

Cooperative Regulation of Fc Receptor γ -Chain Gene Expression by Multiple Transcription Factors, Including Sp1, GABP, and Elf-1*

Received for publication, January 18, 2008, and in revised form, March 5, 2008 Published, JBC Papers in Press, March 31, 2008, DOI 10.1074/jbc.M800498200

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The Fc receptor γ -chain (FcR γ), which was first identified as a constituent of the high affinity IgE receptor, associates with various cell surface receptors to mediate intracellular signals. We identified three transcriptional enhancer elements in the 5' region of the human FcR γ gene; one of the *cis*-elements was recognized by the transcription factor Sp-1 and another was recognized by GABP or Elf-1. The sequence of the other element was similar to a binding motif of the C/EBP family. Overexpression experiments showed that these transcription factors cooperatively activated the FcR γ promoter. Furthermore, inactivation of the GABP-binding site by nucleotide substitutions as well as repression of GABP α expression by RNA interference reduced Sp1-mediated transactivation of the FcR γ promoter, demonstrating that Sp1 and GABP synergistically activated the FcR γ promoter. This synergistic activation was suggested to require physical interaction between the two transcription factors, because the Ets domain of GABP α was demonstrated to directly bind Sp1. On the other hand, GABP and Elf-1, whose recognition sequences overlapped, were shown to bind the FcR γ gene with similar affinity in the context of chromatin, although Elf-1 exerted weaker enhancer activity for FcR γ gene expression than did GABP. Both were thought to compete for binding to the element, because additional expression of Elf-1 in combination with Sp1 and GABP reduced FcR γ promoter activity. Such functional and physical interactions among transcription factors involved in the cooperative regulation of FcR γ gene expression as revealed in this study will become promising targets for medical applications against various immune diseases involving FcR γ .

The Fc receptor γ -chain (FcR γ),² which was first identified as a constituent of the high affinity IgE receptor (Fc ϵ RI) (1), also

* This work was supported in part by a grant-in aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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² The abbreviations used are: FcR γ , Fc receptor γ -chain; Fc ϵ RI, high affinity IgE receptor; Fc γ R, IgG receptor; Fc α R, IgA receptor; GP VI, collagen receptor

associates with other immunoglobulin Fc receptors, including the IgG receptors (Fc γ RI and Fc γ RIII) (2–5) and the IgA receptor (Fc α R) (6–8). FcR γ possesses an immunoreceptor tyrosine-based activation motif and mediates intracellular signals upon stimulation of a wide variety of cell surface receptors. These receptors play essential roles in immune reactions of the host. However, unusual or excess activation of effector cells through FcR γ often leads to various immune diseases. For instance, cross-linking of Fc ϵ RI on mast cells or basophils by antigen (allergen)-IgE complexes triggers an allergic reaction by activating intracellular signal cascades to induce not only the release of various chemical mediators, including histamine and leukotriene in the early phase reaction, but also cytokine gene expression leading to the late-phase reaction. On the other hand, excessive responses to self-antigens through Fc γ RI or Fc γ RIII occur in autoimmune diseases. IgE-mediated passive cutaneous anaphylaxis, experimental hemolytic anemia induced by anti-erythrocyte Ab, and the Arthus reaction induced by immune complexes were all inhibited in FcR γ -deficient mice lacking expression of functional Fc ϵ RI, Fc γ RI, and Fc γ RIII, indicating an essential role of FcR γ for all types of hypersensitivity reactions (9). Therefore, a novel manipulation strategy based on the regulation of FcR γ function is expected to yield medical applications.

In addition to Fc receptors, FcR γ is also reported to associate with the collagen receptor glycoprotein (GP) VI on platelets (10). Binding of collagen to GP VI activates platelets in an FcR γ -dependent manner and induces aggregation of platelets. Moreover, FcR γ was recently reported to associate with the osteoclast-associated receptor on dendritic cells and monocytes, indicating the possible involvement of FcR γ in osteoclast function (11–13).

Because FcR γ associates with various receptors, including Fc receptors, GP VI and osteoclast-associated receptor as described above, functional regulation of FcR γ will control various immune diseases, including allergy, thrombosis, and lupus nephritis involving Fc ϵ RI, GP VI, and Fc α R, respectively. Here we analyzed the regulatory mechanisms of human FcR γ gene expression, as local and specific regulation of FcR γ gene expres-

glycoprotein VI; GST, glutathione S-transferase; nt, nucleotide; ChIP, chromatin immunoprecipitation; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; Ab, antibody; siRNA, small interfering RNA; EMSA, electrophoretic mobility shift assay.

sion is thought to be one reasonable means of controlling FcR γ function.

The genomic structure of the human FcR γ gene has already been determined (14, 15). However, little analysis has been carried out on the regulatory mechanisms of FcR γ gene transcription. The human FcR γ gene consists of five exons, and its 5' region contains a GC box and a reversed CAAT box but not a canonical TATA box. As seen in many genes possessing a TATA-less promoter, transcription of the FcR γ gene starts at multiple positions, including a major site 25 bp upstream from the translation start site and several minor sites within 100 bp of the translation start site (15). Brini *et al.* (16) analyzed the 5' region of the human FcR γ gene over 2.5 kb and revealed that this region was involved in hematopoietic cell-specific transcriptional activation. We here analyzed a 5' region of about 450 bp of human FcR γ gene and identified *cis*-elements within 100 bp upstream of the translation start site. Because synergistic activation by transcription factors binding to these elements was observed, we further analyzed and discussed the synergistic activation of the FcR γ gene promoter by multiple transcription factors.

EXPERIMENTAL PROCEDURES

Cell Culture—THP1 (a human monocyte line), U937 (a human promonocyte line), KU812 (a human basophilic leukemia cell line), and Jurkat cells (a human T cell line) were cultured in RPMI 1640 medium (Sigma) at 37 °C in a humidified incubator with 5% CO₂. HeLa (a human epithelial cell line) was cultured in Dulbecco's modified Eagle's medium (Sigma). Both media contained 10% (v/v) fetal bovine serum (JRH Bioscience, Lenexa, KS), 100 units/ml of penicillin (Banyu Pharmaceutical, Tokyo, Japan), and 100 μ g/ml of streptomycin (Meiji Seika, Tokyo, Japan).

Plasmid Construction—A DNA fragment corresponding to the nt -450/+861 region (nucleotide numbers start at the translation start site) of the human FcR γ gene was obtained by PCR using the human genomic DNA library (Clontech) as a template. Synthetic oligonucleotides of 5'-GGCGTGGTGGTGCATGCCTGTAATGCCAGCTACTC-3' and 5'-ACCAAGGGTGCCTTTGTATATTAACAGCTAGAGA-3' were used as primers. The amplified product was inserted into a pCR2.1 vector (Invitrogen) for cloning and verifying its nucleotide sequence. The resulting plasmid was named pCR2.1- γ . The DNA fragments corresponding to nt -450/-1 and nt -370/-1 of the FcR γ gene were prepared by PCR employing pCR2.1- γ as a template and inserted into pGL3-Basic vector (Promega, Madison, WI) at the KpnI/XhoI site to obtain pGL γ -(-450/-1) and pGL γ -(-370/-1). The primers used for the PCR are as follows: forward primer for -450/-1, 5'-ggggtaccGGCGTGGTGGTGCATGCCTG-3'; forward primer for -370/-1, 5'-ggggtaccTGAGCCGAGATGGCGCCATTGC-ACT-3' (with the nucleotides represented by lowercase letters added to introduce a KpnI site (underlined)). The reverse primer used was 5'-ccgctcgagCTTGGGCTGGAGATCGGC-CGTTCTG-3' (with the nucleotides represented by lowercase letters added to introduce a XhoI site (underlined)).

A BglII-digested fragment from pGL γ -(-450/-1) was inserted at the BglII site of pGL3-Basic vector to yield pGL γ -

(-177/-1). Similarly, a PvuII/BglII-digested fragment from pGL γ -(-450/-1) was inserted at the SmaI/BglII site of pGL3-Basic vector to obtain pGL γ -(-39/-1). The DNA fragments corresponding to nt -133/-1, nt -102/-1, and nt -73/-1 region of FcR γ gene were prepared by PCR using pGL γ -(-177/-1) as a template and introduced into pGL3-Basic vector. The employed PCR primers are as follows: forward primer for -133/-1, 5'-gcggtaccTCTTGTGCAGGAAGGGGAA-GGG-3'; forward primer for -102/-1, 5'-gcggtaccTGGGG-GAAGGCGTGGCAGGA-3'; forward primer for -73/-1, 5'-gcggtaccGACTCTGTGGTCAGGGAAGTCT-3' (with the nucleotides represented by lowercase letters added to introduce a KpnI site (underlined)). The reverse primer used was 5'-gcagatcCTTGGGCTGGAGATCGGCCGTTTC-3' (with the nucleotides represented by lowercase letters added to introduce a BglII site (underlined)).

The amplified products were digested with KpnI and BglII to insert at the KpnI/BglII site of the pGL3-Basic vector. A series of mutant plasmids of pGL γ -(-177/-1) carrying a few nucleotide substitutions (mutA, mutB, mutC, and mutD) was constructed using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used to introduce nucleotide substitutions by expressing the substituted nucleotides as underlined are as follows: mutA, 5'-GGGCAAAGCATGTCT-GAAGGCGTGGCAGGA-3'; mutB, 5'-GGGAAGGCGTGGCACTAAGAGGGGGACTCTG-3'; mutC, 5'-GTGGCAGGAAGAGCGCTACTCTGTGGTCAGG-3'; mutD, 5'-GAGGGGGACTCTGTAGTCAGGGAAGTCTC-3'.

An Sp1 expression plasmid carrying a human Sp1 cDNA on pCL-neo vector (Promega) was kindly provided by Dr. Y. Ikeda (Kochi Medical School, Kochi, Japan) (17). Expression plasmids of GABP α , GABP β , and deletion mutants of GABP α were prepared as described previously (18). The empty vector pCR3.1-Empt was generated by self-ligation of EcoRI-digested pCR3.1 (Invitrogen). The Elf-1 expression plasmid pcDNA3.1-Elf-1 was constructed as follows. A full-length of human Elf-1 cDNA was prepared by reverse transcription-PCR employing total RNA from Jurkat cells as a template and synthetic oligonucleotides of 5'-ATGGCTGCTGTTGTCCAACAGAAC-3' and 5'-CTAAAAAGAGTTGGGTTCCAGCAG-3' as primers and cloned into pcDNA3.1 (Invitrogen). For pGST-GABP α , glutathione *S*-transferase (GST) was fused to the N terminus of human GABP α and its deletion mutants were generated as described previously (18). To construct siRNA expression plasmid of GABP α , a double-stranded DNA generated by annealing synthetic oligonucleotides of 5'-GATCCGTGTAAGCCAGGCCATAGACACTGTGAAGCCACAGATGGGTGTCTATGGCCTGGCTTACACTTTTTTA-3' and 5'-AGCTTAAAAAAGTGTAAAGCCAGGCCATAGACACCCATCTGTGGCTT-CACAGTGTCTATGGCCTGGCTTACACG-3' was inserted into pBasi-hU6 Pur (TAKARA BIO, Shiga, Japan) digested with BamHI/HindIII.

Transfection of Cells for Luciferase Assay—Transfection was performed as described previously (19). Briefly, cells were transfected with 5 μ g of test construct by electroporation at 300 V, 950 microfarads using a Gene Pulser II (Bio-Rad). For overexpression experiments, 3 μ g of expression plasmids of the transcription factors of Sp1, GABP α , GABP β , and Elf-1 or each

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corresponding empty vector as control were co-introduced into the cells with 3 μ g of the reporter construct. For RNA interference experiments, 3 μ g of the expression plasmid of GABP α siRNA was co-introduced into the cells. Twenty five picograms of the plasmid pHRL-CMV (Promega) carrying *Renilla* luciferase gene under the control of the human cytomegalovirus promoter were introduced to normalize transfection and cell lysis efficiency in every experiment. After 20–24 h of culture, cells were harvested and washed with phosphate-buffered saline (PBS) (pH 7.4). Cell lysis and determination of the luciferase activity were carried out using the dual-luciferase assay kit (Promega) according to the manufacturer's instructions. Luminescence was measured with Luminometer (Berthold, Postfach, Germany).

Nuclear Extract Preparation—Nuclear extract was prepared as described previously (20). Briefly, cells were washed with ice-cold PBS and resuspended in ice-cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT)) containing protease inhibitors. Cells were then incubated on ice for 10 min and for an additional 15 min with 0.5% Nonidet P-40. After centrifuging at 6,000 \times *g* for 1 min, the pellet was resuspended in extract buffer (20 mM HEPES (pH 7.9), 400 mM KCl, 4.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT) containing protease inhibitors and incubated on ice for 1 h. The lysate was centrifuged at 10,000 \times *g* for 10 min. After the addition of 15% glycerol, the supernatant was stored at -80°C until use.

Electrophoretic Mobility Shift Assay (EMSA)—Double-stranded DNAs were prepared as probe A and probe B by annealing FITC-labeled synthetic oligonucleotides. Nucleotide sequences of the probes are as follows: probe A, 5'-ATGGGG-GAAGGCGTG-3'; probe B, 5'-GCGTGGCAGGAAGAGG-3'. Nonlabeled, double-stranded oligonucleotides carrying the same sequence as the probe were prepared to use as competitors. Similarly, mutant competitors with a few nucleotide substitutions were generated. Their nucleotide sequences were as follows with the substituted nucleotides underlined: mutant competitor A, 5'-ATGGTCTAAGGCGTG-3'; mutant competitor B, 5'-GCGTGGCAACTAGAGG-3'. Thirty micrograms of nuclear extract and 5 pmol of DNA probe were incubated at room temperature with 5–125 pmol of competitors in 10 mM HEPES buffer (pH 7.9) containing 400 ng of poly(dI-dC), 1 mM MgCl₂, 30 mM KCl, 1 mM DTT, and 5% glycerol for 20 min. For the supershift or inhibition experiments by Abs, 2.0 μ g of Abs were added to the reaction mixtures and incubated for an additional 20 min. Rabbit polyclonal Abs raised against chicken GABP α and GABP β , which cross-react with human GABP, were kindly provided by Dr. S. Toku (University of Ryukyus, Okinawa, Japan). The other Abs were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mixtures were separated by electrophoresis on 4% polyacrylamide gels at 120 V for 2.0–2.5 h in 0.25 \times TBE buffer (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM DTT). FITC fluorescence was detected by Fluor Imager 595 (Amersham Biosciences).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed using the ChIP assay kit (Upstate Biotechnology, Inc., Lake Placid, NY). Cells were exposed to 1% formaldehyde for 10 min to obtain cross-linked chromatin. After

quenching the reaction with 0.125 M glycine, cells were washed with ice-cold PBS, solubilized with SDS lysis buffer containing protease inhibitors, and sonicated to shear the genomic DNA to an average length of 500–1000 bp. After centrifuging, the supernatants were diluted 10-fold in ChIP dilution buffer and immunoprecipitated with anti-GABP α rabbit polyclonal Ab (provided by Dr. S. Toku), anti-Elf-1 rabbit polyclonal Ab (Santa Cruz Biotechnology), or rabbit IgG as control (Santa Cruz Biotechnology) and salmon sperm DNA/protein A-agarose (Upstate Biotechnology, Inc.). The immunoprecipitated chromatin was washed, eluted from beads, and incubated at 65 $^{\circ}\text{C}$ to reverse the cross-links. After treatment with proteinase K, DNA was recovered by QIAquick PCR DNA purification kit (Qiagen, Hilden, Germany) and subjected to PCR analyses using synthetic oligonucleotide primers of 5'-AGCTGCA-CAGTGCTGTCAGAACGGCCGATC-3' and 5'-CCCTCAC-CAAACCCTCTTACCTGCTTGTTC-3' specific for nt $-41/-12$ and nt $+40/+69$ regions of the FcR γ gene, respectively. Primers of 5'-ACCAGGAGACTTACGAGACTCTGAAGC-ATG-3' and 5'-AGAATATAAATATCCGTAAACAGCATC-TGA-3' corresponding to nt $+3576/+3605$ and nt $+3796/+3825$ regions of the FcR γ gene, respectively, were used as control. A thermal cycle of 94 $^{\circ}\text{C}$ for 40 s, 64 $^{\circ}\text{C}$ for 40 s, and 72 $^{\circ}\text{C}$ for 1 min was repeated 32 times to amplify the nt $-41/+69$ region and that of 94 $^{\circ}\text{C}$ for 40 s, 57 $^{\circ}\text{C}$ for 40 s, and 72 $^{\circ}\text{C}$ for 1 min was repeated 32 times to amplify the nt $+3576/+3825$ region.

Western Blotting—Cells were transfected with the expression plasmids of Sp1, GABP α , GABP β , and Elf-1 or each corresponding empty vector as control and cultured for 16 h. After adding G418 to select the transfected cells, cells were cultured for an additional 28–32 h. Nuclear extracts were prepared for immunoblotting with anti-Sp1 (Santa Cruz Biotechnology), anti-GABP α (provided by Dr. S. Toku), anti-GABP β (provided by Dr. S. Toku), anti-Elf-1 (Santa Cruz Biotechnology), and anti- β -actin Abs (Abcam, Cambridge, UK). Alternatively, cells were transfected with the siRNA expression plasmid for GABP α or the empty vector as control and cultured for 24 h. After selecting the transfected cells with puromycin for an additional 24 h, cell lysates were prepared and subjected to immunoblotting with anti-GABP α Ab or anti- β -actin Ab.

Pulldown Assay—Pulldown assay was performed as described previously (18). In brief, GABP α , its deletion mutants, and GABP β fused to GST were respectively expressed in *E. coli*. The recombinant proteins in the sonicated *E. coli* lysates were immobilized on glutathione-Sepharose 4B beads (Amersham Biosciences). Five microliters of beads bound to 10 μ g of GST-fused protein were incubated with [³⁵S]methionine-labeled Sp1 prepared by *in vitro* transcription/translation employing TNT QuickCoupled Transcription/Translation Systems (Promega) in the presence of 100 μ g/ml ethidium bromide. After washing, the proteins bound to the beads were eluted by boiling in SDS sample buffer and subjected to SDS-PAGE.

RESULTS

An Approximate 100-bp Region Upstream of the Translation Start Codon Is Commonly Required for Activation of the

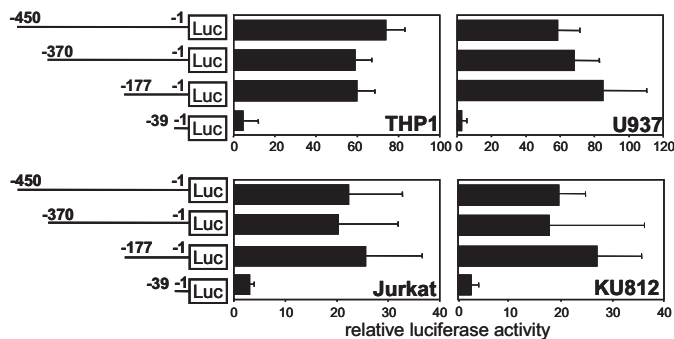


FIGURE 1. The 5' region nt $-177/-1$ is required for transcriptional activation of the human FcR γ gene. Four hundred and fifty bp of the human FcR γ gene 5' region and its deletion mutants were evaluated for their transcriptional regulatory activities by reporter gene assays employing γ -chain expressing human cell lines (THP1, U937, Jurkat, and KU812). Luciferase (LUC) activities relative to the control without promoter are shown. Results are expressed as means \pm S.D. of three or four independent experiments. Nucleotide numbers are counted from the translation start site as +1.

Human FcR γ Gene Promoter in Various Types of FcR γ -expressing Cells—The 5' regions of the human FcR γ gene were inserted upstream of a luciferase gene and analyzed for their transcriptional regulatory activity by reporter gene assays employing various FcR γ expressing human cell lines as follows: THP1 (a monocyte line), U937 (a promonocyte line), Jurkat (a T cell line), and KU812 (a basophilic leukemia cell line). (Note, nucleotide numbers are counted from the translation start site as +1.) The regions nt $-450/-1$, nt $-370/-1$, and nt $-177/-1$ activated the FcR γ gene promoter to almost the same extent but nt $-39/-1$ hardly activated it in all cell lines used for the assay (Fig. 1). Standard deviations were large in some cell lines, because the levels of luciferase activity from FcR γ constructs compared with those from the promoter-less construct varied widely among experiments probably depending on cell conditions, although the relative patterns among the FcR γ constructs were very reproducible in each cell line. These results indicated that the region nt $-177/-40$ contained core enhancer elements required for the activation of the FcR γ gene promoter. We further specified the *cis*-acting elements in the region nt $-177/-40$ by a reporter gene assay employing a series of deletion constructs. About 30–40 bp of the nt $-177/-40$ region of the FcR γ gene was sequentially deleted from the 5' end. Deletion of nt $-102/-74$ resulted in almost undetectable transcriptional activation in all cell lines, indicating that the region of nt $-102/-74$ contained common enhancer elements essential for activation of the FcR γ promoter (Fig. 2). Moreover, the regions nt $-133/-103$ and nt $-177/-134$ were shown to contain additional enhancer elements crucial for THP1, Jurkat, and U937 cells and for U937 and KU812 cells, respectively.

Three Enhancer Elements Are Identified in the Promoter Proximal Region of FcR γ Gene—To identify *cis*-acting elements, we introduced nucleotide substitutions in the sequences similar to transcription factor-binding motifs in the nt $-102/-1$ region (nt $-101/-95$ (motif A), nt $-87/-80$ (motif B), nt $-77/-71$ (motif C), and nt $-67/-57$ (motif D)), which was found by the assistance of the TFSEARCH program which is owned by the TRANSFAC databases (21). As shown in Fig. 3, substitution of nucleotides in motifs A (mutA), B (mutB), and D (mutD) reduced the luciferase activity but that in motif C

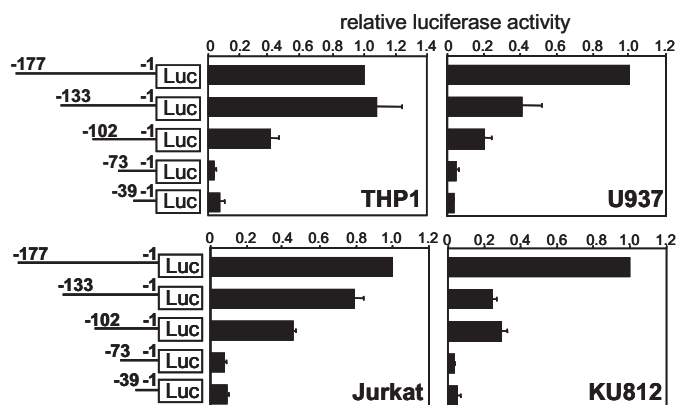


FIGURE 2. The region nt $-74/-102$ of the FcR γ gene contains common transcriptional enhancer elements. Transcriptional enhancer elements in the region nt $-177/-1$ of γ -chain gene were further mapped by reporter gene assays using a series of deletion constructs. Luciferase (LUC) activities are expressed as the ratios to that of pGL γ ($-177/-1$). Results are represented as means \pm S.D. of three independent experiments.

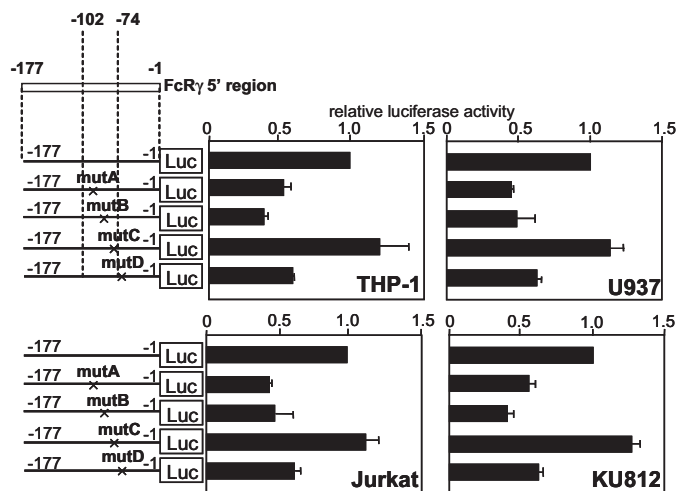


FIGURE 3. The regions nt $-101/-95$ and nt $-87/-80$ act as enhancer elements. Mutant reporter plasmids were constructed by introducing nucleotide substitutions into motif A of nt $-101/-95$ (mutA), motif B of nt $-87/-80$ (mutB), motif C of nt $-77/-71$ (mutC), and motif D of nt $-67/-57$ (mutD) of the FcR γ gene. Cells were transfected with the constructs for a transient expression assay. Luciferase (LUC) activities are expressed as the ratios to those of the wild type construct pGL γ ($-177/-1$). Results are represented as means \pm S.D. of three independent experiments.

(mutC) did not affect it. These results indicated that motif A and motif B functioned as transcriptional enhancer elements in the region of nt $-102/-74$, which was shown to commonly activate the FcR γ promoter in various cell lines. In addition, motif D was also revealed to activate the FcR γ promoter probably by cooperating with the elements in the region of nt $-102/-74$.

Two of the Enhancer Elements Are Recognized by Transcription Factors of Sp1 and GABP/Elf-1, Respectively—We next identified by EMSA the transcription factors binding to these motifs using nuclear extracts from FcR γ expressing cells and FITC-labeled double-stranded oligonucleotide probes containing motif A or motif B (Fig. 4). Among several shifted bands that appeared upon the addition of the nuclear extracts to a probe containing motif A, the band indicated by an *arrow* disappeared when nonlabeled, double-stranded oligonucleotide competitor with the same sequence of the probe was added (Fig. 4a). In

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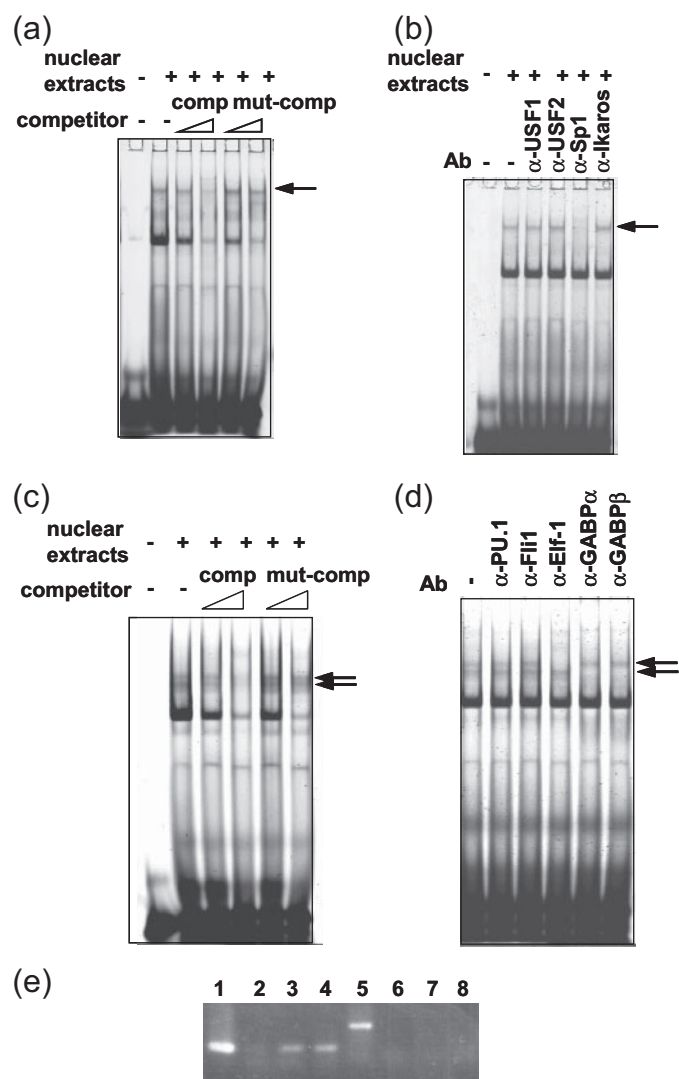


FIGURE 4. Motif A (nt $-101/-95$) is recognized by the transcription factor Sp1, and motif B (nt $-87/-80$) is recognized by GABP or Elf-1. *a-d*, EMSA was performed employing the nuclear extracts prepared from Jurkat cells and FITC-labeled, double-stranded oligonucleotide probes that corresponded to the sequences of nt $-102/-88$, including motif A (*a* and *b*), and nt $-93/-76$, including motif B (*c* and *d*). Unlabeled double-stranded oligonucleotide competitors with the same sequence of the probe (*comp*) and with three nucleotide substitutions in motif A or motif B (*mut-comp*) were used for the assays (*a* and *c*). Antibodies specific for various transcription factors were added in *b* and *d*. Similar results were obtained using the nuclear extracts prepared from THP1, U937, and KU812 cells. *e*, ChIP assays of the endogenous FcR γ gene in Jurkat cells were carried out employing anti-Elf-1 and anti-GABP α Abs. Rabbit IgG was used as control. The region nt $-41/+69$ of FcR γ gene proximal to the element of nt $-87/-80$ was amplified by PCR from the immunoprecipitated chromatins (*lanes 1-4*). As control, nt $+3576/+3605$ region of the FcR γ gene, which was distal to the nt $-87/-80$ element, was amplified (*lanes 5-8*). *Lanes 1* and *5*, input (diluted fractions of nonimmunoprecipitated chromatins were employed as templates for PCR to quantify the amount of DNA present in each sample before immunoprecipitation); *lanes 2* and *6*, rabbit IgG; *lanes 3* and *7*, anti-GABP α Ab; *lanes 4* and *8*, anti-Elf-1 Ab. Similar results were obtained when using THP1 cells.

contrast, this band was not affected by a mutant competitor with the same nucleotide substitutions in motif A as the mutant reporter plasmid (*mutA*) used in Fig. 3, indicating that this band represented a sequence-specific binding of a nuclear protein to motif A. Furthermore, because the band disappeared only upon the addition of anti-Sp1 antibody and was not affected by other antibodies against USF1, USF2, and Ikaros, the binding protein

was identified as Sp1 (Fig. 4*b*). Several shifted bands also appeared on the addition of nuclear extracts when a probe containing motif B was employed for the assay. The double band indicated by the *arrows* was revealed to depend on the sequence-specific binding between motif B and nuclear proteins by a similar competition assay using nonlabeled, double-stranded DNA (Fig. 4*c*). Addition of antibodies specific for some transcription factors, including PU.1, Fli1, Elf-1, GABP α , and GABP β , revealed that the binding factor forming the lower band was an α/β heteromer of GABP, as the band disappeared following the addition of either anti-GABP α or anti-GABP β Ab (Fig. 4*d*). On the other hand, the binding factor forming the upper band was identified as Elf-1 (Fig. 4*d*). Similar results were obtained when employing nuclear extracts from THP1, U937, and KU812 cells. We also tried to identify by EMSA the transcription factor that bound to motif D, but we were unsuccessful in our attempts to do this (data not shown).

Because the sequences required for GABP and Elf-1 binding almost completely overlapped in motif B, it was predicted that these transcription factors could not bind to motif B together. To compare the affinity of GABP and Elf-1 for the nt $-87/-80$ element in the context of chromatin *in vivo*, ChIP assays of the endogenous FcR γ gene were performed employing anti-GABP and anti-Elf-1 Abs (Fig. 4*e*). The nt $-41/+69$ region of the FcR γ gene, proximal to the element of nt $-87/-80$, was amplified from the chromatins immunoprecipitated with anti-GABP and anti-Elf-1 Abs (Fig. 4*e*, *lanes 3* and *4*) to almost the same extent but scarcely amplified from those immunoprecipitated with control rabbit IgG (*lane 2*). In contrast, the region nt $+3576/+3605$, distal to the element of nt $-87/-80$, as control was not amplified from the chromatins immunoprecipitated with either Ab (Fig. 4*e*, *lanes 7* and *8*). These results demonstrated that GABP and Elf-1 bound to motif B with similar affinity *in vivo*. Collectively, these results demonstrated that Sp1 specifically recognized motif A and that Elf-1 or GABP bound specifically to motif B.

Synergistic Activation of the γ -Chain Gene Promoter by Multiple Transcription Factors—To examine whether the identified transcription factors actually activated the FcR γ promoter, expression plasmids of Sp1, GABP α , GABP β , and Elf-1 were introduced into KU812 cells with the reporter plasmid pGL γ ($-177/-1$) carrying the nt $-177/-1$ region of the FcR γ gene upstream of a luciferase gene (Fig. 5*a*). Overexpression of each transcription factor increased the luciferase activity, indicating that these transcription factors respectively activated the FcR γ promoter. Coexpression of Sp1 and GABP caused synergistic activation of the FcR γ promoter, whereas that of Sp1 and Elf-1 only additively activated the promoter, consistent with the result that Elf-1 itself exerted smaller enhancing effect than GABP. Overexpression of the transcription factors by the introduction of expression plasmids was detected by Western blotting as shown in Fig. 5*d*.

To further confirm the synergistic activation by Sp1 and GABP, transactivation of the FcR γ promoter by overexpressed Sp1 was compared by employing the wild type reporter pGL γ ($-177/-1$) and a mutant reporter plasmid that had nucleotide substitutions at the GABP-binding site (Fig. 5*b*). Sp1-mediated transactivation was reduced from 3.61- to 1.76-fold by the

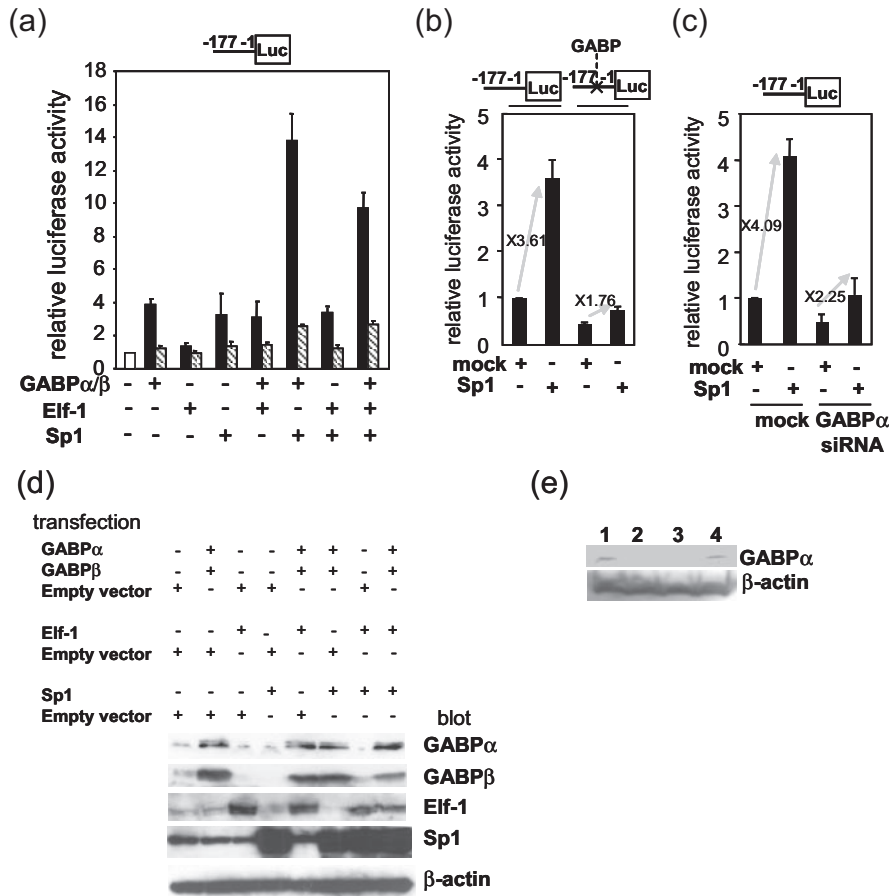


FIGURE 5. Sp1, GABP, and Elf-1 cooperatively activate the FcR γ promoter. *a*, expression plasmids of GABP α , GABP β , Elf-1, and Sp1 (filled bars) or corresponding empty vectors as control (hatched bars) were introduced into KU812 cells with the reporter plasmid pGL γ (-177/-1) carrying the FcR γ nt -177/-1 region upstream of a luciferase gene for a transient expression assay. *b*, KU812 cells were co-transfected with an expression plasmid of Sp1 and the reporter plasmid pGL γ (-177/-1) with or without nucleotide substitutions at the GABP-binding site. *c*, siRNA expression plasmid of GABP α was co-introduced with the Sp1 expression plasmid and pGL γ -177/-1 in KU812 cells. *a-c*, relative luciferase (*Luc*) activities to that of pGL γ (-177/-1) alone are shown. Results are represented as means \pm S.D. of three independent experiments. *d*, expression of GABP α , GABP β , Elf-1, and Sp1 in the cells transfected with indicated combinations of expression plasmids and empty vectors was detected by Western blotting. *e*, expression of GABP α in the cells transfected with GABP α siRNA was analyzed by Western blotting. Lane 1, no treatment; lane 2 and 3, GABP α siRNA; lane 4, control siRNA.

mutation at the GABP-binding site. Furthermore, repression of endogenous GABP α expression by siRNA also reduced Sp1-mediated transactivation (from 4.09- to 2.25-fold) in addition to FcR γ promoter activity itself (Fig. 5*c*). The effect of siRNA on the expression of GABP α was verified by Western blotting (Fig. 5*e*). These results indicated that full activation of the FcR γ promoter by the overexpressed Sp1 was achieved by functional interaction with endogenous GABP. Collectively, Sp1 and GABP were demonstrated to synergistically activate the FcR γ promoter.

GABP α Interacts with Sp1 through Its ETS Domain—Because it was suggested that functional interaction between Sp1 and GABP was required for full activation of the FcR γ promoter in Fig. 5, *b* and *c*, we next analyzed whether the two transcription factors physically interacted by a pull-down assay using 35 S-labeled Sp1 prepared by *in vitro* transcription/translation and recombinant GABP α or GABP β fused to GST. The results demonstrated that GABP α but not GABP β directly interacted with Sp1 (Fig. 6*b*). Sp1 has been reported to bind GABP α through its zinc finger motifs (22), although regions of GABP α

interacting with Sp1 remain to be analyzed. Consequently, a series of deletion mutants of GABP α was constructed and employed to address this (Fig. 6*c*). The amino acid 318–399 region of GABP α corresponding to the ETS domain known to be essential for DNA binding interacted with Sp1. These results showed that GABP α physically interacted with Sp1 through its ETS domain.

Functions of deletion mutants of GABP α were further analyzed by co-introducing their expression plasmids with the reporter plasmid pGL γ (-177/-1) and the expression plasmids of GABP β and Sp1 (Fig. 6*d*). To clearly evaluate the synergistic activation of Sp1 and GABP, Sp1 was expressed at a low level where the expressed Sp1 by itself hardly activated the FcR γ promoter by introducing a small amount of Sp1 expression plasmid into HeLa cells which expressed no hematopoietic transcription factors. GABP α Δ Ets, which lacked the Ets domain, could neither activate the FcR γ promoter by itself nor give synergistic activation of FcR γ promoter with Sp1, whereas the wild type GABP α (GABP α WT) activated the promoter by itself and synergistically with Sp1. On the other hand, GABP α (-318–454), which lacked the N-terminal portion and consisted of the Ets domain

and GABP β binding domain, showed reduced enhancing effects on the FcR γ promoter compared with GABP α WT but caused the synergistic activation of the promoter with Sp1. These results were in accordance with the findings that the Ets domain of GABP α was required for both DNA binding and interaction with Sp1.

DISCUSSION

In this study we revealed that expression of the FcR γ gene is regulated by multiple transcription factors, including Sp1, GABP, and Elf-1, which recognize elements in the 5' region within about 100 bp upstream of the transcription start site. The nucleotide sequence of the region is highly homologous between human and mice, leading us to believe that regulation mechanisms by these transcription factors are therefore possibly common beyond the species.

Sp1 and GABP physically interacted with each other and synergistically activated the FcR γ promoter. It has been reported that Sp1 cooperatively activates transcription with GABP in several types of genes (23–28) and physically interacts with

Regulation of FcR γ Gene Expression

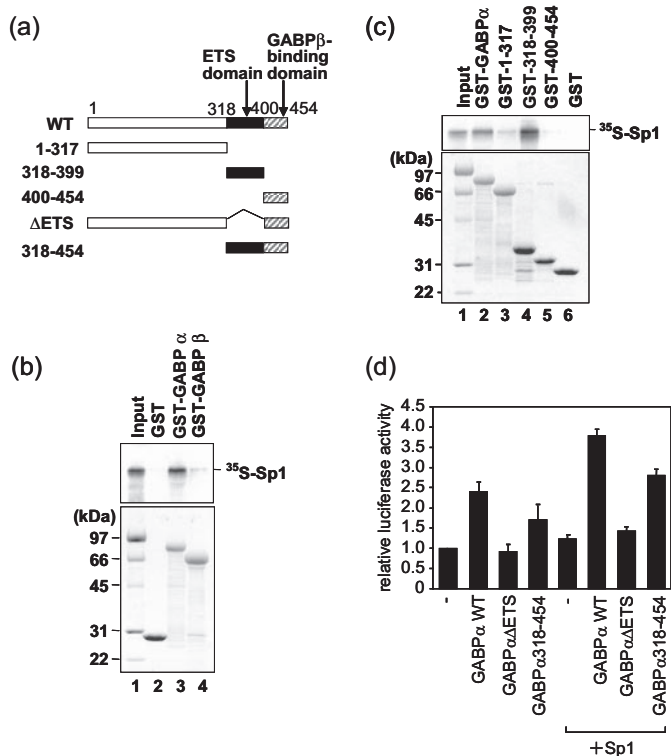


FIGURE 6. The ETS domain of GABP α physically interacts with Sp1. *a*, schematic drawing of wild type GABP α (WT) and its deletion mutants employed for the experiments. *b*, [35 S]methionine-labeled Sp1 was prepared by *in vitro* transcription/translation and mixed with recombinant GABP α or GABP β fused to GST, which was expressed in *E. coli* for a pull-down assay using glutathione-Sepharose beads. The proteins bound to the beads were subjected to SDS-PAGE and analyzed by autoradiography (top) or Coomassie Blue staining (bottom). *c*, [35 S]methionine-labeled Sp1 prepared by *in vitro* transcription/translation and recombinant GABP α or its deletion mutants were mixed for a pull-down assay employing glutathione-Sepharose beads. The proteins bound to the beads were subjected to SDS-PAGE and analyzed by autoradiography (top) or Coomassie Blue staining (bottom). *d*, 3 μ g of expression plasmids of wild type GABP α or its deletion mutants and the reporter plasmid pGL γ (-177/-1) were co-introduced into HeLa cells with or without 0.3 μ g of an expression plasmid for Sp1. Relative luciferase activities to that of pGL γ (-177/-1) alone are shown. Results are represented as means \pm S.D. of more than two independent experiments.

GABP α through its zinc finger motifs (22). We demonstrated that GABP α physically interacts with Sp1 through its Ets domain, which is known to be essential for DNA binding, indicating that the Ets domain of GABP α is required for binding to both of DNA and Sp1. This is the first study to determine the domain of GABP α that is involved in the interaction with Sp1. The amino acid residues crucial for the binding to DNA and those crucial for the binding to Sp1 can be separated, because GABP interacts with DNA and Sp1 at the same time. Determination of those amino acid residues will enable us to modify the transcriptional activation of the FcR γ gene by inhibiting the interaction of GABP α with protein (Sp1) but not with DNA, for example. Because both Sp1 and GABP are essential transcription factors, absolute functional inhibition of Sp1 or GABP will not be acceptable to the host. Therefore, specific and precise modulation of FcR γ expression depending on the partial or moderate inhibition of the function of these transcription factors is required for medical applications. A mutant form of GABP α with amino acid residue substitutions or chemical

compounds that inhibit the protein-protein interaction is expected to yield such a precise modulation of FcR γ expression.

In addition to Sp1, GABP, and Elf-1, a nuclear factor binding to motif D was suggested to be involved in the regulation of FcR γ expression, although we failed to identify it. Although it remains to be analyzed what nuclear factors actually bind to the element in the nucleus of FcR γ -expressing cells, overexpression of C/EBP α increased FcR γ promoter activity (data not shown), suggesting that a nuclear factor similar to C/EBP family bound to motif D and activated the FcR γ promoter. Because substitution of nucleotides in motif D decreased FcR γ promoter activity (Fig. 3) but nt -1/-73 containing motif D hardly activated transcription (Fig. 2), motif D was thought to activate FcR γ promoter by cooperating with motif A recognized by Sp1 and/or motif B recognized by GABP or Elf-1. Actually, cooperative transcriptional enhancing effects by the combination of C/EBP and Sp1 (29-31) and by C/EBP and GABP (18, 32) were previously shown in several reports. The transcriptional enhancing effects of the combination of C/EBP α and GABP on the FcR γ promoter were not only additive but synergistic, with the maximum synergistic effect achieved by the coexpression of Sp1, GABP, and C/EBP α (data not shown). The trimeric transcriptional activating complex consisting of Sp1, GABP, and some C/EBP α -like factor might be formed on the 5' region of the FcR γ gene, as every physical interaction between Sp1 and GABP (22), GABP and C/EBP (18), and Sp1 and C/EBP (33) has been reported. Modification of the formation of such a regulatory complex will be one promising strategy to specifically and moderately modulate FcR γ expression.

On the other hand, because the recognition sequences of GABP and Elf-1 overlapped, GABP and Elf-1 were thought to compete for binding to the element. The two transcription factors seemed to activate the FcR γ promoter at different levels (Fig. 5a), although they were shown to have similar affinity for the element *in vivo* by ChIP assays (Fig. 4e). Elf-1, which exerted weaker enhancing activity than GABP, might reduce the FcR γ promoter activity by competitively inhibiting the binding of GABP to the overlapped element, because additional overexpression of Elf-1 in combination with Sp1 and GABP reduced FcR γ promoter activity compared with the case of the Sp1/GABP coexpression (Fig. 5a). Therefore, the ratio of expression levels of GABP and Elf-1, which differs depending on cell types or conditions, might affect the expression level of FcR γ . Consistent with this, Juang *et al.* (34) recently reported that Elf-1 bound to GGAA elements on the FcR γ promoter and repressed its expression. In their study, Elf-1 bound to multiple elements, including motif A in the region of nt -141/-66 (corresponding to nt -121/-46 in their study), whereas Sp1 bound to motif A in our study. There is a possibility that both Sp1 and Elf-1 can bind to motif B depending on the conditions of cells. GABP activated FcR γ promoter more strongly than Elf-1 in KU812 cells which express FcR γ (Fig. 5a), but GABP and Elf-1 activated the promoter almost equally in HeLa cells which do not express FcR γ (data not shown). The difference of the effects of Elf-1 overexpression on KU812 and HeLa cells is thought to result from the different levels of endogenous expression of Elf-1 in the two cell lines, because the endogenous expression level of Elf-1 is higher in the hematopoietic cell line KU812 than

in the nonhematopoietic cell line HeLa. Moreover, conditions of Elf-1 modification, including its phosphorylation level, might be different among the cell lines.

Because FcR γ is expressed in various but limited types of cells, it is predicted that elements responsible for the cell type-specific expression exist. However, activation of the core promoter was essential to FcR γ expression but was mediated by transcription factors such as Sp1 and GABP that are ubiquitously expressed, indicating the possibility that further upstream 5' regions, introns, and 3' regions include the elements that determine the cell type-specific expression of FcR γ .

More detailed study will reveal the entire regulatory mechanisms of FcR γ gene expression, and concrete and specific information about the molecules and their interactions that participate in the regulation of FcR γ gene expression will contribute to medical applications for various immune diseases.

Acknowledgments—We are grateful to Dr. Y. Ikeda (Kochi Medical School) and Dr. S. Toku (University of Ryukyus) for kindly providing the Sp1 expression plasmid and antisera against GABP. We thank the members of the Department of Molecular Cell Immunology and Allergology for helpful discussions.

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