B. The pCXbsr retrovirus was used as a control 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.

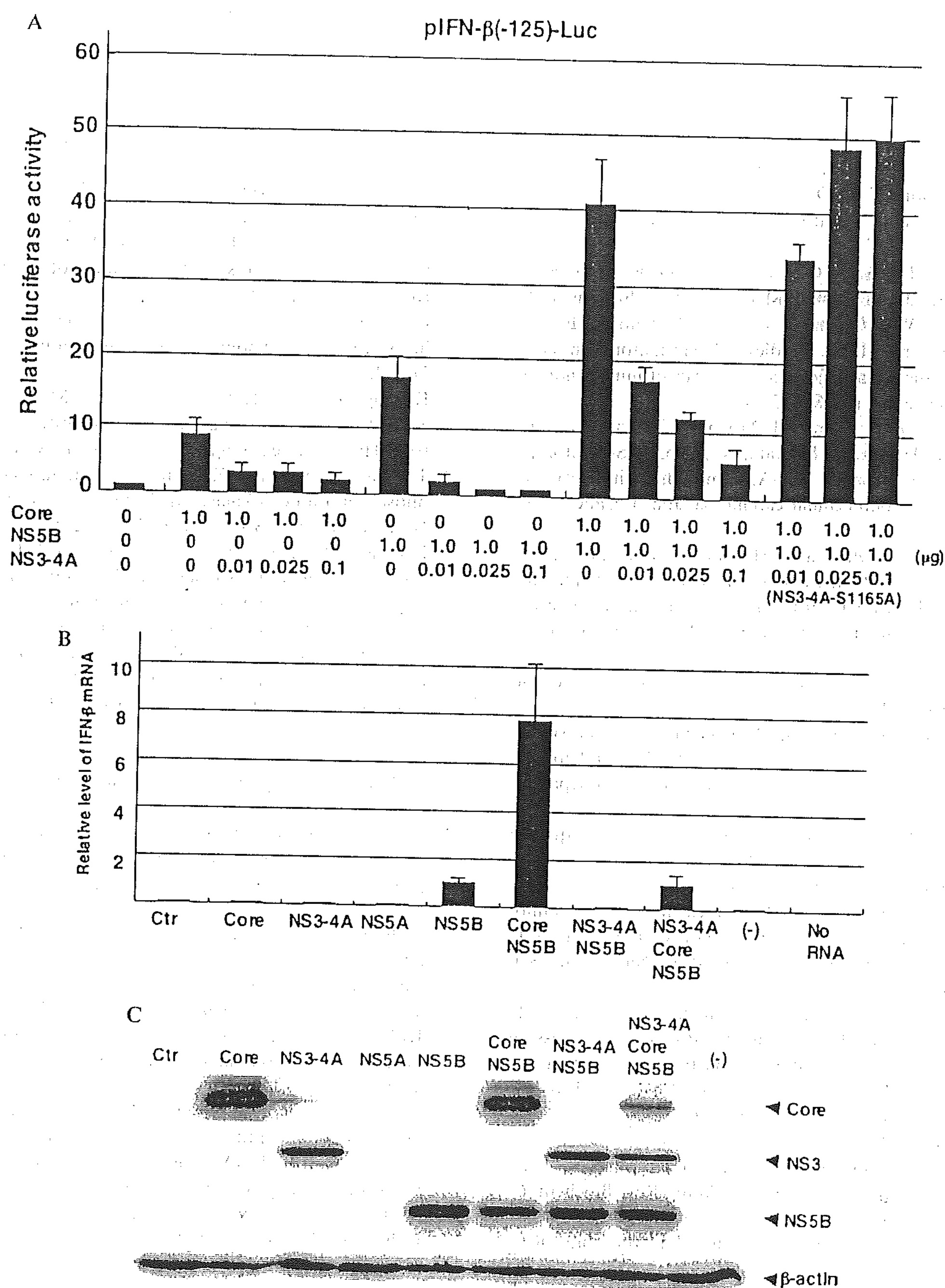

Fig. 7. NS3-4A did not suppress the synergistic effect of Core and NS5B on IFN-β gene expression. (A) Effect of NS3-4A on the activation of the *IFN-β* gene promoter by Core, by NS5B, and by the synergistic effect of Core and NS5B. The dual luciferase reporter assay was performed as described in Fig. 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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Distinct Poly(I-C) and Virus-activated Signaling Pathways Leading to Interferon-β Production in Hepatocytes*

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Innate cellular antiviral defenses are likely to influence the outcome of infections by many human viruses, including hepatitis B and C viruses, agents that frequently establish persistent infection leading to chronic hepatitis, cirrhosis, and liver cancer. However, little is known of the pathways by which hepatocytes, the cell type within which these hepatitis agents replicate, sense infection, and initiate protective responses. We show that cultured hepatoma cells, including Huh7 cells, do not activate the interferon (IFN)-β promoter in response to extracellular poly(I-C). In contrast, the addition of poly(I-C) to culture media activates the IFN-β promoter and results in robust expression of IFN-stimulated genes (ISG) in PH5CH8 cells, which are derived from non-neoplastic hepatocytes transformed with large T antigen. Small interfering RNA knockdown of TLR3 or its adaptor, Toll-interleukin-1 receptor domain-containing adaptor inducing IFN-β (TRIF), blocked extracellular poly(I-C) signaling in PH5CH8 cells, whereas poly(I-C) responsiveness could be conferred on Huh7 hepatoma cells by ectopic expression of Toll-like receptor 3 (TLR3). In contrast to poly(I-C), both cell types signal the presence of Sendai virus infection through a TLR3-independent intracellular pathway requiring expression of retinoic acid-inducible gene I (RIG-I), a putative cellular RNA helicase. Silencing of RIG-I expression impaired only the response to Sendai virus and not extracellular poly(I-C). We conclude that hepatocytes contain two distinct antiviral signaling pathways leading to expression of type I IFNs, one dependent upon TLR3 and the other dependent on RIG-I, with little cross-talk between these pathways.

The innate immune system represents the first line of defense against viral infections in mammalian cells. It senses invading viral pathogens and initiates signaling pathways leading to the induction of protective cellular genes, including type I interferons (IFN-α and IFN-β)[1] and proinflammatory cytokines that directly limit viral replication and also help to shape subsequent adaptive immune responses (1, 2). Recognition of conserved molecular structures that are expressed by large groups of pathogens (pathogen-associated molecular patterns, PAMPs) is carried out by specific pattern recognition receptor (PRR) molecules (3, 4). The Toll-like receptors (TLRs) are a class of PRRs that have been shown to detect infection by many types of pathogens, including viruses (5). TLR3 is engaged specifically by double-stranded (ds) RNA that is present either in viral genomes or generated during viral replication, and is involved in the cellular recognition of RNA viruses and induction of type I IFN responses (6). However, several recent studies indicate that viral infection can also activate host responses through TLR3-independent pathways (7–9). Mouse TLR7 and human TLR8, which are expressed within endosomal membranes, detect GU-rich viral single-stranded RNA (7, 10, 11). Whereas TLR3 signaling requires the adaptor protein, Toll-IL1 receptor (TIR) domain-containing adaptor inducing IFN-β (TRIF/TICAM-1) (12–15), TLR7/8 engagement utilizes the MyD88 adaptor protein and requires endosomal acidification for activation of IFN responses (7, 10). Finally, virally encoded proteins may engage TLR2 or TLR4, activating MyD88-dependent pathways leading to expression of inflammatory cytokines and contributing to both viral clearance as well as pathogenesis (16–18).

The TLRs are not the only class of PAMP receptors that contribute to the recognition of virus infection. Yoneyama and colleagues (19) have demonstrated recently that IFN-β production is induced in response to Newcastle disease virus infection through a pathway that is independent of TLR3 but requires the retinoic acid-inducible gene I (RIG-I, or DDX58), a cellular RNA helicase with homology to caspase-recruitment domain (CARD) proteins.

The ability of these pathways to induce the expression of type I IFNs and subsequently a wide array of IFN-stimulated genes (ISGs) is likely to influence the outcome of infection by many human viruses. Such responses may have direct antiviral effects within the infected cell (2). Type I IFNs also exert

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[1] The abbreviations used are: IFN, interferon; dsRNA, double-stranded RNA; HCV, hepatitis C virus; IRF-3, interferon regulatory factor 3; ISG, interferon-stimulated gene; MDA5, melanoma differentiation associated gene-5; MyD88, myeloid differentiation factor 88; RIG-I, retinoic acid-inducible gene I; SenV, Sendai virus; siRNA, small interfering RNA; TLR, Toll-like receptor; TRIF, Toll-IL1 receptor domain-containing adaptor inducing IFN-β; PRR, pattern recognition receptor; PAMP, pathogen-associated molecular patterns; CARD, caspase-recruitment domain; HBV, hepatitis B virus; pAb, polyclonal antibody; RT, reverse transcription; HAU, hemagglutinin units; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

critically important immunoregulatory effects, including stimulation of natural killer cell cytotoxicity, activation of $\gamma\delta$ T cells, and stimulation of immunoglobulin synthesis. Cytokines and chemokines induced by the activation of these signaling pathways also contribute to the maturation of dendritic cells and influence the priming of T_H1 and cytotoxic T cells (20). These responses thus may play critical roles in shaping subsequent adaptive T cell responses that are required for the ultimate elimination of viruses (21, 22).

Although the liver is a particularly important site of persistent viral infections in humans, very little is known about how these signaling pathways function specifically in hepatocytes. Despite this, there is strong, albeit indirect evidence, that type I IFN responses are important in the pathogenesis of chronic viral hepatitis. Both hepatitis B virus (HBV) and hepatitis C virus (HCV) cause persistent infections involving the hepatocyte, and both have evolved mechanisms to disrupt the induction of type I IFNs. Although not well understood, the core protein of HBV inhibits the transcription of IFN-β (23, 24). Similarly, the NS3/4A protease of HCV blocks Sendai virus (SenV)-induced activation of IFN regulatory factor 3 (IRF-3), a cellular transcription factor that plays a critical role in the expression of IFN-β (25). IRF-3 blockade is dependent upon the protease activity of NS3/4A, suggesting that NS3/4A proteolytically targets one or more cellular proteins residing within the signaling pathways leading to IRF-3 activation and type I IFN production (25). These and other data (26, 27) suggest that a more detailed understanding of the mechanisms by which virus infections trigger IRF-3 activation and IFN production in hepatocytes would be helpful in unraveling the pathogenesis of persistent HBV and HCV infections, and might possibly lead to the design of novel therapeutic interventions. Here, we describe efforts to better define the antiviral signaling pathways that are active in cultured hepatocyte-derived cell lines and that thus may be triggered by HBV or HCV infection *in vivo*.

MATERIALS AND METHODS

Cell Culture and Reagents—Murine macrophage RAW264.7, human hepatoma Huh7, Huh7.5 (kindly provided by C. M. Rice via Apath), HepG2, Hep3B cells, and PH5CH8, a simian virus 40 (SV40) large T antigen-immortalized non-neoplastic human hepatocyte cell line (28), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin G and streptomycin in a humidified 37 °C, 5% CO_2 incubator. Poly(I-C) was purchased from Sigma. Bafilomycin A1 was from Calbiochem. Recombinant human interleukin-1β was from Raybiotech. R-848 was kindly provided by K. A. Fitzgerald.

Plasmids—pIFN-β-luc, pCMV1FlagTLR3, pCMV1FlagTLR3DN, and pEF-Bos Flag-RIG-I were generous gifts of Rongtuan Lin, Ruslan Medizhitov, and Takashi Fujita, respectively. The ISG54 ISRE-Luc and pNFκB-Luc plasmids were from Stratagene. Sequence encoding a dominant negative form of MyD88, which lacks the amino-terminal death domain (29), was amplified from cDNA transcribed from PH5CH8 cell RNA, and cloned into pCDNA3.1 V5-His TOPO (Invitrogen). pPRDII-luc has been described previously (30). pCMV-β-galactosidase (Clontech) was used to normalize transfection efficiencies. Cells were transfected with plasmid DNAs using TransIT-LT1 (Mirus) according to the manufacturer's instructions.

Sendai Virus Infection—Cells were infected with 100 hemagglutinin units/ml of SenV (Charles River Laboratory) and harvested 16 h later for luciferase/β-galactosidase reporter assays or immunoblot analysis as previously described (25).

Poly(I-C) Treatment—Poly(I-C) was added directly to the medium at 50 μg/ml (M-pIC), or complexed with Lipofectin for transfection (T-pIC). Cells were assayed for poly(I-C) induced responses 6 h after exposure by either route.

Reporter Gene Assay—Cells (5×10^4 cells per well in 24-well plates) were transfected with reporter plasmids (100 ng), pCMV-β-galactosidase (100 ng), and the indicated amounts of an expression vector. Twenty-four hours later, cells were mock treated or treated with 20 ng/ml interleukin-1β, or challenged with poly(I-C) or Sendai virus, then subsequently lysed and assayed for luciferase and β-galactosidase activities as indicated. For comparisons, luciferase activity was normalized to β-galactosidase activity. Data are expressed as mean relative luciferase activity ± S.D. for one representative experiment carried out in triplicate, typically from a minimum of three separate experiments. The -fold induction of promoter activity was calculated by dividing the relative luciferase activity of stimulated cells with that of mock-treated cells.

TABLE I
Gene-specific primers for semiquantitative RT-PCR

Gene name		Primer sequence[a]	Product size
			bp
IFN-β	Forward	gattcatctagcactggctgg	186
	Reverse	cttcaggtaatgcagaatcc	
TLR3	Forward	tcacttgctcattctccctt	157
	Reverse	gacctctccattcctggc	
TRIF	Forward	ccagatgcaacctccactgg	339
	Reverse	ctgttccgatgatgattcc	
ISG56	Forward	tagccaacatgtcctcacagac	396
	Reverse	tcttctaccactggtttcatgc	
RIG-I	Forward	cagtatattcaggctgag	389
	Reverse	ggccagttttccttgtc	
MDA5	Forward	agtttggcagaaggaagtgtc	480
	Reverse	ggagttttcaaggatttgagc	

[a] GAPDH primers were purchased from Clontech.

Immunoblot Analysis—Cellular extracts were subjected to immunoblot analysis as described (25). Briefly, protein samples were separated by SDS-PAGE, and transferred to Hybond enhanced chemiluminescent (ECL) nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with 3% nonfat milk in phosphate-buffered saline and processed for immunodetection using the following monoclonal or polyclonal (pAb) antibodies: anti-FLAG M2 and anti-actin monoclonal antibodies (Sigma), anti-TLR3 monoclonal antibody (Imgenex), rabbit anti-MyD88 pAb (Santa Cruz), rabbit anti-IRF-3 pAb (kindly provided by Michael David), rabbit anti-ISG15 pAb (kindly provided by Arthur Haas), rabbit anti-ISG56 pAb (kindly provided by Ganes Sen), rabbit anti-MxA and anti-Sendai virus pAbs (kindly provided by Ilkka Julkunen), peroxidase-conjugated secondary donkey anti-rabbit (Jackson ImmunoResearch), and sheep anti-mouse (Amersham Biosciences) pAbs. Protein bands were visualized using ECL Plus Western blotting detection reagents (Amersham Biosciences), followed by exposure to Kodak Bio-Max film.

RNA Interference—Transfections of siRNAs targeting TLR3, TRIF, or RIG-I were carried out using Oligofectamine (Invitrogen) with a final concentration of siRNA of 80 nM according to the manufacturer's instructions. The target sequences of siRNA used in this study were: TLR3, GGTATAGCCAGCTAACTAG; TRIF (13), GACCAGACGCCACTCCAAC; RIG-I, GGAAGAGGTGCAGTATATT; and MDA5, GGTGAAGGAGCAGATTCAG. siRNAs were purchased from Dharmacon and Ambion. The scrambled negative control siRNA was from Ambion.

RT-PCR—Total cellular RNA was extracted with TRIzol Reagent (Invitrogen), treated with DNase I to remove genomic DNA contamination, and reverse-transcribed using Advantage RT-4PCR kit (Clontech). The resulting cDNA was subjected to PCR using primers (Table I) specific for IFN-β, TLR3, TRIF, ISG56, RIG-I, MDA5, β-actin, and GAPDH, respectively. The quantity of the cDNA template included in these reactions and the number of amplification cycles were optimized to ensure that reactions were stopped during the linear phase of product amplification, permitting semiquantitative comparisons of mRNA abundance between different RNA preparations. To exclude the possibility of contaminating DNA, control reactions were performed in parallel in the absence of reverse transcriptase. PCR products were visualized by agarose gel electrophoresis.

RESULTS

Cultured Hepatocyte Cell Lines Differ in Their Ability to Activate IFN-β Transcription in Response to dsRNA or Virus Infection—The induction of type I IFNs represents an early protective response to many viral infections in mammalian cells. IFN-β induction represents the immediate response of cells to viral infection, and precedes the transcription of most IFN-α species, the induction of which depends on autocrine/paracrine feedback of IFN-β and activation of IRF-7 (31). We thus focused on characterizing the IFN-β response. The molecular basis for induction of IFN-β expression has been exten-

sively studied and shown to be induced by dsRNA or other products of virus infection through coordinate activation of transcription factors IRF-3, NF-κB, and ATF-2/c-Jun (2). Most of these studies were conducted in human embryonic kidney 293 or epithelial cells, and relatively little is known about these events in hepatocytes, which constitute ~80% of the liver cell population (32), and are the primary cells within which both HBV and HCV replicate. Because primary differentiated hepatocytes are difficult to maintain in culture, we studied 3 different continuous cell lines derived from human hepatocellular carcinomas: Huh7 cells, which appear to be particularly permissive for HCV RNA replication (33, 34); HepG2 cells and Hep3B cells, the latter of which contains integrated HBV DNA and express the HBV envelope protein, HBsAg (35). Huh7 cells are not available from any standard repository, but are carried in many different laboratories and different laboratory variants may show significant variation in morphology and perhaps other characteristics. We thus studied 3 different Huh7 sublines: Huh7 SL, cells carried in this laboratory for many years; Huh7 2–3c, cells derived from Huh7 SL that were selected for their ability to support replication of genome-length HCV RNA and subsequently "cured" of the replicating RNA with IFN-α2b treatment (36), and Huh7 MG cells, Huh7 cells carried in the Gale laboratory at the University of Texas Southwestern Medical Center. We also studied the PH5CH8 cell line, which is a clonal variant of a non-neoplastic hepatocyte cell line, PH5CH, immortalized with the simian virus 40 large T antigen (28, 37, 38).

We characterized virus-induced activation of the IFN-β promoter in these various hepatocyte-derived cell lines after transient transfection with a reporter plasmid expressing luciferase under control of the IFN-β promoter. Although the PH5CH8 cell line has been reported to support replication of HCV (28), no cultured cell has been reported to be fully for HCV replication. Thus, to challenge these cells, we exposed them to the dsRNA analog, poly(I-C), or infected them with SenV. Interestingly, when poly(I-C) was added to the culture medium, there was no induction of IFN-β promoter activity in the Huh7, HepG2, or Hep3B hepatoma cells (M-pIC, Fig. 1*A*, *upper panel*). In contrast, the PH5CH8 cells demonstrated a 6-fold up-regulation of IFN-β promoter activity upon exposure to poly(I-C) (Fig. 1*A*, *upper panel*). Consistent with the reporter data, M-pIC treatment significantly up-regulated the expression of ISG15 and ISG56, which are responsive to either IRF-3 or IFN, in PH5CH8 cells, but not in hepatoma cell lines Huh7, HepG2, or Hep3B (Fig. 1*B*). To mimic intracellular dsRNA generated during viral replication, we also transfected poly(I-C) into cells using a liposome-mediated procedure. This resulted in much more potent stimulation of the IFN-β promoter in PH5CH8 cells, leading to a 17-fold increase in activity over basal promoter levels (T-pIC, Fig. 1*A*, *middle panel*). It also resulted in significant activation of the IFN-β promoter in HepG2 (8-fold induction) and Hep3B (4-fold induction) cells. However, under the same conditions, there was little if any activation of the promoter in any of the Huh7 cell sublines. These promoter assay results were confirmed by RT-PCR analysis of endogenous IFN-β mRNA synthesis in cells transfected with increasing concentrations of poly(I-C) (Fig. 1*C*). Whereas transfection of as little as 1 μg/ml poly(I-C) induced IFN-β transcription in HepG2, Hep3B, and PH5CH8 cells, there was no detectable IFN-β mRNA in Huh7 SL cells transfected with up to 100 μg/ml poly(I-C).

A similar pattern of IFN-β induction was observed following infection of these different cell lines with SenV. The greatest induction of IFN-β promoter activity occurred in PH5CH8 cells

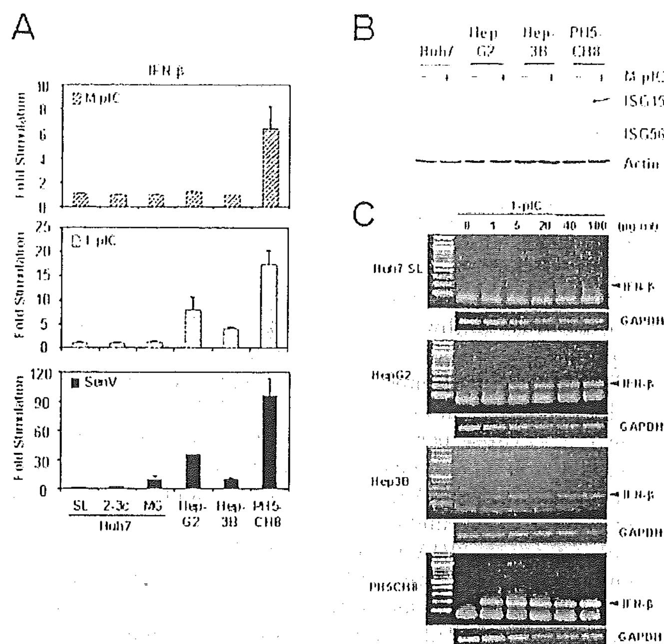

FIG. 1. Activation of IFN-β transcription in various hepatocyte cell lines by dsRNA and virus. *A*, cells grown in 24-well plates were cotransfected with IFN-β Luc and pCMV-β-galactosidase for 24 h before stimulation with 50 μg/ml poly(I-C) directly added to culture medium for 6 h (*M-pIC*, *upper panel*), 5 μg of poly(I-C) transfected with Lipofectin for 6 h (*T-pIC*, *middle panel*), or SenV 100 HAU/ml for 16 h (*lower panel*). Fold induction of IFN-β promoter was calculated by dividing the relative luciferase activity of stimulated cells with that of mock-treated cells. *B*, immunoblot analysis of ISG15 and ISG56 expression in various hepatocyte cell lines either mock-treated or treated with 50 μg/ml M-pIC for 12 h. Actin was included as a loading control. *C*, cells were mock-treated (Lipofectin) or transfected with differing concentrations of poly(I-C) for 6 h before total RNA isolation. IFN-β mRNA was detected by semiquantitative RT-PCR.

(96-fold increase over basal promoter activity), followed by HepG2 (35-fold), and Hep3B (10-fold) (Fig. 1*A*, *lower panel*). However, there were significant differences in the magnitude of the IFN-β promoter activation induced by SenV infection in the different Huh7 cell lines. SenV infection induced IFN-β promoter activity by ~10-fold in the Huh7 MG cells, but only 1.5-fold in Huh7 SL cells and 3-fold in the cured Huh7 2–3c cells (Fig. 1*A*, *lower panel*).

We conclude from these results that cultured hepatoma cells generally have impaired poly(I-C)- and virus-activated IFN responses, compared with the PH5CH8 cells that were established from non-neoplastic hepatocytes. These latter cells retain robust IFN-β responses to extracellular and intracellular poly(I-C) as well as SenV infection. They are likely to more closely resemble normal hepatocytes *in vivo*, and thus may represent a superior cell model for investigation of antiviral responses in hepatocytes. The considerable variation we observed in the IFN response in different Huh7 cell lines mandates caution in comparing studies carried out with these cells in different laboratories, an important point because Huh7 cells are widely used for cell culture studies with HCV.

TLR3 and TRIF Are Not Required for SenV Activation of the IFN-β Promoter in PH5CH8 Cells—TLR3 is the only known TLR that recognizes viral dsRNA and it is expressed both on the cell surface and within intracellular vesicles (39, 40). We were thus interested in determining whether the virus-induced IFN response involves TLR3 in PH5CH8 cells. To investigate this, we transfected the cells with a synthetic siRNA duplex targeting human TLR3, or a scrambled negative-control siRNA, then challenged the cells by adding poly(I-C) to the media or infecting them with SenV prior to measuring IFN-β

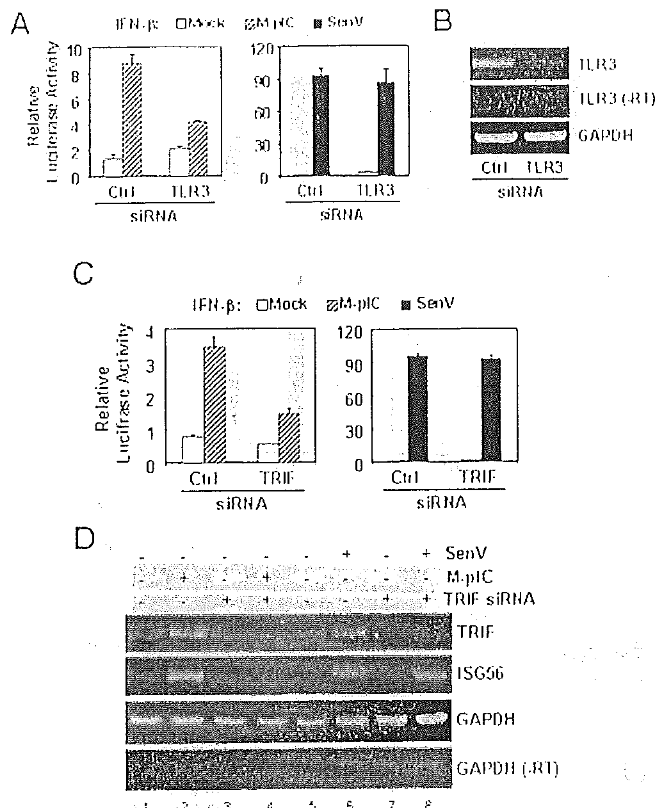

FIG. 2. **Extracellular dsRNA, but not SenV, activates IFN response via TLR3 and TRIF in PH5CH8 hepatocytes.** *A*, TLR3 expression is required for activation of IFN-β promoter by M-pIC but not by SenV. PH5CH8 cells grown in 24-well plates were transfected with control (*Ctrl*) or TLR3 siRNA, and pIFN-β Luc plus pCMV-β-galactosidase. 48 h later, cells were mock-treated or stimulated with 50 μg/ml M-pIC for 6 h or infected with SenV (100 HAU/ml) for 16 h. *B*, semiquantitative RT-PCR detection of TLR3 and GAPDH mRNAs in control/TLR3 siRNA-transfected cells. *C*, TRIF expression is required for activation of IFN-β promoter by M-pIC but not by SenV. PH5CH8 cells were transfected with Ctrl or TRIF siRNA and reporter plasmids and treated similarly as in *A*. *D*, semiquantitative RT-PCR detection of TRIF, ISG56, and GAPDH mRNAs in cells under conditions as described for *panel C*.

promoter activity (Fig. 2). PH5CH8 cells express a readily detectable abundance of TLR3 mRNA, which was reduced to almost undetectable levels upon transfection of TLR3-specific siRNA (Fig. 2*B*). The induction of IFN-β promoter activity by extracellular poly(I-C) was substantially reduced by this siRNA knockdown of TLR3 expression (Fig. 2*A*, *left panel*). In contrast, there was no appreciable effect on SenV-induced IFN-β promoter activation (Fig. 2*A*, *right panel*).

TRIF is an essential adaptor protein that links TLR3 to downstream kinases responsible for IRF-3 activation and IFN-β production (13–15). It may well play additional roles in intracellular signaling events, given its relatively large size (712 amino acids) compared with other TLR adaptors. Mice that are deficient for TRIF function have an impaired response to murine cytomegalovirus infection (12). The liver contains a higher abundance of TRIF than any other organ (14), suggesting that it may play a particularly important role in intrahepatic signaling. To determine whether TRIF is required to mount a type I IFN response against SenV in hepatocytes, we carried out a TRIF knockdown experiment in PH5CH8 cells. As shown in Fig. 2*D*, semiquantitative RT-PCR demonstrated that transfection of the TRIF-specific siRNA efficiently reduced TRIF mRNA abundance. As expected from the TLR3 knockdown (Fig. 2*A*), TRIF knockdown also significantly inhibited the induction of IFN-β promoter activity in response to extracellular poly(I-C) (Fig. 2*C*, *left panel*). It also significantly reduced extracellular poly(I-C) induction of ISG56 mRNA transcription (Fig. 2*D*, compare *lanes 2 versus 4*). In contrast, there was no effect on either IFN-β promoter activity (Fig. 2*C*, *right panel*) or ISG56 mRNA transcription (Fig. 2*D*, compare *lanes 6 versus 8*) triggered by SenV infection. Similar results were obtained in an ISG54 ISRE promoter assay (data not shown). We conclude from these data that exposure to extracellular poly(I-C) triggers activation of the IFN-β promoter in PH5CH8 cells through a TLR3-TRIF dependent pathway, whereas SenV induction of IFN-β transcription is TLR3- and TRIF-independent.

SenV Induction of IFN-β in Hepatocytes Does Not Utilize MyD88 nor Require Endosomal Acidification—In addition to TLR3, recent data indicate that TLR7, TLR8, and TLR9 may sense viral components and lead to type I IFN production. Murine TLR7 and human TLR8 recognize viral ssRNA, whereas TLR9 senses unmethylated CpG DNA present in the murine cytomegalovirus genome (41). A common feature of these three TLRs is that they all localize within endosomes and signal through MyD88 and IRF-7 (42, 43). In addition, the responses induced through these TLRs require intact endocytic pathways and are thus sensitive to endosomal acidification inhibitors, such as chloroquine and bafilomycin A1 (7, 10). Because SenV induction of IFN-β occurs via a TLR3-TRIF independent pathway in PH5CH8 cells, we considered the possibility that TLR7/8 may initiate this response. To determine whether MyD88 is required for SenV activation in the PH5CH8 cells, as would be the case were it mediated by TLR7, TLR8, or TLR9, we measured promoter activation in PH5CH8 cells transfected with a vector expressing a dominant-negative MyD88 mutant that lacks the amino-terminal death domain that is required for interaction with IRF-7 (42) (MyD88DN). Expression of MyD88DN significantly inhibited the activation of the NF-κB-dependent PRDII promoter (44) by interleukin-1β, which is known to signal through MyD88 (29). However, we found that the activity of both PRDII and IFN-β promoters was induced to similar levels by SenV with or without MyD88DN co-expression (Fig. 3*A*). Consistent with these reporter data, SenV-induced hyperphosphorylation of IRF-3 was not affected by overexpression of MyD88DN (Fig. 3*B*). This was also true for SenV-induced expression of ISG56, which is responsive to either IRF-3 or IFN (45), and MxA, which responds only to IFN (46).

To determine whether SenV activation of the IFN-β promoter requires endosomal acidification, PH5CH8 cells were pretreated with the endosomal inhibitor bafilomycin A1 prior to infection with SenV. Although bafilomycin A1 treatment completely ablated NF-κB activation in RAW264.7 cells induced by a TLR7/8 ligand, R-848 (Fig. 3*C*), SenV-stimulated IFN-β and PRDII promoter activity was minimally reduced in cells treated with the compound (Fig. 3*D*). Moreover, immunoblot analyses indicated that there were no differences in SenV-induced hyperphosphorylation of IRF-3, or ISG15 and MxA expression in these cells in the presence or absence of bafilomycin A1 (Fig. 3*E*). Bafilomycin A1 did not alter the expression of SenV proteins.

These data suggest that SenV induction of IFN responses is MyD88-independent in PH5CH8 cells, and does not require active endocytic pathways. Both lines of evidence argue strongly against the involvement of TLR7/8. In addition, because MyD88 is thought to be used by all TLRs other than TLR3 (and TLR4 for signaling to IRF-3), these data, taken in the context of the absence of any inhibition of the SenV response by TRIF knockdown (Fig. 2, *C* and *D*), suggest that the recognition of SenV by PH5CH8 cells is not dependent upon any known TLR.

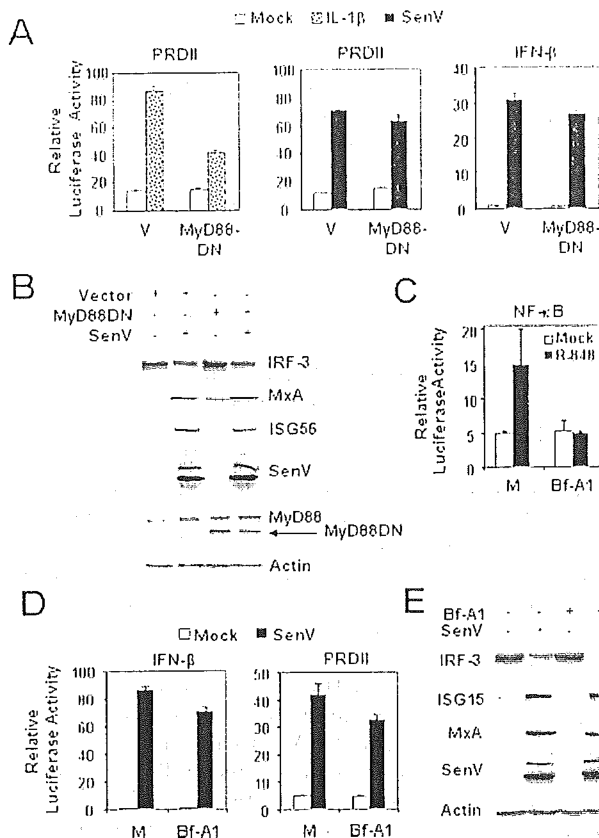

FIG. 3. **SenV activation of type I IFN response in hepatocytes is MyD88-independent and does not require endosomal acidification.** *A*, PH5CH8 cells in 24-well plates were cotransfected with the indicated reporter plasmids (100 ng), pCMV-β-galactosidase (100 ng), and 600 ng of a dominant-negative form of MyD88 (*MyD88DN*) or empty vector (*V*) for 24 h before mock-infected or infected with SenV (100 HAU/ml) for 16 h (*middle* and *right panels*) or mock/treated with 20 ng/ml interleukin-1β for 8 h (*left panel*). *B*, immunoblot detection of IRF-3, MxA, ISG56, SenV, and MyD88 in PH5CH8 cells using the conditions as described for the *middle* and *right panels* of *A*. *C*, NF-κB promoter activity in RAW264.7 cells mock-treated or treated with bafilomycin A1 and then mock-treated with R-848. Cells transfected with pNF-κB Luc and pCMV-β-galactosidase for 24 h were pretreated with medium alone (*M*), or 100 nM bafilomycin A1 (*Bf-A1*) for 1 h and then mock-treated with 1 μM R-848 for 6 h in the presence of M/Bf-A1. *D*, SenV-induced IFN-β and PRDII promoter activity in PH5CH8 cells treated with medium alone or Bf-A1. *E*, immunoblot detection of IRF-3, ISG15, MxA, and SenV in PH5CH8 cells as described for the conditions of *panel D*.

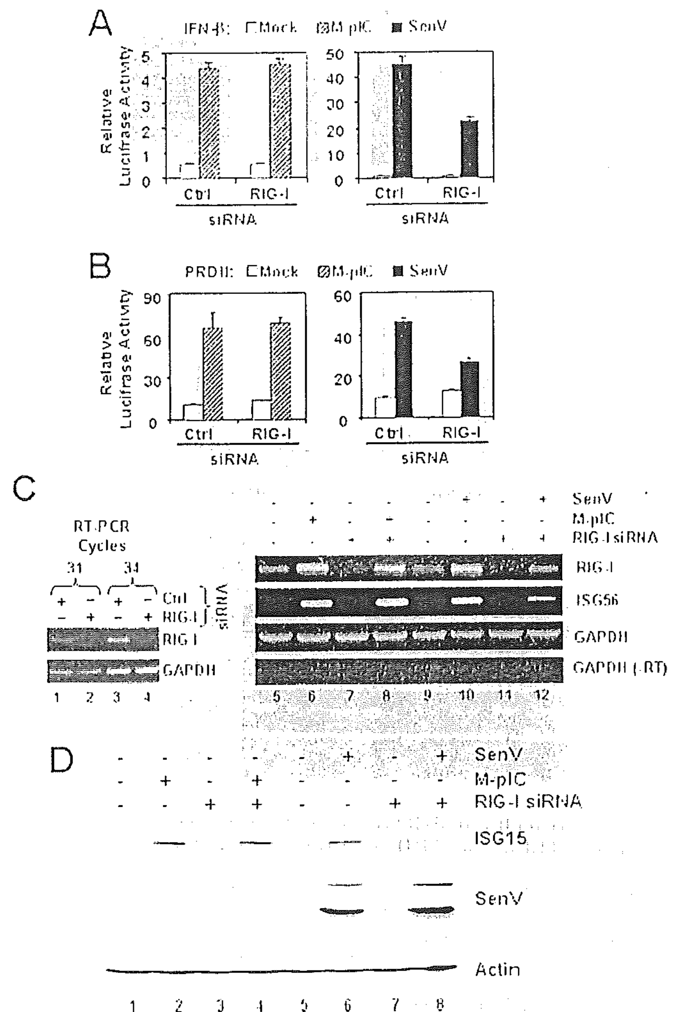

FIG. 4. **SenV, but not TLR3 engagement, activates type I IFN response through RIG-I in hepatocytes.** PH5CH8 cells grown in 24-well plates were transfected with control (*Ctrl*) or RIG-I siRNA, and pIFN-βLuc (*A*) or PRDII Luc (*B*), and pCMV-β-galactosidase. 48 h later, cells were mock-treated or stimulated with 50 μg/ml M-pIC for 6 h or infected with SenV (100 HAU/ml) for 16 h. *C*, semiquantitative RT-PCR detection of RIG-I, ISG56, and GAPDH mRNAs in PH5CH8 cells under conditions of *A* and *B*. The *left panel* shows efficient knockdown of the basal expression of RIG-I in PH5CH8 cells by RIG-I siRNA determined by increasing cycles of RT-PCR. *D*, immunoblot analysis of ISG15 and SenV protein expression in PH5CH8 cells under conditions of *panels A* and *B*. Actin was included as a loading control.

SenV Activates Type I IFN Responses through a RIG-I-dependent Pathway in Hepatocytes—Studies with knock-out mice have indicated that both virus infection and dsRNA can trigger type I IFN responses via TLR3-independent mechanisms (7, 8). Recently, Yoneyama and colleagues (19) demonstrated that RIG-I, a putative DExD/H box RNA helicase containing an N-terminal sequence with CARD-like homology domains, is essential for IFN-β production induced in response to infection with Newcastle disease virus. To determine whether SenV induces IFN-β promoter activity in PH5CH8 cells through a RIG-I-dependent pathway, we utilized RNA interference to knockdown RIG-I expression prior to virus challenge. Transfection of a RIG-I-specific siRNA, but not a scrambled control siRNA, reproducibly caused a ~50% reduction in SenV-induced activation of both the IFN-β (Fig. 4*A*, *right panel*) and PRDII promoters (Fig. 4*B*, *right panel*). Semiquantitative RT-PCR confirmed that the transfection of RIG-I siRNA efficiently knocked down the basal expression of RIG-I (Fig. 4*C*, *lanes 2* and *4* in the *left panel* and *lanes 7* and *11*, in *right panel*), and also significantly blunted the up-regulation of this IFN-induced protein upon SenV infection (Fig. 4*C*, *right panel*, compare *lanes 9* and *10* with *lanes 11* and *12*). SenV-induced transcription of ISG56 mRNA was also significantly reduced in cells transfected with RIG-I siRNA (Fig. 4*C*, *lanes 9–12*). Therefore, RIG-I is an essential component in the TLR-independent pathway by which PH5CH8 cells sense SenV infection and initiate a type I IFN response.

In contrast to these results, PH5CH8 cells transfected with the RIG-I siRNA responded normally when poly(I-C) were added to the media, in terms of activation of both the IFN-β (Fig. 4*A*, *left panel*) and NF-κB-dependent PRDII (Fig. 4*B*, *left panel*) promoters. In contrast to SenV-induced signaling, the poly(I-C) induction of RIG-I transcription was not dramatically reduced by transfection of the RIG-I siRNA (Fig. 4*C*, compare *lanes 5* and *6* with *lanes 7* and *8*). Similarly, there was no inhibition of poly(I-C) induction of ISG56 transcription (Fig. 4*C*, *lanes 5–8*). We also confirmed the RT-PCR results by immunoblot analysis of ISG15 expression under these conditions (Fig. 4*D*). These data indicate that TLR3- and RIG-I-mediated signaling function independently of each other in PH5CH8

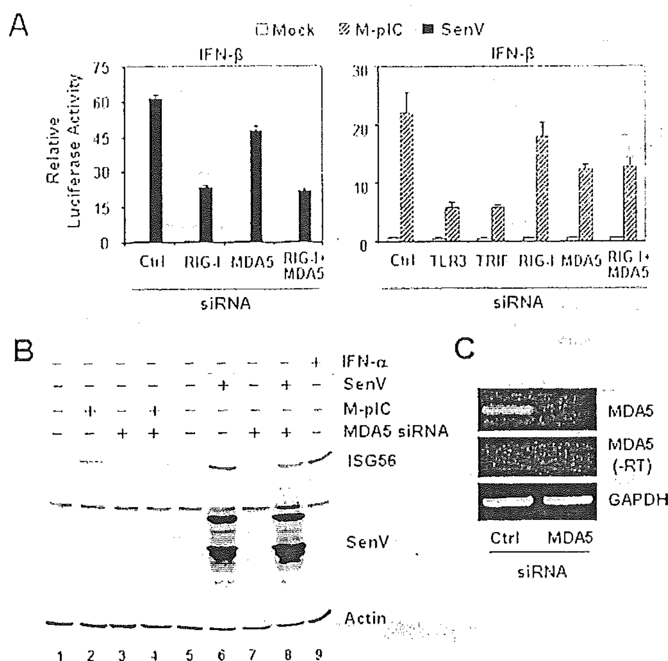

FIG. 5. **Poly(I-C), as well as SenV activation of the IFN-β promoter is partially MDA5-dependent in hepatocytes.** *A*, SenV (*left panel*) and M-pIC (*right panel*)-induced IFN-β promoter activity in PH5CH8 cells transfected with the indicated siRNAs. *B*, immunoblot detection of ISG56 and SenV proteins in PH5CH8 cells transfected with Ctrl (*lanes 1, 2*, and *5, 6*) or MDA5 siRNA (*lanes 3, 4*, and *7, 8*). Where indicated, cells were treated with 50 μg/ml M-pIC for 8 h or infected with 100 HAU/ml SenV for 16 h. In *lane 9*, cells were treated with 500 units/ml of IFN-α2b as a control for ISG56 expression. *C*, semiquantitative RT-PCR detection of MDA5 and GAPDH mRNAs in control (*Ctrl*)/MDA5 siRNA-transfected PH5CH8 cells.

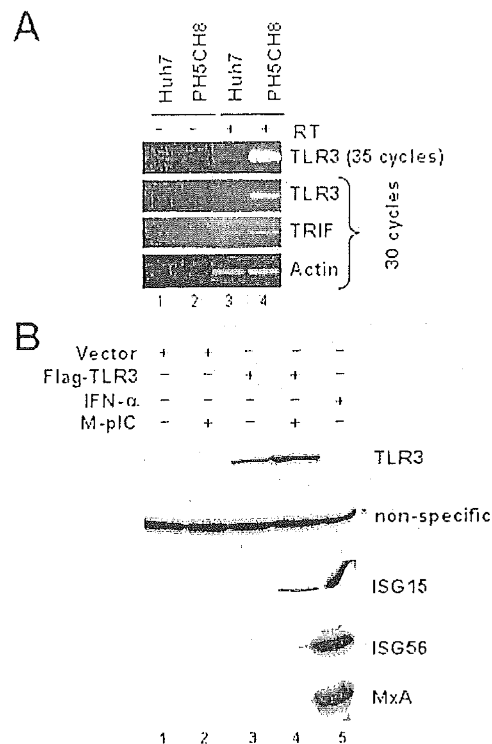

FIG. 6. **The defect of TLR3 signaling in Huh7 cells is because of insufficient expression of TLR3.** *A*, semiquantitative RT-PCR detection of TLR3, TRIF, and β-actin mRNAs in Huh7 and PH5CH8 cells. *B*, ectopic expression a FLAG-tagged TLR3 restored the induction of ISG15, ISG56, and MxA expression by M-pIC (50 μg/ml) determined by immunoblot analysis. A nonspecific band detected by the TLR3 antibody serves as a loading control. In *lane 5*, cells were treated with 500 units/ml of IFN-α2b as a positive control for ISG expression.

cells, similar to what has been reported previously for epithelial HeLa cells (19).

Poly(I-C) Activation of the IFN-β Promoter Is Partially MDA5-dependent—Because siRNA knockdown of RIG-I did not ablate but only partially reduced the IFN-β promoter response to SenV infection in PH5CH8 cells (Fig. 4*A*, *right panel*), we carried out similar experiments to determine whether the human melanoma differentiation associated gene-5 product (MDA5) participates in this signaling pathway, possibly in a redundant role with respect to RIG-I. MDA5, another DExD/H box RNA helicase, is an IFN-inducible protein that shares a subdomain architecture and considerable sequence homology with RIG-I (47). It has been associated with the induction of apoptosis, but not clearly identified as playing a role in activation of IRF-3 similar to that of RIG-I. siRNA-mediated knockdown of MDA5 expression had only a minor, but reproducible, suppressive effect on SenV induction of the IFN-β promoter. The magnitude of the reduction in promoter activity was substantially less than that observed with RIG-I knockdown (Fig. 5*A*, *left panel*), but it was associated nonetheless within a modest reduction in SenV-induced expression of ISG56 (Fig. 5*B*, compare *lanes 6 versus 8*). The suppressive effect of MDA5 knockdown was not additive with RIG-I knockdown, as the degree of suppression of promoter activity was not increased when both RIG-I and MDA5 siRNAs were cotransfected into PH5CH8 cells. The MDA5 knockdown had no effect on SenV protein expression (Fig. 5*B*).

Interestingly, MDA5 knockdown caused ~30% reduction in the induction of IFN-β promoter activity by extracellular poly(I-C) (Fig. 5*A*, *right panel*). This effect was less than that observed with TLR3 or TRIF knockdown, yet nonetheless reproducible and significant. Consistent with this, MDA5 knockdown also caused a moderate reduction in poly(I-C)-induced ISG56 expression (Fig. 5*B*, compare *lanes 2* and *4*). We conclude from these results that MDA5, like RIG-I, may contribute to viral activation of the IFN-β promoter but, unlike RIG-I, plays a greater role in poly(I-C) activation of the promoter than in SenV-induced responses. Whether this occurs in association with, or independent of, the TLR3-TRIF pathway remains to be determined.

TLR3 Overexpression Reconstitutes Poly(I-C)-induced ISG Expression in Huh7 Cells—As shown in Fig. 1, all three Huh7 cell sublines studied were defective in poly(I-C) signaling. This lack of poly(I-C) responsiveness may be explained by the fact that Huh7 cells express a negligible abundance of TLR3 mRNA, in contrast to PH5CH8 cells that demonstrate robust TLR3 expression (Fig. 6*A*). In contrast, TRIF mRNA abundance was approximately equal in these cell lines. Overexpression of FLAG-tagged TLR3 effectively restored the ability of Huh7 cells to respond to poly(I-C) when added to the culture medium (50 μg/ml), as indicated by expression of ISG15, ISG56, and MxA (Fig. 6*B*, compare *lanes 2* and *4*). Thus, Huh7 cells are normally deficient in their ability to respond to externally applied poly(I-C) because of a lack of sufficient expression of TLR3.

TLR3- and RIG-I-mediated Signaling Function Independently in Huh7.5 Cells—TLR3 signaling has been reported not to be adversely affected by overexpression of a dominant-negative form of RIG-I in HeLa cells (19). Consistent with this, the siRNA knockdown experiments shown in Figs. 2 and 4 suggest that these pathways function largely independently of each other in PH5CH8 cells. To confirm this in another hepatocyte-derived cell line, we utilized a Huh7 subline, Huh7.5, which is highly permissive for replication of HCV RNA replicons (48) and which has recently been shown to be defective for RIG-I

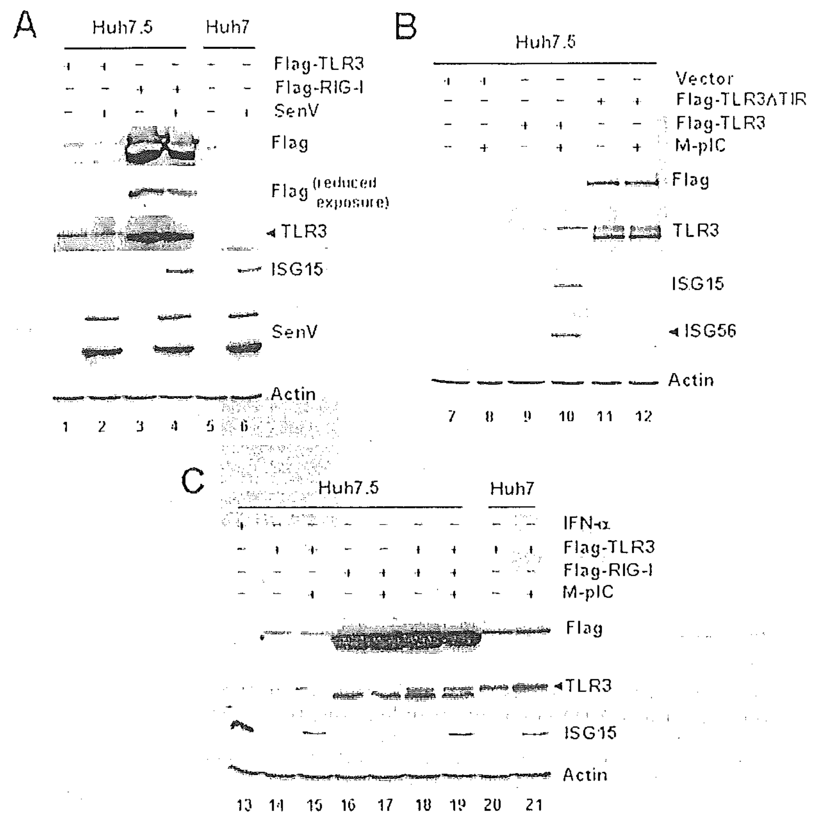

FIG. 7. **TLR3 and RIG-I signaling pathways function independently in Huh7.5 cells.** *A*, the defect of SenV-induced ISG15 expression can be restored by overexpression of RIG-I, but not TLR3 in Huh7.5 cells. *B*, the defect of extracellular dsRNA-induced expression of ISG15 and ISG56 can be restored by overexpression of TLR3, but not a mutant TLR3 with the TIR domain deleted (ΔTIR) in Huh7.5 cells. *C*, ectopic expression of TLR3, but not RIG-I, restored the response to M-pIC in Huh7.5 cells. All panels shown were immunoblot analysis. Where indicated, cells were treated with 50 μg/ml M-pIC for 12 h, or 100 HAU/ml SenV for 16 h, or 500 units/ml of IFN-α2b. Please note that the TLR3 antibody detects transfected FLAG-RIG-I for unknown reasons. The superintense bands of RIG-I detected by anti-FLAG antibody is likely because of the 2× FLAG tag *versus* 1× FLAG tag in TLR3.

signaling because of a point mutation within its CARD-like homology domain (49). Thus, in contrast to PH5CH8 cells in which both TLR3 and RIG-I pathways are intact, neither pathway is functional in Huh7.5 cells. By reconstituting expression of either TLR3 or a functional RIG-I molecule in Huh7.5 cells, we were thus able to assess the role played by each signaling pathway independently in recognition of viral infection while determining the extent to which these pathways act independently of each other.

We first confirmed previous observations (49) that SenV-induced signaling through RIG-I is deficient in Huh7.5 cells, by demonstrating that SenV-induced expression of ISG15 was dependent upon ectopic expression of RIG-I in these cells (Fig. 7*A*). In contrast, ectopic expression of TLR3 did not rescue SenV-induced ISG15 expression in Huh7.5 cells, suggesting that the products of SenV infection do not engage TLR3, or that optimal TLR3 signaling requires functional RIG-I. The latter is unlikely, however, as RIG-I knockdown had no effect on IFN induction by extracellular poly(I-C) in PH5CH8 cells (Fig. 4). Moreover, ectopic expression of TLR3 alone was able to restore responsiveness to extracellular poly(I-C) in Huh7.5 cells (Fig. 7*B*, *lanes 8 versus 10*), to a degree similar to that in normal Huh7 cells (Fig. 7*C*, *lanes 15 versus 21*). The rescue of poly(I-C) responsiveness was specific for TLR3, as overexpression of a TIR domain-deleted TLR3 mutant failed to restore expression of ISG15 and ISG56 in response to poly(I-C) (Fig. 7*B*, *lanes 10 versus 12*), nor did RIG-I (Fig. 7*C*, *lanes 15 versus 17*). Dual ectopic expression of TLR3 and RIG-I only slightly increased the ISG15 induction in response to poly(I-C) (Fig. 7*C*, *lanes 18* and *19*). Taken together, the data suggest that there is little if any cross-talk between the TLR3- and RIG-I pathways in hepatocytes.

DISCUSSION

We have shown here that cultured hepatoma cells generally have impaired poly(I-C) and viral-activated IFN responses compared with PH5CH8 cells that are derived from normal hepatocytes (Fig. 1). These data are thus in agreement with a previous report (50) showing that hepatoma cells have impaired antiviral responses. HepG2 and Hep3B cells are deficient in signaling in response to extracellular poly(I-C), and appear to have diminished responses to either intracellularly delivered poly(I-C) or SenV infection. As described by others (51, 52), we found that Huh7 cells also failed to respond to poly(I-C), irregardless of whether it was added to the culture medium or introduced into cells via transfection. In addition, only a weak response was observed in some Huh7 cell sublines (Huh7 2–3c and MG) after infection with SenV, a more potent IFN inducer. Poly(I-C) signaling could be restored to Huh7 cells by ectopic expression of TLR3 (Fig. 6), indicating that the lesion in Huh7 cells is at the level of the PRR molecule, and that the downstream pathway involving TRIF is intact. In contrast, the non-neoplastic PH5CH8 cells retain robust IFN-β responses to extracellular and intracellular poly(I-C) as well as SenV infection, and thus may be more representative of the antiviral signaling pathways present within hepatocytes *in vivo*.

dsRNA is commonly expressed during the replication of most RNA viruses, and has long been considered a candidate viral PAMP. Treatment of mammalian cells with the synthetic dsRNA analog, poly(I-C), induces type I IFN production through activation of transcription factors IRF-3 and NF-κB (53). TLR3 is well characterized as a PRR that is engaged specifically by dsRNA in many cell types. We have shown here that it is expressed in non-neoplastic hepatocytes, and that it plays an important role in these cells in activation of the IFN-β promoter following exposure to extracellular poly(I-C) (Fig. 2*A*). Furthermore, we have shown that poly(I-C) signaling through TLR3 leading to the induction of ISG expression is dependent upon the adaptor protein TRIF in these cells, as might be expected (Fig. 2*C*). Thus, the data presented demonstrate clearly that the TLR3-TRIF pathway is functional in the non-neoplastic hepatocyte-derived PH5CH8 cells, although it is

generally absent in cultured cells derived from hepatocellular carcinomas (Fig. 1).

Importantly, neither RNA interference directed silencing of TLR3 nor TRIF resulted in any impairment of IFN-β promoter activation or ISG expression in SenV-infected PH5CH8 cells (Fig. 2). This indicates that dsRNA, or possibly another PAMP produced during SenV replication, triggers activation of the IFN-β promoter in these hepatocytes through a distinctly different signaling pathway. This is consistent with several recent reports that dsRNA and/or virus infection can initiate antiviral signaling through TLR3-independent pathways (7, 8). SenV could potentially activate signaling through recognition of viral ssRNA by endosomally located TLR7/8, as reported for murine plasmacytoid dendritic cells and B lymphocytes (7). However, neither endosomal acidification inhibitors nor expression of a dominant-negative MyD88 mutant affected the response in SenV-infected PH5CH8 cells (Fig. 3). Thus, SenV appears to activate a TLR-independent intracellular signaling pathway in hepatocytes that is distinct from the TLR3-TRIF pathway activated by extracellular poly(I-C).

Consistent with a previous study carried out in mouse fibroblasts and human epithelial cells (19), we found that RIG-I is an essential component of this TLR-independent antiviral signaling pathway in cells derived from non-neoplastic hepatocytes (Fig. 4). Similarly, we have recently shown that SenV activation of IRF-3 is dependent upon RIG-I expression in hepatoma cells as well (49). RIG-I is a cytoplasmic RNA helicase that contains tandem motifs near its N terminus with limited homology to CARD domains and a downstream DExD/H-box helicase domain. It putatively binds viral dsRNA within its helicase domain, resulting in activation of IRF-3 and NF-κB through signaling involving the N-terminal CARD-like homology domains (19). MDA5 is a closely related DExD/H box helicase with similar domain architecture, and has been associated with apoptotic signaling in terminally differentiating melanoma cells (47). Its expression is induced by IFN-α as well as infection with human immunodeficiency virus (47, 54). We found that it contributes to the induction of IFN signaling by extracellular poly(I-C) (Fig. 5), but has only a minor, yet clearly discernible, contribution to SenV induction of IFN-β promoter activity and downstream ISG expression. Whether it functions in a fashion similar to that proposed for RIG-I, as a PRR molecule binding dsRNA through its helicase domain, seems likely but remains to be demonstrated. It is also not known whether RIG-I and MDA5 are evolved to preferentially recognize dsRNAs derived from viruses. The relationship of MDA5 to the TLR3-TRIF pathway also remains to be defined.

Although HCV replicon RNAs expressing selectable markers are capable of replicating in some alternative cell types (55), Huh7 cells have proven to be nearly unique in their ability to support the autonomous replication of these viral RNAs (34). In addition, we previously demonstrated that Huh7 cells are uniquely permissive for self-amplification of subgenomic replicons derived from hepatitis A virus, another positive-strand RNA virus (56). Huh7.5, a Huh7 subline that is highly permissive for HCV RNA replication (48), has a lethal mutation in the RIG-I CARD-like homology domain that renders it unresponsive to structured HCV RNA or SenV induced signaling (49). Whereas this accounts for the highly permissive phenotype of Huh7.5 cells, it does not fully explain why parental Huh7 cells are already more permissive than other cell types. Although relatively little is known regarding the cellular factors associated with permissiveness for HCV (34, 57–59), it is intriguing to speculate that the enhanced permissiveness for positive-strand virus RNA replication in normal Huh7 cells may relate to the absence of significant TLR3-mediated antiviral responses in these cells. It remains to be determined, however, whether HCV RNA replication results in specific engagement of TLR3 and activation of the downstream signaling pathway, and whether the TLR3 signaling cascade acts to limit HCV replication. Recent data indicate that expression of the HCV NS3/4A protease inhibits poly(I-C)-induced, TLR3-dependent signaling by directing the proteolytic cleavage of TRIF in osteosarcoma cells as well as in HeLa cells supporting replication of subgenomic HCV RNA replicons (26). However, for reasons that remain unclear, the ectopic expression of NS3/4A does not appear to block TLR3 signaling in PH5CH8 cells.[2]

In summary, hepatocytes contain two distinct antiviral signaling pathways leading to expression of type I IFNs, one dependent upon TLR3 and the other on RIG-I, and with little evidence of significant cross-talk between them. However, although the RIG-I and TLR3 pathways function independently in hepatocytes (Figs. 4 and 7), it is very likely that during viral infection *in vivo* both pathways are activated in a coordinate manner to enhance innate immune responses. Activation of the RIG-I pathway by the intracellular presentation of dsRNA or another viral PAMP produced by replicating viruses may contribute to the initial induction of type I IFNs. Subsequently, viral dsRNA released by lysis of cells in later stages of the infection may engage TLR3 and induce TRIF-dependent signaling, resulting in further amplification of the antiviral response (53). Importantly, type I IFNs induced through either pathway would have a positive feedback on signaling through both pathways, as both RIG-I and TLR3 are ISGs (19, 60).

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