Table 2 *Sequence analysis of (CA) repeat region obtained from the pur^R colonies*

The numbers in the table indicate the actual number of plasmid clones obtained and sequenced.

PCR product		Colony no. 1	2	3	4	5	6	7	8	9	10
LS174T (resistant to puromycin 10 μg/ml)											
$(CA)_{17}$	out-of-frame	1	1			1					1
$(CA)_{16}$	in-frame	3	3	4	4	3	4	3	4	4	3
$(CA)_{13}$	in-frame							1			
HCT116 (resistant to puromycin 10 μg/ml)											
$(CA)_{19}$	in-frame				3						
$(CA)_{17}A$	in-frame					3					
$(CA)_{16}$	in-frame	3	4	3	1	1	4	2			
$(CA)_{10}$	in-frame							2			
$(CA)_7$	in-frame	1		1							
PH5CH8 (resistant to puromycin 10 μg/ml)											
$(CA)_{17}$	out-of-frame							1	1	1	
$(CA)_{16}$	in-frame	4	4	3	2	2	4	3	2	1	2
$(CA)_{15}$	out-of-frame								1		
$(CA)_{14}$	out-of-frame									2	
$(CA)_{13}$	in-frame			1	2						
$(CA)_9$+CC	in-frame										2
$(CA)_7$	in-frame					2					

pared with the results from LS174T and HCT116 cells, PH5CH8-derived colonies showed a variety of mutation patterns. Although the $(CA)_{16}$ sequence was obtained from all colonies, $(CA)_{13}$ and $(CA)_7$ resulting in in-frame were obtained from two colonies and one colony, respectively, and $(CA)_9CC$ resulting in in-frame was also obtained from one additional colony. In addition, $(CA)_{15}$ and $(CA)_{14}$ resulting in out-of-frame were obtained from a single colony, respectively, and the original $(CA)_{17}$ without mutation was also obtained from the three colonies. These results suggest that at least three copies of retrovirus were initially infected and integrated in a single target cell. In summary, sequence data on the (CA) repeat region indicated that the pur^R colonies possessed the frameshift mutation (2-bp deletion) resulting in in-frame in the open reading frame of pur^R gene. Taken together with these results, we concluded that our method can be used as an MSI assay at the cell-culture level.

HCV Core Protein Promoted MSI in PH5CH8 Cells. Because PH5CH8 cells did not show any tumorigenic potential when inoculated s.c. into thymic nude mice (23), we were surprised by the result that PH5CH8 cells showed the RER+ phenotype, as did the human colon cancer cell lines. Although the mechanism responsible for this finding is unclear, we speculate that HCV proteins may have further promoted MSI in PH5CH8 cells. Therefore, to evaluate this possibility, we initially prepared PH5CH8 cells stably expressing HCV protein [core(1b-P), E1(1b-P), E2(1b-P), or NS5A(1b-P)] as the recipient cells for the pCXpur/$(CA)_{17}$/out-of-frame retrovirus infection, by the pCXbsr/core(1b-P), pCXbsr/E1(1b-P), pCXbsr/E2(1b-P), or pCXbsr/NS5A(1b-P) retrovirus infection and following selection with blasticidin. As control recipient cells, we prepared PH5CH8 cells infected with retrovirus pCXbsr and selected with blasticidin. After retrovirus infection and following selection with blasticidin for 7 days, we monitored the growth curve of these blasticidin-resistant PH5CH8 cells, and we observed that the growth rates of these cells were almost the same (data not shown). We also confirmed by Western blot analysis the stable expression of core(1b-P), NS5A(1b-P), E1(1b-P), and E2(1b-P) proteins in PH5CH8 cells at day 10 and day 19 post-infection with retrovirus pCXbsr encoding HCV proteins (data not shown). Using these PH5CH8 cells, we performed an MSI assay, and found that the number of pur^R colonies obtained from the cells expressing the core(1b-P) protein was approximately 1.5-fold (selection with 5 μg/ml of puromycin) and approximately 2.5-fold (selection with 10 μg/ml of puromycin) higher than that from the control cells, as shown in Fig. 3. As compared with the core(1b-P) protein, the E1(1b-P), E2(1b-P), and NS5A(1b-P) proteins did not increase the number of pur^R colonies, although NS5A(1b-P) protein slightly decreased the number of pur^R colonies. Because the increase of pur^R colonies in PH5CH8 cells expressing the core(1b-P) protein was reproducibly observed, it was suggested that core(1b-P) protein was able to further promote the MSI in PH5CH8 cells.

Promotion of MSI by the Core Protein Depends on HCV Genotype or Strain. Because the core protein is known to show some aa sequence heterogeneity among HCV genotypes (7, 8), we examined whether or not HCV core proteins other than the core(1b-P) protein are able to promote the MSI, using pCXbsr/core(1a), pCXbsr/core(2a), pCXbsr/core(2b), and pCXbsr/core(3a) retrovirus vectors

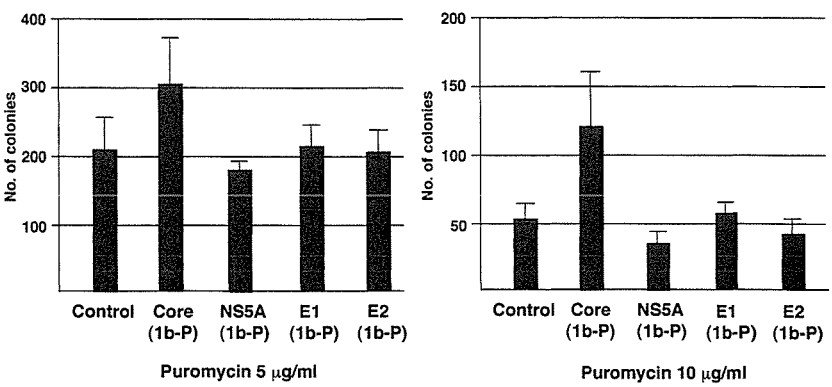

Fig. 3. Hepatitis C virus core protein promoted microsatellite instability in PH5CH8 cells. Microsatellite instability assay using pCXbsr/$(CA)_{17}$/out-of-frame was carried out in PH5CH8 cells stably expressing core(1b-P), NS5A(1b-P), E1(1b-P), or E2(1b-P) protein. The culture period from retrovirus infection to addition of puromycin was 9 days. The puromycin-resistant colonies were counted by the method described in Fig. 2. *Control*, PH5CH8 cells infected with retrovirus pCXbsr.

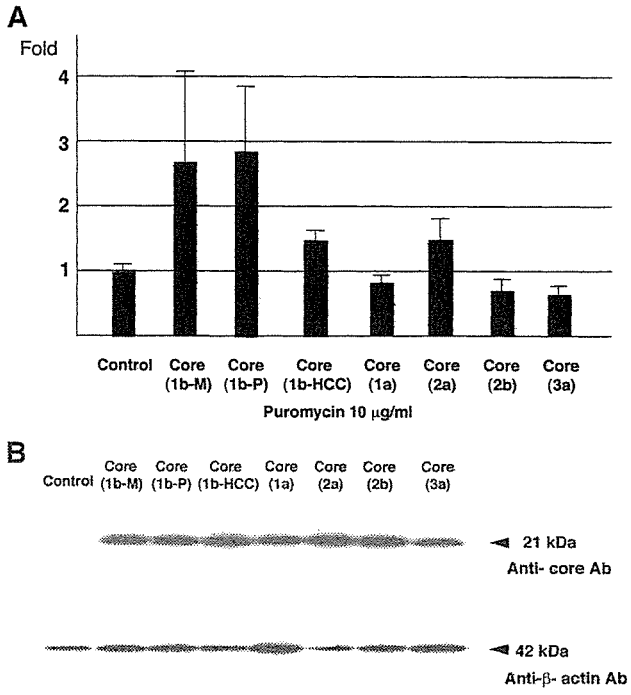

Fig. 4. *A*, promotion of microsatellite instability by the core protein depends on hepatitis C virus genotype. Microsatellite instability assay using pCXbsr/$(CA)_{17}$/out-of-frame was carried out in PH5CH8 cells stably expressing the core protein derived from various hepatitis C virus genotypes. The culture period from retrovirus infection to addition of puromycin was 9 days. The puromycin-resistant colonies were counted by the method described in Fig. 2. *Control*, PH5CH8 cells infected with retrovirus pCXbsr. *B*, stable expression of the core protein in PH5CH8 cells. PH5CH8 cells were infected with retrovirus pCXbsr encoding the core protein belonging to various genotypes, and at 19 days postinfection, the lysate of cells was used for the detection of core protein and β-actin by Western blot analysis. *Control*, PH5CH8 cells infected with retrovirus pCXbsr.

encoding the core(1a), core(2a), core(2b), and core(3a) protein, respectively. In addition, pCXbsr/core(1b-HCC) was also used as a retrovirus vector encoding the core(1b-HCC) protein, which was derived from a cancerous HCC lesion. The pCXbsr/core(1b-M) retrovirus vector (32) encoding core(1b-M) protein, which possessed the consensus sequence of genotype 1b, was also used for the MSI assay. The core(1b-P), core(1b-HCC), core (1a), core(2a), core(2b), and core(3a) proteins differed by 1, 6, 3, 14, 22, and 17 aa from the core(1b-M) protein, respectively (30). Using these retrovirus vectors, including pCXbsr as a control vector, we initially prepared PH5CH8 cells stably expressing the core(1b-M), core(1b-P), core(1b-HCC), core(1a), core(2a), core(2b), and core(3a) proteins, respectively. We performed an MSI assay using these core protein-expressing cells. As shown in Fig. 4*A*, the results revealed that the number of pur^R colonies obtained from the cells expressing the core(1b-M), core(1b-P), core(1b-HCC), or core(2a) protein was 1.5- to 2.8-fold higher than that obtained in the control, whereas the number of pur^R colonies obtained from the cells expressing the core(1a), core(2b), or core(3a) protein was similar to that obtained in the control. Western blot analysis confirmed that these core proteins were stably and equally expressed in PH5CH8 cells at day 19 postinfection with the retrovirus pCXbsr expressing the core protein (Fig. 4*B*). These results suggest that the effectiveness of the core protein in promoting MSI, that is, in down-regulating MMR, is dependent on the HCV genotype or strain.

Expression Level of MMR-Related Genes in PH5CH8 Cells Expressing the Core Protein. To investigate the possibility that the core protein represses the expression of genes functioning in MMR, we examined the effect of the core protein on the expression level of MMR-related genes, including *hMLH1* and *hMSH2*, the frequent genetic mutations of which have been observed in the hereditary nonpolyposis colorectal cancer and a variety of sporadic cancers (25). As shown in Fig. 5, we were not able to find any significant differences in the expression level of *hMLH1*, *hMSH2*, *hMSH6*, *hPMS2*, *hMSH3*, and *hPMS1* genes between PH5CH8 cells expressing the core(1b-P) or NS5A(1b-P) protein, and PH5CH8 cells infected with retrovirus pCXbsr. This result suggests that the down-regulation of MMR by the core protein occurs by an as yet unknown mechanism other than the repression of MMR-related genes.

DISCUSSION

In this study, we first demonstrated that HCV core proteins were able to further repress the down-regulation of MMR activity in cultured human non-neoplastic hepatocytes, by a newly developed MSI assay system using a microsatellite sequence consisting of $(CA)_{17}$.

Regarding the MSI assay system developed in this study, we used retrovirus infection as a method for transduction of a microsatellite (CA) repeat sequence to the cells. However, it remains possible that the RER of pCXpur/$(CA)_{17}$/out-of-frame occurs in the packaging of Bosc23 cells and results in the production of the retrovirus possessing the (CA) repeat sequence altered in-frame. Although we cannot absolutely exclude this possibility, it is unlikely that such an event occurs in Bosc23 cells, because we observed a good correlation between the RER+ and RER− phenotypes of the examined cell lines with respect to the number of pur^R colonies obtained. In addition, we observed that the number of pur^R colonies increased in a culture-time-dependent manner. Therefore, the MSI assay developed in this study will be a useful method at the cell culture level.

The fact that non-neoplastic PH5CH8 cells showed remarkable RER+ phenotype was an unexpected result. Although the PH5CH8 cell line was cloned from PH5CH cells as an HCV-susceptible clone (24), we observed that not only the PH5CH8 cells but also the parental PH5CH cells showed the RER+ phenotype (data not shown). PH5CH cells were established from the non-neoplastic liver as a SV40 large T antigen-immortalized cell line and express hepatocyte characteristics (23). Therefore, the activity of p53 and pRb, two tumor suppressor

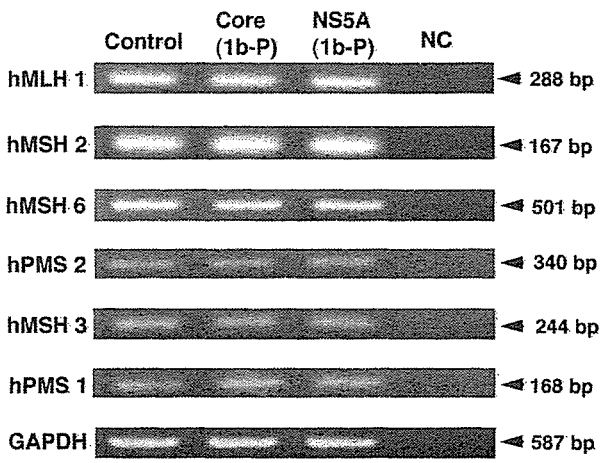

Fig. 5. Effect of the core protein on the expression level of mismatch-repair-related genes in PH5CH8 cells. PH5CH8 cells were infected with retrovirus pCXbsr/core(1b-P) or pCXbsr/NS5A(1b-P), and the cells were used for reverse transcription-PCR analysis of mismatch-repair-related genes. As a control, PH5CH8 cells infected with retrovirus pCXbsr were also used for the analysis. *Control*, PH5CH8 cells infected with retrovirus pCXbsr; *Core(1b-P)*, PH5CH8 cells stably expressing core(1b-P) protein; *NS5A(1b-P)*, PH5CH8 cells stably expressing NS5A(1b-P) protein; *NC*, no RNA.

proteins, in PH5CH cells should be partially repressed by the physical binding of the SV40 large T antigen (42). By complex with p53, the SV40 large T antigen blocks the apoptotic function of p53 and allows proliferation (43), and by binding pRb, the SV40 large T antigen induces the release of the E2F transcription factor, which activates the promoters of genes required for the S-phase transition (44). The functional repression of p53 or pRb may be involved in the repression of MMR activity, although no data suggesting such a relation has yet been reported. As an alternative possibility, the SV40 large T antigen may bind and repress some proteins that function in the MMR system, because it was reported recently that the SV40 large T antigen bound MRE11-NBS1-RAD50 complex, which was involved in homologous recombination, and, as a consequence, perturbed the double-strand break repair (45). Preliminary experiments using NKNT-3 cells (SV40-large T antigen immortalized non-neoplastic human hepatocytes) derived from primary normal human hepatocytes (46) and Saos-2 cells (derived from p53-deficient human osteogenic sarcoma; Ref. 47) revealed that NKNT-3 cells, like PH5CH8 cells, also showed the RER+ phenotype, but Saos-2 cells showed the RER− phenotype in our MSI assay. These results suggest that the activity of MMR is influenced by the SV40 large T antigen but not by p53; however, in addition to PH5CH8 cells, the analysis of cell lines derived from HCV-related HCC cases will be necessary to clarify the reason that PH5CH8 cells show the RER+ phenotype.

Although we found that the core protein promoted MSI in PH5CH8 cells, it is difficult to prove our findings in an HCV replication system because of the lack of a sufficiently reproducible and efficient HCV proliferation system (14). Alternatively, several HCV subgenomic replicons containing NS3-NS5B regions have been established using a human hepatoma cell line Huh-7 (48–50). These subgenomic replicon systems may be useful for the functional evaluation of the core protein. However, our preliminary results revealed that these subgenomic replicon cells showed the RER− phenotype and that no pur^R colonies were obtained from these subgenomic replicon cells stably expressing the core(1b-P) protein. These results suggest that these replicon cells have an intact MMR system that is not influenced by the core protein. To reproduce the promotion of MSI by the core protein in cells in which the HCV genome is replicated, we are currently establishing an HCV subgenomic replicon using PH5CH8 cells.

Our observation that the core proteins belonging to genotypes 1b and 2a, but not those belonging to genotypes 1a, 2b, and 3a, may promote MSI in human hepatocytes is interesting. Although it is not yet defined which region of the core protein is responsible for the promotion of MSI, comparison of aa sequences among these core proteins revealed that aa position 91 was a Cys residue in the core(1a), core(2b), and core(3a) proteins, whereas this position was a Leu residue in the core(1b-M), core(1b-P), and core(2a) proteins and a Met residue in the core(1b-HCC) protein. Only this aa position showed good correlation with the effect of the core proteins in the MSI assay. To clarify whether or not aa position 91 is important to promote MSI, further analysis using chimeric core proteins will be necessary. On the other hand, several studies have described an increased risk of HCC in patients infected with HCV genotype 1b (51, 52), although the contradictory result has also been reported (53). The fact that the core protein belonging to genotype 1b was most effective at promoting the MSI in hepatocyte cells may be related to the increased risk of HCC in patients infected with HCV genotype 1b. To examine this possibility, further MSI analysis using various core proteins derived from many HCV strains belonging to different genotypes will be needed. In addition, our preliminary experiment showed that the number of pur^R colonies in PH5CH8 cells increased approximately 1.5-fold in the presence of $FeSO_4$ (100 μM), suggesting that the Fe(II) compound promotes microsatellite mutations. Although the mechanism of this phenomenon has not yet been clarified, it has been reported that Nickel(II) also induces microsatellite mutations in human lung cancer cell lines (39). Future studies on the relationship between the core protein and these cation compounds will also be important to clarify their roles during the process of hepatocarcinogenesis.

Because we could find no effect of the core protein on the expression level of MMR-related gene, the mechanism by which the core protein promotes MSI in human hepatocytes is still unclear. However, it remains possible that the core protein directly interacts with these components involved in MMR and then suppresses their functions. An alternative possibility—that the core protein affects the functions of the other proteins involved in MMR, including DNA polymerase δ/ϵ, exonuclease 1, and endonuclease FEN1—remains to be examined. Future analyses to evaluate these possibilities may clarify the mechanism of the down-regulation of the MMR system by the core protein.

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cDNA microarray analysis to compare HCV subgenomic replicon cells with their cured cells

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Abstract

The hepatitis C virus (HCV) replicon system carrying autonomously replicating HCV subgenomic RNA in human hepatocyte cells is a potent tool for basic studies of HCV, such as viral replication and drug development. Recently, we developed two HCV subgenomic replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively. Since the expression of HCV proteins is thought to affect the host cells' gene expression profiles, we attempted to identify target genes of HCV proteins using microarray analysis (9970 genes) by comparing 50-1 and 1B-2R1 replicon cells with their "cured cells", from which the replicons had been eliminated by prolonged treatment with interferon-α. The results showed that HCV replicons could have a variety of expression profiles in human hepatocytes. The results also showed that 2 and 6 genes were commonly up-regulated (more than 2.0-fold) and down-regulated (less than 0.50-fold), respectively, in both 50-1 and 1B-2R1 replicon cells compared with their cured cells. The differential expression profiles of genes selected by the microarray analysis were confirmed with standard RT-PCR and real-time LightCycler PCR. It was noteworthy that the commonly down-regulated genes contained large multifunctional proteases 2 and 7, which are known as catalytic subunits of immunoproteasome, and serine proteinase inhibitor clade C. Our microarray analysis demonstrated that HCV subgenomic replicons can change the gene expression profiles of host cells, and it allowed us to compile the first list of genes that the replicons transcriptionally regulate.

Keywords: Hepatitis C virus; HCV subgenomic replicon; Cured cells; cDNA microarray; Gene expression profile

1. Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis (Choo et al., 1989; Kuo et al., 1989), which progresses to liver cirrhosis and hepatocellular carcinoma (Ohkoshi et al., 1990; Saito et al., 1990). HCV belongs to the family Flaviviridae, whose genome consists of a positive-stranded RNA molecule of about 9.6 kb and encodes a large polyprotein precursor of about 3000 amino acids (Kato et al., 1990; Tanaka et al., 1995). This precursor protein is cleaved by the host and viral proteases to generate at least ten proteins: the core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. Although many hypotheses have been proposed over the past decade regarding the functions of the viral proteins (Bartenschlager and Lohmann, 2000; Kato, 2001), the lack of reproducible and efficient HCV proliferation in cell cultures (Kato and Shimotohno, 2000) has been a serious obstacle in understanding those proteins' actual functions.

However, in 1999, an HCV replicon system carring autonomously replicating HCV subgenomic RNA containing the NS3-NS5B regions was first established using a human hepatoma cell line, Huh-7 (Lohmann et al., 1999). Since then, several additional replicons have also been established (Blight et al., 2000, 2003; Ikeda et al., 2002; Kato et al., 2003b). In these systems, replicated HCV RNAs were detected by Northern blot analysis, and the HCV proteins pro-

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duced were detected by Western blot analysis. Therefore, the system of HCV replicons has become a powerful tool for basic studies in HCV, such as viral replication and drug development (Bartenschlager, 2002).

Recently, we also established two HCV subgenomic replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively, using Huh-7 cells (Kato et al., 2003a; Kishine et al., 2002). We demonstrated that the 50-1 and 1B-2R1 subgenomic replicons (Kato et al., 2003a) were sensitive to interferon (IFN)-α, IFN-β and IFN-γ as are the other replicons (Frese et al., 2001, 2002). The nucleotide sequences of the NS3-NS5B regions in the 50-1 subgenomic replicon showed differences of 8.1% from those in the1B-2R1 subgenomic replicon (Kato et al., 2003a), although both the 1B-1 and 1B-2 strains belong to genotype 1b. Although the efficient replication of an HCV subgenomic replicon expressing HCV proteins is considered to affect the gene expression profiles of host cells (Bartenschlager and Lohmann, 2000; Kato, 2001), few reports have demonstrated inclusive searches for HCV's target genes (Zhu et al., 2003). Therefore, we thought a comprehensive search for HCV subgenomic replicon-regulated cellular genes would be important in understanding the molecular interplay exerted by HCV in vivo.

In the present study, to obtain the candidates of HCV's target genes, we performed cDNA microarray analysis by comparing two types of HCV subgenomic replicon cells with their "cured cells", from which the replicons had been eliminated by prolonged treatment with IFN-α. Here we report on the differential gene expression profiles in the replicon cells, and we first provide a list of genes that the replicons transcriptionally regulate.

2. Materials and methods

2.1. Cell cultures

50-1 and 1B-2R1 cells possessing 50-1 and 1B-2R1 subgenomic replicons, respectively, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and G418 (300 mg/ml; Geneticine, Invitrogen). The 50-1 and 1B-2R1 cells were known to possess the G418-resistant phenotype, because neomycin phophotransferase (NEOR) was produced by the efficient replication of HCV subgenomic replicon in the cells. When an HCV subgenomic replicon is excluded from the cells or its level is decreased, the cells are killed by the presence of G418. Therefore, the cured cells obtained from 50-1 and 1B-2R1 cells were maintained in the absence of G418.

2.2. IFN treatment

To prepare the cured cells, 50-1 and 1B-2R1 cells (each 1×10^6) were plated onto 10-cm plates and were cultured for 1 day immediately before IFN treatment. Human IFN-α (Sigma) was added to the cells at a final concentration of 3000 IU/ml as described previously (Kato et al., 2003a). The incubation in the absence of G418 was continued for 3 weeks with the addition of IFN-α (3000 IU/ml) at 4-day intervals. The cured cells obtained from 50-1 and 1B-2R1 cells were named 50-1C and 1B-2R1C cells, respectively.

2.3. Northern blot analysis

Total RNAs from the cultured cells were prepared using the RNeasy extraction kit (Qiagen). Three micrograms of total RNA was used to detect the HCV replicon RNA and β-actin. Northern blotting and hybridization were performed as described previously (Ikeda et al., 2002; Kato et al., 2003a). As a molecular length marker, replicon RNA synthesized in vitro from replicon cassette plasmid pNSS1RZ2RU (Kato et al., 2003a) was also utilized.

2.4. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting analysis with a polyvinylidene difluoride membrane were performed as previously described (Hijikata et al., 1993). The antibodies used in this study were those against NS3 (Novacastra Laboratories, UK), NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science), and β-actin (Sigma). Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences).

2.5. cDNA microarray analysis

The 50-1, 50-1C, 1B-2R1, and 1B-2R1C cells (each 1×10^6 cells) were plated onto 10 cm plates, and each plate was cultured for 5 days in the absence of G418. The confluent cells were harvested and total RNAs were prepared using the RNeasy extraction kit (Qiagen). Using the obtained total RNAs, cDNA microarray analysis (CodeLink™, Uniset human I containing 9970 spots of 30-mer oligonucleotides; Amersham Biosciences) was performed by Kurabo Industries Ltd. (Osaka, Japan) with the authorization of Amersham Biosciences.

2.6. Analysis of mRNA expression by RT-PCR

The total RNAs (each 2 μg) that were the same as those subjected to cDNA microarray analysis were reverse-transcribed with Superscript II using an oligo dT primer (Invitrogen). One-tenth of the synthesized cDNA was subjected to PCR. The PCR primers are listed in Table 1. After 10 min at 98 °C, PCR was performed with Taq DNA polymerase (TaKaRa, Japan). Each cycle consisted of annealing at 60 °C (64 °C for LMP2 and LMP7 only) for 45 s, primer extension at 72 °C for 1 min, and denaturation at 94 °C for 20 s. The cycle numbers and the size of PCR products were also

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

Genes	Orientation	Nucleotide sequence	Product (bp)	Cycles
Large multifunctional protease2 (LMP2)	Forward	ATGGAACCCTGGGAGGAATGCTG	145	30
	Reverse	GCAATAGCGTCTGTGGTGAAGCG		
Large multifunctional protease 7 (LMP7)	Forward	CTGGGATAAGAAGGGTCCTGGAC	293	27
	Reverse	TACTGGTGCAGCAGGTCACTGGAC		
Serine proteinase inhibitor (serpin) clade C	Forward	TGGATGAATTGGAGGAGATGATGC	249	25
	Reverse	CAATCACAACAGCGGTACTTGCAG		
S100-type calcium binding protein A14	Forward	CAGAGGATGCTCAGGAATTCAGTG	256	27
	Reverse	CTCTTGGCCGCTTCTCCAATGAG		
Latent transforming growth factor β binding protein 1 (LTBP1)	Forward	GCCTTGGTTGACTTCAGTGAACAG	325	27
	Reverse	CAGAAGGCACGTAGCCTGGCAG		
Weakly similar to zinc finger protein 91	Forward	CCAGAACCACATCCAAACCATCC	299	33
	Reverse	CCATCCCTTCGAAGCTGTGCTC		
Transgelin	Forward	GATTCTGAGCAAGCTGGTGAACAG	254	25
	Reverse	AGTGCCCATCATTCTTGGTCACTG		
Annexin A1	Forward	GATGCCAGGGCCTTGTATGAAGC	264	25
	Reverse	AACACCTTTCATGGCTTGATGAAGC		
Solute carrier family 7	Forward	AGTCCTTCGCTGGAAGAAGCCTG	314	27
	Reverse	CCATGTCCTCATTAGCCTCCTCTG		
Protein phosphatase 1 regulatory subunit 1A	Forward	CCACGGCAACGGAAGAAGATGAC	302	27
	Reverse	GCTCCCTTGGAATCCAGTGGTGG		
Phosphatidylserine-specific phospholipase A1α	Forward	GAGAAACAAGGACACCAACATCGAG	288	28
	Reverse	GTCACACTTGCTTGTAAGTTCACTG		
Oncostatin M receptor	Forward	CAGAAAAGAGTCACTCTGGCCCTG	292	27
	Reverse	GGTGCCTCTACTGGGTTTGTTGG		
Similar to interferon-induced protein 35	Forward	CCGTATGTGAATGGGGAGATCCAG	222	27
	Reverse	GCCTGACTCAGAGGTGAAGACTG		
Caspase 1	Forward	AGAAACACTCTGAGCAAGTCCCAG	278	30
	Reverse	AACATTATCTGGTGTGGAAGAGCAG		
Neutrophil cytosolic factor 2	Forward	GACATGGTGTCTAAGAAACTGGAG	277	27
	Reverse	CTCATAACTGAAGAGTGCCTCCAC		
Putative secred protein ZSIG13	Forward	CTGGTTATGACAATGACCGACCAG	272	25
	Reverse	GCAGATCTGGGCATATTTGAGAGG		
GAPDH	Forward	GACTCATGACCACAGTCCATGC	334	22
	Reverse	GAGGAGACCACCTGGTGCTCAG		

listed in Table 1. The PCR products were detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels. RT-PCR was performed in duplicate experiments. The mRNA levels of target genes were monitored by a ChemiImager 4400 (Alpha Innotech), which measured the intensities of bands stained with ethidium bromide as described previously (Kato et al., 2003a). As an internal control, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was amplified by RT-PCR, and the products were used to normalize the mRNA levels of the target genes.

2.7. LightCycler PCR

One-twentieth of the cDNA synthesized above was subjected to real-time LightCycler PCR as described previously (Nozaki and Kato, 2002; Nozaki et al., 2003). The primers listed in Table 1 were also used for LightCycler PCR. Temperature cycling conditions for each primer set consisted of 10 min at 95 °C followed by 35 cycles for 1 s at 94 °C, 5 s at 60 °C (64 °C for LMP2 and LMP7 only), and 6–14 s (25 bp per second) at 72 °C. All reactions were performed in a LightCycler™ Quick System 330 (Roche) using Fast-Start DNA Master SYBR Green I mix (Roche) according to the manufacturer's instructions. The experiments were performed in at least triplicate. The relative mRNA expression ratios of the target genes were calculated based on crossing-point analysis using a second derivative maximum method (LightCycler analysis software version 3.5). To correct for differences in RNA quality and quantity between the samples, data were normalized using the ratio of the target cDNA concentration to that of GAPDH. This ratio was assessed by a different reaction in the same experimental round.

Table 2
Genes whose expression levels were commonly altered in 1B-2R1 and 50-1 cells compared with their cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
Up-regulation (more than 2-fold)			
Phosphatidylserine-specific phospholipase A1α[a]	2.2	2.9	NM_015900
Oncostatin M receptor[a]	2.1	2.2	NM_003999
Down-regulation (less than 0.50-fold)			
LMP2[a]	0.14	0.30	NM_002800
LMP7[a]	0.21	0.44	NM_004159
Similar to interferon-induced protein 35[a]	0.31	0.32	BC001356
Weakly similar to zinc finger protein 91[a]	0.36	0.42	AK027354
Protein phosphatase 1, regulatory subunit 1A[a]	0.40	0.32	NM_006741
Serpin clade C[a]	0.49	0.31	NM_000488

[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

3. Results

3.1. Preparation of the cured cells from 50-1 and 1B-2R1 cells

To obtain cured cells for the microarray analysis, 50-1 and 1B-2R1 cells were cultured with prolonged IFN-α treatment as described Section 2. After 3 weeks of this treatment, we demonstrated by Northern blot analysis that the replicon RNAs were not detected in the IFN-α-treated (50-1C and 1B-2R1C) cells, although approximately 10^8 copies of replicon RNA were detected in the total RNA (3 mg) extracted from 50-1 and 1B-2R1 cells (Fig. 1A). We further confirmed by RT-nested PCR (Mizutani et al., 1996) for the detection of the 5′-untranslated region that the replicon RNAs were absolutely excluded from the cells (data not shown). Western blot analysis also showed that the NS3 and NS5B proteins were no longer detected in 50-1C and 1B-2R1C cells, but were detected in 50-1 and 1B-2R1 cells, as shown in Fig. 1B.

3.2. cDNA microarray analysis

To examine the effects of HCV replicons on gene expression in host cells, cDNA microarray analyses (CodeLink™, Amersham Biosciences; 9970 human genes) were performed by comparing 1B-2R1 with 1B-2R1C cells and 50-1 with 50-1C cells. The majority of genes examined showed only small differences, with ratios ranging between 2.0 and 0.50 (data not shown). There were 55 and 101 up-regulated genes (those

Table 3
Genes whose expression levels were up-regulated (more than 3-fold) in either 1B-2R1 or 50-1 cells compared with the cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
AU62G04.X1	**8.5**	1.4	AI929792
Homeobox 1(HESX1)	**4.2**	0.50	NM_003865
Microsomal NAD+ dependent retinol dehydrogenase 4	**3.4**	0.92	NM_003708
Advillin	**3.3**	0.61	NM_006576
SSFV proviral integration oncogene Spi1	**3.1**	1.0	NM_003120
Napsin 2 precursor	**3.1**	0.94	AF098485
Transgelin[a]	0.85	**8.5**	NM_003186
Uncharacterized bone marrow protein BM040	0.81	**5.8**	AF217516
Annexin A1[a]	1.0	**4.2**	NM_000700
Putative secreted protein ZSIG13[a]	1.7	**3.9**	AF193611
Protease serine 23	1.2	**3.8**	NM_007173
Colon cancer antigen NY-CO-45	1.3	**3.7**	AF039442
HSPC157 protein	1.1	**3.5**	NM_014179
Uronyl-2-sulfotransferase	1.0	**3.5**	NM_005715
Cadherin, EGF lag seven-pass G-type receptor 2	0.68	**3.5**	NM_001408
Hypothetical protein (LOC51321)	1.1	**3.4**	NM_016627
Kidney-specific membrane protein (NX-17)	1.0	**3.3**	NM_020665
Neutrophil cytosolic factor 2[a]	1.8	**3.2**	NM_000433
Amphiregulin	1.4	**3.1**	NM_001657
Fibrillin 1	0.83	**3.1**	NM_000138
LTBP1[a]	1.6	**3.0**	NM_000627

The numbers of more than 3-fold were indicated by bold letters.
[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

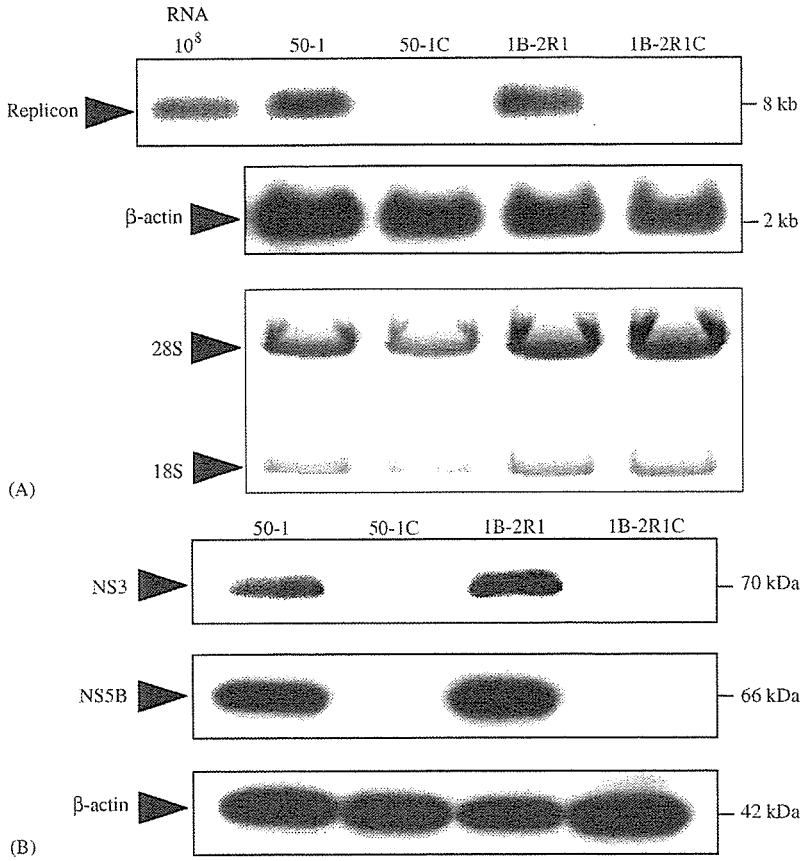

Fig. 1. Characterization of the replicon cells and their cured cells. (A) Northern blot analysis. Total RNAs from 50-1 and 1B-2R1 cells, as well as total RNAs from the cured cells, were analyzed by Northern blotting using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β-actin-specific RNA probe (middle panel). RNA samples were equalized for 28S and 18S ribosomal RNAs stained with ethidium bromide (lower panel). A synthetic RNA transcribed from pNSS1RZ2RU (10^8 genome equivalents spiked into normal cellular RNA) was used as a positive control. (B) Western blot analysis. Productions of NS3 and NS5B in 50-1 and 1B-2R1 cells were analyzed by immunoblotting using anti-NS3 and anti-NS5B antibodies, respectively. 50-1C and 1B-2R1C cells were also analyzed to confirm the lack of NS3 and NS5B proteins. β-actin was used as a control for the amount of protein loaded per lane.

with ratios of more than 2.0) in 1B-2R1 and 50-1 cells, respectively. Between the two types of replicon cells, only two genes were commonly up-regulated. There were 56 and 74 down-regulated genes (those with ratios of less than 0.50) in 1B-2R1 and 50-1 cells, respectively, of which 6 genes were commonly down-regulated in both types of replicon cells. Table 2 summarizes the genes that the replicons commonly affected. Among these genes, it is noteworthy that large multifunctional proteases 2 (LMP2) and LMP7, which have been known as catalytic subunits in immunoproteasome (Akiyama et al., 1994; Tanaka and Kasahara, 1998), and serine proteinase inhibitor (serpin) clade C (Gettins, 2002) were down-regulated in both types of replicon cells (discussed below). However, no common genes were directly linked to the transformation of the cells. Since the standard of selection seemed to be rather strict, we further selected the genes whose expression levels were up-regulated or down-regulated with ratios of more than 3.0 or less than 0.33, respectively, in either 1B-2R1 or 50-1 cells. By this method, we selected 6 and 15 genes as up-regulated genes in 1B-2R1 and 50-1 cells, respectively (as shown in Table 3); and 6 and 9 genes as down-regulated genes in 1B-2R1 and 50-1 cells, respectively (as shown in Table 4). These selections allowed us to find several additional genes, including latent transforming growth factor β binding protein 1 (LTBP1) and caspase 1, that were commonly regulated in both types of replicons.

3.3. RT-PCR confirmation of the alteration of gene expression by HCV replicons

To confirm the results of our microarray selection, we examined the levels of several mRNAs by RT-PCR in duplicate. As shown by the stars in Tables 2-4, 16 genes (7 up-regulated and 9 down-regulated) were subjected to RT-PCR analysis. As shown in Fig. 2, RT-PCR confirmed that the expressions of most of these genes changed. This result suggests that the relative mRNA expression ratio obtained by the microarray analysis reflects the differential expression profiles of the replicon and its cured cells. Of the 16 genes, 9 (4 up-regulated and 5 down-regulated) were fur-

Table 4
Genes whose expression levels were down-regulated (less than 0.33-fold) in either 1B-2R1 or 50-1 cells compared with the cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
Hephaestin	**0.14**	1.7	NM_014799
Solute carrier family 7[a]	**0.15**	0.62	NM_003982
Caspase 1[a]	**0.18**	0.65	NM_033292
Protease inhibitor 3	**0.19**	1.1	NM_002638
Collagen type II α1	**0.31**	1.6	NM_033150
C-terminal binding protein 2	**0.31**	0.71	NM_022802
ATPase α polypeptide (ATP 12A)	0.57	**0.26**	NM_001676
Hypothetical protein FLJ20043	0.79	**0.27**	NM_017637
CM2-HT0948-070900-368-D08 cDNA	1.0	**0.28**	BF089733
S100-type calcium binding protein A14[a]	0.62	**0.30**	NM_020672
Hypothetical protein MGC2827	0.65	**0.31**	NM_023940
EGFL6	2.4	**0.32**	NM_015507
ISL1 transcription factor	0.94	**0.32**	NM_002202
Pre-α globulin inhibitor	1.2	**0.32**	NM_002217
Regulator of G-protein signalling 16	0.65	**0.33**	NM_002928

The numbers of less than 0.33-fold were indicated by bold letters.
[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

ther subjected to real-time LightCycler PCR analysis in order to obtain the actual ratios of mRNA expression. As shown in Table 5, the resultant relative mRNA expression ratios actually correlated with those obtained by our microarray analysis. Regarding the selected genes in this study, we confirmed by RT-PCR the reproducibility of the relative mRNA ratios using different lots of RNA specimens derived from 1B-2R1 and 1B-2R1C cells (data not shown). Taken together, our results suggest that these altered mRNA expressions are caused by the multiplication of HCV subgenomic replicons.

4. Discussion

This study yielded evidence of alterations in gene expression by HCV subgenomic replicons in human hepatocytes, as observed through microarray analysis (9970 genes), and first provided a list of genes including LMP2, LMP7, and serpin clade C that the replicons transcriptionally regulate.

To date, only one report of cDNA microarray analysis (832 cytokine-related genes) has been conducted by comparing HCV subgenomic replicon cells with parental Huh-7 cells (Zhu et al., 2003). That analysis obtained 14 up-regulated genes (those with ratios of more than 2.0) in the replicon cells. However, the parental Huh-7 cells may not be appropriate for use as control cells in such microarray analyses, because the HCV subgenomic replicon cells used are derived from a single cloned cell. Therefore, it is very important to avoid the clone-based differences for microarray analysis. From this principal reason, we used two types of cured cells derived from 50-1 and 1B-2R1 cells as the control cells for our microarray analysis. The cured cells are considered to have the same background as the replicon cells. The possibility remains that the genes selected in this study were obtained by the effect of IFN-α that was used to

Table 5
LightCycler RT-PCR analysis of genes whose expression levels were altered by HCV replicons

Genes	Relative mRNA expression ratio (mean ± S.D.)	
	1B-2R1/1B-2R1C	50-1/50-1C
Up-regulation		
Phosphatidylserine-specific phospholipase A1α	2.03 ± 0.09	3.09 ± 0.74
Oncostatin M receptor	2.58 ± 0.20	2.46 ± 0.49
Transgelin	0.83 ± 0.11	13.72 ± 0.56
Annexin A1	1.19 ± 0.17	4.23 ± 0.72
Down-regulation		
LMP2	0.06 ± 0.00	0.40 ± 0.12
LMP7	0.09 ± 0.02	0.33 ± 0.08
Serpin clade C	0.39 ± 0.11	0.37 ± 0.11
Solute carrier family 7	0.13 ± 0.08	0.77 ± 0.18
S100-type calcium binding protein A14	0.37 ± 0.21	0.32 ± 0.17

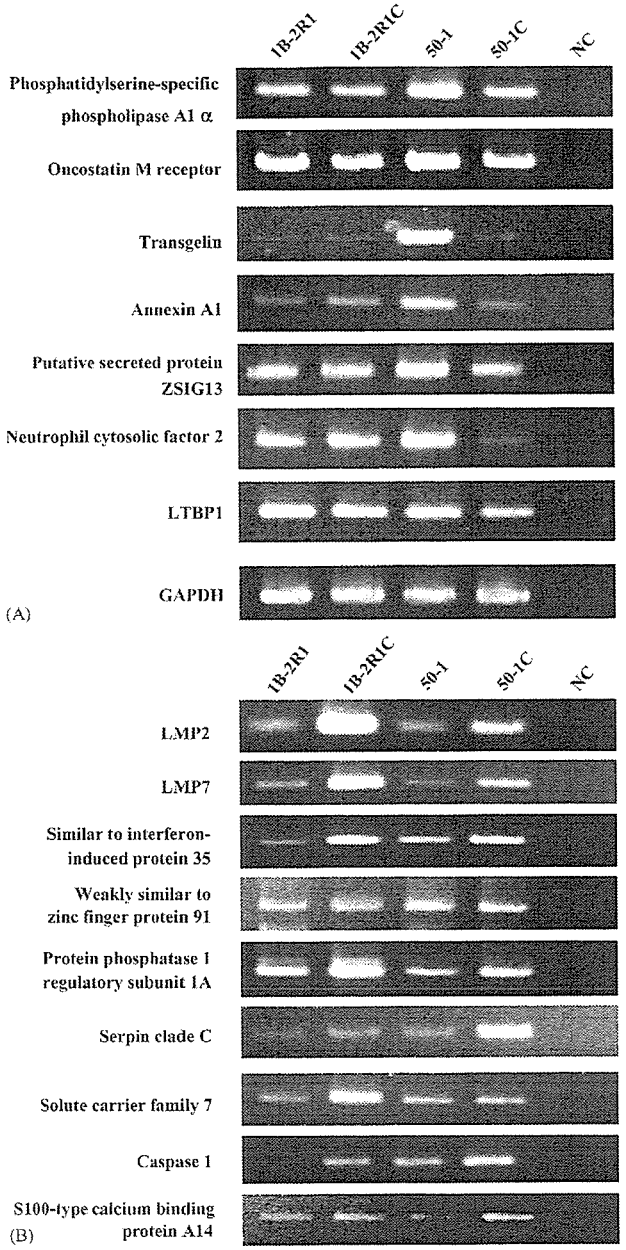

Fig. 2. RT-PCR analysis of mRNA expression of selected genes based on the microarray results. The total RNAs extracted from 50-1, 50-1C, 1B-2R1, and 1B-2R1C cells were subjected to RT-PCR, and the mRNA levels of target genes were monitored as described in Section 2. The primers used, PCR product lengths, and PCR cycle numbers are listed in Table 1. GAPDH was used as a control. (A) Up-regulated genes. (B) Down-regulated genes.

eliminate replicon RNA. However, this possibility is quite low because most of the genes regulated by IFN-α were not transcriptionally altered according to our microarray analysis, and it is unlikely that IFN-α treatment irreversibly altered the expression of genes that it either induced or suppressed. In actuality, most genes selected by our cDNA microarray analysis were not related to the genes regulated by IFN-α, although the selected genes in this study did not show any common characteristics involved in the progression of hepatic diseases. Very recently, after most of the present study was finished, a cDNA microarray analysis using full-length HCV RNA replicating cells and their cured cells was reported by Scholle et al. (2004). Although those authors found dozens of genes whose expression levels were altered by the replication of HCV RNA derived from HCV strain N, we had no information on the genes they had selected and therefore we could not compare them with ours. Therefore, we are currently establishing full-length HCV RNA replicating cells derived from 1B-1 and 1B-2 HCV strains. Further cDNA microarray analysis using such full-length HCV RNA replicating cells will help to identify HCV's target genes.

Among the genes selected in this study, LMP2 and LMP7 are quite interesting. Both are known as important catalytic subunits in immunoproteasome induced by IFN-γ (Akiyama et al., 1994; Tanaka and Kasahara, 1998). In the presence of IFN-γ, the three catalytic subunits of vertebrate proteasomes are replaced by their homologous subunits, LMP2, LMP7, and MECL1, to form immunoproteasome, which increased the ability to produce peptides with a proper motif for efficient MHC binding (Fehling et al., 1994; Van Kaer et al., 1994). A number of peptides that were poorly processed by the standard proteasome were recently found to be more effectively produced by the immunoproteasome (Van den Eynde and Morel, 2001). Therefore, down-regulation of LMP2 and LMP7 expressions in HCV subgenomic replicon cells will reduce the production efficiency of viral antigenic peptides presented to CD8+ T cells (Van den Eynde and Morel, 2001), and may subsequently help to cause the persistent viral infection. In contrast with the expression of LMP2 and LMP7, that of the MECL1 gene was not altered regardless of the presence of HCV replicon. The molecular mechanism by which HCV replicon cells suppress LMP2 and LMP7 remains unknown. This phenomenon is considered to be caused by one of the HCV NS proteins in the replicon cells. As a first step toward identifying the responsible NS protein, we carried out a preliminary experiment using 1B-2R1C cells that stably expressed NS3, NS4A, NS4B, NS5A, NS5B, or NS3-NS5B protein by retrovirus-mediated gene transfer. Unfortunately, however, this experiment failed to identify the responsible NS protein. This result suggests that either the replication of replicon RNA or replicon RNA itself is necessary to suppress LMP2 and LMP7 gene expression. To clarify this point, further analysis will be necessary, using HCV subgenomic replicon cells derived from the other HCV strains or HCV subgenomic replicon cells re-established by the transfection of 50-1 or 1B-2R1 subgenomic replicon RNA.

A third interesting gene obtained in this study was serpin clade C. Although the expression of the serpin clade C gene was down-regulated to approximately one-third in HCV subgenomic replicon cells, those of the other eight clades of the serpin family were not quite altered. Since serpins are a unique class of proteinase inhibitors that irreversibly neu-

tralize target proteinases by a mechanism that conformationally distorts the proteinase (Gettins, 2002), the relationship between serpin clade C and HCV serine proteinase is interesting. To clarify this relationship, further analysis, such as that of the compulsory expression of serpin clade C in the replicon cells, will be necessary.

In this study, we demonstrated that microarray analysis to compare HCV subgenomic replicon cells with their cured cells was useful for screening and selecting HCV's target genes. Also, we compiled the first list of genes transcriptionally regulated by the multiplication of HCV subgenomic replicons. Although we need to clarify the mechanisms underlying transcriptional regulation by HCV subgenomic replicons, we believe that the genes involved in viral replication and multiplication are among the genes listed in this study. Further analysis using new experimental systems, such as the full-length HCV RNA replicating system, will be useful to clarify this point.

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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′-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-

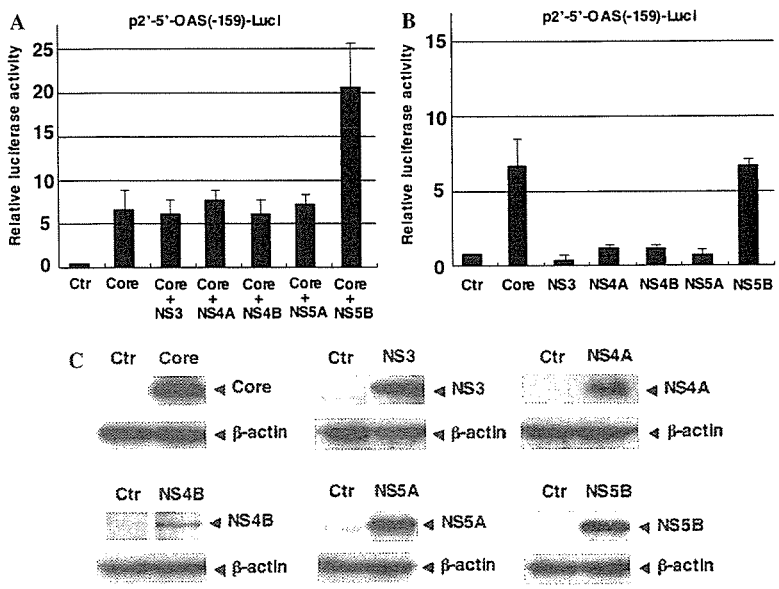

Fig. 1. NS5B synergistically enhanced Core's activation of the human *2′-5′-OAS* gene promoter in PH5CH8 cells. (A) NS5B enhanced Core's activation of the *2′-5′-OAS* gene promoter. The dual luciferase reporter assay was performed as described in Materials and methods. The relative luciferase activity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

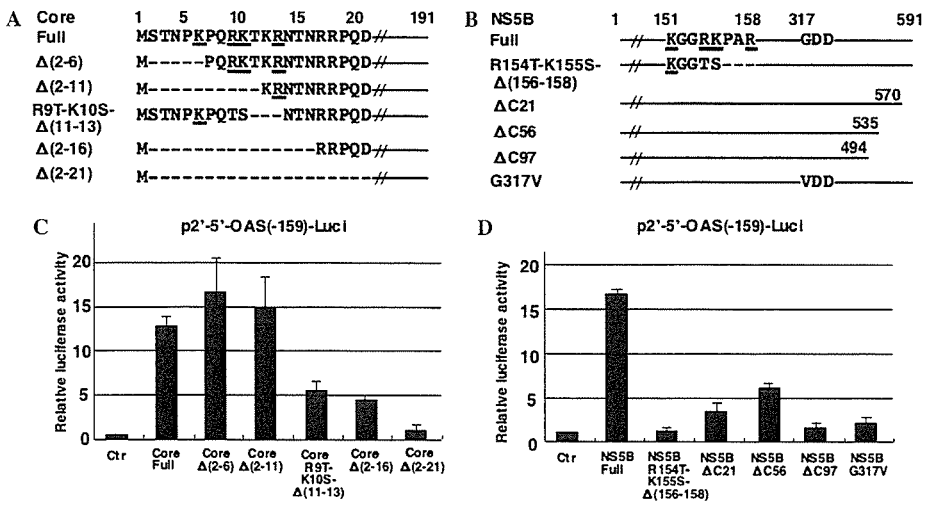

Fig. 2. Deletion analysis of the Core and NS5B. (A) Schematic presentation of the Core mutants used. The aa sequences of aa 1–21 in the Core (1b-P) [15] are indicated. The bars indicate the deleted aa residues, and K and R in the KxxRKxxR motif are underlined. (B) Schematic presentation of the NS5B mutants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

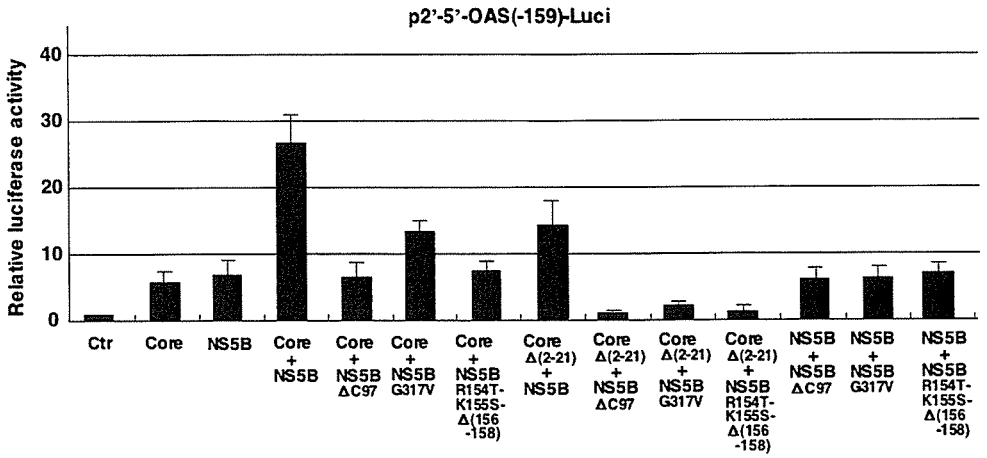

Fig. 3. Characterization of the synergistic effect of Core and NS5B on the *2'-5'-OAS* gene promoter. The dual luciferase reporter assay was performed as described in Fig. 1A. One microgram of each HCV protein expression effector plasmid was used.

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

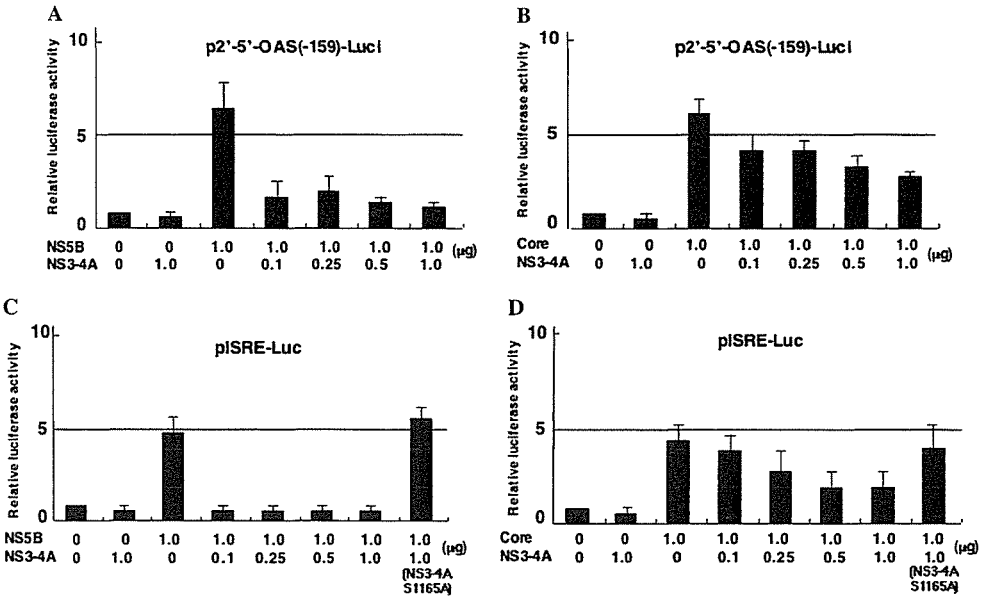

Fig. 4. NS3-4A differentially suppressed the promoter activations by Core and NS5B. The dual luciferase reporter assay was performed as described in Fig. 1A. (A) Effect of NS3-4A on NS5B's activation of the *2′-5′-OAS* gene promoter. (B) Effect of NS3-4A on Core's activation of the *2′-5′-OAS* gene promoter.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

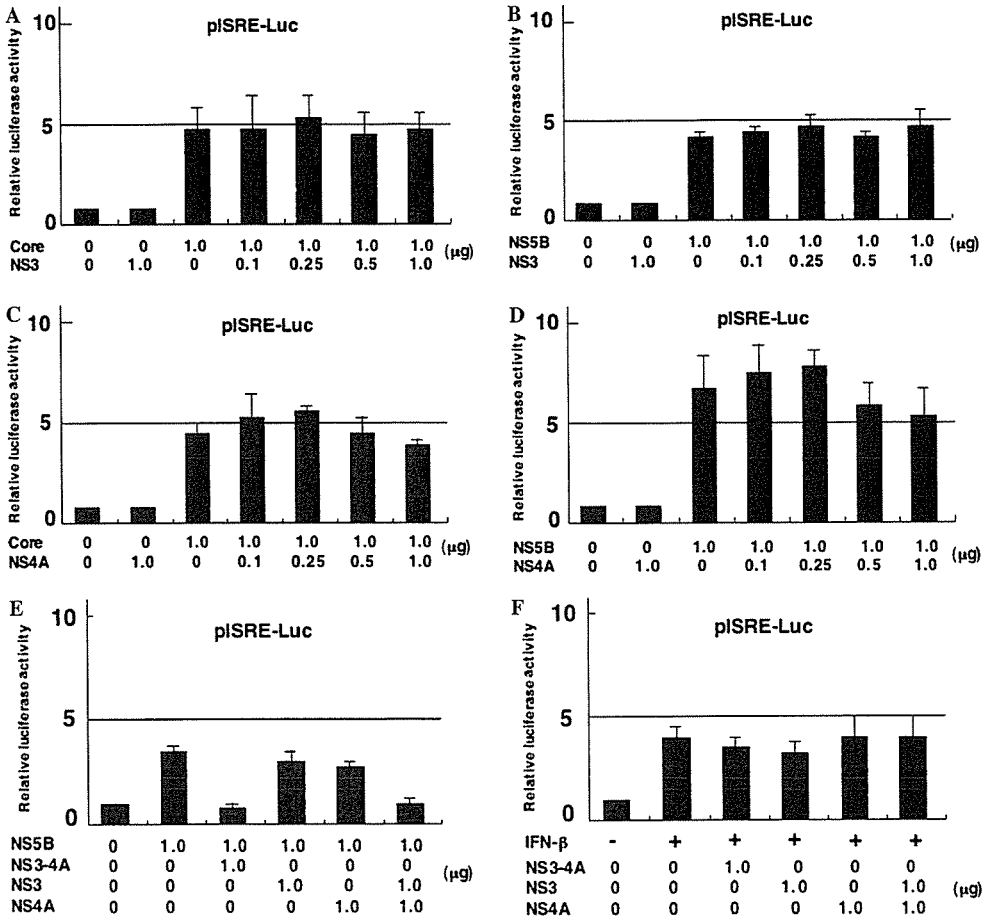

Fig. 5. The synthetic ISRE promoter activation by Core or NS5B is not suppressed by only NS3 or NS4A. Dual luciferase reporter assay was performed as described in Fig. 1A. (A) Effect of NS3 on Core's activation of the synthetic ISRE promoter. (B) Effect of NS3 on NS5B's activation of the synthetic ISRE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