Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 103–108, January 1997
Biochemistry

Interferon γ-induced transcription of the murine ISGF3γ (p48) gene is mediated by novel factors

(gene transcription/transcriptional element/inducible transcription factors)

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Communicated by George R. Stark, The Cleveland Clinic Foundation, Cleveland, OH, October 1, 1996 (received for review August 8, 1996)

ABSTRACT **In this investigation, we show that the gene encoding p48, a subunit of transcription factor ISGF3, is transcriptionally induced by interferon γ (IFN-γ). We have identified a novel IFN-γ-activated response element in the p48 gene promoter. This motif, notated as *g*amma-*a*ctivated *t*ranscriptional *e*lement (GATE), has no significant resemblance to either pIRE (palindromic IFN-response element) or GAS (the IFN-γ-activated sequence) but has partial homology to ISRE (IFN-stimulated response element). When fused to a neutral promoter, GATE, a 24-bp element, induced the expression of reporter genes following IFN-γ treatment. In murine RAW cells, two IFN-*g*amma-*i*nducible *f*actors (GIF) bind to GATE. Binding of these factors to GATE is inhibited by cycloheximide and staurosporine. Although p48 gene induction is dependent on STAT1 and JAK1, activated STAT1 does not bind to GATE. Thus, GIFs appear to be novel trans-acting factors in the IFN-signaling pathway.**

Interferon-stimulated gene factor 3 (ISGF3) is a crucial regulator of interferon (IFN)-stimulated gene (ISG) expression (1, 2). This factor, consisting of a 48-kDa DNA-binding protein and three signal transducers and activators of transcription (STAT) proteins (STAT1α, STAT1β, and STAT2), binds to the IFN-stimulated response element (ISRE) and induces gene transcription in response to IFN-α/β (1, 2). Since p48 is IFN-γ inducible, it is also known as ISGF3γ (3, 4). In addition, ISRE also binds IRF-1 (ISGF-2; IRF1 is IFN gene regulatory factor 1), a transcription factor involved in the regulation of IFN-β gene and apoptosis (5, 6). Although the majority of ISGs are dependent on ISGF3 for their induction (1), certain others such as those of inducible NO synthase (7) and murine guanylate binding protein (8) require IRF-1. Gene knock-out studies have shown that p48 and IRF-1 regulate distinct genes and are not redundant in the IFN-signal transduction pathway (9). Ligand-activated IFN-α/β receptor(s) using Janus tyrosine kinases tyk2 and JAK1 stimulates rapid phosphorylation of cytoplasmic STAT1 and STAT2 proteins that then migrate to the nucleus and bind to DNA in association with p48 to stimulate transcription (1). Similarly, ligand-stimulated IFN-γ receptor using Janus kinases JAK1 and JAK2 activates STAT1 protein by tyrosine phosphorylation, which then moves to the nucleus and binds to GAS (IFN-γ-activated site) or GAS-like elements to stimulate gene transcription. These events occur within minutes of receptor occupancy of IFNs without requiring new protein synthesis, and are down-regulated within few hours of initial stimulus (1). Unlike IFN-α/β, gene induction by IFN-γ is variable in terms of regulatory elements, kinetics, and requirement of protein synthesis (1, 10).

It is known from variant cell studies that activation of ISGs and induction of the antiviral state by IFN-α/β requires pretreatment of cells with IFN-γ for several hours (11), which elevates p48 levels (3, 12). Furthermore, expression of p48 is severely impaired in cells expressing adenovirus E1A, thus leading to a global loss of IFN responses (13–15). Therefore, it is necessary to understand how the p48 gene is regulated. Here, we show that the p48 gene is regulated at the transcriptional level by IFN-γ. We have identified a novel regulatory element in the promoter of murine p48 gene and cognate factors that bind to it.

MATERIALS AND METHODS

Cell Culture and Reagents. Murine macrophage RAW (RAW264.7) cell line was grown in RPMI medium 1640. Mutant cell lines U3A (STAT1 deficient), U4A (JAK1 deficient), their corresponding rescued partners U3AR and U4AR, and parent cell line 2fTGH (16, 17) were a gift from George Stark (Cleveland Clinic Foundation). Rabbit anti-STAT1 (p84/p91) and anti-STAT2 antibodies were provided by Chris Schindler (Columbia University, New York). Rabbit anti-ISGF3γ antibody (p48) was provided by David Levy (New York University). Rabbit antibodies against IRF-1 and IRF-2 were gifts from Keiko Ozato (National Institutes of Health, Bethesda). Rabbit anti-IRF-3 antibody (18) was a gift from Paula Pitha-Rowe (Johns Hopkins University School of Medicine, Baltimore). Recombinant murine (Boehringer Mannheim) and human (Genzyme) IFN-γ were used for induction.

Gene Expression Analyses. Northern blot, run-off transcription, transfection, and electrophoretic mobility-shift analyses (EMSAs) were performed as described (15, 19). The following oligonucleotides were used for EMSA, site-directed mutagenesis, and reporter gene analyses: GATE, 5′-CCCGGAGAGAATTGAAACTTAGGG-3′; GATE-Mu, 5′-CCCGGAGAGAATTACTCCTTAGGG-3′; Myc*pm*, 5′-AGACCACGGAGTTTC-3′; Short GATE, 5′-GAGAGAATTGAAACTT-3′; and ISRE, 5′-TAGTTTCACTTTCCC-3′. Mutated bases are underlined. Four clones were identified in a screening of a BALB/c mouse liver genomic library (CLONTECH) in EMBL3 phage vector (5×10^6 plaque-forming units) with a ^{32}P-labeled human p48 cDNA (ref. 4, provided by David Levy). All of them contained the same 13-kb insert as

Abbreviations: GATE, gamma-activated transcriptional element; IFN, interferon; GIF, IFN-γ-inducible factor; GAS, IFN-γ-activated site; ISRE, IFN-stimulated response element; ISGF, IFN-stimulated gene factor; STAT, signal transducers and activators of transcription; pIRE, palindromic IFN-response element; IRF, IFN gene regulatory factor; EMSA, electrophoretic mobility-shift assay; *pm*, point mutation.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U72760).

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analyzed by restriction digestion and Southern blotting. A 7.5-kb *Xho*I fragment containing the upstream regulatory region and the first exon and intron was detected when probed with a 249-bp fragment representing the 5′ end of the cDNA. It was subcloned into pGL-3 basic vector in which luciferase gene served as reporter and did not contain any promoter elements (Promega). Clone A6 was generated by PCR amplification, using an upstream primer (at −1045 bp relative to transcription start site) and a downstream primer (located 42 bp upstream of ATG codon). These primers are shown in Fig. 3. Construct A8 was generated by deleting the sequences up to *Pst*I site in A6. All heterologous promoter-driven constructs contained the simian virus 40 early promoter in pGL3-promoter vector. These vectors lacked all cryptic start sites and yielded high luciferase activities (Promega). Deletion and point mutations were constructed (20) using a PCR-based kit (Stratagene). All constructs were confirmed by DNA sequencing.

RESULTS

IFN-γ Induces the Transcription of p48 Gene. To understand the IFN-γ-regulated expression of p48, we performed Northern blot analyses of RNA from RAW cells, because these cells were exquisitely sensitive to IFN-γ. No detectable level of p48 was observed in untreated cells (Fig. 1*A*, lane 1) but IFN-γ induced expression of this gene (lane 2). Cycloheximide inhibited such induction (lane 3). Thus, induction of p48 gene by IFN-γ was dependent on *de novo* protein synthesis. β-Actin expression was unaffected, suggesting a specific inhibition of p48 gene expression. Nuclear runoff transcription assays demonstrated that IFN-γ up-regulated the transcription after 6 h of treatment compared with untreated RAW cells (Fig. 1*B*). Cycloheximide inhibited IFN-γ-induced transcription. These observations indicated that p48 gene expression was regulated at the transcriptional level, although additional posttranscriptional mechanisms might also contribute to the overall levels of mRNA in the cells.

Because IFN-γ-induced expression of other IRF-family members was regulated by STAT1 and was independent of protein synthesis (21, 22), we next examined whether the induction of p48 occurred in mutant human cell lines (16, 17) that lacked STAT1 and JAK1 (Fig. 1*C*). Expression of p48 mRNA was induced only in the IFN-treated parent cell line 2fTGH but not in the mutants U3A and U4A, which lacked STAT1 and JAK1 genes, respectively. Longer exposure of this blot revealed basal level of p48 mRNA in untreated 2fTGH cells (data not shown). Transfection of JAK1 and STAT1 genes into these cells restored normal induction of p48 gene. Thus, JAK1 and STAT1 were essential for p48 gene expression. Since JAK1 and STAT1 were essential, the activation of STAT1 was quite rapid and independent of protein synthesis, it was surprising that the induction of p48 gene was slow (6–8 h) and sensitive to cycloheximide. These observations prompted us to hypothesize that IFN activation of p48 was dependent on the synthesis of a factor that in association with STAT1 acted as a transcriptional regulator. Alternatively, the p48 promoter might contain a novel response element that did not directly bind STAT1 but rather bound a specific factor whose synthesis was dependent on IFN-activated JAK–STAT pathway.

Isolation and Characterization of the Murine p48 Promoter. To elucidate whether the transcriptional induction was mediated by novel regulatory elements, we isolated the murine p48 gene promoter. Reporter construct A6, upon transfection into RAW cells, strongly responded to IFN-γ treatment and expressed 18-fold more luciferase activity compared with untreated cells (Fig. 2*A*). To further define the region of IFN response, we created the deletion mutant A8 that contained up to −351 bp region of the promoter. Transfection assays revealed that the A6 reporter construct, but not A8, responded to IFN-γ. Two other mutants that contained −177 and −29 bp

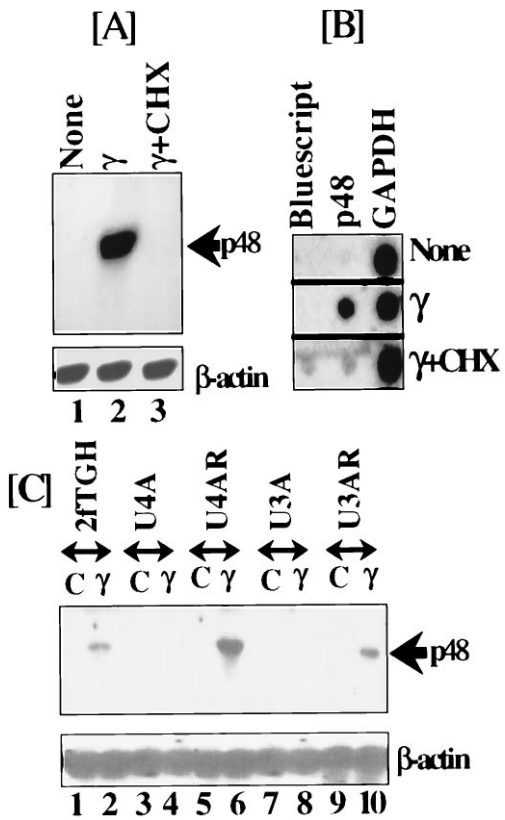

FIG. 1. (*A*) Northern blot analysis of p48 gene expression in RAW cells. Poly(A)$^+$ RNA (1.5 μg) from IFN-γ-treated cells (1000 units/ml for 18 h) was hybridized with a ^{32}P-labeled human p48 cDNA (4).The same blot was probed with a labeled human β-actin to ensure the presence of equal amount RNA in each lane. Blots were exposed for 24 and 6 h to detect p48 and β-actin mRNAs, respectively. Where indicated, cells were treated with cycloheximide for 30 min before the addition of IFN-γ. (*B*) Nuclear runoff transcription. Cells were treated with indicated agents as in *A* except that IFN-γ treatment was performed for 8 h, and runoff transcription assays, in the presence of [α-^{32}P]UTP, were performed as described elsewhere. Labeled RNAs were hybridized to indicated cDNAs immobilized on nylon filters. Bluescript and human GAPDH (glyceraldehyde-phosphate dehydrogenase) were used as negative and positive controls for transcription, respectively. (*C*) Induction of p48 mRNA in mutant cell lines. 2fTGH, U3A, U3AR, U4A, and U4AR cell lines were treated with human IFN-γ (1000 units/ml). Total RNA (40 μg) was Northern blotted and probed as above. C, no treatment; γ, IFN-γ.

upstream sequence also did not respond to IFN-γ (data not shown). These data indicated that the IFN-responsive region was present upstream of the *Pst*I site, between −351 and −1045 bp of the promoter. Similar results were obtained with these reporters in HeLa cells (data not shown). Primer extension analysis revealed the transcriptional start site at 370 nucleotides upstream of ATG codon (data not shown). DNA sequence analysis (Fig. 3) revealed a TATA-box and NF-κB site at −12 and −22 positions, respectively. More importantly, a sequence with partial homology to ISRE and a myc/max binding site were identified at positions −507 and −556, respectively. Since the ISRE-like sequence had no homology to either ISRE or GAS or pIRE (Fig. 4), it was named *g*amma-*a*ctivated *t*ranscriptional *e*lement (GATE).

To further characterize the IFN-responsive element, we created a deletion mutant ΔM1 that lacked the GATE. The wild-type construct A6 but not ΔM1 was strongly up-regulated by IFN-γ. To examine the role of myc/max binding site and GATE in IFN induction, we created two point mutants (*pm*): Myc *pm* and GATE *pm* (20). Transfection analyses showed that Myc *pm* but not GATE *pm* was inducible by IFN-γ (Fig.

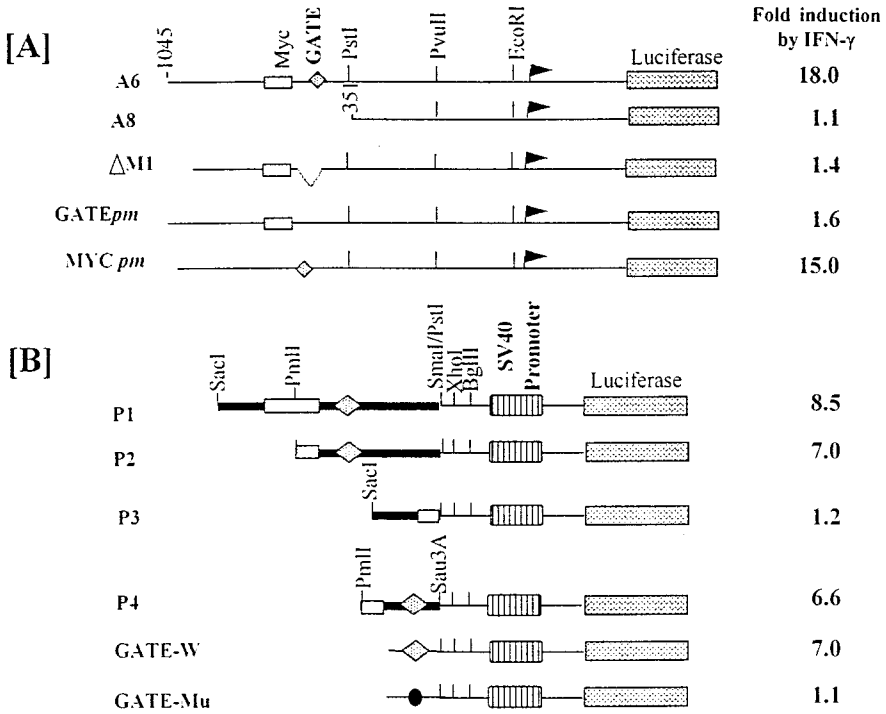

FIG. 2. Murine p48 promoter constructs used in this study. (*A*) Constructs cloned into pGL3-basic vector. Selected restriction sites were indicated. Number of the last nucleotide from the +1 site was indicated on the left. Myc, binding site for myc/max; ΔM1, a deletion mutant that lacked GATE; *pm*, point mutant. The arrow shows the transcription start site. (*B*) Constructs prepared in pGL3 promoter vector, in which simian virus 40 early promoter drives the expression of luciferase gene. Sequences derived from p48 gene promoter are indicated with thick lines. Fold induction by IFN-γ is indicated on the right.

2*A*). Thus myc/max binding site was not essential for gene induction by IFN-γ. We next determined whether fusion of this region to a neutral promoter would impart IFN inducibility. As shown in Fig. 2*B*, the P1 construct that carried the 694 bp sequence that included the GATE and myc/max binding site, in pGL3-promoter vector was stimulated by IFN-γ. The P2 reporter plasmid that carried a deletion in the myc/max site was induced by IFN-γ, but not the P3 reporter that lacked GATE. These data confirmed that GATE was essential for IFN-γ response. Further, a 76-bp element (P4) was IFN responsive in RAW cells. Similar data were obtained with these constructs in HeLa cells (data not shown).

Although disruption of the GATE region resulted in a loss of IFN-γ response, insertion of a synthetic minimal 16 bp element (see Short GATE sequence in *Materials and Methods*) upstream of simian virus 40 promoter did not result in the induction of luciferase gene expression by IFN-γ (data not shown). Therefore, we included four additional nucleotides of flanking sequence from both sides in the synthetic elements. This 24 bp sequence, as a single copy in pGL3-promoter vector, responded to IFN-γ treatment, resulting in a 7-fold increase in luciferase activity (Fig. 2*B*, GATE-W). A similar construct with the mutant element (GATE-Mu) was unresponsive to IFN-γ. More importantly, this sequence had no homology to GBP-GAS or pIRE (Fig. 4*A*). A computer-aided sequence analysis did not reveal a strong homology between GATE and ISRE (Fig. 4*B*). A 10-bp gap was observed in sequence alignment maps. Best fit analysis of this sequence suggested a homology with consensus ISRE in the core area but not with natural ISREs. However, in the best-fit analysis the adjacent sequences were placed away from the core element (Fig. 4 *C*), making it an unlikely site for the interaction of known ISRE binding proteins. These data thus defined GATE as a novel IFN-responsive element.

Binding of Trans-Acting Factors to GATE. To determine if GATE bound to specific factors, EMSA was performed using a ^{32}P-labeled GATE and nuclear extracts of IFN-γ-treated RAW cells. Two trans-acting factors bound to this element in IFN-treated RAW cells (Fig. 5*A*). We named these factors GIFs (IFN-γ-inducible factors). Although a low level of GIF-2 binding was detected in untreated cells, it was enhanced with IFN treatment. A new factor, GIF-1, appeared in IFN-treated cells. GIF-1 binding was detected after 2 h of IFN exposure, which continued to increase for 8 h, remained stable until 16 h, and disappeared by 24 h. Detectable but reduced GIF-2 binding was present at 24 h. Since GATE had a partial homology to ISRE, we tested whether binding of trans-acting factors to ISRE was also affected by IFN-γ. The same nuclear extracts (used in Fig. 5*A*) were employed in this experiment except that ^{32}P-labeled ISRE of ISG-561 was used as probe. A distinct pattern of binding was observed with ISRE (Fig. 5*B*). In RAW cells, two trans-acting factors bound to ISRE. The levels did not change significantly with IFN treatment. The upper band was IRF-1 (see below). Further, an additional ISRE-binding protein(s) with faster mobility was present in RAW cells, whose DNA binding did not change with IFN-γ treatment. ISRE did not bind factors similar to GIF-1 under these conditions. We next determined the specificity of GATE binding factors (Fig. 5*C*). In these experiments, nuclear extracts were incubated with several different competitor oligonucleotides before the addition of probe. GIF binding was competed by wild-type GATE (lanes 6 and 7) but not by mutant GATE (lanes 4 and 5). At higher concentrations, ISRE was able to displace binding (lanes 2 and 3), probably due to its sequence homology with GATE. Neither pIRE nor guanylate-binding protein–GAS could displace the binding of GIFs. Thus, GATE bound to specific transacting factors.

Effect of Inhibitors of Protein Synthesis and Protein Kinases on GIFs. Since the transcription of p48 gene was inhibited by cycloheximide treatment, we asked whether the binding of GIFs was affected similarly. IFN-γ treatment of RAW cells resulted in an activation of GIF-1 and GIF-2 (Fig. 6*A*). IFN-γ-induced binding of GIF-1 and GIF-2 was inhibited in cycloheximide-pretreated cells (Fig. 6*A*, lane 3). Interest-

-1050

TTCCAGCACCAATAAAGAGGCATCTTATGGCCTAGGCTTCTTGGTGGTCCTT

Upstream primer

CTTCCTGGCCTCAGCATCCCGGGGCATTAGGGAAAGGGAGGGGGGAGAGGGACAGA
ATCCTGTTGGAAGGGCAGCCTACCGTAATCCAACCACCCCTGGACTCCTTCCTGGCT
GGGAAGATGTGAAACACCGCGGAGGAACAACAGAGACCCTCCACATCTAGCCACCT
GGCTTGGGCTCAGCGTCCTCTTTTCCCATCATTGACTTCGGCTGCTCTGTGAGAATAA
CCTAACATTTACCAAGCAGACACGGTTGGGGAGCACACATCAGCCTCCGGGTCTGAT
CTGGAGATAACCTTCCTGGGAACCAGACACGGGGCGTAGTTCTGGGAGGAAGGA
GGAGTCTCACAGAGCCACAGGCCCAAGGTGCTACTGCTGACTGAGGCTGCTTCCCTC

PmlI

TGGTGGTAACCGCTTGCCCTGCAACTC*AGACCACGTGGTTT*CTAGGAAATGCAC

Myc/Max site

CTC*CCCGAGGAGAATTGAAACTTAGGG*TGGGGTACTGTAGAAAGGGGAGGA

GATE

GAGATCAGAAAAAGCCTAGAACCTGCTAAGCCTGGGACCTTTTCAGCGGCTCAGGT
AAGAGCAACTCTGCGTCTGCTTCTTTTACCAACTGGGAGCGATGAGAGGGGGCTGGT
GAGGGCTTCCAGAGCCCTGAG*CTGCAG*GGAGAGTCAGAGTGGCTTGTGAATCTGA

PstI

AGTAGATGTGGCCTGAGAGACTGATATCACAGGGCCAGACTGGCTGCCAACTTAAC
TGTTGAGGAAGGTCTACAAAGGCCTTAGTTGAGGTTTGTTTTTCTTGCCTGAGCCCTG
CCCATTTCTTCACGTCCGCCCTTGCCC*CAGCTG*CTGCCCCACCTACCCCTCTAGCAA

PvuII

AGAGGGGTATGGTAAGGAGAAGGTCAGTAGGCTGGGGAGATAGATGCCTTAGGAGC
AAATGTTGCCTCTGTAGATGCTTGGGAGCTAAAACTCTGCCACAAGGAACAAGTCAT

EcoRI

TAAG*GGGAATTCTC*CTAAA*TAATA*CATGTCTGAAAC^{+1}ACTTCAACTTATCAAG

NF-κB

ACCAAATGTCTGAGGTTTCCTTTCAGGTGTAATAACGAGGAATATTCAAAAGAAGTT
TTCCGCCCTAGTTTTTAACCTCTGGGGATGTTTCTAGAGAGAAAAGAACATGGAAGG
GTCTTCAGGGAAGGGCTTCTGAGTGAAGCTGCCCCGGTGGCCAGGGGTTTGCAAGTT
GTTGGTTCTCCCTGGGCATGATGGCGGCAGATATCTTTGATCCCAGCACTTCAGAGG
CAGGTGGATCTGTTGAGTTTAAGTCCAGTTGGTGTATACTGTGAGTCCCAGGCCAAT
CAGGATAACATAGTGAAGTCTGGTCTCGAAATGAAAAGAGTTCTCATATATCTTGCT

Downstream primer

TCCTCTTCCTAGG**ATG**

FIG. 3. Nucleotide sequence of murine p48 gene promoter. GATE is in underlined italics. Selected DNA elements are indicated. Restriction sites within a transcription factor binding site were identified on the top with a line. Upstream and downstream primers used for the PCR amplification of insert in clone A6 are underlined. Transcriptional start site is indicated with a +1 sign.

ingly, even the basal level of GIF-2 was undetectable, which suggested that it may be a labile factor. Synthesis of GIF-1 appeared to be initiated in response to IFN-γ. We also examined the effect of cycloheximide on the ISRE binding factors using the same nuclear extracts. Binding of factors to ISRE was unaffected (Fig. 6*B*). Further, cycloheximide had no effect on STAT1 activation in RAW cells (data not shown). Thus, cycloheximide specifically inhibited the binding of GIFs. A GIF-1-like factor was observed in HeLa-tk cells, whose binding to GATE was inhibited by cyclohemide treatment However, no GIF-2-like factor was detected in these cells (data not shown).

Since IFN-γ activates transcription factors by phosphorylation (17, 23, 24), the effect of protein kinase inhibitors on the activation of GIFs by IFN-γ was examined. RAW cells were incubated with several protein kinase inhibitors: genistein and herbimycin (tyrosine kinase inhibitors), 2-aminopurine (a double-stranded RNA-dependent serine threonine kinase inhibitor), and staurosporine (a protein kinase C inhibitor) during IFN-γ treatment. None of these inhibitors except staurosporine were able to inhibit the binding of GIF-1 and GIF-2 (Fig. 6*C*). Thus, a staurosporine-sensitive kinase may be necessary for the activation of GIFs. These inhibitors had no effect on ISRE-binding proteins (data not shown).

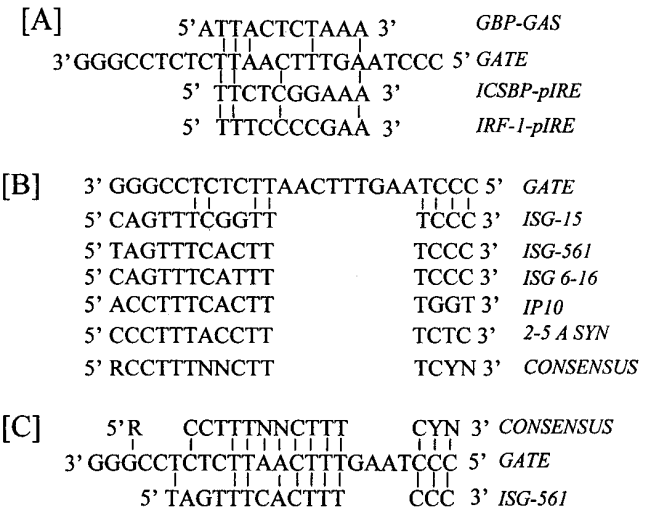

FIG. 4. Comparison of GATE with other IFN-regulated elements. (*A*) Homology between GATE and GAS or pIRE. (*B*) Computer-assisted alignment of the GATE with ISRE sequences of several known genes. (*C*) Best fit alignment of consensus and a natural ISREs (from ISG-561) with GATE. Similarity is noted with a vertical line between the nucleotides.

GIFs Are Not Related to Known ISRE-Binding Proteins. To further identify the factors that interact with the GATE, we used antibodies against various members of IRF and STAT proteins involved in IFN signal transduction pathway (Fig. 7*A*). Antibodies against p48, STAT1, IRF-1, IRF-2, and IRF-3 had

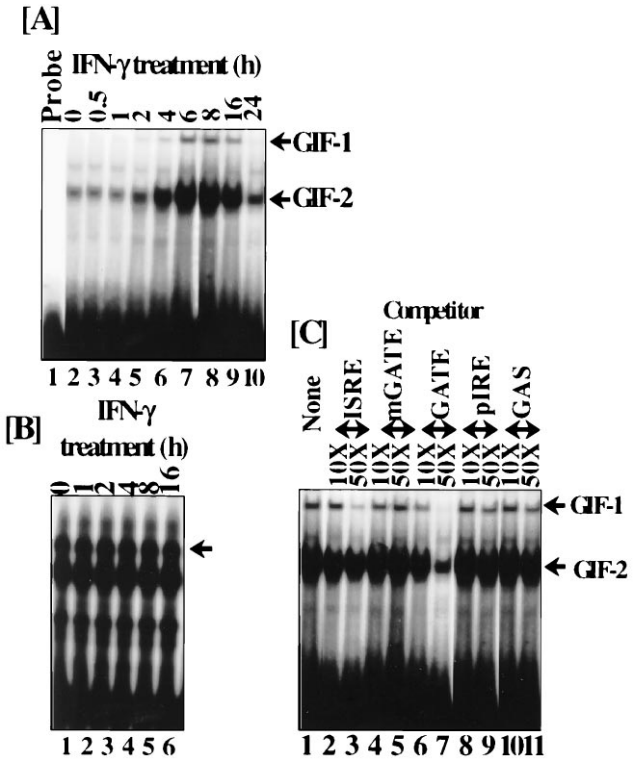

FIG. 5. (*A*) Kinetics of GIF activation by IFN-γ. IFN-γ (1000 units/ml)-treated RAW cells nuclear extracts (15 μg) were analyzed in EMSA using GATE probe. (*B*) The same nuclear extracts were used with a labeled ISRE (from ISG-561) probe. Arrow shows the position of IRF-1 (ISGF-2). (*C*) Specific binding of GIFs to GATE. EMSA was carried out except that nuclear extracts were incubated with indicated molar excesses of various unlabeled oligonucleotides corresponding the IFN-response elements as competitors for 20 min before the addition of labeled GATE probe.

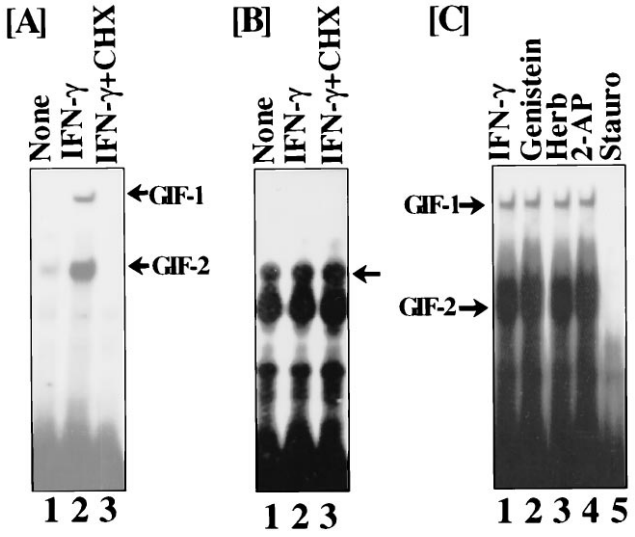

FIG. 6. Effect of cycloheximide (CHX) on IFN-stimulated transcription factors in RAW cells. (*A*) Binding of GIFs to GATE. Treatments were indicated on the top of the figure. *B* was identical to *A* except that ISRE probe was employed. Position of the IRF-1 was indicated with an arrow. (*C*) Effect of protein kinase inhibitors on GIF activation. RAW cells were incubated with various protein kinase inhibitors before IFN-γ-treatment, and EMSA was performed with GATE. Genistein (100 μg/ml) and herbimycin (Herb; 3 μM) were added to the cells at least 10 h before IFN-γ (1000 units/ml) treatment for 6 h. Other inhibitors, 10 mM 2-amino purine (2-AP) and 0.5 μM staurosporine, were added 30 min before incubation with IFN-γ. EMSA with GATE probe was performed as above. Positions of GIF complexes were indicated.

no effect on the GIF-1 mobility. Similar results were obtained with three different anti-STAT1 antibodies (data not shown). Thus, lack of reactivity with GIFs may not be due to differential affinity of the antibodies used in this study. Interestingly, IRF-1 antibodies were able to reduce the DNA binding of GIF-2. Thus, GIF-2 might be an IRF-1-related factor. Effects of anti-IRF antibodies were also checked on the ISRE binding

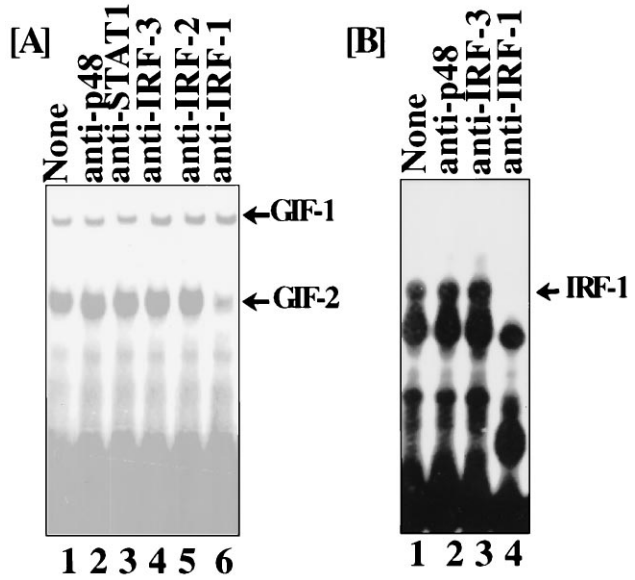

FIG. 7. (*A*) Effect of antibodies against various IFN-regulated proteins on the binding of GIFs to GATE in RAW cells. Nuclear extracts (20 μg) were incubated with indicated antibodies for 40 min at room temperature before the addition of EMSA reagents and ^{32}P-labeled GATE. *B* was similar to *A* except that ISRE probe was used.

complexes in RAW cells (Fig. 7*B*). Anti-IRF-1 antibody, but not the others, completely inhibited the DNA binding of the most upper complex formed with ISRE (lane 4). This observation was consistent with the presence of ISGF-2/IRF-1 in this complex. These data suggested that GIFs are novel IFN-regulated transcription factors.

DISCUSSION

In several variant cells, IFN-γ pretreatment results in a stronger induction of ISGs by IFN-α/β mainly due to an increase in the levels of ISGF3γ (3, 12). Similarly, in RAW cells IFN-γ enhanced the DNA binding of ISGF-3 (data not shown). In this investigation, we have demonstrated that the induction of p48 gene by IFN-γ is mediated by a novel 24-bp regulatory element, GATE. Promoters of other IFN-inducible IRF proteins such as IRF-1 and ICSBP contain pIRE (21, 22, 25) and that of IRF-2 has an ISRE (26, 27). These elements have no strong homology to GATE. Although GATE has a 9-bp central core element like the consensus ISRE (Fig. 4*B*), it does not contain two TTT repeats spaced by three nucleotides, like most ISREs (1, 10). Even in the best fit alignment, the adjacent sequences are spaced farther from the core element, which makes it an unlikely site for the binding of known ISRE-binding proteins. Mutagenesis of TTT residues results in a loss of GIF binding to GATE and transcriptional response (Figs. 2 and 5*C*), which indicates that they are essential for DNA binding of GIFs. Thus, interaction of GATE with its cognate factors requires a longer sequence than the DNA-binding sites of known IFN-regulated transacting factors. Further, neither GAS nor pIRE competes with GATE for GIFs (Fig. 5*C*). Taken together, these data suggest that GATE is a original response element.

Since GATE is a novel element, GIFs that bind to it may also be unique. Several observations suggest that GIFs are novel: (*i*) Induction of GIFs is slower than the other IFN-regulated factors. (*ii*) Although ISRE is partially related to GATE, it does not bind to GIFs. (*iii*) The binding of GIFs to GATE is increased with prolonged IFN-γ treatment (Fig. 5*A*), in contrast to the binding of ISGF1 and IRF-1 to ISRE (Fig. 5*B*). (*iv*) Binding of ISGF2 and ISGF1 to ISRE, or STAT1 to pIRE is independent of protein synthesis (data not shown) and insensitive to protein kinase inhibitors, while that of GIFs to GATE is highly sensitive to cycloheximide and protein kinase C inhibitor staurosporine (Fig. 6 *A* and *C*). (*v*) Antibodies against other known IFN-regulated transacting factors do not affect the DNA binding properties of GIFs (Fig. 7*A*). Although anti-IRF-1 antibodies are able to recognize GIF-2 (Fig. 7*A*, lane 6), it does not appear to be IRF-1 for the following reasons: (*i*) GIF-2 binding to GATE increases with IFN-γ treatment, but IRF-1 binding to ISRE remains unchanged under the same conditions. (*ii*) IRF-1 binding is insensitive to protein synthesis inhibitors as well as protein kinase inhibitors, whereas GIF-2 binding to GATE is inhibited by these agents. (*iii*) p48 gene expression is normal in IRF-1$^{-/-}$ mice (9).

If GIFs are regulators of the p48 gene, and protein kinase C-like activities are necessary for their activation and STAT proteins do not bind to GATE, why would mutations in JAK1 and STAT1 inhibit (Fig. 1*C*) p48 gene induction? Our hypothesis is that p48 is regulated by secondary factors such as GIFs, whose expression is controlled by the JAK–STAT pathway. Once GIFs are induced by IFN, their functional activity is modulated by a staurosporine-sensitive protein kinase. This kinase may be an IFN-activated enzyme or an ISG. Consistent with this, protein kinase C-ε gene is an ISG (28). In this connection, p48 gene regulation is similar to that of major histocompatibility complex class II. IFN-γ induction of major histocompatibility complex class II genes is also quite slow and regulated by a secondary regulatory factor, class II transcrip-

tional activator (CIITA). Expression of GIFs, like CIITA, may also be regulated by IFN-γ (29, 30).

We thank all the colleagues who have generously provided several reagents used in this study; Ernest Borden, Daniel Lindner, Kyunghee Choi, and Padma Vanguri for critical reading of this manuscript; and the National Cancer Institute for grant support.

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