lation initiation process. To evaluate this hypothesis, our established replicon cells (1B-2R1 and 50-1) will be useful, and the establishment of IFN-resistant replicon cells will also be necessary.

Although dozens of reports using HCV subgenomic replication systems have been reported to date, HCV genomic sequences in these subgenomic replicons are limited to only 4 HCV isolates (Con1, N, and 1B-1 belonging to the 1b genotype, and H77 belonging to the 1a genotype). Since HCV genomes are known to show remarkable genetic heterogeneity and their functional differences in host cells are also well known [12,13,28–30], the establishment of replicons possessing viral genomes derived from different HCV isolates is extremely important for the advancement of current HCV studies. In conclusion, we have established a 1B-2R1 subgenomic replicon system in this study, and this system should prove to be useful for basic studies of HCV replication as well as HCV–host cell interactions. In addition, this system may help lead to the development of novel anti-HCV reagents.

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Differential activation of interferon-inducible genes by hepatitis C virus core protein mediated by the interferon stimulated response element☆

Hiromichi Dansako[a,b], Atsushi Naganuma[a,1], Takashi Nakamura[a], Fusao Ikeda[a], Akito Nozaki[a], Nobuyuki Kato[a,*]

[a] *Department of Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*
[b] *Department of Internal Medicine II, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*

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Abstract

We previously found that hepatitis C virus (HCV) core protein, which possesses the consensus sequence of genotype 1b, transcriptionally activates the interferon (IFN)-inducible 2′-5′-oligoadenylate synthetase (2′-5′-OAS) gene in human hepatocyte cells. To clarify the mechanism of this activation, we further characterized the core protein as an activator of the 2′-5′-OAS gene. We demonstrated that the activation of the 2′-5′-OAS gene by the core protein is a general phenomenon, regardless of HCV genotype and strain. We showed that the 20 N-terminal amino acids (aa) of the core protein were important to the activation of the 2′-5′-OAS gene, although this N-terminal region did not have any effect on the subcellular localization of the core protein. We demonstrated that the core protein was able to activate all promoters possessing the IFN-stimulated response element (ISRE) examined. However, we found that the level of activation of the 2′-5′-OAS gene promoter possessing a particular variant type of ISRE was significantly higher than that of other IFN-inducible gene promoters. This phenomenon was confirmed using synthetic promoters possessing five repeats of the consensus or a 2′-5′-OAS-type ISRE. In addition, we showed that gene activation induced by the core protein is mediated by the ISRE. These results imply that the core protein prefers a subclass of IFN-inducible genes, the promoters of which possess the 2′-5′-OAS-type ISRE. Accordingly, we found that the IFN-inducible double-stranded RNA-specific adenosine deaminase gene promoter, possessing a 2′-5′-OAS-type ISRE sequence, was also efficiently activated by the core protein. The exact mechanism by which the core protein enhances gene expression was not determined, but we could find no effects of core protein on gene expression and phosphorylation status of the components of the JAK-STAT signaling transduction pathway.

Keywords: Hepatitis C virus; Interferon; ISRE; Core protein; 2′-5′-Oligoadenylate synthetase; Double-stranded RNA-specific adenosine deaminase

1. Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis (Choo et al., 1989; Kuo et al., 1989) and can progress to liver cirrhosis and hepatocellular carcinoma (HCC) (Ohkoshi et al., 1990; Saito et al., 1990). HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the Flaviviridae family (Kato et al., 1990; Miller and Purcell, 1990; Tanaka et al., 1995). The HCV genome shows remarkable genetic heterogeneity, and at least six major HCV genotypes, which have been further grouped into more than 50 subtypes, have been identified to date (Bukh et al., 1995; Kato, 2000; Purcell, 1997; Simmonds, 1995). The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acid (aa) residues, and this precursor protein is cleaved by the host and viral proteinases to generate at least 10 proteins in the following order: NH_2-core-envelope 1 (E1)-E2-p7-nonstructural protein 2 (NS2)-NS3-NS4A-NS4B-NS5A-NS5B–COOH (Grakoui et al., 1993; Hijikata et al., 1991, 1993; Lin et al., 1994; Mizushima et al., 1994). These HCV proteins not only play a role in viral replication but also affect a variety of cellular functions (Bartenschlager and Lohmann, 2000;

☆ The nucleotide sequence data of core(1a), core(2a), core(2b), and core(3a) genes first used in this study have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers AB092962 to AB092965, respectively.

* Corresponding author. Tel.: +81-86-235-7385; fax: +81-86-235-7392.
E-mail address: nkato@md.okayama-u.ac.jp (N. Kato).
[1] Present address: First Department of Internal Medicine, Gunma University School of Medicine, Maebashi 371-8511, Japan.

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Kato, 2001). In particular, it appears that the core protein exerts an effect on a variety of cellular functions, including gene expression, signal transduction, and apoptosis (Lai and Ware, 2000).

The core protein is a 21 kDa protein produced by host cell signal peptidase(s) from the N-terminal portion of the precursor protein (Hijikata et al., 1991). The core protein appears to reside primarily in the cytoplasm and is localized on the endoplasmic reticulum and on lipid droplets; however, a portion of the core protein also appears to be localized in the nucleus (Barba et al., 1997; Yasui et al., 1998). Although the core protein binds to the 5′ untranslated region of the HCV genome and E1 protein that from the nucleocapsid in virus particles, the core protein can apparently bind to several cellular proteins, including lymphotoxin-β receptor, TNF receptor 1 and RNA helicase (Bartenschlager and Lohmann, 2000; Kato, 2001; Lai and Ware, 2000). Furthermore, the oncogenic potential of the core protein was identified, using a cell culture system in co-operation with H-ras (Ray et al., 1996) and a system of transgenic mice showing constitutive expression of the core protein (Moriya et al., 1998). Thus, the core protein is currently considered to be a multifunctional protein that plays an important role in persistent infection and hepatocellular carcinogenesis.

We previously examined whether or not the core protein possessing the consensus sequence of genotype 1b can affect signal transduction pathways (NF-κB, AP-1, CRE, etc.) in human non-neoplastic PH5CH8 hepatocytes, which can support HCV replication (Ikeda et al., 1998). Although most signal transduction pathways were not significantly affected by the core protein, we found that the core protein transcriptionally activated the promoters of both exogenous and endogenous interferon (IFN)-inducible 40/46 kDa 2′-5′-oligoadenylate synthetase (2′-5′-OAS) genes (Naganuma et al., 2000). The E1, E2, and NS5A proteins did not activate the 2′-5′-OAS gene promoter. This activation by the core protein occurred in a dose-dependent manner in different human hepatocytes cell lines (PH5CH8, HepG2 and PLC/PRF/5) was further enhanced in cells treated with IFN-α and was suppressed by antisense RNA complementary to core-encoding RNA. Based on these findings, it has been speculated that the core protein could be involved in the maintenance of low levels of virus load by the activation of the 2′-5′-OAS genes (Naganuma et al., 2000).

In this study, we further characterized the core protein as an activator of 2′-5′-OAS genes. We demonstrated that the activation of 2′-5′-OAS genes by the core protein occurred, regardless of HCV genotype and strain, and that the 20 N-terminal aa of the core protein were important for the activation of the 2′-5′-OAS gene. Furthermore, we demonstrated that activation of 2′-5′-OAS genes by the core protein was mediated through the IFN-stimulated response element (ISRE). We also found that a variant type of ISRE, present in the 2′-5′-OAS gene promoter, was preferred for the activation by the core protein and that a double-stranded RNA-specific adenosine deaminase (ADAR1) gene promoter possessing a 2′-5′-OAS type ISRE sequence was efficiently activated by the core protein. We describe here the involvement of the core protein in the IFN signal transduction pathway.

2. Materials and methods

2.1. Cell lines

Non-neoplastic human PH5CH8 hepatocytes, which are susceptible to HCV infection and supportive of HCV replication (Ikeda et al., 1998), were maintained as described previously (Noguchi and Hirohashi, 1996).

2.2. Construction of expression vectors

The core genes belonging to HCV genotypes 1a, 2a, 2b, and 3a were amplified by reverse transcription-nested polymerase chain reaction (RT-nested PCR) using genotype-specific primers and sera (Sugiyama et al., 1995) containing HCV genotypes 1a, 2a, 2b, and 3a, respectively, according to a previously described method using proofreading KOD-plus DNA polymerase (Toyobo, Japan) (Alam et al., 2002; Naganuma et al., 2000). The amplified PCR products were cloned into the *Eco*RI and *Not*I sites of pCXbsr (Georgescu et al., 1999) as described previously (Naganuma et al., 2000). The obtained clones were referred to as pCXbsr/core(1a), pCXbsr/core(2a), pCXbsr/core(2b), and pCXbsr/core(3a), respectively. The core gene belonging to genotype 1b was also amplified by RT-nested PCR using RNA derived from a cancerous portion of HCC (Alam et al., 2002). pCXbsr/core(1b-HCC) was constructed by using the core gene (Alam et al., 2002; accession number: AB062173) encoding a variant core protein with a 6 aa difference from the core(1b-M) protein possessing the consensus sequence of HCV genotype 1b. The nucleotide sequences of the core genes in these expression vectors were determined by Big Dye termination-cycle sequencing using an Applied Biosystem 310 automated sequencer (Applied Biosystems, Norwalk, CT). pCXbsr/core(1b-P)ΔN20 and pCXbsr/core(1b-P)ΔN40, which have 60 and 120 bp deletions, respectively, at the 5′-end of pCXbsr/core(1b-P) were constructed according to the previously described method (Naganuma et al., 2000) and were confirmed by nucleotide sequencing. pCXbsr/core(3a) ΔN20 and pCXbsr/core(3a) ΔN40, which have 60 and 120 bp deletions, respectively, at the 5′-site of pCXbsr/core(3a), were also constructed. An IFN-induced double-stranded RNA-activated protein kinase (PKR) promoter region (−234 to −29) was amplified by KOD-plus DNA polymerase using genomic DNA derived from PH5CH8 cells and a primer set (primers PKRPF and PKRPR; Table 1) designed from the published sequence data (Kuhen and Samuel, 1999; accession no. HSU51035). An amplified PKR promoter was replaced by the 2′-5′-OAS

Table 1
Oligonucleotides used in this study

Oligonucleotide	Sequence	Direction
PKRPF	attatGGTACCACGTGGGTGCCAAGCCCG	Forward
PKRPR	attatAGATCTGCTTCGGGAGAGCTGGTTCT	Reverse
ADAR1F	attatGGTACCGGCTTTCCGAGGAAACGAAAGC	Forward
ADAR1R	attatAGATCTGCGCGCCGGGCCCAAGATGG	Reverse
BSRF	ATGTGGGAGCGGCAATTCGTACG	Forward
BSRR	CATACCACAAGGACTTACCACTCG	Reverse
IFNAR1F	GCTTTCAACTTCTGAGGAACAAATCA	Forward
IFNAR1R	GCTCCCAGTGTAACTCCTGCTG	Reverse
IFNAR2cF	CAGTCAGAGGGAATTGTTAAGAAGC	Forward
IFNAR2cR	GTGCATTATGACTGCACCTGTGAAT	Reverse
STAT1F	AAGTGTTATGGGACCGCACC	Forward
STAT1R	TGCCATTGGTGGACTCCTCC	Reverse
STAT2F	GCGGATCCCTGAGCCAATGGAAATCT	Forward
STAT2R	TGGCTCAGCATCTGTTCT	Reverse
GAPDHF	GATGCTGGCGCTGAGTACGTCG	Forward
GAPDHR	GAGGAGACCACCTGGTGCTCAG	Reverse
CIEA	attatGAATTCCGCCACCATGAGCACAAATCCTAACC	Forward
CBR191	attatGGATCCTCACTAAGCGGAAGCTGGGATGGTA	Reverse
CPN20	attatGAATTCCGCCACCATGGTCAAGTTCCCGGGCGGTGGT	Forward
CPN40	attatGAATTCCGCCACCATGCCCAGGTTGGGTGTGCGCGCG	Forward

The recognition sites of restriction enzymes are underlined.

promoter by cloning to the *Kpn*I and *Bgl*II sites of the p2′-5′-OAS(−159)-Luci (Benech et al., 1987), and resulted in pPKR(−34)-Luci. The nucleotide sequence of the pPKR(−234)-Luci revealed that T at position −215 was substituted with CC in the PH5CH8 cells, most likely due to polymorphism. The ADAR1 gene promoter region (−23 to +38) was amplified by KOD-plus DNA polymerase using genomic DNA derived from HepG2 cells and a primer set (primers ADAR1F and ADAR1R, Table 1) designed from the published sequence data (George and Samuel, 1999). The amplified ADAR1 promoter region was replaced by the 2′-5′-OAS promoter by cloning to the *Kpn*I and *Bgl*II sites of the p2′-5′-OAS(−159)-Luci (Benech et al., 1987). The nucleotide sequence of the obtained pADAR1(−23)-Luci was confirmed to be identical to that in the published sequence data (George and Samuel, 1999).

2.3. Western blot analysis

The FuGENE 6 transfection reagent (Boehringer Mannheim) was used for plasmid DNA transfection into hepatocytes according to the manufacturer's protocols. The preparation of cell lysates at 48 h post-transfection, 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). Two anti-core monoclonal antibodies (Mabs) were used in this study; one (no. 29) was the generous gift from A. Takamizawa, Osaka University, and the other (2ZCP9) was obtained from the Institute of Immunology Co., Tokyo. The epitopes of Mabs 29 and 2ZCP9 were located in the N-terminal region (probably within the first 40 aa, unpublished data) and within amino acid positions 39–74 of the core protein (Takahashi et al., 1992), respectively. Anti-β-actin antibody (AC-15, Sigma) was also used for the detection of β-actin as the internal control. To monitor the expression levels and phosphorylation status of STAT1, STAT2, JAK1 and Tyk2, PH5CH8 cells were infected with retroviruses derived from HCV protein expression vectors (pCXbsr series) (Naganuma et al., 2000). Retrovirus infection was performed by a previously described method (Georgescu et al., 1999). Anti-STAT1, STAT2, JAK1 and Tyk2 antibodies (BD Transduction Laboratories) were used for the detection of STAT1, STAT2, JAK1 and Tyk2, respectively, in PH5CH8 cells at 48 h postinfection. The PH5CH8 cells at 48 h postinfection were cultured for 30 min with or without IFN-α (500 IU/ml, Sigma), and then phosphorylation status (was monitored in cell lysates). Anti-*p*-STAT1(Tyr 701) (Cell Signaling Technology), *p*-STAT2(Tyr 689) (Upstate Biotechnology), *p*-JAK1(Tyr1022/1023) (Sigma) and *p*-Tyk2(Tyr1054/1055) (Cell Signaling Technology) antibodies were used to monitor the phosphorylation status of these proteins. Immunocomplexes on the filters were detected by enhanced chemiluminescence assay (Renaissance; NEN, Boston, MA).

2.4. RT-PCR

RT-PCR for the detection of core mRNA was performed as follows. After the transfection of the core expression vector (2 μg) to PH5CH8 cells (5×10^5), total RNA at 48 h post-transfection was extracted using an ISOGEN extraction kit (Nippon Gene Co., Toyama, Japan). Before RT, the RNA was treated with RNase-free DNase I (Promega) to remove the transfected plasmid DNA, and was then extracted with

phenol-chloroform and precipitated with ethanol. The obtained DNA-free RNA (2 μg) was used as template for RT by SuperScript II (Invitrogen) with an oligo dT primer according to the manufacturer's instructions. One-tenth of the synthesized cDNA was used for the amplification of the core gene using primers BSRF and BSRR (Table 1). Twenty PCR cycles were performed, each cycle consisting of annealing at 60 °C for 45 s, primer extension at 72 °C for 1 min, and denaturation at 94 °C for 45 s; *Taq* DNA polymerase (Takara, Japan) was used. The amplified DNA (215 bp) was detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels. PCR (20 cycles) without the RT step was also performed to confirm the complete removal of the transfected plasmid vector.

RT-PCR for the detection of cellular mRNA was performed. Briefly, total RNA (2 μg) was reverse-transcribed with the SuperScript II using an oligo dT primer. One-tenth of the synthesized cDNA was used for the PCR. The sequences of IFNAR1, IFNAR2c, STAT1, STAT2 and GAPDH, obtained from previous studies (Uze et al., 1990; Novick et al., 1994; Schindler et al., 1992; Bluyssen and Levy, 1997; Hanauer and Mandel, 1984, respectively) were used to design specific primers listed in Table 1. PCR (20 cycles for GAPDH; 25 cycles for STAT1; 30 cycles for IFNAR1, IFNAR2c and STAT2) was performed with Taq DNA polymerase (Takara, Japan), each cycle consisting of annealing at 60 °C for 45 s, primer extension at 72 °C for 2 min, and denaturation at 94 °C for 10 s. The amplified DNA was detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels.

2.5. Dual luciferase reporter assay

The following firefly luciferase reporter vectors were used in this study: p2′-5′-OAS(−159)-Luci (Benech et al., 1987) containing the −159 to +82 region of the 2′-5′-OAS gene, pPKR(−234)-Luci containing the −234 to −29 region of the PKR gene, pGBP-1(−216)-Luci (Lew et al., 1991) containing the −216 to +19 region of the human guanylate-binding protein (GBP-1) gene, pADAR1(−23)-Luci containing the −23 to +38 region of the human *ADAR1* gene, pISRE-Luci (Stratagene) containing five repeats of the consensus ISRE sequence, pISRE(V1)-Luci containing five repeats of ISRE(V1) sequence, and pISRE(V2)-Luci containing five repeats of ISRE(V2) sequence. The dual luciferase reporter assay was carried out as previously described (Naganuma et al., 2000). Briefly, a total of 1.5×10^5 cells was seeded in a six-well plate 24 h before transfection. Then, 0.5 μg of firefly luciferase reporter plasmid (Promega), 2 μg of HCV protein expression effecter plasmid (pCXbsr series) and 1 ng of phRL-CMV (Promega), which expresses *Renilla* luciferase under the control of cytomegalovirus promoter, as an internal control reporter, were transfected into PH5CH8 cells with FuGENE 6. The cells were cultured for 42 h, and then for an additional 6 h with or without human IFN-α (Sigma, 500 IU/ml). The dual luciferase assay was performed according to the manufacturer's protocol (Promega). Triplicate transfection experiments were repeated at least three times in order to verify the reproducibility of the results. A manual Lumat LB 9501/16 luminometer (EG&G BERTHOLD, Bad Wildbad) was used for the detection of luciferase activity.

2.6. Enhanced green fluorescent protein (EGFP)-fluorescence analysis

Several plasmids which express EGFP-core fusion proteins were constructed for the EGFP-fluorescence analysis. pEGFP-core(1b-P), an expression vector for EGFP-fused core(1b-P) protein, was created by inserting the PCR product obtained with primers CIEA and CBR191 (Table 1) on pCXbsr/core(1b-P) as a template, into the *Eco*RI-*Bam*HI sites of pEGFP-C1 (Clontech). The construction of the pEGFP-core(1b-P)ΔN20 and the pEGFP-core(1b-P)ΔN40 was carried out in an analogous way, using the primers CPN20 and CPN40 (Table 1), respectively, instead of the CIEA primer. The pEGFP-core(3a) series was created by a method similar to that used for the pEGFP-core(1b-P) series, using pCXbsr/core(3a) as a template. PH5CH8 cells (5×10^5 cells) were transfected by FuGENE 6 reagent with 2 μg of pEGFP-core(1b-P) or pEGFP-C1, a control vector. pEGFP-core(1b-P)ΔN20, pEGFP-core(1b-P)ΔN40, pEGFP-core(3a), pEGFP-core(3a)ΔN20, and pEGFP-core(3a)ΔN40 were also used as expression vectors. At 48 h post-transfection, the cells were fixed with 4% paraformaldehyde and were photographed under a fluorescence microscope (Axiovert 25CFL, Carl Zeiss).

3. Results

3.1. Activation of the 2′-5′-OAS gene by HCV core protein is a general phenomenon, regardless of genotype and strain

We previously reported that the HCV core(1b-M) protein, possessing the consensus sequence of genotype 1b, specifically activated the IFN-inducible 2′-5′-OAS gene at the transcriptional level (Naganuma et al., 2000). The core(1b-P) protein, showing one amino acid difference at position 70 from that of the core(1b-M) protein, has also been shown to be an activator of the 2′-5′-OAS gene (Naganuma et al., 2000). Since the core protein shows some aa sequence heterogeneity among HCV genotypes (Bukh et al., 1995; Kato, 2000; Purcell, 1997; Simmonds, 1995), we examined whether or not HCV core proteins, other than core(1b-M) and core(1b-P) proteins, are able to activate the 2′-5′-OAS gene. First, we obtained several core genes from sera containing HCVs belonging to genotypes 1a, 2a, 2b, and 3a by RT-nested PCR using proofreading DNA polymerase. Based on the obtained PCR products, pCXbsr/core(1a), pCXbsr/core(2a), pCXbsr/core(2b), and pCXbsr/core(3a) were constructed as the expression

```
                  10        20        30        40        50        60        70
1b consensus  MSTNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATRKTSERSQPRGRRQPIPKARR
Core(1b-M)    ---------------------------------------------------------------------
Core(1b-P)    ---------------------------------------------------------------------Q
Core(1b-HCC)  ---------------------------------------------------------------------Q
Core(1a)      ---------------------------------------------------------------------
Core(2a)      ----------------------------------------------T--------------------D--
Core(2b)      ---------------------------------------------------------------LRD--
Core(3a)      ---L----------I-------------------V-----------------------------------

                  80        90        100       110       120       130       140
1b consensus  PEGRAWAQPGYPWPLYGNEGLGWAGWLLSPRGSRPSWGPTDPRRRSRNLGKVIDTLTCGFADLMGYIPLV
Core(1b-M)    ---------------------------------------------------------------------
Core(1b-P)    ---------------------------------------------------------------------
Core(1b-HCC)  --------------------M---------H--------N-------------------------------
Core(1a)      ----T---------------C-------------------------------------------------
Core(2a)      ST-KS-GK-----------------------------------H----V-------------------V-
Core(2b)      ST-KS-GK------------C--------------T-------H-----------I--SL--------VI
Core(3a)      S---S---------------C------------------N-------------------------------

                  150       160       170       180       190
1b consensus  GAPLGGAARALAHGVRVLEDGVNYATGNLPGCSFSIFLLALLSCLTIPASA
Core(1b-M)    ---------------------------------------------------
Core(1b-P)    ---------------------------------------------------
Core(1b-HCC)  ------V--V--------------------------------------------
Core(1a)      ---------------------------------------------V----
Core(2a)      ------V-------------------------------------V-V--
Core(2b)      ---V--V-----------------------------------I-V-V--
Core(3a)      ---V--V---------A----I-F----------------F---IH--AS
```

Fig. 1. Amino acid sequence alignment of the HCV core proteins used in this study. The sequences are indicated by the single letter code. The aa sequences of core(1b-M) and core(1b-P) proteins indicate the representative sequences derived from MT-2C cells and PH5CH7 cells inoculated with HCV-positive serum 1B-2 (genotype 1b), respectively (Naganuma et al., 2000). The aa sequence of core(1b-HCC) protein indicates the representative sequence derived from a cancerous HCC lesion (genotype 1b) (Alam et al., 2002). The aa sequences of core(1a), core(2a), core(2b), and core(3a) proteins indicate representative sequences derived from sera (Sugiyama et al., 1995) containing HCVs belonging to genotypes 1a, 2a, 2b, and 3a. Amino acids differing from the consensus sequences of genotype 1b are shown. Although the alignments of aa sequences are indicated until aa 191, aa 173, 179, and 182 have been suggested as C-terminal residues of the mature core protein in vivo (Hussy et al., 1996; Yasui et al., 1998).

effector plasmids. In addition, pCXbsr/core(1b-HCC) was also constructed as an expression vector of core(1b-HCC) protein, which was derived from a cancerous HCC lesion. As shown in Fig. 1, core(1a), core(1b-HCC), core(2a), core(2b), and core(3a) proteins differed by 3, 6, 14, 22, and 17 amino acids, respectively, from the core(1b-M) protein. We performed a dual luciferase reporter assay in human hepatocyte PH5CH8 cells, which were cotransfected with p2′-5′-OAS(−159)-Luci, phRL-CMV (internal control), and the individual core protein expression vector (pCXbsr series). As shown in Fig. 2, all core proteins examined activated the 2′-5′-OAS gene promoter, although the level of activation by the core(1a) protein was lower than that of the other core proteins. IFN-α at 500 IU/ml induced further luciferase activity enhancement (approximately two to fivefold) (Fig. 2). To evaluate the degree of enhancement

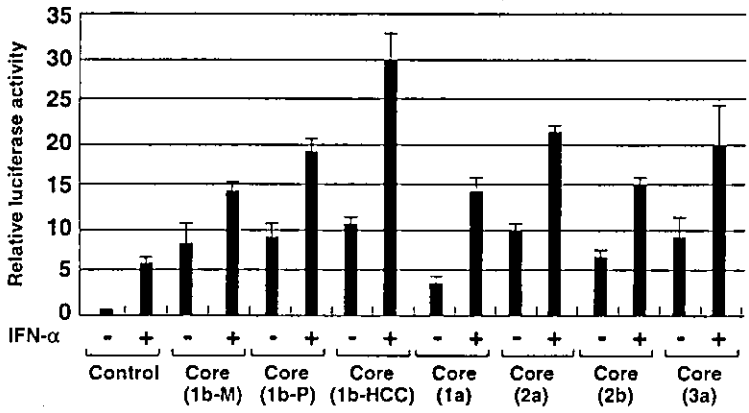


Fig. 2. Effect of core protein production on human 2′-5′-OAS gene promoter activity in PH5CH8 cells treated with and without IFN-α. DNA transfection, IFN-α treatment, and a dual luciferase reporter assay were carried out as indicated in Section 2. The relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). The lysate of cells transfected with expression vector pCXbsr was used as a control.

of luciferase activity by the core protein, we examined the expression level of core proteins in PH5CH8 cells transfected with core expression vectors. Western blot analysis revealed that all core proteins (21 kDa) were expressed at a similar level, although the detected band of core(1a) protein was slightly weaker than that of the other core proteins (Fig. 3A). To further check the expression levels of the core genes, we examined RNA levels from each core expression plasmid by RT-PCR. The result of RT-PCR using the DNase I-treated RNA samples revealed that the level of each core mRNA was roughly equal in the cells transfected with core expression plasmid (Fig. 3B). No attempt was made to quantify the data further at this stage. These results indicate that the activation of the 2′-5′-OAS gene by core protein is a general phenomenon, regardless of HCV genotype and strain. The present findings also suggest that

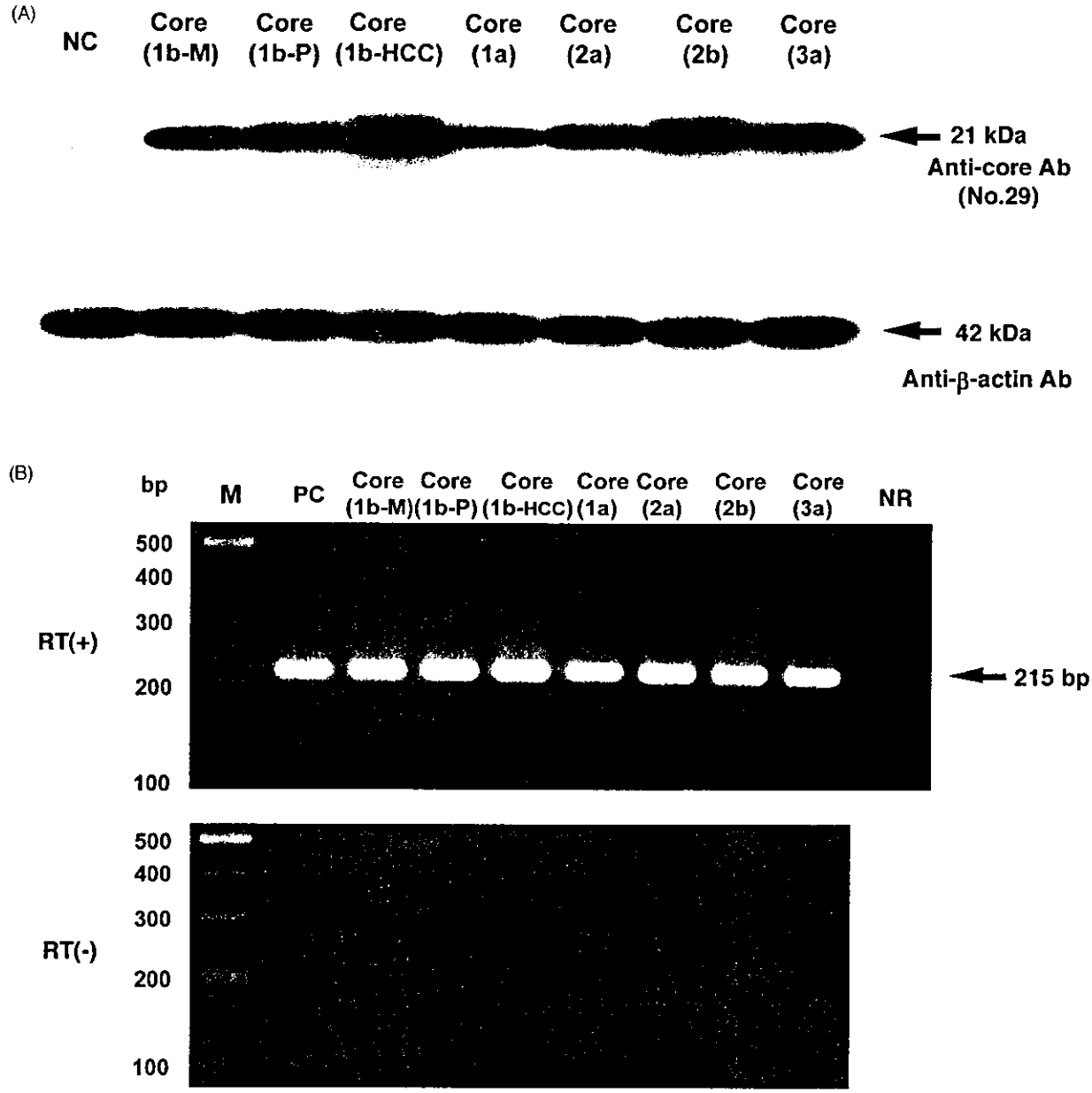


Fig. 3. Expression of core genes in PH5CH8 cells. (A) Western blot analysis of core protein and β-actin expressed in PH5CH8 cells. Core protein and β-actin expressed in the cells were detected on an immunoblot using the anti-core antibody (no. 29; upper portion) and anti-β-actin antibody (AC-15; lower portion). Proteins were resolved by SDS-PAGE (15%). The lysate of cells transfected with core expression vector (pCXbsr series) was used for the detection of the core protein. The lysate of cells transfected with expression vector pCXbsr was used as a negative control (lane NC). (B) Detection of HCV RNA by RT-PCR in samples from PH5CH8 cells transfected with core expression vector (pCXbsr series). At 48 h post-transfection, total RNA was extracted and treated with RNase-free DNase I, and then the core-encoding region of HCV RNA was amplified by RT-PCR. RT-PCR products (215 bp) were detected by staining with ethidium bromide after electrophoresis on a 3% agarose gel. RT-PCR with (upper portion) or without (lower portion) RT (SuperScript II) was performed. Lane M, 100 bp ladder as a size marker; lane PC, PH5CH8 cells inoculated with HCV-positive serum 1B 2; lane NR, no RNA.

the conserved region among core proteins derived from different genotypes is involved in this type of gene activation.

3.2. Twenty N-terminal aa of the core protein are critical for the activation of the 2′-5′-OAS gene

We previously attempted to identify the region responsible for the activation of the 2′-5′-OAS gene promoter using several C-truncated forms and internal deleted forms of the core(1b-P) protein (Naganuma et al., 2000). However, at that time, we failed to identify the essential region, because the C-truncated forms of the core protein were unstable in PH5CH8 cells and the internal deleted forms of the core protein still possessed the ability to activate the 2′-5′-OAS gene promoter (Naganuma et al., 2000). Therefore, we constructed two pCXbsr vectors expressing N-truncated forms [core(1b-P) ΔN20 and core(1b-P) ΔN40] of the core(1b-P) protein. Two additional plasmid vectors [pCXbsr/core(3a) ΔN20 and pCXbsr/core(3a) ΔN40] were also constructed in order to express the N-truncated forms of the core(3a) protein, because within the 40 N-terminal aa, only three aa of the core(3a) protein differ from those of the other genotypes (Fig. 1). Western blot analysis indicated that these N-truncated forms were stably expressed with the expected size in PH5CH8 cells (data not shown). We performed a dual luciferase reporter assay in PH5CH8 cells, which were cotransfected with p2′-5′-OAS(−159)-Luci, phRL-CMV (internal control) and the plasmid vector expressing the N-truncated form of the core protein. In this experiment, PH5CH8 cells were also treated with IFN-α for 6 h at 42 h after transfection. As shown in Fig. 4A, the results clearly showed that the activities of both core(1b-P) ΔN20 and core(1b-P) ΔN40 proteins were completely abolished. A similar result was obtained with core(3a) ΔN20 and core(3a) ΔN40 proteins (Fig. 4B). These results suggest that the 20 N-terminal aa of the core protein are required for the activation of the 2′-5′-OAS gene promoter. In addition, as shown in Fig. 4A, co-expression of core(1b-P) ΔN20 had no effect to the activity of core(1b-P), suggesting that the truncated core protein is not a competitive or dominant-negative inhibitor for IFN-α signaling pathway. To evaluate the possibility that the loss of activity of N-truncated core proteins is due to differences in subcellular localization, we compared the subcellular localizations of N-truncated core proteins [core(1b-P) ΔN20 and core(1b-P) ΔN40] and core(1b-P) protein using the plasmid vectors expressing EGFP-fused core proteins. Core(1b-P) ΔN20 and core(1b-P) ΔN40 proteins the same perinuclear localization as that of the core(1b-P) protein (Fig. 5), although the control EGFP-C1 protein was localized diffusely in the cytoplasm. This result

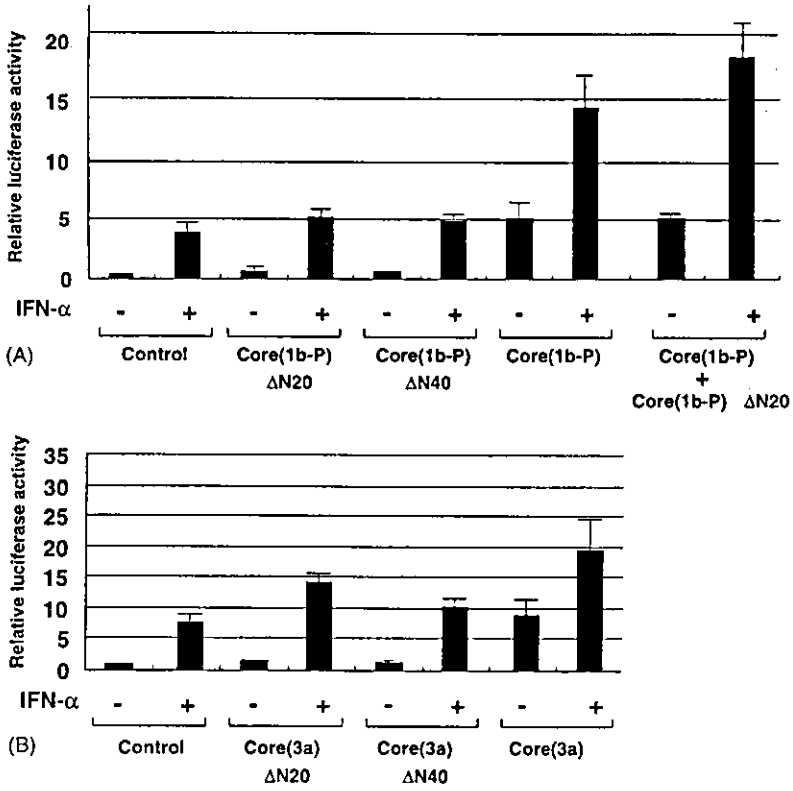


Fig. 4. Deletion analysis of the core protein. (A) Effect of N-truncated core(1b-P) proteins on 2′-5′-OAS gene promoter activity in PH5CH8 cells with and without IFN-α. DNA transfection, IFN-α treatment, and dual-luciferase assay were carried out as indicated in Section 2. The lysate of cells transfected with expression vector pCXbsr was used as a control. (B) Effect of N-truncated core(3a) proteins on 2′-5′-OAS gene promoter activity in PH5CH8 cells with and without IFN-α. DNA transfection, IFN-α treatment, and dual-luciferase assay were carried out as indicated in Section 2. The lysate of cells transfected with expression vector pCXbsr was used as a control.

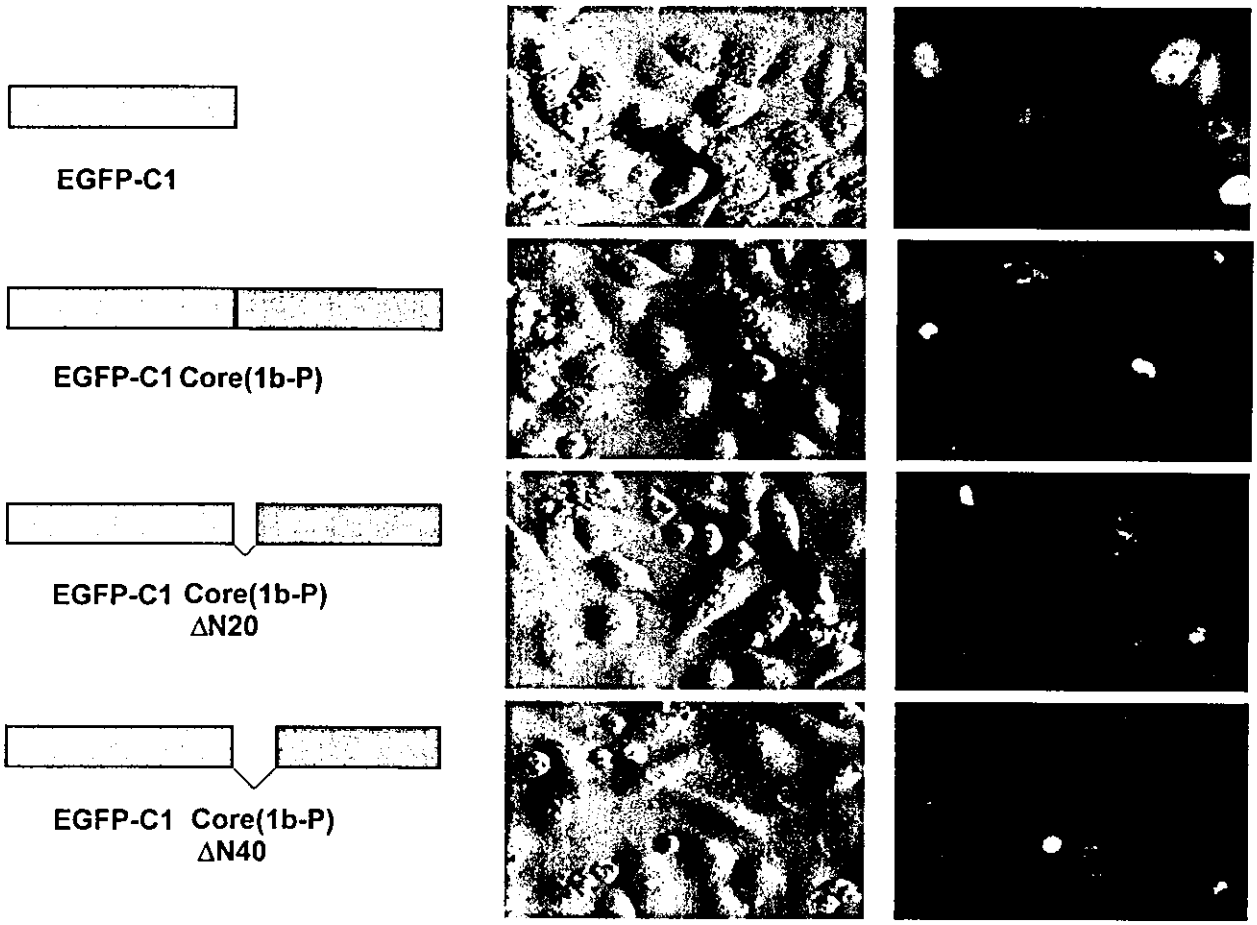
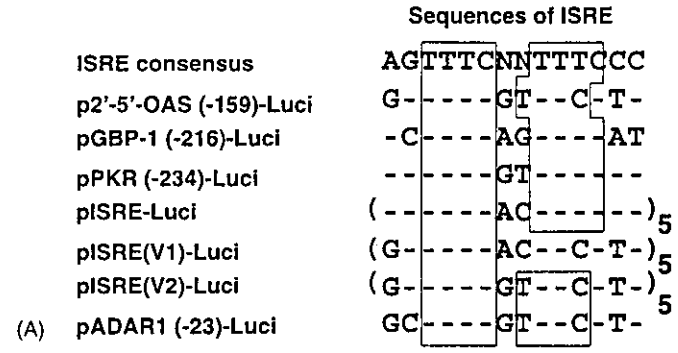


Fig. 5. Subcellular localization of the core protein. The subcellular localization of N-truncated core(1b-P) proteins and core(1b-P) protein was examined by EGFP-fluorescence analysis using plasmid vectors expressing EGFP-fused core proteins. The EGFP-C1 was used as a control. The PH5CH8 cells were transfected with the expression vectors. At 48 h post-transfection, the cells were observed with a fluorescence microscope. The panels at the left show phase contrast images of the cells; the panels at the right show the fluorescence derived from EGFP-C1.

indicates that the 40 N-terminal aa of the core protein do not affect the subcellular localization of the core protein. We confirmed that EGFP-core(1b-P) fusion protein also activated the 2′-5′-OAS gene promoter as well as core(1b-P) protein alone (data not shown). Moreover, these findings suggest that some factor(s), which may interact with the N-terminal portion of the core protein, is (are) necessary for activation of the 2′-5′-OAS gene promoter.

3.3. The core protein prefers the 2′-5′-OAS-type ISRE sequence to the consensus ISRE sequence

Using an ISRE deletion mutant of the 2′-5′-OAS gene promoter, we had previously obtained data suggesting that the activation of the 2′-5′-OAS gene promoter by the core protein is mediated through the ISRE in the promoter (Naganuma et al., 2000). If this proposed mechanism is correct, then the core protein should be able to activate all promoters possessing ISRE. On the other hand, it has already been demonstrated that the consensus sequence of ISRE is AGTTTCNNTTTCCC (N = A, G, C, or T) and that two repeats of TTTC (underlined) are important for ISRE function (Kessler et al., 1988; Stark et al., 1998). However, it has also been found that the ISRE sequences are rather heterogeneous among IFN-inducible gene promoters (Stark et al., 1998). Since the ISRE sequence of the 2′-5′-OAS gene promoter differs at three nucleotides from the those of the consensus ISRE sequence (Fig. 6A), we assumed that the core protein would be able to discriminate between classes of ISRE sequences. To evaluate this possibility, we examined the effect of the core(1b-P) protein on the PKR gene promoter (−234 to −29 region of the gene) possessing one copy of the consensus ISRE sequence and the GBP-1 gene promoter (−216 to +19 region of the gene) possessing a variant ISRE sequence (three nucleotides different from the consensus sequence) (Fig. 6A). As shown in Fig. 6B, the results revealed that these two promoters were marginally activated by the core(1b-P) protein, but not by the NS5A(1b-P) protein. To further characterize this phenomenon, we examined the effects of core protein on the ISRE sequence using pISRE-Luci (Fig. 6A), which possesses five repeats of the consensus ISRE



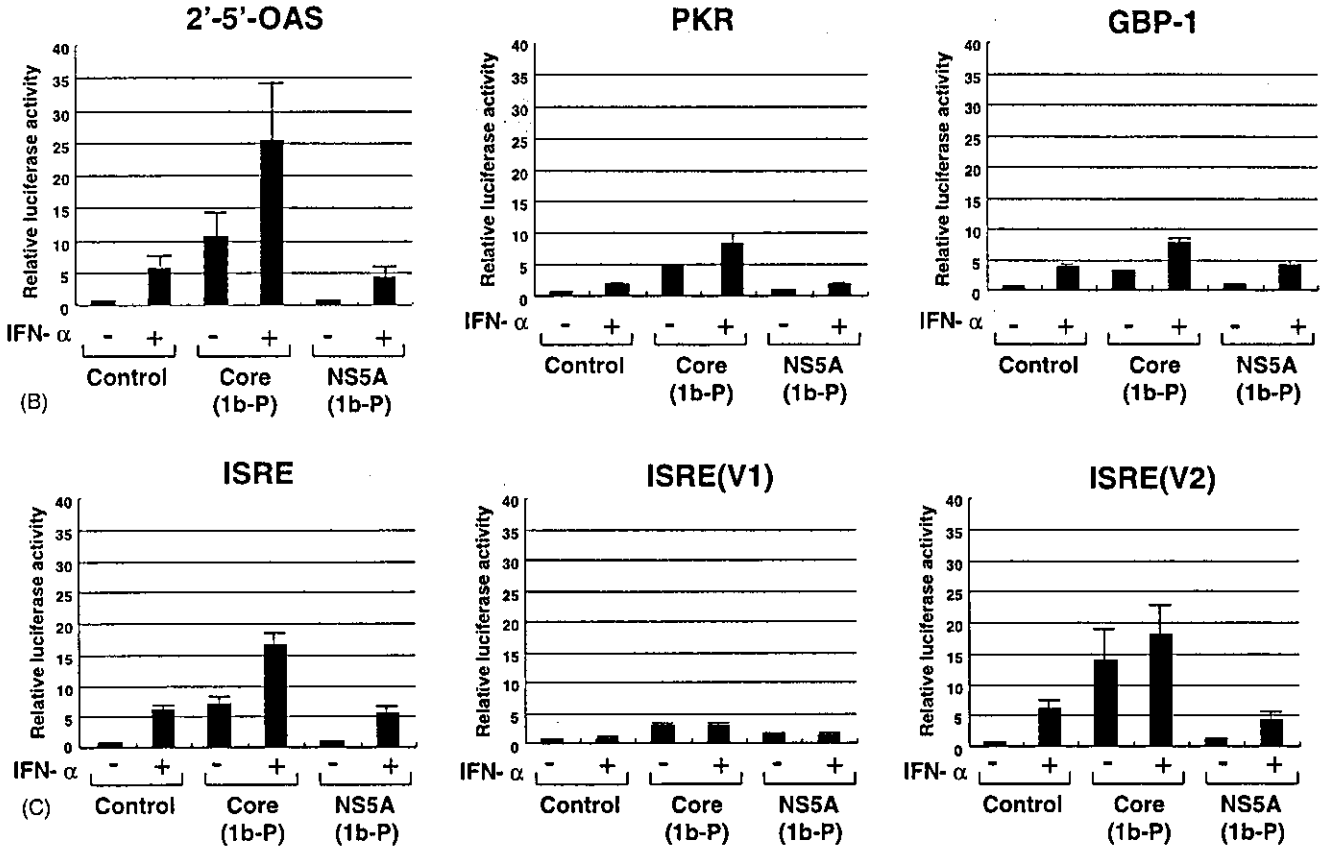


Fig. 6. The core protein prefers the 2′-5′-OAS-type ISRE sequence to the consensus ISRE sequence. (A) Nucleotide sequence alignment of ISREs in the reporter plasmids used in this study. Nucleotides differing from the consensus ISRE sequence are shown. N is equivalent to A, G, C, or T. Two TTTC motifs, essential for the functional ISRE (Lew et al., 1991), are in boxes. (B) The effects of core(1b-P) or NS5A(1b-P) production on human 2′-5′-OAS, PKR, and GBP-1 gene promoter activities in PH5CH8 cells treated with or without IFN-α. DNA transfection, IFN-α treatment, and dual-luciferase assay were carried out as indicated in Section 2. The lysate of cells transfected with expression vector pCXbsr was used as a control. (C) Effects of core(1b-P) or NS5A(1b-P) production on synthetic ISRE promoter activities in PH5CH8 cells with or without IFN-α treatment. pISRE(V1)-Luci was constructed by the conversion from a five repeats of the consensus ISRE sequence (AGTTTCACTTTCCC) of pISRE-Luci (Stratagene) to a five repeats of the ISRE(V1) sequence (GGTTTCACTTCCTC) using a PCR method described previously (Tanaka et al., 2000). This ISRE(V1) sequence is probably a non-functional ISRE, because this sequence does not contain the essential duplicate of TTTC (Kessler et al., 1988), although its sequence is very close to that of the ISRE of the 2′-5′-OAS promoter. Based on pISRE(V1)-Luci, pISRE(V2)-Luci was also constructed using a previously described PCR method (Tanaka et al., 2000). The pISRE(V2)-Luci possessed a five repeats of the ISRE(V2) sequence (GGTTTCGTTTCCTC), which was the same as the ISRE sequence of the 2′-5′-OAS promoter. The nucleotide sequences of pISRE(V1)-Luci and pISRE(V2)-Luci were confirmed by nucleotide sequencing. PISRE-Luci, pISRE(V1)-Luci and pISRE(V2)-Luci were used for the assay. DNA transfection, IFN-α treatment, and dual-luciferase assay were carried out as indicated in Section 2. The lysate of cells transfected with expression vector pCXbsr was used as a control.

sequence (AGTTTCACTTTCCC), as a reporter plasmid. The results revealed that the core(1b-P) protein enhanced the luciferase activity approximately sevenfold, suggesting that transcriptional activation by the core protein is mediated through the ISRE sequence. To confirm this point, we constructed pISRE(V1)-Luci, which possesses five repeats of the ISRE(V1) sequence (GGTTTCACTTCCTC) converted from the consensus ISRE sequence. Although pISRE(V1)-Luci possesses five repeats of an ISRE-like sequence, which approximates the ISRE sequence of the

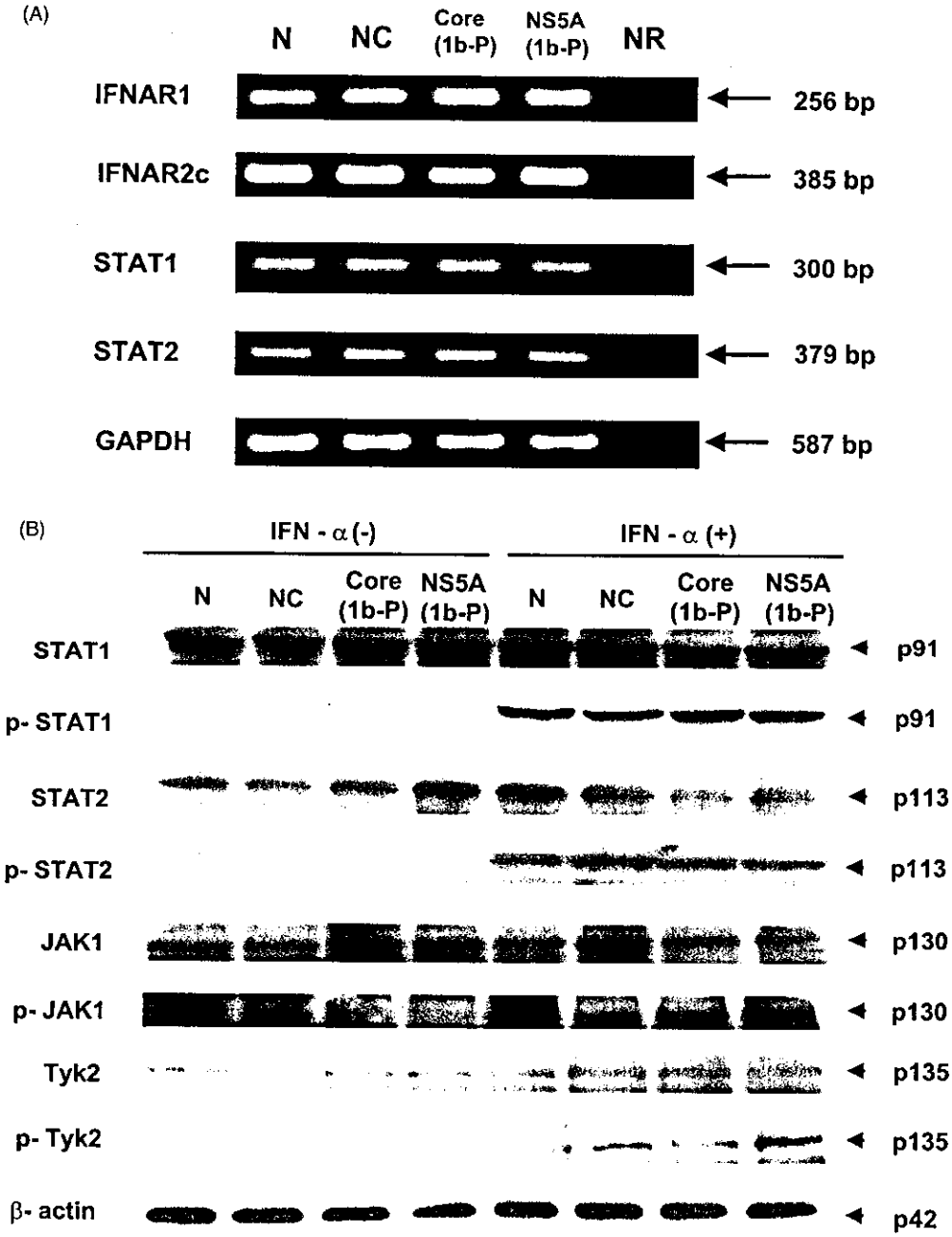


Fig. 7. Effect of core protein to the components involving in the IFN signal transduction pathway in PH5CH8 cells. (A) RT-PCR analysis of IFN-α/β receptors (IFNAR1 and IFNAR2c), transcriptional factors (STAT1 and STAT2) and GAPDH in PH5CH8 cells infected with core or NS5A retrovirus expression vector (pCXbsr series). At 48 h postinfection, total RNA was extracted, and then RT-PCR was performed. RT-PCR products (256 bp for IFNAR1; 385 bp for IFNAR2c; 300 bp for STAT1; 379 bp for STAT2; 587 bp for GAPDH) were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. Lane N, PH5CH8 cells without retrovirus infection; lane NC, PH5CH8 cells infected with retrovirus vector pCXbsr; lane NR, no RNA. (B) Western blot analysis of STAT1, STAT2, JAK1, Tyk2, and β-actin in PH5CH8 cells infected with core or NS5A retrovirus expression vectors (pCXbsr series). At 48 h postinfection, PH5CH8 cells were stimulated with or without IFN-α (500 IU/ml) for 30 min, and then Western blot analysis was performed with antibodies to STAT1, STAT2, JAK1, Tyk2 and β-actin. Phosphorylation status of these proteins (except β-actin) was then determined by Western blot analysis using the *p*-STAT1(Tyr 701), *p*-STAT2(Tyr689), *p*-JAK1(Tyr1022/1023) and *p*-Tyk2(Tyr 1054/1055) antibodies. Lane N, PH5CH8 cells without retrovirus infection; lane NC, PH5CH8 cells infected with retrovirus vector pCXbsr.

2′-5′-OAS gene promoter, a second TTTC motif, essential for the functional ISRE (Lew et al., 1991), is lost in pISRE(V1)-Luci (Fig. 6A). The latter finding suggests that pISRE(V1)-Luci does not function as an ISRE reporter plasmid. Thus, the ISRE-like sequence of pISRE(V1)-Luci did not function as an ISRE, because no enhancement of luciferase activity occurred with IFN-α treatment. Moreover, the enhancement of luciferase activity by the core(1b-P) protein also decreased to one-third of that of the pISRE-Luci (Fig. 6C). However, when pISRE(V2)-Luci, which was converted to possess the same ISRE sequence as that of the 2′-5′-OAS gene promoter from the ISRE(V1) sequence, was used as a reporter plasmid, luciferase activity was again significantly enhanced (approximately 14-fold) by the core(1b-P) protein (Fig. 6C), but not by the NS5A(1b-P) protein. These results indicate that the activation of the 2′-5′-OAS gene by the core protein is mediated through the ISRE, and that the core protein prefers a 2′-5′-OAS-type ISRE sequence to the consensus ISRE sequence.

3.4. Effect of core protein on the components involving in the IFN signal transduction pathway

To examine the mechanisms of activation by the core protein, we evaluated whether it influences the components involving in the JAK-STAT signal transduction pathway. First, we examined the expression levels of IFN-α/β receptors (IFNAR1 and IFNAR2c) and transcription factors (STAT1 and STAT2) in PH5CH8 cells using a RT-PCR method. To increase the efficiency of gene transfer, retrovirus (pCXbsr series) infection was performed in PH5CH8 cells, which showed high efficiency (more than 90%) of gene transfer to the cells (data not shown). The expression of these genes was not enhanced by the transient expression of core protein in PH5CH8 cells (Fig. 7A). As a second possibility, we examined whether the core protein may stimulate the phosphorylation of STAT proteins, JAK1 and Tyk2 kinases involved as downstream effectors in the IFN signal transduction pathway. As shown in Fig. 7B, the results revealed that the phosphorylation status of these proteins was not affected by the transient expression of core protein in PH5CH8 cells treated with or without IFN-α, suggesting that the activation of 2′-5′-OAS gene by the core protein occurs by a mechanism other than those of enhancements of gene expression and phosphorylation status of these signaling components.

3.5. The core protein activates ADAR1 gene promoter

Since the core protein seems to be able to discriminate between ISRE sequences, it was considered that the core protein targets might be the genes possessing the 2′-5′-OAS-type ISRE in the promoter region. The most characteristic feature of the 2′-5′-OAS-type ISRE is a single nucleotide deletion in the NN region of the consensus ISRE sequence (AGTTTCNNTTTCCC), in addition to sequence

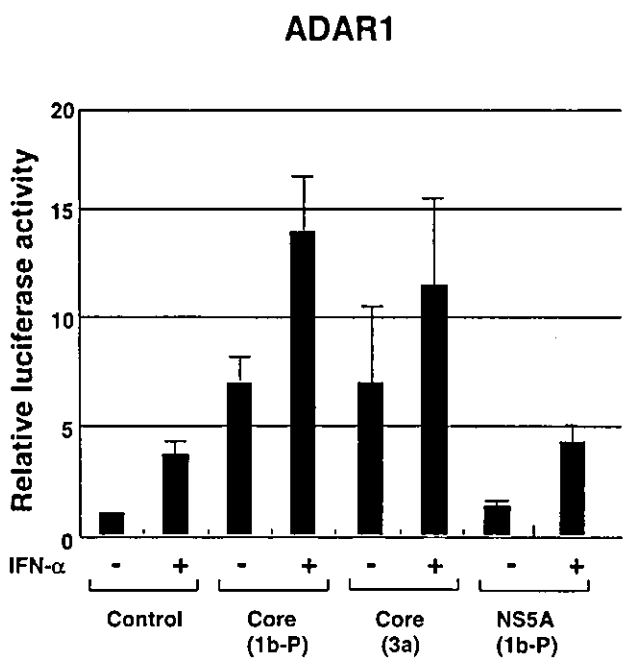


Fig. 8. Effects of core(1b-P), core(3a), or NS5A(1b-P) production on human ADAR1 gene promoter activity in PH5CH8 cells treated with or without IFN-α. DNA transfection, IFN-α treatment, and dual-luciferase assay were carried out as indicated in Section 2. The lysate of cells transfected with expression vector pCXbsr was used as a control.

variations. Using this feature as an index, we compared ISRE sequences of various IFN-inducible gene promoters. It was found that the 2′-5′-OAS-type ISRE was in the promoters of the MxA gene (two of three ISREs), STAT1 gene (one of two ISREs), and the ADAR1 gene. It is of particular interest that the ISRE sequence of the ADAR1 gene promoter was very similar to that of the 2′-5′-OAS gene promoter (Fig. 6A). Based on this information, we assumed that the core protein prefers the ISRE in the ADAR1 gene promoter. Since it appears that the 63 bp minimal promoter (−23 to +38 region of the ADAR1 gene) possessing ISRE was sufficient to drive IFN-inducible transcription (George and Samuel, 1999), we constructed pADAR1(−23)-Luci as a reporter plasmid, as described in Section 2. As shown in Fig. 8, the results revealed that the core(1b-P) and core(3a) proteins could enhance luciferase activity approximately sevenfold via the ADAR1 gene promoter, suggesting that the core protein prefers a 2′-5′-OAS-type of ISRE sequence.

4. Discussion

Based on our previous observation that the core protein transcriptionally activates the 2′-5′-OAS gene (Naganuma et al., 2000), we further characterized the core protein as an activator of the 2′-5′-OAS gene and demonstrated that the gene activation induced by the core protein occurred through an ISRE present within IFN-inducible gene promoters. Furthermore, we found that the core protein prefers the

2′-5′-OAS-type ISRE sequence to the consensus ISRE sequence.

Our observations can shed some light on the mechanism of activation by the core protein. Binding of IFN-α to the receptors, IFNAR1 and IFNAR2c (Stark et al., 1998) causes activation of the receptor activated tyrosine kinases, JAK1 and Tyk2, which in turn recruit and activate the transcription factors STAT1 and STAT2 via tyrosine phosphorylation. Following these activations, STAT1 associates with STAT2, and then the STAT1-STAT2 heterodimer associates with a third DNA binding protein, ISGF3γ-p48, to form ISGF3 (Fu et al., 1992). Finally, ISGF3 binds to the ISRE in the promoter region of the target gene. In this study, we were not able to observe that the core protein had influence on the gene expression and phosphorylation status of the components involving in IFN signal transduction pathway. However, the most likely possibility is that the core protein interacts with STAT1, STAT2, or ISGF3γ-p48, in order to regulate or modify the functions of these proteins. This assumption is likely because ISGF3 can directly bind to the ISRE sequence (Dale et al., 1989; Stark et al., 1998). However, it remains unclear how ISGF3 reaches the nuclei of IFN-treated cells (Stark et al., 1998). The most meaningful contacts between ISGF3 and the ISRE involve STAT1, because it has been determined that the region between aa residues 400 and 500 of STAT1 provide the binding-site specificity to ISRE (Horvath et al., 1995). Therefore, the core protein may modify the function of STAT1 in order to increase the binding affinity to the 2′-5′-OAS-type ISRE sequence. Currently, we are trying to determine whether or not the core protein not only interacts with ISGF3, resulting in the functional enhancement of ISGF3, but also modifies the binding specificity of ISGF3 to the ISRE sequence.

Since the 2′-5′-OAS gene plays a major role in the antiviral activity of host cells by activating ribonuclease L (RNase L) to cleave viral RNA (Baglioni, 1979), the activation of the 2′-5′-OAS gene by the core protein seems to induce unfavorable circumstances for HCV proliferation. It has been reported recently that HCV RNA activates the 2′-5′-OAS/RNase L pathway (Han and Barton, 2002). In contrast, NS5A and E2 proteins have been shown to inactivate PKR, resulting in IFN resistance (Gale et al., 1998; Taylor et al., 1999) and a favorable environment for HCV proliferation. Such contradictory functions of viral proteins have also been observed in other viruses such as human immunodeficiency virus-1 (Schroder et al., 1990) and human T-cell leukemia virus-1 (Mordechai et al., 1995; Schroder et al., 1990). From the present data, taken together with those of other related reports, it is assumed that some viral components act to increase or decrease virus doses by means of the activation or inhibition of host proteins such as 2′-5′-OAS and PKR thereby involving host defense mechanisms. Therefore, HCV core, E2, and NS5A proteins are probably involved in the maintenance of a low steady state of virus in the infected cells, enabling HCV to escape from the host immuno-surveillance system, and facilitating persistent viral infection. To date, it has proven difficult to clarify whether or not the activation of the 2′-5′-OAS gene by the core protein contributes to the degradation of HCV RNA; this ambiguity has been due to the lack of a reproducible and efficient HCV proliferation system (Kato and Shimotohno, 2000). Alternatively, an HCV subgenomic selectable replicon containing NS3–NS5B regions has been recently established using a human hepatoma cell line Huh-7 (Lohmann et al., 1999), and our group also independently established a subgenomic HCV replicon using Huh-7 cells (Kishine et al., 2002). These subgenomic replicon systems will be useful for the functional evaluation of the core protein. However, since our preliminary results using a cDNA expression array showed that the ability of IFN to IFN-target genes in Huh-7 cells was significantly lower than that of PH5CH8 cells, the IFN signal transduction pathway may be partially deficient or abnormal in Huh-7 cells. Therefore, we are currently establishing an HCV subgenomic replicon using PH5CH8 cells for use in a functional assay of the core protein. In the present study, we found that the core protein activated the ADAR1 gene promoter possessing a 2′-5′-OAS-type ISRE; thus, such a subgenomic HCV replicon system will be useful for the functional study of ADAR1, which is the IFN-inducible RNA editing enzyme and is considered to be one of key enzymes in the antiviral activity of IFN (Samuel, 2001).

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RESEARCH ARTICLE

Adenovirus-mediated gene transfer of interferon α improves dimethylnitrosamine-induced liver cirrhosis in rat model

K Suzuki[1,3], K Aoki[2], S Ohnami[1], K Yoshida[1], T Kazui[3], N Kato[4], K Inoue[5], M Kohara[5] and T Yoshida[1]

[1]*Genetics Division, National Cancer Center Research Institute, Tokyo, Japan;* [2]*Section for Studies on Host-immune Response, National Cancer Center Research Institute, Tokyo, Japan;* [3]*First Department of Surgery, Hamamatsu University School of Medicine, Shizuoka, Japan;* [4]*Department of Molecular Biology, Okayama University Medical School, Institute of Cellular and Molecular Biology, Okayama, Japan; and* [5]*Department of Microbiology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan*

Several lines of evidence suggest that interferon (IFN)-α is effective in suppression of liver cirrhosis (LC) as well as hepatitis C virus (HCV) infection, which is a major cause of LC in Japan. However, IFN-α often causes systemic toxicity such as flu-like symptoms, which precludes the IFN-α dose escalation required for clinical efficacy. Since IFN-α is rapidly degraded in the blood circulation, only a small amount of subcutaneously injected IFN-α protein can reach the target organ, the liver. It is expected that on-site IFN-α production in the liver overcomes the limitation of the conventional parenteral IFN-α administration. An adenovirus vector expressing the rat IFN-α gene (AxCA-rIFN) was injected intravenously into rats with dimethylnitrosamine-induced LC. While the subcutaneous IFN-α protein injection led to a transient elevation of the cytokine both in the liver and serum, the vector-mediated IFN-α gene transduction induced a significant amount of IFN-α detected in the liver but not in the serum. The injection of AxCA-rIFN prevented the progression of the rat LC, and improved the survival rate of the treated rats. Although no significant toxicity was noted in the animals, we showed that IFN-α gene expression in the liver can be efficiently downregulated by the Cre/loxP-mediated shut-off system, in case the IFN-α overdose becomes a problem. The study suggested for the first time the advantage and feasibility of IFN-α gene therapy for LC.
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Keywords: *adenovirus; gene transfer; interferon; liver cirrhosis; hepatitis C virus*

Introduction

Liver cirrhosis (LC) is a worldwide problem. In North America and Europe, the prevalence is approximately 1000 per million population, and mortality is approximately 100 per million, which is also the case in Japan.[1–3] Among many etiological factors such as hepatitis virus infection, alcohol and drug abuse, chronic infection by the hepatitis C virus (HCV) is the major cause of LC in Japan and in some southern European countries such as Italy and Spain. HCV-carrier frequency is estimated at 2% worldwide, and chronic hepatitis C progresses to LC and then hepatocellular carcinoma at a high incidence within two or three decades.

While several therapeutic approaches have been investigated for HCV infection, such as iron reduction, antioxidants and antiviral agents (Amantadine, Ribavirin),[4] the most effective therapy is currently considered to be IFN treatment.[2,5,6] Among interferons (IFNs), IFN-α and IFN-β are widely used to treat HCV infection in clinics, whereas IFN-γ does not have a significant antiviral effect. In addition to the antiviral effect, several lines of evidence suggested that IFN-α is also effective in curtailing LC in experimental animal models and clinical studies.[6–13] IFN-α is therefore expected to be particularly powerful in preventing the development of LC in patients with HCV infection.

In the conventional IFN regimen, the recombinant IFN-α protein is subcutaneously injected for several months. However, the overall sustained virologic response rate is still limited to 20–30%.[2,6] Although dose escalation is expected to be effective in a resistant case,[6] it is often impossible because of the severe adverse effects such as flu-like symptoms, leukopenia and mental depression. Since the half-life of the IFN-α protein is 3 h in the blood circulation and only 0.01% of subcutaneously injected IFN-α can reach the target organ (the liver),[14] the administration of IFN-α through a subcutaneous route requires higher IFN-α levels in the serum than in the liver. Therefore, vector-mediated local IFN-α production in the liver may be able to overcome the limitations of conventional IFN-α protein therapy.

In this study, we examined whether a gene therapy strategy of targeting IFN-α gene expression to the liver can be a better alternative to conventional IFN-α protein administration to the subcutaneous space with respect to the distribution of the IFN-α protein, period of expression and development of any adverse effect. An adenovirus vector was employed, since intravenous injection of the vector could express transgenes efficiently in the liver,[15] and the biological effect of the adenovirus-mediated IFN-α gene therapy was also

Correspondence: Dr T Yoshida, Genetics Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
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evaluated in rats with dimethylnitrosamine (DMN)-induced LC.[16–18] This model showed the confinement of the IFN-α expression to the liver and the therapeutic efficacy of suppressing liver injury and fibrosis. Moreover, we demonstrated the usefulness of the Cre-mediated regulation system for shutting off the IFN-α transgene expression efficiently *in vitro* and *in vivo*. The study suggests for the first time the feasibility of IFN-α gene therapy for HCV-associated liver diseases.

Results

Intravenous adenovirus vector injection into rats with DMN-induced cirrhotic liver

In rats with DMN treatment for 3 weeks, all liver specimens from 14 rats, which were killed for examination of LC development (n=5) and vector distribution (n=9), showed the collapse of parenchymal cells and the formation of regenerative nodules separated by extensive fibrous septa, which are similar to the characteristic pathological changes observed in human LC (Figure 1a). To examine the adenovirus-mediated gene transduction efficiency into the liver, AxCA-lacZ or ADVCA-AP was intravenously injected into rats with DMN-induced LC. The expression of lacZ and AP genes was mainly observed in the liver, especially in septal cells but not in the hepatocytes (Figure 1c and inset).[15] Except for a faint expression in the spleen and kidney, X-gal staining was not detected in major organs such as the lung, heart, small intestine and testis. To confirm the preferential transgene expression in the liver, we examined the lacZ gene expression in various organs of cirrhotic rats transduced with AxCA-lacZ by the RT-PCR method.

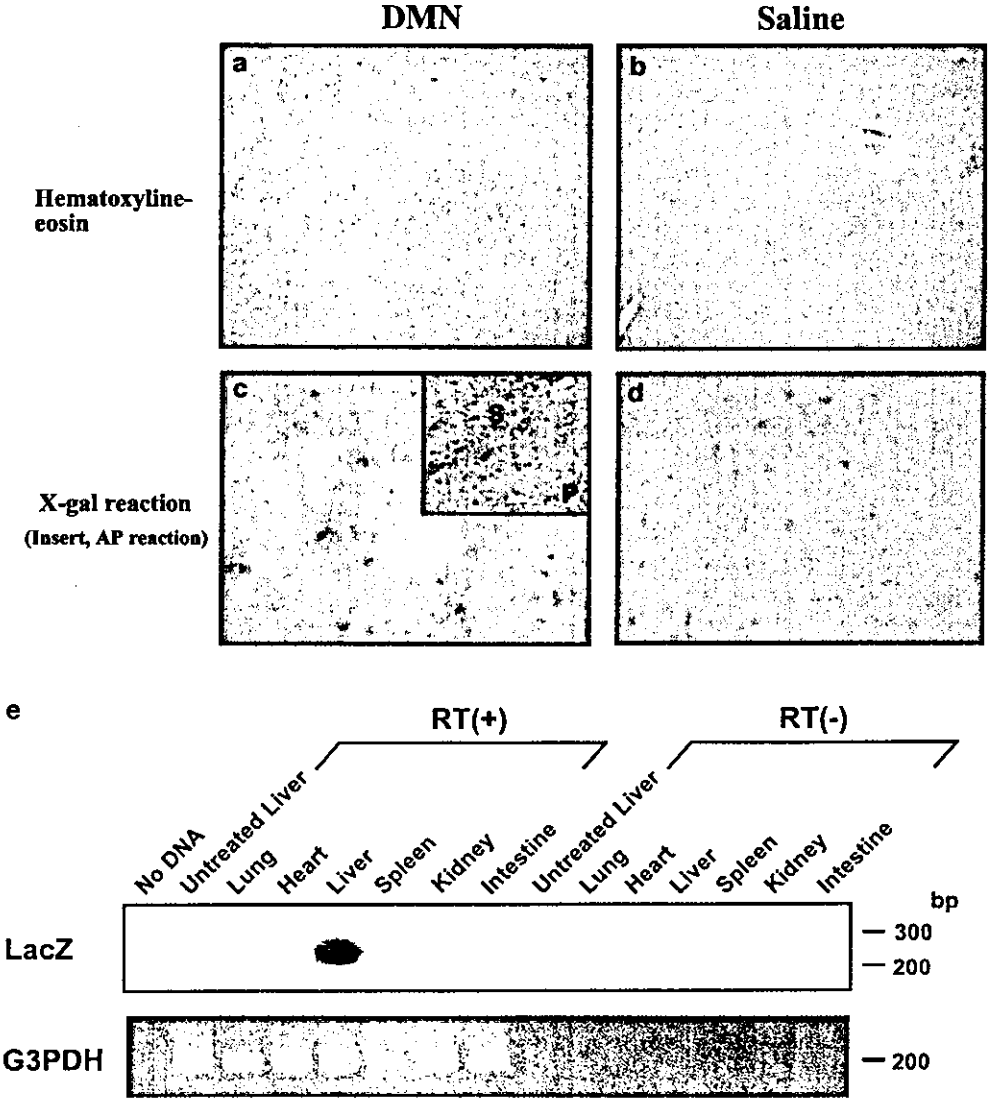

Figure 1 Intravenous injection of AxCA-lacZ into rats. (a, b) Hematoxylin–eosin staining (× 40) of liver from rats treated with DMN (a) or with saline (b) for 3 weeks. (c, d) X-gal reaction (× 100) of liver from rats treated with DMN (c) or with saline (d) for 3 weeks followed by AxCA-lacZ transduction. Rats were infused once via tail vain with AxCA-lacZ (1×10^7 PFU). (c, inset) AP staining of the DMN-induced LC after the intravenous injection of ADVCA-AP (1×10^7 PFU) (× 400). P, parenchyma; S, fibrous septa. Brown-stained cells were observed in fibrous septa. X-gal and AP staining was performed as described.[47,49] (e) RT-PCR analysis of organ distribution of lacZ gene expression 2 days after the intravenous injection of AxCA-lacZ. The untreated normal liver did not show any PCR band.

The expression of the lacZ gene was mainly observed in the liver, and faint expression was detected in the lung, kidney and small intestine (Figure 1e). In normal rats injected with saline, X-gal staining was detected in septal cells as well as in hepatocytes of the liver (Figure 1d) and faint staining was observed in the spleen. The lacZ gene expression data were consistent with the immunostaining of IFN-α in the rats with DMN-induced LC or normal rats after the injection of AxCA-rIFN (data not shown).

IFN-α levels in the liver and serum following AxCA-rIFN injection

Based on *in situ* evidence suggesting the preferential expression of INF-α in the liver, the IFN-α levels and time course of its clearance were measured in the liver and serum following the intravenous injection of AxCA-rIFN. In total, 120 IU of the rat IFN-α per gram of tissue was produced in the liver of DMN-injected rats 3 days after the injection of AxCA-rIFN, whereas no IFN-α was detected in the serum (Figure 2). On the other hand, a significant level of rat IFN-α transiently appeared in the liver and in the serum as well by the subcutaneous injection of 1×10^5 IU of the recombinant rat IFN-α (Figure 2), which corresponds to the dose range of conventional IFN-α treatment in clinics. To assess the toxicity of the adenovirus-mediated IFN-α gene therapy, hepatic transaminases were measured in DMN-injected rats 2 days after the injection of AxCA-rIFN. No significant difference was observed among the rats injected either with PBS, AxCA-lacZ or AxCA-rIFN (AST; 257.0 ± 135.2, 267.7 ± 171.7, 264.2 ± 70.9, ALT; 226.3 ± 122.1, 233.3 ± 135.9, 211.7 ± 64.3, respectively), suggesting that the IFN-α gene expression did not cause significant hepatic toxicity.

Improvement of LC in DMN-treated rats by intravenous injection of AxCA-rIFN

We examined whether hepatic INF-α expression could improve liver fibrosis in a DMN-injected rat, because the antifibrotic effect of IFN-α protein has been shown in experimental animal models and clinical studies.[6–13] The AxCA-rIFN-treated rats survived significantly longer than the rats injected with either AxCA-lacZ or PBS (P=0.0001), and five of nine IFN-α-transduced rats survived more than 100 days (Figure 3). Histological examination clearly revealed less fibrous connective tissue components in Glisson's sheath and pseudolobule formations in the livers of control and AxCA-rIFN-transduced rats than in the liver of the AxCA-lacZ-transduced rats, in which the extensive formation of fibrotic septa and thickened reticulin fibers joining central areas were observed (Figures 1a, 4a and b). The collagens were detected by Sirius red staining[19,20] in the bridging fibrous septa in the liver of AxCA-lacZ-transduced rats, whereas the liver of AxCA-rIFN-transduced rats was almost devoid of collagens (Figure 4c and d). The expression of α-smooth muscle actin was reduced in the activated stellate cells of the DMN-treated rats transduced with AxCA-rIFN (Figure 4e and f). To quantitatively assess the liver fibrosis, we measured the hydroxyproline content of the liver[21] and serum levels of transaminases (AST and ALT). The hydroxyproline and transaminases in AxCA-rIFN-treated rats 70 days after the injection were significantly lower than those in AxCA-lacZ- or PBS-injected rats 10 days after the injection (Table 1). Since it is known that the DMN-induced histopathological changes and hepatic transaminase elevation do not regress spontaneously in the rat LC model,[16,18,22] our observation suggested that IFN-α

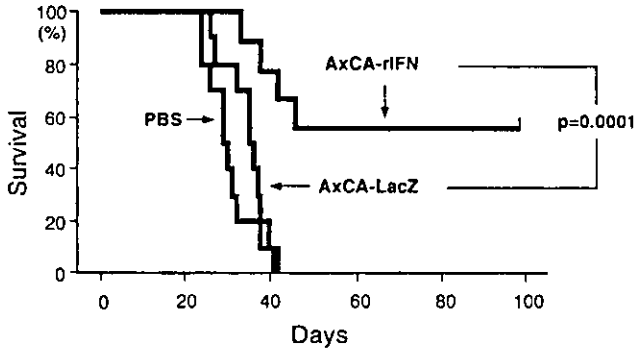

Figure 3 Survival of rats transduced with AxCA-rIFN. DMN-treated rats were intravenously injected once with PBS (n=10), AxCA-rIFN (1×10^7 PFU, n=9), or AxCA-lacZ (1×10^7 PFU, n=10) after treatment of DMN for 3 weeks. Life-table analysis is presented as a Kaplan–Meier plot.

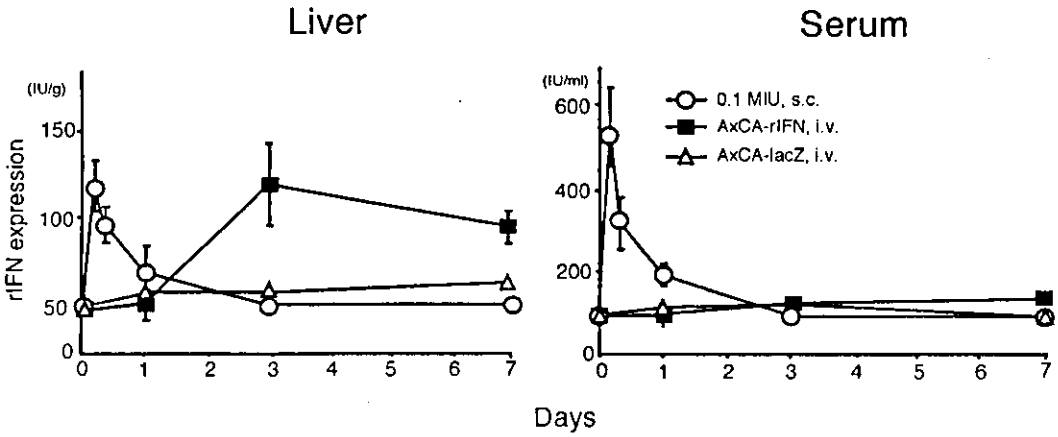

Figure 2 Time course of the rat IFN-α expression in the liver and serum. After treatment with DMN for 3 weeks, rats were injected once with recombinant rat IFN-α (open circle; 0.1 MIU, s.c., n=3), AxCA-rIFN (solid square; 1×10^7 PFU, i.v., n=3), or AxCA-lacZ (open triangle; 1×10^7 PFU, i.v., n=3).

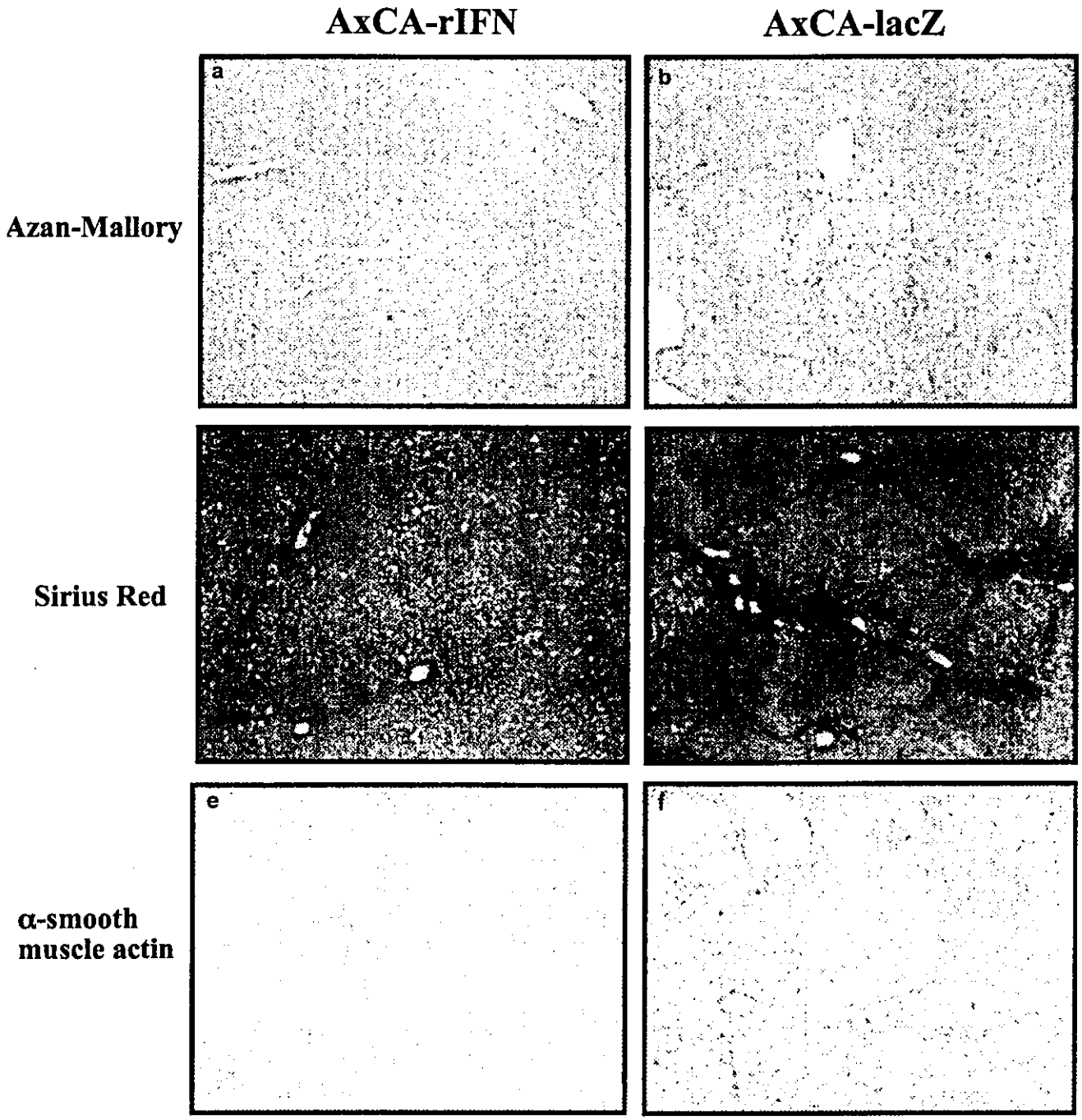

Figure 4 Improvement of DMN-induced rat LC by the injection of AxCA-rIFN. (a, b) Liver sections stained with Azan-Mallory (× 40). (c, d) Differential staining of collagenous and noncollagenous proteins with Sirius red and Fast green (× 100). (e, f) Immunohistochemical staining of the liver using antibody against α-smooth muscle actin (× 40). (a, c, e) A rat injected with AxCA-rIFN, and survived from the DMN-induced LC. Killed 70 days after the vector injection. (b, d, f) A rat treated with DMN and then injected with AxCA-lacZ. Killed 10 days after the vector injection.

gene expression can rescue the rats from DMN-induced hepatic damage and improve the histopathology of LC.

Expression of MMPs and TGF-β in the liver transduced with AxCA-rIFN

To examine the antifibrotic action of IFN-α gene therapy, the expression of metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMP) related to matrix degradation during liver fibrosis was analyzed by the RT-PCR method (Figure 5). Interstitial collagenase (MMP-13) has been considered an essential enzyme for collagenolysis in liver fibrosis, and it was reported that expression of MMP-13 is elevated at peak fibrosis and drops rapidly in the recovery periods.[23,24] The MMP-2, which is stimulated by TGF-β, is necessary for proliferation and infiltration of hepatic stellate cells in the process of fibrosis formation.[25–27] The expression of TIMP-1