A Novel Transactivating Factor That Regulates Interferon-γ-dependent Gene Expression

We have previously identified a novel interferon (IFN)-stimulated cis-acting enhancer element, γ-IFN-activated transcriptional element (GATE). GATE differs from the known IFN-stimulated elements in its primary sequence. Preliminary analysis has indicated that the GATE-dependent transcriptional response requires the binding of novel transacting factors. A cDNA expression library derived from an IFN-γ-stimulated murine macrophage cell line was screened with a 32P-labeled GATE probe to identify the potential GATE-binding factors. A cDNA coding for a novel transcription-activating factor was identified. Based on its discovery, we named it as GATE-binding factor-1 (GBF-1). GBF-1 homologs are present in mouse, human, monkey, and Drosophila. It activates transcription from reporter genes carrying GATE. It possesses a strong transactivating activity but has a weak DNA binding property. GBF-1 is expressed in most tissues with relatively higher steady-state levels in heart, liver, kidney, and brain. Its expression is induced by IFN-γ treatment. GBF-1 is present in both cytosolic and nuclear compartments. These studies thus identify a novel transactivating factor in IFN signaling pathways.

The interferon (IFN) family of cytokines regulates antiviral, antitumor, and immune defenses in the vertebrates by inducing expression of a number of cellular IFN-stimulated genes (ISGs) (1). ISG induction has been attributed largely to the activation of the well established Janus tyrosine kinase-signal transducing activators of transcription (STAT) pathway, wherein tyrosine-phosphorylated STATs regulate the expression of downstream genes directly (2–4). In addition to STATs, some members of the IFN gene regulatory factor (IRF) family regulate IFN responses (5–7). It is now well established that Janus tyrosine kinase 1 and Tyk2, recruited to the ligand-engaged IFN-α/β receptor, induce the phosphorylation of STAT1 and STAT2. These then associate with ISGF3γ (p48 or IRF-9), a member of the IRF family of transcription factors (8). Although the published literature describes the ISGF3γ protein as p48, it will be referred to as IRF-9 in the light of the unified nomenclature suggested by the International Society for Interferon and Cytokine Research. This trimeric complex, known as ISGF3, migrates to the nucleus and stimulates transcription after binding to the IFN-stimulated response element (ISRE) (2, 3, 8). In a number of tumors and viral oncogene-expressing cell lines, down-regulation of the physical levels or inactivation of the components of ISGF3 serves as a mechanism for evading the action of IFNs (9, 10). ISGF3 is targeted by adenovirus (10), human papilloma (11, 12), Epstein-Barr (13), and hepatitis C viruses for evading the antiviral action of IFNs.

IRF-9 is an IFN-regulated gene, like certain other members of the IFN family (14). Pretreatment of cells with IFN-γ causes a robust activation of IFN-α-induced gene expression and consequent biological responses (15–17). This latter effect is achieved through an elevation of cellular IRF-9 levels (14, 16, 17); however, its delayed expression and sensitivity to protein synthesis inhibitors, unlike other IRFs, suggest that the IRF-9 gene is regulated in an atypical manner (18). Previous investigations from this laboratory have revealed the molecular basis for such regulation. Analysis of the murine IRF-9 promoter led to the discovery that an unusual IFN-γ response element, GATE, and its cognate transacting factors regulate the transcription (18). These studies did not reveal the molecular nature of these factors. In the present study using the GATE binding property as a basis, we have identified a GATE-binding factor (GBF) from an IFN-γ-stimulated macrophage cDNA library. Sequence analysis revealed that it codes for a novel protein, which we have named GBF-1. It regulates GATE-dependent gene expression in an IFN-dependent manner. GBF-1 homologs are present in other species. Although recombinant GBF-1 alone does not bind DNA on its own, its expression in cultured cells permits DNA binding. Thus, GBF-1 is a novel factor that mediates the pleiotropic effects of IFN-γ. This study uncovers a novel mechanism of IFN action.

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‡ These authors contributed equally to this work.

§ Present address: Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Peoples Republic of China.

¶ To whom correspondence should be addressed: Greenebaum Cancer Center, University of Maryland School of Medicine, 655 W. Baltimore St., BRB 9th Floor, Baltimore, MD 21201.

1 The abbreviations used are: IFN, interferon; CBS, C/EBP-binding site; C/EBP, CCAAT enhancer-binding protein; C/ITA, class II trans-activator; CMV, cytomegalovirus; DAPI, 4,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay(s); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GATE, γ-IFN-activated transcriptional element; GBF, GATE-binding factor; GFP, green fluorescent protein; GM, GATE mutant; GW, GATE wild type; H2K, human embryonic kidney; IC/EBP, IFN-consensus sequence-binding protein; IPTG, isopropyl-β-D-galactopyranoside; IRF, IFN gene regulatory factor; ISG, IFN-stimulated gene; ISGF, IFN-stimulated gene factor; ISRE, IFN-stimulated response element; Luc, luciferase; MEF, mouse embryo fibroblast; M, monkey; MSA, Myc-Max-stimulated element; Mu, murine; Nt-NTA, nickel-nitritrotriacetic acid; RACE, rapid amplification of cDNA ends; STAT, signal transducing activator of transcription; TK, thymidine kinase; pIRE, palindromic IFN response element; GAS, gamma IFN-activated site.
A Novel IFN-inducible Transcription Factor

Materials and Methods

Reagents—Recombinant murine and human IFN-γ, mouse IFN-β (Pestka Biomedical Laboratories, Piscataway, NJ), IPTG, 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (X-gal; Invitrogen) cDNA synthesis kits (Stratagene), mouse kidney cDNA library (CLONTECH), restriction and modifying enzymes (New England Biolabs), and nitrocellulose membranes (Schleicher & Schuell) were used in these studies. Rabbit polyclonal antibodies specific for c-Jun and Bcl2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies, FLAG epitope tag, and actin were obtained from Sigma. Murine multiple tissue Northern blots, pEGFP, and GFP-specific antibodies were purchased from CLONTECH Inc.

Cell Culture and Plasmids—The murine macrophage cell line RAW (RAW264.7) was grown in RPMI 1640 supplemented with 10% fetal bovine serum. Human embryonic kidney 293 (HEK-293) cells were grown in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum. Primary mouse embryo fibroblasts were derived from 9-day-old embryos of C57Bl/6 mice. pIRE-tk-Luc and ISRE-tk-Luc constructs were provided by Keiko Ozato, National Institutes of Health, Bethesda, MD, and Nancy Reich, SUNY, Stony Brook, NY, respectively. Wild type murine and monkey GBF-1 open reading frames were also cloned into the mammalian expression vector pCMV-FLAG. Initial experiments also used monkey GBF-1 cloned into the mammmalian expression vector pME1SS under the control of the SV40 promoter. The expression vector for C/EBP-β was provided by Richard W. Hanson, Case Western Reserve University, Cleveland, OH. Murine IRF-9 promoter and its mutants were described previously (18). This promotor was included in a pGEM promoter element.

The A6 construct was derived by inserting the native promoter elements of the murine IRF-9 gene upstream of a luciferase reporter in the promoterless pGL3-Basic vector (Promega). The GATE pm and MSE pm constructs bear mutations in the respective elements in the context of the native promoter. The P4 construct is generated by inserting a 74-bp element of the IRF-9 gene, harboring GATE, upstream of the SV40 early promoter element (Promega). The P3 mutant is similar to P4 but lacks the GATE sequence. The GW, GM, GM-1, GM-2, GM-3, and MSE constructs, bearing minimal wild type or mutant GATE, were generated by cloning synthetic complementary double-stranded oligonucleotides upstream of the SV40 early promoter in the pGL3 promoter vector. The terms GW and GM refer to wild type and mutant GATE sequences, respectively. The sequences of a single-stranded oligonucleotide are as follows:

GW: 5′-GATCTTCCGAGGAGAATTTAACATAGGTGGGGTAA-3′
GM-1: 5′-GATCTTCCGAGGAGAACATTTAGGGGTA-3′
GM-2: 5′-GATCTTCCGAGGAGAACATTTAGGGGGTA-3′
GM-3: 5′-GATCTTCCCAGGAGGAACATTTAGGGGTA-3′

Gene Expression Analyses—Southern, Northern, and Western blot analyses, transfection, β-galactosidase and luciferase assays, electrophoretic mobility shift assays (EMSAs), SDS-PAGE, and sequence analysis were performed as described in our earlier publications (18). Luciferase activities were normalized to the activity of a cotransfected CMV-β-galactosidase reporter. Triplicate transfections/sample were performed to evaluate the statistical significance of stimulation. In some experiments 4 μg of pCMV-FLAG-GFP-1 and 1 μg of pEGFP vectors were electroporated into cells, and the fluorescent cells (green) were sorted out using flow cytometry. Three separate transfections were sorted to generate a sufficient number of cells. Sorted cell lysates were prepared for monitoring the expression of endogenous IRF-9, GFP-1, and GFP using specific antibodies after Western blotting. Experiments were repeated at least three times to obtain consistent results. In vitro 4 μg of pCMV-FLAG-GFP-1 and 1 μg of pEGFP vectors were electroporated into COS cells, and the fluorescent cells were stained using the commercially available RiboMax system (Promega).

cDNA Libraries—A cDNA library was generated from poly(A)+ mRNA isolated from RAW cells stimulated with 400 units/ml murine IFN-γ for 0, 4, 8, and 12 h. A commercially available kit (Stratagene) was employed for cDNA synthesis with pooled poly(A)+ RNA as template. The cDNA library was cloned into the EcoRI and XhoI sites of the λ-ZAPII vector (Stratagene). The resultant library was picked in vitro and was used to infect Escherichia coli to obtain the final library. This library consisting of more than 99% recombinants was used for identifying the GATE-binding proteins. The induction of the protein encoded by the cloned cDNA was achieved by IPTG treatment. Three million plaques were screened using a 32P-labeled, cotransamirized GATE as Nimino oligonucleotide (19). In each case a corresponding column was subjected to two more rounds of screening with wild type 5′-CCCCAGAGGAATTTAATTTTTAGG-3′ and mutant GATE probes 5′-CCCCAGAGGAATTTAATTTTTGAGG-3′. The mutated bases are italicized and underlined. Nucleotide changes were introduced primarily on the basis of our earlier observation that this sequence exhibited a high homology (18) to ISRE. In each case a complementary oligonucleotide was synthesized and annealed. These double-stranded oligonucleotides were cotransamirized, labeled with 32P using a nick translation kit (Amersham Biosciences), and used in the experiments. At the end, 13 independent phage clones expressing GATE-binding proteins were isolated. These were grouped into three based on Southern blot analyses of the rescued inserts and partial sequence analysis. Among these, four clones expressing various sizes of the same cDNA were grouped as GBF-1. None of the clones contained the full-length sequence and lacked the 5′-end. Inserts in the phage were rescued by in vivo excision of the inserts, which allowed the transfer of cDNA into pBluescript phagemid. Inserts were sequenced and used for further analysis. A commercially available mouse kidney cDNA library (CLONTECH) was further to screen for the full-length transcribion factor.

Bacterial Expression—The partial cDNA was used for generating the recombinant GBF-1 protein in E. coli. An upstream primer (5′-CCGATTCCGGGTGGTCCAGAGAGGG-3′) and a downstream primer (5′-CGGGTTCTTCGTTGCTGGCTTGGG-3′) were used for amplifying the coding region, and the resultant PCR product (850 bp) was cloned into the EcoRI and XhoI sites of the bacterial expression vector pET29(+) (Novagen). After transformation into the E. coli BL21 codon plus strain, the cells were induced with 1 mM IPTG in the growth medium for 2 h. Sonicated cell extracts were prepared and passed though a Ni-NTA-agarose column for purification the recombinant protein. The protein was eluted using 50 mM imidazole. The protein was dialyzed and passed through the Ni-NTA-agarose column again to remove the trace contaminants. The expression tag was removed by thrombin digestion, and the recombinant GBF-1 was collected in the unbound fraction, after passing the digest through a Ni-NTA-agarose column.

Immunization—Electrophoretically pure recombinant murine GBF-1 was used for immunization. 20 μg of the antigen was prepared in Freund’s complete adjuvant and administered into the footpads of three mice. Two weeks later, a booster immunization, at biweekly intervals, were performed with subcutaneous injection of 10 μg of antigen prepared in Freund’s incomplete adjuvant. Serum was collected after tail vein bleeding, and the presence of high titer antibody was determined using ELISA with recombinant GBF-1 as antigen. A negative control ELISA with similarly produced unrelated recombinant GRIM-19 protein was also performed. The antisera did not react significantly with the negative control. Blood collected after cardiac puncture of animals and serum was saved. Nonimmune serum was also collected prior to immunization.

Immunofluorescence—Cells were grown on sterilized cover glasses in six-well dishes (Nalgene) and then fixed with 2% paraformaldehyde in phosphate-buffered saline for 10 min and then with methanol for 20 min at room temperature. The cells were then washed three times with phosphate-buffered saline containing 0.15% glycine and 0.5% bovine serum albumin. The cells were then incubated with antibodies specific for GBF-1 (1:250 dilution) in the same buffer for 1 h at 37 °C. Cells were then washed with phosphate-buffered saline and then incubated with Texas Red-labeled goat anti-mouse IgG (1:1,000 dilution) for 1 h. They were then washed with phosphate-buffered saline and observed under a Nikon fluorescence microscope. These cells were also counterstained with 0.1 μg/ml DAPI to visualize the nuclei. In some experiments, FLAG-GFP-1 was introduced into cells by transfection, and cells were fixed and stained as described above 16 h after transfection.

Expression in Mammalian Cells—The mouse GBF-1 was PCR amplified using a forward primer (5′-CCGAATTCATGGCAGGAGGC-3′) and a reverse primer (5′-CGCCTTGGCATTGCTGGCT-3′), digested with EcoRI and XhoI, and cloned into the pCMV-FLAG vector. A full-length GFP-1 cDNA was isolated by the Japanese consortium on the monkey (Macaca fuscata) genome. The open reading frame of monkey GBF-1 (here forth Mk-GBF-1) was PCR amplified by using an upstream primer (5′-CCGAATTCGGGCG- GAGGAAATTTAATTTTTGAGG-3′) and a downstream primer (5′-CCGG- GATCCCTAGACATTCAGTGCGCTG-3′). The resultant PCR product...
(1.1 kb) was cloned into the pcMV-FLAG vector between EcoRI and BamHI for expressing as an N-terminal FLAG-tagged protein.

Subcellular Fractionation—HeLa cells were transfected with the FLAG-tagged GBF-1 construct. 36 h later cells were lysed in an ice-cold buffer consisting of 20 mM HEPES, pH 7.4, 0.5% Nonidet P-40, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a commercially available protease inhibitor mixture (Sigma) as described previously (20). The lysates were overlaid on a 50% sucrose cushion. After centrifugation at 2,000 g for 25 min, the overlaid fraction was collected and centrifuged at 10,000 g for 25 min. The supernatant was collected and used as the cytosolic fraction. The pellet formed after sucrose cushion centrifugation was collected, washed, and examined under a phase-contrast microscope to ensure the presence of nuclei. It contained greater than 99% pure nuclei. This fraction was extracted further with high salt and dialyzed.

The dialed fraction was centrifuged at 10,000 g for 30 min, and the supernatant was collected as the nuclear fraction. An aliquot from the initial lysate was saved for determining the total GBF-1 content. Typically, 70–90 μg of total protein extract from each sample was analyzed by Western blot analysis.

RESULTS

Isolation of GBFs—The GBFs were identified using a Southwestern technique based on the DNA binding property of a protein (19). An expression library was prepared using the pooled poly(A)-RNA isolated from the IFN-γ-stimulated murine macrophage cell line RAW and cloned into λ-ZAPII vector (see “Materials and Methods”). Protein encoded by the cloned cDNA in the phage plaques was induced by IPTG treatment. The plates were overlaid with nitrocellulose membranes, and the membranes were probed with a 32P-labeled, concatamerized GATE. Clones identified in the first round, using wild type GATE probe, were subjected to two more rounds of screening. Replica membranes from the same plate were probed with mutant or wild type GATE, separately. Because GATE exhibits a partial homology (18) to the ISRE, a mutant GATE was designed by mutating the residues that were homologous to ISRE. In particular, the GAA and AAACTT nucleotides in the middle of the sequence were changed to CTG and CTCGGC, respectively. A phage clone from the first round, named GBF-1, scored positive with wild type GATE and did not bind to mutant GATE in the second and third round analyses (Fig. 1).

Clones positive in the second round are indicated with arrows. These differences were not the result of differential labeling of the two probes because both sequences were labeled to the same efficiency (~10^9 cpm/μg). Positive phage clones were purified. Three independent plaques coding the same insert were identified. Sequence analysis revealed that they coded for a novel protein and carried only the partial cDNAs. All of them lacked the 5'-end including the open reading frame area, and the longest one contained a 1.04-kb insert.

Identification of Full-length GBF-1, a Novel Protein—A 1.43-kb long incomplete cDNA was obtained after screening a mouse kidney cDNA library. An extremely GC-rich nature of the 5'-end prevented the isolation of a full-length cDNA. Therefore, two rounds of 5'-RACE were performed to obtain the 5'-region. The 5'-RACE products were fused to the cDNA to generate the final cDNA (~1.6 kb). BLAST analyses identified highly homologous expressed sequence tag cDNAs from mouse (GenBank accession no. BC004846), human (AK024100 and BC011613), monkey (M. fascicularis, AB046026), and Drosophila melanogaster (AJ253037). The sources of these cDNAs were quite disparate. The mouse cDNA was isolated from kidney, mammary gland, and blastocysty granules. The monkey cDNA was identified from a brain cDNA library. The human cDNAs with variable sizes (1.6–1.8 kb) were isolated from uterine and neuroblastoma cell lines. The longest human cDNA (BC011613) had 190- and 450-nucleotide 5'- and 3'-untranslated regions, whereas the shortest human cDNA (FLI14038) had 19- and 450-nucleotide 5'- and 3'-untranslated regions, respectively. The mouse cDNA in the data bases had 17- and 389-nucleotide 5'- and 3'-untranslated regions, respectively. The monkey cDNA (2.041 kb) had 451-, and 456-nucleotide 5'- and 3'-untranslated regions, respectively. The mouse and human cDNAs map to chromosomes 2 and 9 in their respective species. The precise location of the human GBF-1 is 9q34.13.

The mouse, human, and monkey GBF-1 cDNAs encode 384, 377, and 377 amino acids, respectively. An assignment of the name GBF-1 was based primarily on amino acid homologies. The monkey and mouse proteins functions as GBF-1s (see below). The mouse and human GBF-1 polypeptides are 73% identical and 77% similar. Most of these cDNAs were capable of encoding 38–40-kDa polypeptides. The mouse and Drosophila homologs exhibit a 38% identity and 58% similarity. Most of the homology resides between amino acids 96 and 371. The high sequence homology between monkey and human (97%) predicts that the human cDNA codes for a GBF-1-like function, although this was not tested experimentally.

ClustalW alignment of the mouse, monkey, human, and Drosophila sequences showed extensive homology throughout their polypeptide backbones (Fig. 2A). The mouse and Drosophila sequences have a longer C terminus than those of human and monkey. Drosophila has a 14-amino acid internal peptide between positions 105 and 120, which is absent in the mamalian GBF-1. A glutaredoxin-like motif is present between amino acids 103 and 119 (Fig. 2B). BEAUTY analysis (21) indicates that GBF-1 has a homologous domain located between amino acids 146 and 220 (32% identity and 49% similarity) to a domain present in mouse, human protein-tyrosine phosphatases κ and μ. In addition, regions between amino acids 182 and 315, 167 and 242, and 183 and 246 have similarities to the regions conserved in eukaryotic RNA polymerases, eukaryotic and viral ribonucleoside diphosphate re-
ductases, respectively. These features are consistent with its DNA binding property. Phylogenetic analysis revealed a close relationship among the protein sequences (Fig. 2C). As expected, the vertebrate homologs cluster together, whereas the invertebrate homolog formed a distal branch.

Sequence analysis revealed that mouse GBF-1 codes for a novel protein with an apparent molecular mass of 38 kDa. To test whether the cDNAs code for predicted proteins, the cDNAs were cloned into the pGEM7Zf(+) vector, under the control of the SP6 promoter. Using SP6 RNA polymerase, an mRNA was generated in vitro from the cloned inserts and programmed into rabbit reticulocyte lysates in the presence of [35S]methionine. The resultant products were separated on a 10% SDS-PAGE and fluorographed. The mouse and monkey cDNAs yielded proteins of 39 and 42 kDa, respectively (Fig. 2D). The difference in sizes could be caused by post-translational modifications.

**GBF-1 Induces GATE-dependent Gene Expression**—Because the GBF-1 cDNA has been isolated by virtue of its interaction with GATE, it is necessary to determine whether it is a transcriptional activator or repressor. Therefore, we have subcloned the GBF-1 open reading frame under the control of a constitutive enhancer in the mammalian expression vector pCMV-FLAG and tested whether it induces the luciferase re-

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**Fig. 2. GBF-1 codes for a novel protein.** A, sequence analysis of GBF-1. A number of complete DNA sequences from expressed sequence tag databases were identified using Blast analysis. A conceptual translation of the cDNA sequences was performed, and the resultant open reading frames were compared in a ClustalW analysis. The mouse and monkey cDNAs were characterized in this study, and their GBF properties are confirmed. An assignment of GBF-1 to human and Drosophila cDNAs is primarily on the basis of sequence homology but not function. B, a domain map of mouse GBF-1. Various predicted domains and their locations are indicated on the polypeptide backbone. PTP, protein-tyrosine phosphatase; RDR, ribonucleotide diphosphate reductase. Numbers flanking the lines show amino acid coordinates. C, phylogenetic relationships of GBF-1 homologs. Using the GCG software, the sequence divergence among various GBF-1 homologs was compared. All GBF homologs seem to have a common ancestry. D, in vitro translation of full-length GBF-1 and the cloning vector pGEM7zf(+). 5 μg of plasmid DNA linearized with XhoI was used as a template to generate RNA using SP6 RNA polymerase. The resultant RNA was programmed into nuclease-treated rabbit reticulocyte lysates (Promega), and translation was carried out for 2 h at 30 °C in the presence of 100 μCi of [35S]methionine (Amersham Biosciences). 20-μl reaction components were separated on a 10% SDS-polyacrylamide gel and visualized after fluorography. Positions of the molecular mass standards (kDa) are indicated on the right.
porter. Initial studies used a construct P4, driven by a 74-bp enhancer element consisting of GATE region of p48. GFB-1 expression vector but not the control vector induced the luciferase expression, in a dose-dependent manner. Although at a higher molar ratio GFB-1 slightly inhibited the gene expression, luciferase activity was significantly more than the vector alone at that dose (Fig. 3A). This inhibitory effect may be the result of a competition for limited amounts of general transcriptional coactivators such as histone acetylases available in the cell. A similar construct, P3, lacking GATE did not respond to GFB-1 (Fig. 3C; see below).

Because GATE was an IFN-γ-inducible element and GFB-1 induced GATE-dependent gene expression, we next determined the influence of IFN-γ on GATE-dependent gene expression (Fig. 3B). We also compared the gene-inductive effects of monkey and mouse GFB-1 in these experiments. RAW cells were transfected with the P4 reporter, along with an empty expression vector or the corresponding vector with mouse or monkey GFB-1 (Fig. 3B). Cells were exposed to IFN-γ for 16 h, and luciferase activity was measured. Both mouse and monkey GFB-1 caused an elevation of basal expression, and IFN-γ treatment led to further induction. These data suggest that either an IFN-γ-dependent post-translational modification such as phosphorylation or an increase in GFB-1 levels (see Fig. 8) or both contribute to the transcriptional up-regulation. Monkey GFB-1 was marginally better at inducing gene expression in several independent experiments.

The effect of Mu-GFB-1 was also determined in the context of wild type promoter. The A6 construct carried a 1,056-bp upstream sequence derived from the mouse IRF-9 promoter. This promoter carries GATE and a MSE. Two other mutants, each bearing the targeted mutations in the GATE (GATE pm) and MSE (MSE pm), were also included in this experiment. Luciferase gene expression from the wild type and MSE pm was induced by GFB-1 (Fig. 3B). In contrast, the GATE mutant (GATE pm) did not respond to GFB-1 under these conditions. GFB-1 also induced transcription from the P4 construct. In contrast the P3 construct, which lacked GATE, did not respond to GFB-1. Only those reporters that possessed a wild type GATE responded further to IFN-γ, significantly (Fig. 3C). These results, together with the binding data (Fig. 1), indicate that GFB-1 mediates both basal and inducible expression from GATE-driven promoters.

We next examined the effect of GFB-1 on the endogenous IRF-9. Our attempts to overexpress this protein stably did not yield productive transfectants. This may be caused by the toxic effects of overexpressed GFB-1. Therefore, we used transient transfection to examine the influence on the expression of endogenous IRF-9 protein. Mouse embryo fibroblasts were transfected with an empty expression vector (pCMV-FLAG) or the same vector expressing FLAG-GFB-1 along with the pEFGP plasmid. After treating the cells with IFN-γ for 16 h, transfected cells (on the basis of green fluorescence) were sorted out using flow cytometry. Cell lysates were prepared from the sorted transfectants and monitored for IRF-9, GFB-1, and GFP using specific antibodies. Although IFN-γ induced the expression of endogenous IRF-9 in the vector-transfected cells, overexpression of GFB-1 further augmented the IFN-γ induced expression of IRF-9, significantly (Fig. 3D). IFN-γ had no effect on the expression of GFB-1 and GFP. There was marginal induction of the basal IRF-9 level. A quantification of the IRF-9 expression is shown in Fig. 3E. A modest but significant stimulation of endogenous IRF-9 protein occurred in the presence of GFB-1. The difference in the extent of the stimulatory effects of GFB-1 on the luciferase reporters (Fig. 3, B and C) and the endogenous IRF-9 (Fig. 3, D and E) could be the result of different conformations and locations of these promoters: the former is located on a plasmid, and the latter is part of the genome.

GFB-1 Does Not Stimulate GAS- or ISRE-dependent Transcription—Because GATE possesses a partial homology to

![Fig. 3](image-url)

**Fig. 3. Induction of gene expression by GFB-1.** Transfection was carried out using LipofectAMINE reagent (Invitrogen) and normalized to an internal control, the β-actin-β-galactosidase reporter gene (18). A, expression vector (pCMV-FLAG) or its derivative expressing GFB-1 was transfected along with the 0.4-μg P4-Luc construct into RAW cells, and the expression of luciferase was measured using 30 μg of total cell protein at 30 h post-transfection. Numbers on the x axis refer to fold of the expression vector or GFB-1 relative to the P4 reporter. B, a comparison of the gene-inductive effects of mouse (Mu-GFB-1) and monkey (Mk-GFB-1). RAW cells were transfected with the indicated GFB-1 expression plasmid or the corresponding empty vector. Cells were treated with 200 units/ml IFN-γ for 16 h. Each bar represents the mean ± S.E. of triplicate measurements. C, GATE-dependent induction of gene expression by GFB-1. Cells were transfected with 0.4 μg of the indicated reporter genes and 0.1 μg of GFB-1 expression vector. The fold induction of luciferase gene was calculated, relative to that of vector (pCMV-FLAG) transfected in each case. Data represent the mean of triplicate measurements. Construction of luciferase reporters was described earlier (18). A6, wild type p48 promoter; MSE pm, mutant of Myc-stimulated element; GATE pm, mutant of GATE. The P4 and P3 constructs contain the p48 promoter sequences (thick black bar), placed upstream of a heterologous promoter (SV40). Cells were treated with 200 units/ml IFN-γ, where indicated. D, effect of GFB-1 on endogenous IRF-9 expression. Primary MEFs were electroporated with the indicated expression vectors along with a pEGFP expression vector. GFP-expressing cells were sorted by flow cytometry, and the cell lysates were monitored for IRF-9, FLAG, and GFP expression using specific antibodies in Western blot analysis. E, a quantification of IRF-9 expression. Empty and filled bars correspond to the vector and GFB-1-transfected, respectively. Each bar represents mean densitometric intensity ± S.E. of triplicates. Cells were stimulated with 200 units/ml IFN-γ for 16 h.
ISRE, and it promotes IFN-γ-responsive gene expression, we next tested whether GBF-1 could stimulate transcription from other known IFN-stimulated elements. Two luciferase reporters, one driven by ISRE and the other by pIRE (GAS) were used for this study. RAW cells were transfected with the reporters along with pCMV-FLAG or pCMV-Mu GBF1. pIRE-Luc-transfected cells were exposed to 200 units/ml IFN-γ for 16 h. ISRE-Luc-transfected cells were treated with 200 units/ml IFN-γ or 200 units/ml mouse IFN-β for 16 h, respectively. Luciferase activity was measured as described under “Materials and Methods.”

Mutant GATE Does Not Support GBF-1-stimulated Transcription—In the next part of this study we aimed at defining the nucleotides required for the gene stimulatory activity of GBF-1 via GATE. Four mutants were used for this purpose. Oligonucleotides bearing wild type GATE or specific base changes were synthesized and cloned upstream of the SV40 early promoter into the pGL3-promoter vector. The sequences of wild type GATE (GW) and mutants GM-1, GM-2, and GM-3 are shown under “Materials and Methods.” One of these mutants, GM-13, was generated spontaneously during the cloning of wild type GATE. It is identical to wild type GATE except for a deletion of the T residue at the 13th position. The reporter constructs bearing GATE mutations were introduced into RAW cells along with murine and monkey GBF-1 via transfection and tested for induction of the luciferase reporter (Fig. 5A). GM-1, GM-2, and GM-3 were unresponsive, whereas GM-13 indicated GBF-1 or C/EBP-β expression. RAW cells were transfected with the indicated GFB-1 or C/EBP-β expression vectors (0.25× relative to the reporter) along with the GM-13 reporter. Cells were stimulated with 200 units/ml IFN-γ for 16 h as described in A. Luciferase activity was determined as described under “Materials and Methods.”

![Fig. 4](image)

**Fig. 4.** Effect of GBF-1 on ISRE- and GAS-dependent gene induction. RAW cells were transfected with expression vector (pCMV-FLAG) or the same vectors carrying GBF-1 (pCMV-GBF-1) (0.25× molar concentration with respect to the reporter) along with 0.4 g/ml luciferase reporters driven by ISRE or pIRE (GAS). 0.2 μg of a CMV-galactosidase reporter was also included for normalizing luciferase activity. An empty vector (pCMV-FLAG) was cotransfected for a control. Where indicated with a + sign pIRE-Luc or ISRE-Luc-transfected cells were stimulated with 200 units/ml mouse IFN-γ or 200 units/ml mouse IFN-β for 16 h, respectively. Luciferase activity was measured as described under “Materials and Methods.”

![Fig. 5](image)

**Fig. 5.** GATE sequences necessary for stimulating gene expression. A, RAW cells were transfected with luciferase vectors driven by various mutant GATE sequences and CMV-β-galactosidase. Along with these reporter plasmids the pCMV-FLAG or the same vector carrying Mu-GBF-1 or Mk-GBF-1 was cotransfected, and luciferase activity was measured. Empty bars, no treatment; filled bars, 200 units/ml IFN-γ for 16 h. Luciferase activity was normalized to β-galactosidase activity. Because the scale is too large for distinguishing the effects of GBF-1 on GM-13, these data were replotted on a different scale and are presented in the inset. B, a comparison of wild type and mutant GATE sequences to the CBS of other cellular genes. The core CBS is indicated with a line over the sequences. The conserved T residue has also been underlined in CBS and wild type GATE. Mutated bases in GATE have been italicized and underlined. The Δ sign in GM-13 indicates the deleted T residue. C, effect of C/EBP-β and Mu-GBF-1 on the GM-13 reporter. RAW cells were transfected with the indicated GFB-1 or C/EBP-β expression vectors (0.25× relative to the reporter) along with the GM-13 reporter. Cells were stimulated with 200 units/ml IFN-γ for 16 h as described in A. Luciferase activity was determined as described under “Materials and Methods.”

Several cellular genes, is present in the center of GATE (Fig. 5B). Because GM-1, GM-2, and GM-3 did not respond to IFN, and the mutations were within the CBS, the location of a GBF-1 response element was not revealed. These mutants also did not respond to C/EBP-β (data not shown). GM-13 has a highly reduced response to IFN-γ (Fig. 5A). A well conserved T residue, present in most C/EBP binding sites, is absent in GM-13 (Fig. 5B). This mutation is within the CBS of GATE. Therefore, we tested whether this mutant responded to
C/EBP-β or GBF-1. RAW cells were transfected with the GM-13 reporter along with an empty expression vector, C/EBP-β expression vector, or GBF-1 expression vector (Fig. 5C). They were then treated with IFN-γ for 16 h, and luciferase activity was measured. In vector- and C/EBP-β-transfected cells a similar extent of IFN-stimulated expression of luciferase gene was noted. In contrast, GBF-1 enhanced both basal and IFN-stimulated response significantly. By comparing the sequences of the GATE mutants, we have inferred that GBF-1 requires both part of the CBS and the adjacent sequences for stimulating gene expression. Thus, the residues required for GBF-1 response appear to be GAACCTTAG in the context of full-length GATE.

**Binding of GBF-1 to GATE**—Initial studies showed that recombinant GBF-1 alone did not bind to [32P]-labeled GATE (data not shown). Therefore, for conducting these experiments we have expressed the mouse and monkey GBF-1s as FLAG epitope-tagged proteins using the pCMV-FLAG vector. The plasmid was transfected into HEK-293 cells, and the nuclear lysate was employed for EMSA. To ensure the production of the FLAG-tagged protein in the cells, the lysates were subjected to a Western blot analysis with FLAG epitope-specific antibodies (Fig. 6A). The FLAG antibody detected an appropriate size band in the extracts from mouse GBF-1 protein-expressing cells. The control vector expressed only the FLAG epitope tag (1.5 kDa), which was too short to be retained on the gel. Therefore, this band was not detected in lanes loaded with vectortransfected cell lysates.

After confirming the expression, the cell extracts were incubated with [32P]-labeled GATE, and EMSA was performed to detect DNA binding (Fig. 6B). Protein extracts from vectortransfected cells formed two complexes with GATE in EMSA, denoted as a and b (lane 1). Of these two, complex b is less prominent, and its formation is variable. In contrast, lysates from the FLAG-GBF-1-expressing cells formed an additional complex that migrated above complex a (lanes 3 and 4). This complex can be seen only after a long exposure of the blots (48 h). This observation suggests that GBF-1 interacts weakly with GATE. This complex corresponds to FLAG-GBF-1 because a similarly transfected pCMV vector expressing cell lysates did not form the same complex (lane 2). A fast migrating complex, denoted as NSC (nonspecific complex), is detected in all lanes. Formation of the band is variable in different experiments. Preincubation of the nuclear extracts with an excess of unlabeled wild type GATE probe resulted in the disappearance of complexes GBF-1, a and b (lane 5). Unlabeled GM-1 oligonucleotide failed to inhibit the formation of this complex. In addition, GBF-1-transfected cell lysates failed to form the same unique complex when incubated with [32P]-labeled GM-1, GM-2, or GM-3 (data not shown). The GATE-binding complexes formed with IFN-γ-treated HEK-293 cells is different from those observed in RAW cells. In the human cell lines we have observed the formation of weaker complexes (data not shown), presumably because of species differences. Only one of them was slightly inducible. Furthermore, HEK-293 and RAW cells represent human kidney and mouse macrophages, respectively. Thus, these differences could account for variations in the EMSA banding patterns.

The effect of IFN-γ on the formation of the GBF-1 complex was analyzed in the next experiment. Cells transiently transfected with FLAG-GBF-1 expression vector were treated with IFN-γ for 1 h, and the extract was used for EMSA (Fig. 6C). A short treatment time was chosen to permit the post-translational modifications. This also precludes the induction of endogenous GBF-1. Cell extracts from empty expression vector formed complexes a and b (lanes 2 and 3). Lysates from GBF-1-transfected cells formed a specific slow migrating complex (lane 4) whose binding was intensified after IFN-γ treatment (lane 5). In addition, the binding of complex a was also intensified. The enhancement of DNA binding was not caused by differential amounts of expressed protein in the treated and untreated lysates. Western blot analysis of the extracts used for EMSA with FLAG epitope-tag-specific antibodies revealed that both untreated and IFN-γ-treated cells had a comparable expression of GBF-1 (Fig. 6D, compare lanes 3 and 4). As expected no such band could be detected in the vectortransfected cell lysates (lanes 1 and 2).

Similarly, we determined the DNA binding specificity of muGBF-1, using transfected HEK-293 cells (Fig. 6E). As expected, the GBF-1 complex was formed only with the lysates from muGBF-1 expression vector-transfected cells but not those transfected with empty vector (compare lanes 2 and 3 with lane 7). In lane 2, cells were treated with IFN-γ for 1 h before preparing nuclear lysates. Therefore, its binding was enhanced compared with the untreated cells. Formation of the GBF-1 complex was inhibited by an excess of GW but not GM-1 and GM-3 oligonucleotides (lanes 5 and 6). This complex was “supershifted” by the monoclonal FLAG epitope but not Myc epitope-specific antibodies. Vector transfectants did not form any complex different from that of mock transfected cells (compare lanes 1 and 7). Expression of the transfected gene product was confirmed by Western blot analysis of the lysates with FLAG antibodies. No change in GBF-1 protein expression was noted after IFN-γ treatment.

**Expression of the GBF-1 Gene**—We next determined whether GBF-1 was expressed in normal tissues. A commercially available mouse multiple tissue Northern blot was probed with a [32P]-labeled GBF-1 cDNA (Fig. 7). These blots contained 2 μg/lane poly(A)+ RNA from each indicated tissue. GBF-1 probing revealed that the gene was expressed at a higher steady-state level in heart, brain, liver, and kidney. Its expression was low in spleen, lung, skeletal muscle, and testis. Prolonged exposure revealed two other weakly cross-hybridizing mRNAs in testis and kidney (indicated by arrows). The identity of these cross-hybridizing bands remains to be determined. This blot was stripped and probed with a [32P]-labeled β-actin to confirm equal loading. All lanes had a comparable amount of RNA. Because the cardiac and skeletal muscles express an additional form of actin, an extra band can also be seen in those lanes.

**GBF-1 Is an IFN-stimulated Gene**—Given the observation that GBFs are synthesized in response to IFN-γ (18), we next determined whether IFN-γ induced the expression of GBF-1. RAW cells were treated with IFN-γ, poly(A)+ RNA was isolated, and the expression of GBF-1 mRNA was examined by Northern blotting. As shown in Fig. 8A, GBF-1 mRNA was induced in a time-dependent manner with maximal induction occurring at 8 h poststimulus (5-fold) and a decline thereafter. A minor species of mRNA (1.3 kb) is also in these cells, and it is not significantly stimulated. This band was seen under low stringency washing conditions or after a long exposure of the blots. Under these conditions the expression of a housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was not altered with IFN-γ treatment (Fig. 8A, bottom panel).

We next determined whether induction of GBF-1 mRNA also resulted in a corresponding rise in protein levels. IFN-γ-stimulated RAW cell extracts were monitored for GBF-1 levels by Western blot analyses with mouse polyclonal antibodies raised against recombinant mouse GBF-1 protein. Indeed, IFN-γ caused a time-dependent increase in the levels of GBF-1 protein (Fig. 8B). A small, inducible, faint band can also be seen in this blot. It may be a proteolytic product or a cross-reacting
A Novel IFN-inducible Transcription Factor

Fig. 6. DNA binding property of GBF-1. A, expression of FLAG-tagged monkey and mouse GBF-1. HEK-293 cells were transfected with pCMV-FLAG or the same vector carrying monkey or mouse GBF-1. After transfection of 2 μg of each plasmid DNA for 24 h, equal quantities of cell lysates (50 μg) were prepared and Western blotted. The blot was probed with a monoclonal antibody that recognizes the FLAG epitope tag. Numbers on the right indicate the migrating positions of size markers in kDa. B, 50 ng of a wild type GATE, GW oligonucleotide was labeled with 32P and used (70,000 cpm/reaction) in an EMSA (for the sequence, see “Materials and Methods”). About 50 μg of protein from transfected HEK-293 cells was employed for EMSA. None, probe alone in the binding buffer; NSC, nonspecific complex; a and b, endogenous complexes. The GBF-1 complex is indicated. Where indicated, an excess (50×) of GW or GM-1 oligonucleotides was incubated with cell extracts prior to the addition of 32P-labeled GW to the reaction. C, IFN-γ-enhanced binding of GBF-1 to GATE. Plasmid-transfected (indicated above the panel) HEK-293 cells were stimulated with 200 units/ml human IFN-γ for 1 h and then used in EMSA, where indicated. D, Western blot analysis of GBF-1 expression in the transfected cell lysates before and after treatment with 200 units/ml IFN-γ for 1 h. HEK-293 cells were transfected with 2 μg of pCMV-FLAG-GBF-1. 30 h later one plate was stimulated with IFN-γ, and the other was left untreated. Cells were lysed, and nuclear extract was prepared. 30 μg of nuclear extracts were separated on a 10% SDS-PAGE, Western blotted, and probed with anti-FLAG antibodies. E, specificity of mouse GBF-1 binding to GATE. HEK-293 nuclear lysates after transfection with 2 μg of Mu-GBF-1 were employed in EMSA. None, probe alone in the binding buffer; NSC, nonspecific complex; a and b, endogenous complexes. The GBF-1 complex is indicated. Where indicated, an excess (50×) of GW or GM-1 oligonucleotides was incubated with cell extracts prior to the addition of 32P-labeled GW to the reaction. C, IFN-γ-enhanced binding of GBF-1 to GATE. Plasmid-transfected (indicated above the panel) HEK-293 cells were stimulated with 200 units/ml human IFN-γ for 1 h and then used in EMSA, where indicated. D, Western blot analysis of GBF-1 expression in the transfected cell lysates before and after treatment with 200 units/ml IFN-γ for 1 h. HEK-293 cells were transfected with 2 μg of pCMV-FLAG-GBF-1. 30 h later one plate was stimulated with IFN-γ, and the other was left untreated. Cells were lysed, and nuclear extract was prepared. 30 μg of nuclear extracts were separated on a 10% SDS-PAGE, Western blotted, and probed with anti-FLAG antibodies. F, expression of Mu-GBF-1 in the transfected cell lysates. Western blot analysis of cell lysates was performed with FLAG antibody. 35 μg of the cell lysate from each sample was used for Western blot.

Fig. 7. Expression of GBF-1 gene in normal mouse tissues. A commercially available multiple tissue Northern blot bearing 2 μg/lane poly(A)+ RNA from the indicated tissues was probed with a 32P-labeled GBF-1. Skel Muscle, skeletal muscle. The position of GBF-1 is indicated with a thick arrow. The upper and lower arrows indicate the positions of weakly cross-hybridizing RNAs from testis and kidney. These bands can only be seen after prolonged exposure (3 days). The bottom panel shows the same blot stripped and probed with β-actin cDNA. The cardiac and skeletal muscles express two forms of actin, and both can be seen in the specific lanes of these blots. All other tissues express a single form of actin.

Fig. 8. IFN-γ-induced expression of GBF-1. A, Northern blot analysis of 2.5 μg of poly(A)+ RNAs from 200 units/ml IFN-γ-stimulated RAW cells using a 32P-labeled GBF-1 probe. This blot was stripped and probed with 32P-labeled GAPDH to ensure the presence of equal loading of RNA. B, Western blot of RAW cells probed with polyclonal antibodies raised against mouse GBF-1. This blot was stripped and reprobed with an actin-specific monoclonal antibody to confirm equal loading of protein in all lanes. C, densitometric quantification of GBF-1 protein expression. Each bar represents the mean band intensity ± S.E. of triplicates. The bars in this graph correspond to the lanes of B. protein. This blot was reprobed with an antibody raised against actin to confirm equal loading (Fig. 8B, lower panel). A quantification of Western blot is shown in Fig. 8C. Al-
mice were injected with 50,000 units of IFN-γ and the expression of GBF-1 was normalized to that of GAPDH.

lanes shown).

Primary mouse embryo fibroblasts and HeLa cells (data not shown).

2 h after IFN-γ treatment. Similar induction of GBF-1 protein was observed in mouse embryonic fibroblasts and HeLa cells (data not shown).

To provide further evidence that GBF-1 is an IFN-γ-inducible gene, we have determined its induction in vivo. BALB/c mice were injected with 50,000 units of IFN-γ via the tail vein, and total RNA from various tissues was extracted after 12 h. These RNAs were used for Northern blotting, and the blots were probed with 32P-labeled GBF-1 cDNA. As shown in Fig. 9A, GBF-1 mRNA was readily induced by IFN-γ treatment in all tissues examined, whereas a basal level of its expression was seen in most tissues. Probing of this blot with 32P-labeled GAPDH showed the presence of a comparable amount of RNA in all lanes (Fig. 9B). The band intensities were quantified using a PhosphorImager. GBF-1 expression data were normalized to that of GAPDH.

Although a significant stimulation of GBF-1 protein occurred at 2 h after IFN-γ treatment, an 6-fold induction was observed at 16 h. Similar induction of GBF-1 protein was observed in primary mouse embryo fibroblasts and HeLa cells (data not shown).

FIG. 9. Induction of GBF-1 mRNA by IFN-γ in vivo. Where indicated with a plus sign, BALB/c mice were treated with 50,000 units/ml of IFN-γ for 12 h by tail vein injection. A minus sign indicates saline treatment. 50 µg of total RNA from each sample was loaded in the lanes. The blot has been probed with a 32P-labeled GAPDH to quantify RNA loading in the tracks (B). C, quantification of GBF-1 mRNA. The expression of GBF-1 was normalized to that of GAPDH.

bottom portion of the panel, these antibodies recognized proteins in the appropriate fractions.

In the second approach we stained cells with mouse antibodies specific for GBF-1. Permeabilized MEF and HeLa cells were first incubated with nonimmune or anti-GBF-1-specific antibodies and then with a secondary antibody tagged with Texas Red. Similar results were obtained with both cell types. The nonimmune antibody did not detect any signals. In contrast, the GBF-1-specific antibodies readily stained the nuclear and cytosolic compartments. The nuclear compartment was more intensely stained. IFN-γ treatment caused a high intensity nuclear staining compared with the untreated cells. This observation is consistent with the rise in GBF-1 protein levels. These data indicate that GBF-1 is primarily a nuclear protein, although a significant portion of it can be found in the cytosol. Nonimmune antibody did not detect any signals. In contrast, the GBF-1-specific antibodies readily stained the nuclear and cytosolic compartments. The nuclear compartment was more intensely stained. IFN-γ treatment caused a high intensity nuclear staining compared with the untreated cells. This observation is consistent with the rise in GBF-1 protein levels. These data indicate that GBF-1 is primarily a nuclear protein, although a significant portion of it can be found in the cytosol.

Intracellular Localization of GBF-1—To determine the intracellular location of GBF-1 we took two different approaches. In one, primary mouse embryo fibroblasts (MEF) were transfected with a FLAG-tagged GBF-1 or the control vector lacking GBF-1. The cells were incubated with FLAG tag-specific antibody and then with a second antibody labeled with Texas Red. The cells were counterstained with DAPI to visualize the nuclei. As shown in Fig. 10A, the FLAG epitope tag-specific antibodies recognize a protein both in the cytosol and nucleus. The vector-transfected cells were not stained significantly with Texas Red. This result was further confirmed using fractionated nuclear and cytosolic extracts (Fig. 10B). HeLa cells were transfected with FLAG vector or FLAG-tagged GBF-1. Cell lysates were prepared and fractionated into nuclear and cytosolic extracts. The fractionated extracts were analyzed by Western blot with FLAG tag-specific antibodies. GBF-1 was present in both nuclear and cytosolic extracts. These extracts were also probed with antibodies that specifically recognize c-Jun (nuclear protein) and Bcl2 (cytosolic). As shown at the

FIG. 10. Intracellular location of GBF-1. A, primary MEFs were transfected with pCMV-FLAG (Vector) or the same vector-expressing mouse GBF-1 (GBF-1). Cells were fixed, permeabilized, and incubated with a monoclonal antibody against the FLAG epitope tag (1:250 dilution). They were then washed and incubated with a goat anti-mouse IgG tagged with Texas Red (1:2,000 dilution). The cells were also counterstained with 0.1 µg/ml DAPI to detect the nuclei. A representative field is shown. Magnification is ×40. B, fractionation of cell extracts to define the intracellular locus of GBF-1. GBF-1- or vector-transfected MEFs were fractionated into nuclear (Nucl) and cytoplasmic (Cyto) compartments as described under “Materials and Methods.” A fraction of the total extract was saved and also used in Western blots. These blots were first probed with FLAG tag-specific antibodies to detect GBF-1 (B1). They were then stripped and probed with c-Jun- (B2) or Bcl2- (B3) specific antibodies. C and D, localization of endogenous GBF-1 in HeLa and MEFs. Cells were grown on cover glasses, fixed, permeabilized, and incubated with a polyclonal antibody against GBF-1. They were then washed and incubated with a goat anti-mouse IgG or the appropriate monoclonal antibody against the FLAG epitope tag (1:250 dilution). These RNAs were used for Northern blotting, and the blots were probed with 32P-labeled GBF-1 cDNA. As shown in Fig. 9A, GBF-1 mRNA was readily induced by IFN-γ treatment in all tissues examined, whereas a basal level of its expression was seen in most tissues. Probing of this blot with 32P-labeled GAPDH showed the presence of a comparable amount of RNA in all lanes (Fig. 9B). The band intensities were quantified using a PhosphorImager. GBF-1 expression data were normalized to that of GAPDH and are shown in Fig. 9C. A 3-4-fold induction of the mRNA was noted in most tissues, although the overall magnitude of expression varied among the tissues.

Intracellular Localization of GBF-1—To determine the intracellular location of GBF-1 we took two different approaches. In one, primary mouse embryo fibroblasts (MEF) were transfected with a FLAG-tagged GBF-1 or the control vector lacking GBF-1. The cells were incubated with FLAG tag-specific antibody and then with a second antibody labeled with Texas Red. The cells were counterstained with DAPI to visualize the nuclei. As shown in Fig. 10A, the FLAG epitope tag-specific antibodies recognize a protein both in the cytosol and nucleus. The vector-transfected cells were not stained significantly with Texas Red. This result was further confirmed using fractionated nuclear and cytosolic extracts (Fig. 10B). HeLa cells were transfected with FLAG vector or FLAG-tagged GBF-1. Cell lysates were prepared and fractionated into nuclear and cytosolic extracts. The fractionated extracts were analyzed by Western blot with FLAG tag-specific antibodies. GBF-1 was present in both nuclear and cytosolic extracts. These extracts were also probed with antibodies that specifically recognize c-Jun (nuclear protein) and Bcl2 (cytosolic). As shown at the
oration of Janus tyrosine kinase-STAT signaling pathways, a transiently activated process (2, 3). Although the activation of STATs and subsequent expression of certain ISGs are independent of de novo RNA or protein synthesis, induction of several IFN-γ-regulated genes requires the synthesis of other protein factors (1, 28). Furthermore, unlike IFN-α/β, the IFN-γ-stimulated genes are induced with variable kinetics (1) and often require new protein synthesis. Additionally, induction of certain ISGs does not correspond to the kinetics of STAT-activation and down-regulation (1, 28). Thus, factors other than STATs regulate the expression of these "late induced genes." Indeed, recent analyses have shown that IFNs can induce some genes in cells lacking STAT1 (29, 30). Although most IFN-α-induced genes are not stimulated in the absence of Janus kinase Tyk2, a fraction of the IFN-β response is retained in the U1A cells that lack Tyk2 (31). Recent studies with TYK2−/− mice also showed only a partial impairment IFN-α/β signaling in the absence of Tyk2 (32, 33). These observations indicate the presence of other potential IFN-regulated pathways and genes, although these could be a minor population relative to the number of total ISGs. Indeed a number of such factors, such as xhXBP1, RF-X, IRF-1, class II transactivator (CIITA), and IFN-consensus sequence-binding protein (ICSBP) represent the secondary mediators of signals (7, 34–40). However, these factors can only partially account for the pleiotropic nature of IFN response, and additional undefined IFN-induced regulators may exist.

We have shown earlier that the murine IRF-9 gene is regulated by a novel IFN-γ response element GATE (18). Others have shown IFN-induced expression of the chemokine RANTES (regulated on activation normal T cell expressed and secreted) (41) and major histocompatibility complex class IB (42) are mediated by novel elements that appear to be dependent on transcription factors other than ISGF3. To define the transacting factors that interact with GATE we have screened a cDNA expression library derived from an IFN-γ-stimulated mouse macrophage cell line, using a method based on the Southwestern approach (Fig. 1), and we identified the candidate cDNAs. One of these is a known transcription factor, C/EBP-β (22). An independent study also confirmed that C/EBP-β stimulates GATE-regulated transcription (23). In this study we have characterized a novel factor, GFB-1. GFB-1 exhibits the characteristics of a GATE-binding protein: it binds to GATE, it is an IFN-γ-inducible protein, and it activates transcription. It also activates only GATE-driven reporters, not other IFN-responsive promoters. The last observation indicates the specificity of GFB-1 in mediating IFN-γ-induced transcriptional responses.

GFB-1 exhibits a weak DNA binding property. Bacterially expressed GFB-1 does not bind to GATE. There could be several reasons for this inability: the recombinant protein lacks appropriate post-translational modifications; it requires other cellular factors for binding to GATE; it may have lower affinity for GATE under the conditions of EMSA experiments. Because we have isolated GFB-1 on the basis of its GATE property (Fig. 1), it is surprising to note that it does not bind to DNA in an EMSA. The DNA binding screen differs from EMSA in the following respects. In the former method a multimerized GATE is used to detect DNA binding. In contrast, a monomeric GATE is employed in the latter. Multimerized GATE may support a low affinity binding of GFB-1, whereas a monomeric GATE might not. Furthermore, the Southwestern detection of DNA-binding proteins (Fig. 1) is an in situ method, whereas EMSA is an in vitro method. However, after expression in the mammalian cells, GFB-1 was able to bind to DNA. Our previous studies identified two GATE-binding complexes, GIF-1 and GIF-2, in the murine macrophage RAW cells (18). We were unable to assign which of these two complexes contains GFB-1 because our antibodies are incapable of neutralizing or supershifting. A better quality antibody is required for examining this aspect. A number of other IFN-regulated transcription factors such as ICSBP (IRF-8) and CIITA do not bind to DNA on their own but can activate transcription very well. They form complexes with other factors to activate transcription (43–45). CIITA additionally possesses histone acetyltransferase and GTP binding activities required for its transactivating function (46, 47). Like CIITA, GFB-1 activates both basal and inducible responses (48). Interestingly, ICSBP was also discovered (49) using a method similar to that described here. Although defined as a repressor of ISRE-dependent transcription (50), subsequent studies showed that it activates transcription in a context-specific manner (51–53). Previously, we have shown that transcription factor C/EBP-β binds to GATE and stimulates transcription. A C/EBP-β consensus sequence (CBS) is present in GATE (Fig. 5B) and is necessary for stimulating transcription in a variety of cells (22, 23). The fact that GM-1, GM-2, and GM-3 do not respond to IFN-γ and have a disrupted CBS indicates that C/EBP-β binding site plays a large role in regulating this promoter. The fact that GM-1, GM-2, and GM-3 do not respond to GFB-1 indicates that it acts as part of the complex formed on CBS with C/EBP-β for promoting transcription (Figs. 1 and 5). The weak but significant transactivation of GM-13 construct by GFB-1 suggests that it mediates a minor fraction of the IFN-γ response in association with a weak complex formed with another protein at GATE. Thus, GFB-1 requires part of the CBS and the adjacent sites for driving gene expression. Consistent with this, GFB-1 synergistically induces GATE-dependent reporter in the presence of C/EBP-β. However, it is also possible that GFB-1 may interact with other proteins to generate unknown biological responses, which need to be investigated further. This may include both transcriptional and nontranscriptional functions, given its location in cytoplasmic and nuclear compartments. The observation that IFN-γ stimulates GFB-1-dependent gene expression significantly further suggests that an inducible post-translational modification regulates its activity. This observation is consistent with our earlier studies, which showed that inhibitors of protein kinases block IFN-γ-induced expression from GATE (18). Our recent studies show that inhibitors of extracellular signal-regulated kinase 1/2 activation suppress IFN-induced gene expression (54). Preliminary studies with mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2-specific and p38 mitogen-activated protein kinase-specific pharmacologic inhibitors do not significantly inhibit GFB-1-dependent IFN-γ-stimulated induction of the GM-13 reporter (data not shown). This observation is also consistent with the absence of any potential mitogen-activated protein kinase-specific phosphorylation sites in GFB-1 protein. It is likely that other factors may control its phosphorylation status. This aspect is currently being studied.

The immunocytochemical analyses in mouse and human cells revealed that GFB-1 was present both in the nucleus and cytoplasm. This observation suggests that GFB-1 might mediate other unknown biological responses in the cells. We have described one of its functions in this study, namely, the GATE-dependent transcriptional activation. GFB-1 protein has a well conserved redox center. This domain may play a role in regulating the transcription. Interestingly, other transcription factors also possess redox domains or are modulated by redox factors. For example, the upstream stimulating factor (55), D0, a RIPE3b1-like transacting factor (56), Hox B5 (57), and thy-

3 Q. Meng, J. Hu, S. K. Roy, A. Raha, J. Hu, J. Zhang, K. Hashimoto, and D. V. Kalvakolanu, in preparation.
roid transcription factor (58) possess redox-sensitive domains. Furthermore, a number of transcription factors of the Fos-Jun/AP-1 family (59, 60), p53 (61, 62), hypoxia-inducible factor-1α (63), and PEB-2 (64) are regulated via a modulation of their redox status. Ref-1, a redox protein, maintains the redox status of some of transcription factors described above (58, 62, 64–66).

Future studies are required for an understanding of the contribution of the glutaredoxin-like domain to the transactivating function of GBF-1.

GBF-1 is also an IFN-inducible gene (Fig. 8). The induction of the GBF-1 gene is not a cell line-specific effect because it is also induced in various tissues by IFN-γ, in vivo (Fig. 9). Preliminary studies show that GBF-1 protein is also induced by other proinflammatory cytokines, interleukin-1 and -6 (data not shown). Therefore, its expression may be uniquely regulated by IFN-γ and other cytokines. A computer-aided search of published interleukin-1- or -6-inducible gene promoters for GATE-like elements did not reveal any strong homologies. The published interleukin-1- or -6-inducible gene promoters for other proinflammatory cytokines, interleukin-1 and -6 (data not shown) was in review a new report showed that monkey GBF-1 has

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Finally, other transcriptional targets and binding partners of activated transcription needs to be investigated further. Finally, of some of transcription factors described above (58, 62, 64–66) redox status. Ref-1, a redox protein, maintains the redox status of some of transcription factors described above (58, 62, 64–66).

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