Journal of Cellular Biochemistry Supplement 36:191–200 (2001)

Interferon Regulatory Factor 1 Promoter Polymorphism and Response to Type 1 Interferon

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Abstract The clinical success of interferon-treatment has been found to vary in different individuals. To explain this, we hypothesized that responses to type 1 interferons could be partly determined by interferon regulatory factor-1 gene transcription, because the latter is an important transcription factor in the interferon system. We demonstrated that the antiproliferative effect of type 1 interferons on human liver cancer cells correlates with levels of transcription of the interferon regulatory factor-1 gene in parallel with those of $p21^{WAF-1}$ expression. Here, we investigated whether mutations in the interferon regulatory factor-1 gene cause different responses to type 1 interferons. DNA from several human liver cancer cell lines and peripheral blood mononuclear cells was investigated. Nucleotide sequences of the interferon regulatory factor-1 gene and polymerase chain reaction products of its upstream region were determined directly and after cloning. The promoter activity of the upstream region of this gene was measured by the luciferase reporter assay. We found 4 point mutations in the upstream ($-1 \sim -495$) region, and the luciferase promoter assay demonstrated that these mutations did modify promoter activity. Analysis of DNA from healthy volunteers showed that these mutations are single nucleotide polymorphisms. These results suggest that single nucleotide polymorphisms of the interferon regulatory factor-1 promoter contribute, at least in part, to determining responses to type 1 interferons. J. Cell. Biochem. Suppl. 36:191–200, 2001.

Key words: interferon; IRF-1; single nucleotide polymorphism; response to IFNs; promoter activity

Interferon (IFN)-induced signaling pathways are known to be complex. In the regulation of IFNs in response to viral infection, viruses can activate the type I IFN (IFN-α and β) genes in their transcription levels, and in turn, IFNs stimulate a variety of signaling pathways that activate genes involved in antiviral activities in their transcription levels [Castelli et al., 1997]. The IFN regulatory factor (IRF) genes represent some of these and comprise positive and negative transcription factors involved in the activation of the type I IFN genes [Fujita et al., 1989] by regulating a variety of IFN-inducible genes [Reis et al., 1992]. IRF-1 and IRF-2 were originally identified as transcription factors that bind to the same sequence, IFN-stimulated response element (ISRE), in the promoters of type I IFN and other cytokine-inducible genes [Miyamoto et al., 1988; Harada et al., 1989; Pine, 1992]. Recently, the precise mechanism of binding between the IRF-1 factor and ISRE has been clarified [Escalante et al., 1998]. These two genes, IRF-1 and IRF-2, exhibit an approximately 60% homology in the N-terminal region that confers DNA binding specificity. They are, however, functionally distinct in their regulation of growth, cell cycle progression and apoptosis [Tamura et al., 1995]. The IRF-1 gene may function as a tumor suppressor gene, while the IRF-2 gene may function as a counter-regulatory gene. Malignant transformation induced by c-*myc* or *fos* can be suppressed by IRF-1 gene transfection [Tanaka et al., 1994]. IRF-2-induced transformation can be reversed

Abbreviations used: IFN, interferon; IRF, interferon regulatory factor; ISRE, interferon-stimulated response element; SNP, single-nucleotide polymorphism; Th, T-helper.

Grant sponsor: Japanese Ministry of Education, Science and Culture; Grant sponsor: Keio University.

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Received 2 August 2000; Accepted 7 December 2000

This article published online in Wiley InterScience, March 22, 2001.

by concomitant overexpression of the IRF-1 gene [Harada et al., 1994b]. Point mutations in and/or deletion of the IRF-1 gene, and exon skipping in IRF-1 mRNA, have been frequently detected in hematopoietic malignancies [Willman et al., 1993a; Harada et al., 1994a]. The cell cycle regulator $p21^{WAF-1}$ is a target gene for IRF-1 and IRF-2, which can be regulated by the balance between the levels of these two factors [Tada et al., 1998; Coccia et al., 1999]. These observations suggest a potential link between IRF-1 gene transcription and its anti-oncogenic potential.

A growth inhibitory effect of IFN-α has been demonstrated in various normal and malignant cells in vitro including human liver cancer cells. However, the precise mechanism of growth inhibition is not well understood. Yano et al. [1999] showed that IFN-α inhibits cell cycle progression of several liver cancer cell lines with or without inducing apoptosis, but its growth suppressive effect varies between the cell lines regardless of the level of IFN-α receptor 2 expressed on the cell surface. This study indicates that the growth suppression levels induced by IFN-α do not depend on the expression levels of the receptor; differences in the efficiency of the signal transduction pathways of the type I IFNs have been postulated to account for this. We have recently studied the anti-proliferative effect of the type I IFNs on several human liver cancer cell lines [Tada et al., 1998]. IFN-α dose-dependently inhibited cell growth, whereby these cell lines could be divided into two types based on sensitivity to IFN-induced growth inhibition. The degree of growth inhibition of cancer cells seemed to depend on their level of $p21^{WAF-1}$ induction, which regulates cell cycle arrest, and these levels were found to parallel the IRF-1 transcription levels. IRF-1 gene rearrangement by BamHI digestion was observed in the less sensitive cell lines, but no significant difference in point mutations was found between the cell lines in their whole cDNA sequences. These results suggest that growth inhibitory efficiency of the type I IFNs is associated with IRF-1 gene abnormalities that modify the IRF-1 transcription levels.

Single-nucleotide polymorphisms (SNPs) are the most common form of human genetic variation. This variation may contribute to the sensitivity of the patient to various medicines, but the molecular mechanism underlying the genetic heritability of the SNPs is largely unknown [Marth et al., 1999]. Sequence inspection suggests that some of the variants will have a functional impact on either expression or activity levels of enzymes [van't Hooft et al., 1999]. Recently, the promoter of the human IRF-1 gene has been isolated and characterized [Sims et al., 1993]. The IRF-1 promoter is very rich in GC and contains various nuclear factor-binding domains such as CpG islands, GC boxes, putative NF-κB binding sites, and many positive regulatory domains for IFNs. These observations suggest a relationship between SNPs and the modification of IRF-1 gene transcription levels by IFN stimulation.

In the present report, we investigated SNPs of the IRF-1 gene in human liver cancer cell lines and DNA from healthy volunteers. We focused on the relationship between SNPs and differences in IRF-1 promoter activity in the different cell lines. Finally, we show that the IRF-1 promoter activity was different between the promoter regions with different SNPs.

MATERIALS AND METHODS

Cell Lines and DNA Extraction

We investigated HCC-T [Saito et al., 1989], HCC-M [Watanabe et al., 1983] and PLC/PRF/5 [Alexander et al., 1976]; the latter two cell lines were used as DNA sources for the IRF-1 promoter. HLE [Dor et al., 1975], HLF [Dor et al., 1975], HepG2 [Aden et al., 1979], and Chang cells [Tada et al., 1998] were used in some experiments. These cell lines showed different sensitivities to the growth inhibitory effects of IFN-α. We also investigated DNA of peripheral blood mononuclear cells from ten healthy volunteers to confirm SNPs in the IRF-1 gene. DNA was extracted according to the procedure described previously [Saito et al., 1996].

Verification of the IRF-1 Gene Rearrangement by BamHI Digestion

Because DNA rearrangement of the IRF-1 gene after digestion with BamHI was observed, but no significant contributing mutation was detected in the cDNA sequence in our previous study, we investigated intron DNAs in this study. The IRF-1 introns of three cell lines (HCC-T, HCC-M, and PLC/PRF/5) were amplified using primers designed in the exons (Fig. 1) and BamHI site in each PCR product was examined.

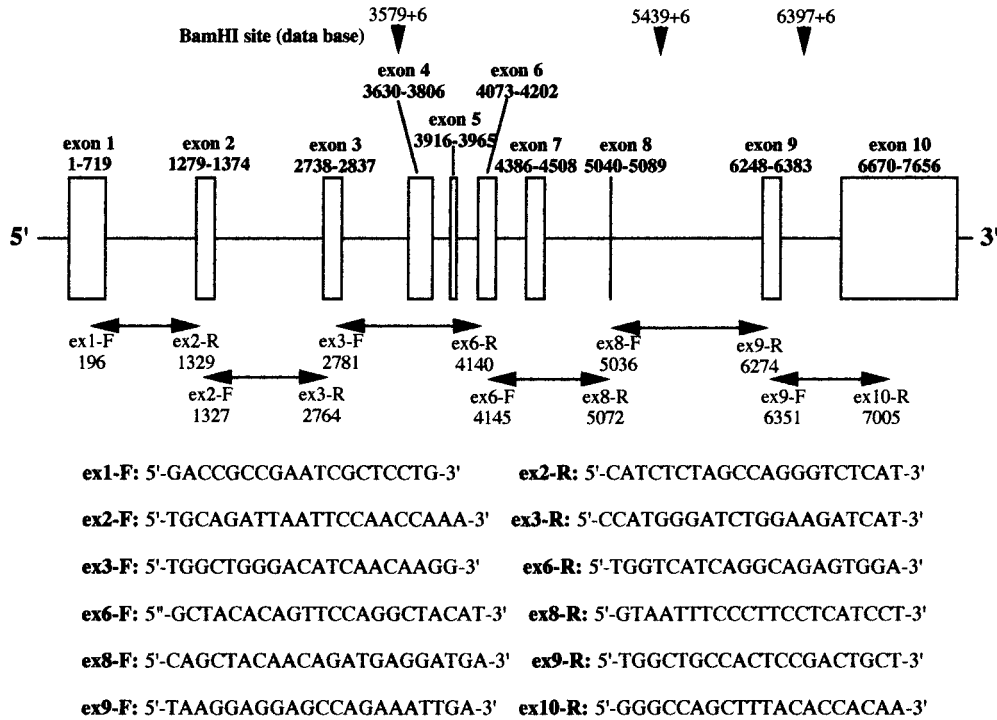

Fig. 1. Genomic structure of the IRF-1. PCR primers for the investigation of introns designed in the exons are shown in this figure. Each nucleotides of the primers are shown in the lower part of the figure. BamHI cleavage sites (from database) are shown by arrrow-heads in the upper part of the figure.

Amplification of the IRF-1 Promoter

The human IRF-1 promoter was amplified by PCR. The PCR primers were synthesized on an Applied Biosystems model 392 (Applied Biosystems), lyophilized, resuspended in PBS, and quantified by spectrophotometry. The primers were designed from the data base (GenBank No. X53095) to amplify 669 bp of the IRF-1 promoter (nucleotide position at -492 to $+177$). These primers contained MluI and XhoI sites for cloning to the vector; IRF-1P-F: 5′-GGG ACG CGT AAG CTT GAG GAG CCA GGC TGC CAG T-3′ and IRF-1P-R: 5′-GGG CTC GAG CTG CAG TGA GGG CGC GCG GAG C-3′. This promoter region has GC-rich sequences, so that we used the Advantage®-GC 2 PCR Kit and the Advantage®-GC 2 Polymerase Mix (Clontech, Palo Alto, CA), which facilitate PCR amplification of virtually all GC-rich sequences that are difficult to amplify by conventional methods. The reaction mixture consisted of 1 μl of sample DNA, 4 μl of 5XPCR buffer, 2 μl of 5M GC-Melt (an additional reagent further weakening base-pairing in GC-rich sequences), 0.2 μl of each primer, 0.4 μl of 50XdNTP mixture, 0.4 μl of 50XAdvanTaq DNA polymerase, and 11.8 μl of distilled water. The reaction mixture was incubated at 94°C for 3 min, and then 35 cycles of PCR reaction (94°C for 15 sec and 68°C for 1 min) was performed followed by further 3 min incubation at 68°C. The PCR product was purified by Sephacryl S-400 HR (Amersham Pharmacia Biotech, Tokyo, Japan) and ligated to pGL3/XhoI-MluI (Promega, Madison, WI) using DNA Ligation Kit Ver. 2 (Takara Shuzo Co.). DNA sequencing was performed using the sequence primers in the genome of plasmid vector (5′-CTA GCA AAA TAG GCT GTC CC-3′ and 5′-CTT TAT GTT TTT GGC GTC TTC CA-3′), followed by comparison with the data base (GenBank) as described above. The sequences of PCR products were examined several times and we confirmed the IRF-1 promoter sequences of DNAs from HCC-M and PLC/PRF/5 that showed different sensitivity to IFN-α-stimulation [Tada et al., 1998].

Luciferase Reporter Assay of the IRF-1 Promoter

Transfection experiments and luciferase reporter assays were performed using the Dual-Luciferase® Reporter Assay System (Promega)

and two promoter regions obtained from HCC-M and PLC/PRF/5 cells. We selected more than one cell line as transfection targets because IFN-stimulated signal transduction pathways might differ between various cell types and the difference in sensitivity to IFN may be correctly evaluated only by using different lines. We first determined optimal transfection conditions for HCC-M and PLC/PRF/5 cells using the FUGENE 6 transfection reagent (Clontech). HCC-M cells were plated at a density of 1.0×10^5 or 2.0×10^5/35 mm culture dish in 2 ml of growth medium (RPMI-1640 with 5% fetal bovine serum [FBS]). PLC/PRF/5 cells were plated at a density of 1.5×10^5 or 3.0×10^5/35 mm culture dish in 2 ml of growth medium. The cells were at 50 – 80% of confluence on the day of the experiment. We added 3 to 6 μl of FUGENE 6 directly into 100 μl of serum-free medium with 12.5 mM Hepes buffer in one tube per 35 mm culture dish, and incubated at room temperature for 5 min. This FUGENE 6 preparation was dropped onto 1 – 2 μg of DNA (0.1 – 2.0 μg/μl) and gently mixed by tapping. The mixture of FUGENE 6 and DNA was then incubated at room temperature for 15 min, and dropped into a 35 mm culture dish. The dish was incubated in a CO_2 incubator. Sampling was carried out at 24 or 48 h after the transfection, and a medium was changed 3 – 5 h after the transfection. pRL-TK vector (Promega) was used as a control that contains a herpes simplex virus thymidine kinase promoter in the upstream region of the Renilla (*Renilla reniformis*) luciferase gene. The IRF-1 promoter regions obtained from both HCC-M and PLC/PRF/5 were cloned into a pGL3-Enhancer vector (Promega), and IRF-1-promoter-luciferase reporter constructs (HCC-M-pGL3 and PLC/PRF/5-pGL3) were obtained. In each experiment, transfection was performed in triplicate.

Cells were lysed in Passive Lysis Buffer®, which is formulated to minimize the effect of lysate composition on coelenterazine autofluorescence, and analyzed for luciferase activity by using a Dual-Luciferase Reporter Assay kit (Promega). In this reporter assay system, the activities of firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*) luciferases are measured sequentially from a single sample. Both luciferase activities were determined in the linear range for each assay, and the activity of the co-transfected "control" reporter provides an internal control, which serves as the baseline response. pGL3 without promoter co-transfected with pRL-TK (pGL3/pRL-TK) was used as a control and the luciferase activity obtained by transfection with HCC-M-pGL3/pRL-TK and PLC/PRF/5-pGL3/pRL-TK with and without the type I IFN (1000 IU/ml of IFN-α2a, a gift from Nippon Rosche Co., Ltd., Tokyo, Japan and IFN-β, a gift from Toray Industries Inc., Tokyo, Japan) was measured. The firefly luciferase activity (photon signals) was normalized by the Renilla luciferase activity (photon signals) (Normalized inducibility). Photon intensity due to luciferase activity was measured by an ATTO-luminometer AB-2100 (Atto, Tokyo, Japan).

Direct Sequence of the PCR Products

The PCR products in some experiments were directly sequenced using an ABI BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) according to the manufacturer's instructions, and an ABI PRISM 377XL DNA Sequencer (PE Applied Biosystems). Nucleotide sequence of the PCR product was analyzed using affiliated software in the DNA sequencer (Sequence Analysis 3.3, Basecaller SemiAdaptive, and Sequence Navigator 1.0.1; PE Biosystems) and also FASTA or TFASTA as described previously [Saito et al., 1996]. DNA sequencing of the IRF-1 promoter was done with primers in that region; IRF-1-5′/U: 5′-AGG CTG CCA GTC GGG AGA TTC-3′ and IRF-1-5′/L: 5′-GCC ACC GAG CAA TCC AAA CAC-3′.

Putative secondary structure of the IRF-1 promoter region postulated from their nucleotide sequences was made using Genetyx-Mac Ver 8.0 (The Software Development Co., Ltd., Chiba, Japan).

RESULTS

SNPs in Intron 8

Amplification of the introns of the IRF-1 gene from the three human liver cancer cell lines showed no difference between them in the size of PCR products, suggesting absence of significant deletions, insertions, or translocations in the IRF-1 introns. To verify the cause of DNA rearrangement in the IRF-1 gene when DNA was digested with BamHI, we digested the PCR products and examined modifications of three specific BamHI sites in the IRF-1 genome, as predicted from the database (Fig. 1). Figure 2 shows the BamHI digestion pattern of the PCR

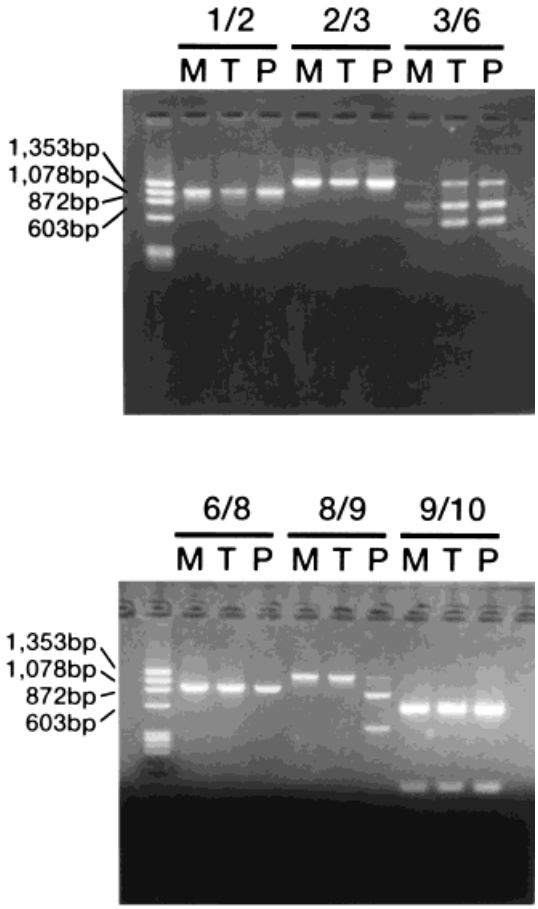

Fig. 2. Agarose gel electrophoresis of the PCR products after digestion with BamHI. The PCR products of the introns of IRF-1 gene as shown in Figure 1 were digested with BamHI and electrophoresed on a 2% agarose gel. No significant deletion, insertion, and translocation was detected. In the ex8-F/ex9-R region in this figure, there is one band in HCC-M and HCC-T, but 2 bands are detected in PLC/PRF/6. There is a BamHI site in this region (see Fig. 1) and the result shows that deletion of this BamHI site exists in HCC-M and HCC-T and PLC/PRF/5 has a wild-type sequence. 1/2, PCR products using ex1-F and exR-R; and other numbers are also those using the primer sets indicated in Figure 1. M, T, and P, PCR products from DNA of HCC-M, HCC-T, and PLC/PRF/5, respectively. Molecular markers shown at the left lane is fl 174/HaeIII digestion (1,353, 1.079, 872, 603 bp from the top).

products from three cell lines (2% agarose). The PCR products of the IRF-1 intron 8 from several human liver cancer cell lines showed a mutation at position 5445 (GenBank L05078) (C to T) causing a change from GGATCC to GGATCT that deleted the BamHI site in this region. Some other mutated positions were also found in this region and the detected mutations seemed to be SNPs, because the mutation site and pattern was restricted in seven different cell lines. Since such SNPs were found in the IRF-1 gene, we then analyzed upstream region of the IRF-1 exon 1 that is considered to be its promoter region, which may directly affect the IRF-1 transcription activity.

The IRF-1 Promoter Sequence

Because the sequence data for the IRF-1 upstream region were different between GenBank L05078 and X53095, we used X53095 as the database throughout the study. The nucleotide sequence upstream of the IRF-1 gene was confirmed in six experiments (6 vectors), and the results are shown in Figure 3A point mutation possessed by both cell lines (or possibly a shared nucleotide different from that in the database) was found at position − 388 upstream of the IRF-1 gene. There are seven GC boxes that may be potential Sp1 binding sites, and two putative NF-κB binding sites in the sequence. AARKGA hexamers and GAAANN motifs, which are commonly found in the type I IFN gene promoters, were also located in this sequence. No point mutation was found in these GC boxes, NF-κB binding sites, AARKGA hexamers or GAAANN motifs. We found 3 point mutations at positions − 300 (G to A), − 410 (G to A), and − 415 (A to C) in the genome of PLC/PRF/5.

We examined these mutations of the IRF-1 promoter in DNA from the peripheral blood mononuclear cells of ten healthy volunteers. Amplification of the IRF-1 promoter region was performed as described above. Table I shows the mutation pattern of this region in each case. We found both homozygosity and heterozygosity of the nucleotide at the same positions found in the liver cancer cell lines; however, no mutations at any other positions were detected at all. These results suggest that the point mutations of the nucleotide positions at − 300, − 388, − 410 and − 415 in the IRF-1 promoter represent nucleotide polymorphism of this gene.

Luciferase Reporter Assay

Preliminary experiments showed no significant differences between the luciferase reporter assay with and without a medium change after transfection. In HCC-M, the maximum transfection efficiency was achieved with a cell density of 1.0×10^5/35 mm dish, sampling 24 h after transfection. In PLC/PRF/5, the best efficiency was obtained with a cell density of 1.5×10^5/

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                -495
X53095          AAGCTTCAGG  AGCCAGGCTG  CCAGTCGGGA  GATTCGGCCC  AGTGTTCCCA
HCC-M           **********  **********  **********  **********  **********
PLC/PRF/5       **********  **********  **********  **********  **********
                                                -415 -410
X53095          CTGGAGAGGG  CGGCAAGTGC  CCGGGCGATC  ACCTCGCCTG  CGTTCGGGAG
HCC-M           **********  **********  **********  **********  **********
PLC/PRF/5       **********  **********  **********  C****A****  **********
                     -388
X53095          ATATACCTCC  GCCCCCGCCC  CGCCAGGAGG  GTGAAAAGAT  GGCCCCAGGA
HCC-M           *******C**  **********  **********  **********  **********
PLC/PRF/5       *******C**  **********  **********  **********  **********
                                                                    -300
X53095          GCCAGCCGGC  TGGGACAAGG  CGGAGTGAGA  GGACAGGCTG  GGGCCGGGGG
HCC-M           **********  **********  **********  **********  **********
PLC/PRF/5       **********  **********  **********  **********  *****A****

X53095          CGCTGGGCTG  TCCCGGGCAG  CCCTCCTCCG  GGCAAGCCGG  AGCAGGGGTG
HCC-M           **********  **********  **********  **********  **********
PLC/PRF/5       **********  **********  **********  **********  **********

X53095          GATTGGGAGC  GCTCGGGGCG  GGCCCGCGGT  GGCCCCGGGG  CGGTGGCGCC
HCC-M           **********  **********  **********  **********  **********
PLC/PRF/5       **********  **********  **********  **********  **********

X53095          CGGCCGGAGA  GGGTGGGGCG  GAGCAGCCGC  CCTGTACTTC  CCCTTCGCCG
HCC-M           **********  **********  **********  **********  **********
PLC/PRF/5       **********  **********  **********  **********  **********

X53095          CTAGCTCTAC  AACAGCCTGA  TTTCCCCGAA  ATGACGGCAC  GCAGCCGGCC
HCC-M           **********  **********  **********  **********  **********
PLC/PRF/5       **********  **********  **********  **********  **********

X53095          AATGGGCGCC  CGCGCGGCTG  TCCGGGGGCG  GGGCCGGCCA  GGGCTGGGGA
HCC-M           **********  **********  **********  **********  **********
PLC/PRF/5       **********  **********  **********  **********  **********
                                                                    1
X53095          ATCCCGCTAA  GTGTTTGGAT  TGCTCGGTGG  CGCCGCTGCC  CTGGCAGAGC
HCC-M           **********  **********  **********  **********  **********
PLC/PRF/5       **********  **********  **********  **********  **********
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Fig. 3. Nucleotide sequences in the promoter region of IRF-1 gene. There are four mutations in this region and one is the same mutation between HCC-M and PLC/PRF/5. The nucleotides from the data base (L053095) are shown at the top of the lines. The same nucleotides are indicated with asterics.

TABLE I. Variations of the IRF-1 Promoter

Position	– 300	– 388	– 410	– 415
Data base	G	T	G	A
Case 1	G/A	C	G/A	C/A
2	G	C	G	A
3	A	C	A	C
4	G/A	C	G/A	C/A
5	G/A	T	G/A	C/A
6	G	C	G	A
7	G/A	C	G/A	C/A
8	G/A	C	G/A	C/A
9	G	C	G	A
10	G	C	G	A
HCC-M	G	C	G	A
PLC/PRF/5	A	C	A	C

The number of position indicates the number from the data base in the Gen Bank (X53095).

35 mm dish, sampling 48 h after transfection. Preliminary experiments showed that the luciferase activity with pGL3/pRL-TK after addition of IFN-β reached a plateau 4 h after the addition of 1000 IU/ml of IFN-β. For example, when HCC-M was used as a recipient cell, the normalized inducibility of the promoter from HCC-M and PLC/PRF/5 was 85.02 and 107.83 (mean of triplicate) without IFN-stimulation, respectively, and that 4 h after IFN-stimulation (1,000 IU/ml) was 130.98 and 152.25, respectively. When PLC/PRF/5 was used as a recipient cell, the normalized inducibility of the promoter from HCC-M and PLC/PRF/5 was 137.99 and 155.47 without IFN-stimulation,

respectively, and that after IFN-stimulation was 284.06 and 400.60, respectively. Those activities increased to about 120% at 4 h with FBS, while this increase was about 240% at 4 h without FBS in the medium. Therefore the following experiments were conducted under these optimal conditions, as established above.

Figure 4 shows one of the results of the luciferase reporter assay 4 h after the addition of 1000 IU/ml each of IFN-α and -β using either HCC-M or PLC/PRF/5. Although the normalized inducibility was different between experiments, IRF-1 promoter activity was increased by both IFN-α and IFN-β. Those activities were always higher in PLC/PRF/5 cells, regardless of the promoter origin, and those obtained with the PLC/PRF/5 promoter were always higher, regardless of the target cell line. The promoter activity without IFN-stimulation in PLC/PRF/5 was 1.415 ± 0.162-fold higher than those in HCC-M ($P < 0.01$, Student's t-test, from six experiments). Those with IFN-stimulation in PLC/PRF/5 were 1.367 ± 0.123-fold higher than those in HCC-M ($P < 0.01$, Student's t-test, from six experiments).

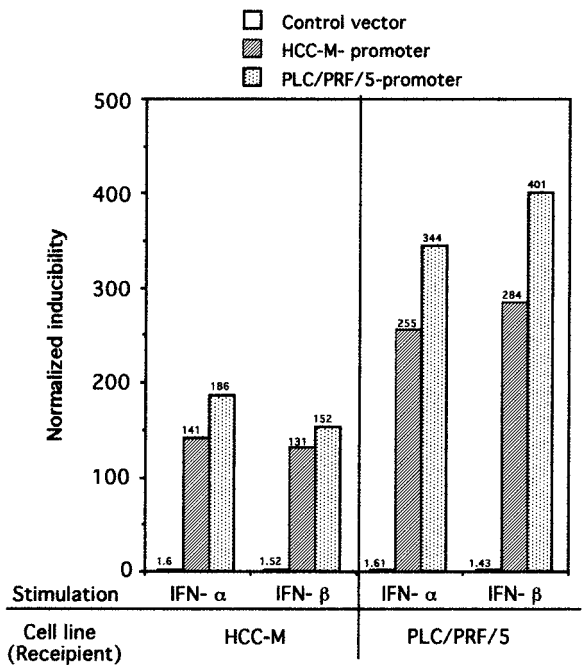

Fig. 4. Promoter activity of the upstream region of the IRF-1. Luciferase reporter assay was carried out and normalized inducibility was calculated as described in the text. IRF-1-promoter-luciferase reporter constructs were made with promoter regions from HCC-M and PLC/PRF/5 into a pGL3-Enhancer vector and HCC-M-pGL3 and PLC/PRF/5-pGL3 were obtained. They were co-transfected with pRL-TK. Control vector indicates pGL3/pRL-TK co-transfection. HCC-M-promoter and PLC/PRF/5-promoter indicate HCC-M-pGL3/pRL-TK and PLC/PRF/5-pGL3/pRL-TK co-transfection, respectively. They were transfected into HCC-M cells or PLC/PRF/5 cells, and IFN-α or IFN-β (1000 IU/ml) was applied, and then luciferase reporter assay was performed 4 h after the stimulation.

Figure 5 shows putative secondary structures of the IRF-1 promoter region of HCC-M and PLC/PRF/5. These secondary structures seem that there is a stick in a bowl, which would receive some molecules, and a short stick at the bottom of a bowl. This stick-like structure has a tilt to the down in HCC-M and to the up in PLC/PRF/5. It seems that tilting of a stick would cause different affinity of molecules to this region.

DISCUSSION

IRF-1 is a key transcription factor in the regulation of growth, cell cycling, and apoptosis, and functions as an anti-oncogene. Ectopic overexpression of IRF-1 results in strong inhibition of cell growth, and deletions of the IRF-1 gene have been demonstrated in human leukemias and myelodysplasias [Harada et al., 1993; Willman et al., 1993a]. A variety of IFN-stimulated genes has been identified as involved in the regulation of cell growth and as target genes of IRF-1. 2′,5′-adenylate oligonucleotide synthetase and double-stranded RNA-dependent protein kinase (PKR) are members of this family of IFN-stimulated genes, which mediate not only resistance to virus infection, but also inhibition of cell proliferation [Wreschner et al., 1981; Rysiecki et al., 1989; Hassel et al., 1993]. In addition to these IFN-stimulated genes, the $p21^{WAF-1}$ gene, a member of the family of cyclin-dependent kinase inhibitors which primarily regulate the cell cycle, is itself regulated in response to DNA damage by both IRF-1 and p53 [Tanaka et al., 1996]. Others and we ourselves have reported p53-independent activation of $p21^{WAF-1}$ in response either to growth factor stimuli or exposure to differentiation-inducing agents, suggesting a role for $p21^{WAF-1}$ in the growth arrest associated with terminal differentiation [Zeng and el-Deiry, 1996; Saito et al., 1998]. Several IRF-1-binding sites have been found in the murine $p21^{WAF-1}$ promoter [Tanaka et al., 1996] and a recent study has directly demonstrated that the $p21^{WAF-1}$ gene is transactivated by IRF-1 [Coccia et al., 1999]. It is postulated that growth inhibitory effects of type 1 IFNs partly depend on levels of expression of

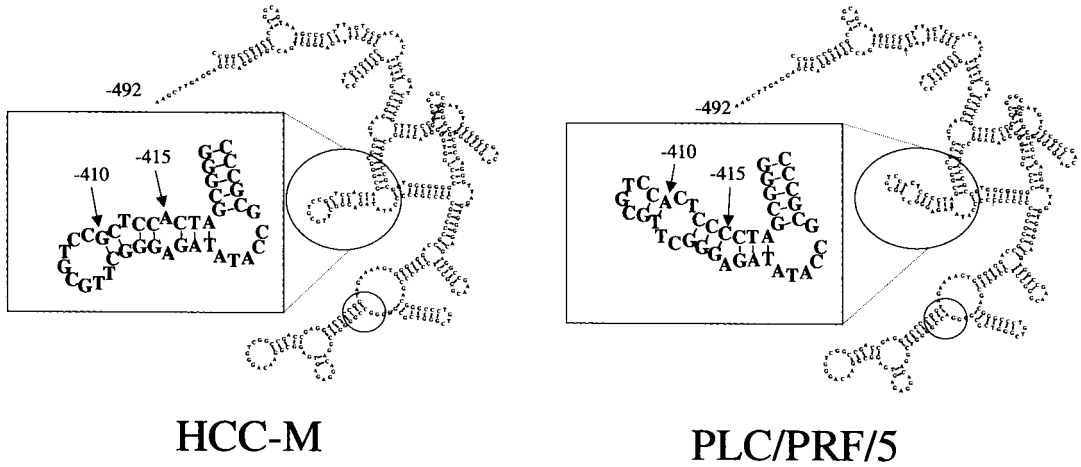

Fig. 5. Secondary stuructures from nucleotide sequences in the promoter regions of IRF-1 gene in HCC-M and PLC/PRF/5.

$p21^{WAF-1}$, which themselves depend on IRF-1 transcription levels. Thus, transcription seemed to vary between individual cell lines. We then hypothesized that polymorphisms in the IRF-1 gene contributed to DNA rearrangement in the BamHI digestion site that was shown in the previous study. We therefore investigated variation of the BamHI sites in the IRF-1 gene. The nucleotide at position 5445*C (GenBank L05072) was found to change to T, which then resulted in the disappearance of the BamHI site (AGGATCC to AGGATCT) in intron 8. This variation causes the DNA rearrangement seen in our previous study by Southern blotting [Tada et al., 1998].

We also hypothesized that polymorphisms in the promoter region of the IRF-1 gene are responsible for interindividual variation in the response to type I IFNs which in turn leads to individual variations in susceptibility to IFN treatment. We screened the proximal promoter of the IRF-1 gene in search of common genomic variants with distinct effects on the transcription activity of the gene. We identified four positions of SNPs in the IRF-1 promoter that have a CpG island. There are eight GC boxes (GGGCGG) within the CpG island of the IRF-1 promoter and seven of them are included in the position from -495 to exon 1, as shown in Figure 3 [Sims et al., 1993]. Sp1 binds to elements containing a GC box and facilitates transcription [Kadonaga et al., 1986], so that the IRF-1 gene may be constitutively expressed. Indeed, IRF-1 promoter activity was detected without IFN-stimulation in the present study, suggesting that various transcription factors including Sp1 may constitutively stimulate the expression of the IRF-1 gene in liver cancer cell lines. There are also κB motifs in the IRF-1 promoter, indicating that a number of agents can activate this gene [Baeuerle, 1991]. However, we found no SNPs in these known transcription factor-binding sites in the IRF-1 promoter. We have shown that the point mutations at -300*G, -388*T, -410*G, and -415*A are linked; another set was -300*A, -388*C, -410*A, and -415*C where heteromers were also detected. These data suggest that, as indicated by others, novel transcription factors may bind to the site, including the SNPs found in this study. Alternatively, another possibility is that SNPs could change the conformation of this promoter region, which then changes the binding capacity of various transcription factors. The finding that constitutive expression levels of luciferase reporter activity were different in the different cell lines without IFN-treatment suggested that a conformational change of the binding sites could affect basal levels of expression of IRF-1 mRNA. Actually, putative secondary structure postulated from their nucleotide sequences showed a little difference in their shape. As mentioned in the result section, it seems that a short stick is standing at the bottom of a bowl. This bowl seems to form a receiver of some molecules, and a stick at the bottom of a bowl tilted to up and down in PLC/PRF/5 and HCC-M, respectively. Such a little conformational difference might modify response to various transcription factors, although the difference does not directly exist on the known binding sites of transcription

factors. The general transcription factor, Sp-1, is known to bind to elements containing a GC box (GGGCGG) to facilitate transcription [Kadonaga et al., 1986]. This box almost certainly contributes to the constitutive expression of the IRF-1 gene [Sims et al., 1993] and GC boxes exists near the structure (root of the stick) ($-376 \sim -387$ and $-433 \sim -438$). The promoter activity of PLC/PRF/5 was always 1.4-fold higher than that of HCC-M, and no difference in this ratio was noted between those with and without IFN-stimulation, although the basal activities were significantly enhanced by IFNs. These results indicates that IFN-α/β generally activates two different IRF-1 promoters equally but IFN-stimulated transcription factors, which are induced after IFN-stimulation, might not enhance this difference in their promoter activities. Some feedback mechanism not to enhance the difference might also be postulated. The difference in their promoter activities was not so large and some feedback mechanisms might exist that the IRF-1 promoter polymorphisms may not primarily contribute to final efficiency of various IFN-treatments. This is probably because such SNPs have been maintained in the long human history and if the functional difference was so large, he or she could not maintain homeostasis of the system. The promoter differences, however, may contribute to inter-individual difference in the response to IFN-treatments.

Thus, our findings imply that heterogeneity in the IRF-1 promoter has a role in determining the response to IFN-α/β treatment. Recently, Edwards-Smith et al. [1999] reported that IL-10 promoter polymorphism correlates with the initial response of chronic hepatitis C patients to IFN-α. They showed that patients genetically predisposed to high IL-10 production respond poorly to IFN-α therapy, because the elevated levels of IL-10, a T-helper type 2 (Th2) cytokine, in the serum of patients with untreated chronic hepatitis C virus infection are thought to compromise the host immune response to the virus. A Th1-dominant immune balance may be beneficial for viral elimination. Several reports have suggested that the Th1-type immune response requires IRF-1 transcription and is regulated by IRF-1 [Lohoff et al., 1997; McElligott et al., 1997; Taki et al., 1997], suggesting that IRF-1 promoter polymorphisms could also determine responses of chronic hepatitis C to IFN-α therapy. However, there is no information on hepatic cytokine expression and response to treatment with IFN-α. It is possible that genetic variations govern differences in the response to IFN-treatment, and further investigation is necessary to elucidate whether IRF-1 promoter SNPs correlate with the response to IFN-treatment in various effector systems.

ACKNOWLEDGMENTS

Authors thank Dr. Mamiko Soga (Biological Science Laboratories, Toray Research Center Inc.) for her technical assistances and fruitful discussions.

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