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Interferon Regulatory Factor 1 Is Required for Mouse *Gbp* Gene Activation by Gamma Interferon

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Full-scale transcriptional activation of the mouse *Gbp* genes by gamma interferon (IFN-γ) requires protein synthesis in embryonic fibroblasts. Although the *Gbp-1* and *Gbp-2* promoters contain binding sites for transcription factors Stat1 and IFN regulatory factor 1 (IRF-1), deletion analysis revealed that the Stat1 binding site is dispensable for IFN-γ inducibility of *Gbp* promoter constructs in transfected fibroblasts. However, activation of the mouse *Gbp* promoter by IFN-γ requires transcription factor IRF-1. Transient overexpression of IRF-1 cDNA in mouse fibroblasts resulted in high-level expression of *Gbp* promoter constructs. Unlike wild-type cells, IRF-$1^{0/0}$ embryonic stem cells lacking functional transcription factor IRF-1 contained very low levels of *Gbp* transcripts that were not increased in response to differentiation or treatment with IFN-γ. Treatment of IRF-$1^{0/0}$ mice with IFN-γ resulted in barely detectable levels of *Gbp* RNA in spleens, lungs, and livers, whereas such treatment induced high levels of *Gbp* RNA in the organs of wild-type mice. These observations suggest two alternative pathways for transcriptional induction of genes in response to IFN-γ: immediate response that results from activation of preformed Stat1 and delayed response that results from induced de novo synthesis of transcription factor IRF-1.

Impressive progress has recently been made towards understanding the mechanistic details of gene activation in response to gamma interferon (IFN-γ). This cytokine binds with high affinity to IFN-γ surface receptors whose two polypeptide chains were cloned and sequenced previously (1, 16, 49). Once stimulated by binding of IFN-γ, the receptor activates the protein tyrosine kinases JAK1 and JAK2, which in turn phosphorylate the latent cytoplasmic transcription factor Stat1 (29, 33, 47). Phosphorylated Stat1 is translocated to the nucleus, where it binds to the GAS sequence motif of IFN-γ-responsive promoters, thereby inducing enhanced gene expression (11, 48). Pioneering work on Stat1 function was performed with the promoter of the human *GBP-1* gene for which the GAS motif was originally described (24). The *GBP-1* gene of humans encodes an IFN-inducible protein capable of binding to agarose-immobilized guanine nucleotides (5, 6). It was recently shown to be an unconventional GTPase that converts GTP to GMP (45). Human and mouse *Gbp* genes are induced by IFN-α and more strongly induced by IFN-γ (7, 10). Some mouse strains are refractory to the induction of *Gbp-1* mRNA in response to IFN-α and IFN-γ (50, 51), and this genetic trait was mapped to mouse chromosome 3 (37). Mouse *Gbp-1* cDNAs were independently cloned from IFN-α-induced embryonic fibroblasts (7) and IFN-γ-induced macrophages (56).

IRF-1 is a transcription factor that binds to sites within the promoters of IFN-α, IFN-β, and several IFN-inducible genes (14, 15, 28, 36). The IFN regulatory factor 1 (IRF-1) gene is induced by IFNs and some other cytokines (12, 13, 15, 36, 54). Mice with homozygous targeted disruptions of the IRF-1 gene (IRF-$1^{0/0}$) show no overt abnormalities other than reduced levels of $CD8^+$ T cells (27, 39). However, IRF-$1^{0/0}$ mice show enhanced susceptibility to challenge with encephalomyocarditis virus (22). IFN was fully inducible in such mice by virus and poly(I) · poly(C) (39). Although embryonic fibroblasts from IRF-$1^{0/0}$ mice produced less IFN-α/β in response to double-stranded RNA than did wild-type cells, this defect was overcome if the cells were primed with IFN-α/β prior to induction (27, 39). Induction by IFN-α/β of several inducible genes in IRF-$1^{0/0}$ embryonic stem cells and fibroblasts was not altered (27, 41). However, induction by IFN-γ of the iNOS gene and the *Gbp-2* gene is impaired in macrophages (20) and embryonic fibroblasts, respectively, of IRF-$1^{0/0}$ mice (22).

The promoter of the *Gbp-1* gene of BALB/c mice has recently been cloned and sequenced (30). Some 60 bp located immediately upstream of the transcription start site was found to be necessary and sufficient for IFN-γ inducibility in a macrophage cell line (30). We independently cloned the promoters of the mouse *Gbp-1* and *Gbp-2* genes and found that sequences upstream of position −219, including a GAS element, are dispensable for IFN-γ inducibility of *Gbp* promoter constructs in 3T3 fibroblasts. Our experiments with cultured cells and animals lacking functional IRF-1 showed that IFN-γ inducibility of the mouse *Gbp* promoters is highly dependent on transcription factor IRF-1.

MATERIALS AND METHODS

Nuclear run-on assays. Confluent monolayers of BALB/c embryonic fibroblasts (51) were treated for 0, 2, or 4 h with 1,000 U of rat IFN-γ per ml in the presence or absence of 75 μg of cycloheximide (CHX) per ml. A control culture was kept for 4 h in medium lacking IFN-γ but containing 75 μg of CHX per ml. Nucleus preparation and run-on assays were done as described elsewhere (10).

Library screening. Approximately 6×10^5 plaques of a BALB/c mouse genomic library constructed in phage lambda EMBL3 Sp6/T7 (Clontech, Palo Alto, Calif.) were screened with a radiolabeled fragment from the 5′ end of *Gbp-1* cDNA (7), and one positive clone was isolated. Southern blot analysis revealed that a 1.4-kb *Eco*RI fragment of this phage contained the target se-

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quence. This fragment was subcloned into pBluescript SK (Stratagene, La Jolla, Calif.), and its complete sequence was determined by the dideoxy sequencing method (42) by using the T7 sequencing kit (Pharmacia).

PCR amplification and cloning of the *Gbp-1* and *Gbp-2* promoters. The promoter regions of the mouse *Gbp-1* and *Gbp-2* genes were amplified by PCR from mouse liver DNA from our local BALB/c subline. Oligonucleotides GBP-4, 5′CACACTGAATTCTGAAGAGAAATCCTTTAGAGAAG3′ (positions +148 to +125), and GBP-5, 5′CCGTGGGTAAATCAGCATGAGG3′ (positions −1021 to −1000) (30), served as PCR primers. Amplification was performed over 30 cycles for 30 s at 94°C, for 2 min at 58°C, and for 90 s at 72°C. PCR products were digested with *Eco*RI and *Hin*dIII and cloned into pBluescript SK. Partial sequencing of the resulting clones was done with oligonucleotide GBP-4.

Electrophoretic mobility shift assays. Various restriction fragments of the *Gbp-2* promoter and a double-stranded oligonucleotide corresponding to its GAS motif (sense, 5′TCGAACAAGCTAGCTGATTTCCCAGCATTTGAC ATGT3′; antisense, 5′TCGAACATGTCAAATGCTGGGAAATCAGCTAG CTTGT3′) were tested for their abilities to inhibit the interaction of transcription factor Stat1 with a radiolabeled oligonucleotide from the human *GBP-1* promoter. The experimental conditions were as described elsewhere (52).

Luciferase constructs. The promoterless luciferase reporter plasmid pHSIIα (44) was prepared by deleting the foreign promoter by *Spe*I and *Xho*I digestion, polishing the ends with Klenow polymerase, and religating. The *Hin*cII-*Sma*I fragment of the *Gbp-2* promoter and the *Hin*dIII-*Sma*I fragments of both *Gbp* promoters were inserted into the promoterless reporter vector. To create unidirectional deletions of the *Gbp-2* promoter, the *Hin*cII-*Sma*I fragment of the *Gbp-2* promoter was cloned into vector pGL2-Basic (Promega, Madison, Wis.) upstream of the promoterless luciferase gene. The resulting plasmid was digested with either *Sac*I and *Nco*I or *Sac*I and *Nde*I, and samples were incubated with exonuclease III, treated with mung bean nuclease, and religated essentially as described elsewhere (17). The 5′ end points of the deletions were determined by sequencing.

Determination of luciferase activities in transfected 3T3 cells. Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Semiconfluent monolayers of 3T3 cells in six-well dishes were transfected with 5 μg of the various plasmid DNAs per well by the calcium phosphate coprecipitation method. At 24 h posttransfection, the cultures were treated for 16 h with mouse IFN-α_2 or rat IFN-γ at a concentration of 1,000 U/ml. Overexpression of IRF-1 was achieved by transfecting 2.5 μg of expression plasmid pIRF-L (28) per well. This DNA was cotransfected with 2.5 μg of the *Gbp-2* promoter-luciferase construct per well. Control transfection mixtures contained (per well) 2.5 μg of vector pCDM8 (2) and 2.5 μg of the *Gbp-2* promoter-luciferase construct. The cells were harvested and subjected to three cycles of freezing and thawing, and luciferase activities in the cell extracts were determined with a Lumat LB 9501 apparatus (Berthold, Wildbad, Germany) by standard procedures (3).

Differentiation and induction of ES cells for Northern (RNA) blot analysis. A clone of embryonic stem (ES) cells (D3) with two disrupted IRF-1 alleles (IRF-$1^{0/0}$) and a wild-type clone with two intact IRF-1 alleles (IRF-$1^{+/+}$) (described in reference 41) were used for the induction experiments. An aliquot of feeder-free undifferentiated ES cells was thawed and cultured in a gelatinized tissue culture flask either in DMEM containing 20% FCS or in 60% buffalo rat liver cell (BRL) conditioned medium (41). The cells were trypsinized after 15 to 18 h and transferred in DMEM with 10% FCS to nongelatinized tissue culture dishes. For experiments with undifferentiated ES cells, 2.8×10^6 cells (thawed in DMEM with 20% FCS) were transferred to 5-cm-diameter dishes and induced immediately with IFN for 12 h. For experiments with differentiated ES cells, 4×10^5 cells (thawed in 60% BRL conditioned medium) were transferred to 9-cm-diameter dishes, grown for 4 days, and then induced with IFN for 6 h.

Induction of *Gbp* genes by IFN-γ in vivo. A male IRF-1 knockout founder mouse (39), which was generated from manipulated ES cells that originated from a 129 mouse, was crossed to females of strain 129 to create offspring of pure genetic background. Wild-type (+/+) mice and animals with heterozygous (+/0) or homozygous (0/0) disruptions of the IRF-1 locus were selected. One mouse of each group was given 50,000 U of rat IFN-γ by the intraperitoneal route. Six hours later, the animals were killed and RNA was extracted from spleens, lungs, and livers. Organs from animals that were not treated with IFN-γ served as controls.

RNA analysis. Northern blot analysis was done with 20-μg samples of total RNA isolated from ES cells or organs by the acid phenol method (8). After electrophoresis through a 1.2% agarose gel in 4% formaldehyde running buffer, the RNA was transferred to a nitrocellulose membrane. The membrane was hybridized sequentially with various ^{32}P-labeled probes generated by nick translation or random primer labeling. The probes were the full-length insert of *Gbp-1* cDNA (7), a fragment from the 3′ noncoding region of IRF-1 cDNA (41), and a fragment of GAPDH cDNA (41). Hybridization signals were quantified with the digital autoradiograph LB286 (Berthold).

RESULTS

Protein synthesis is required for full-scale induction by IFN-γ of the mouse *Gbp* genes. To determine whether induction of the mouse *Gbp* genes by IFN-γ was a transcriptional response, we performed run-on experiments with isolated nuclei of mouse embryonic fibroblasts. These cells were derived from BALB/c mice which have the *Gbp-1*^{a} genotype (51) and therefore express both *Gbp* genes. Treatment of confluent cell monolayers for either 2 or 4 h with 500 U of rat IFN-γ (which is highly active on mouse cells) per ml resulted in strong activation of the *Gbp* genes (Fig. 1). The degree of activation was comparable to that of the IRF-1 gene, a well-characterized IFN-γ-regulated gene (35), which served as a reference. This picture changed when the induction was performed in the presence of the protein synthesis inhibitor CHX. The IRF-1 gene responded to the combined treatment with IFN-γ and CHX by enhanced gene transcription (Fig. 1). After normalizing the signals to the corresponding actin controls, we calculated that IFN-γ induced the IRF-1 gene about fourfold better in the presence of CHX than in its absence. By contrast, the *Gbp* genes responded less well to IFN-γ in the presence of CHX (Fig. 1). Quantitation showed that the normalized *Gbp* signals were three- to fourfold weaker when the induction was performed in the presence of CHX. These results showed that the mouse *Gbp* genes are regulated at the transcriptional level, like IRF-1 and other IFN-γ-activated genes. But unlike IRF-1, the *Gbp* genes required protein synthesis for full-scale induction by IFN-γ. Thus, regulation by IFN-γ of the mouse *Gbp* genes in fibroblasts was fundamentally different from that of the human *GBP* gene in the same cell type (24).

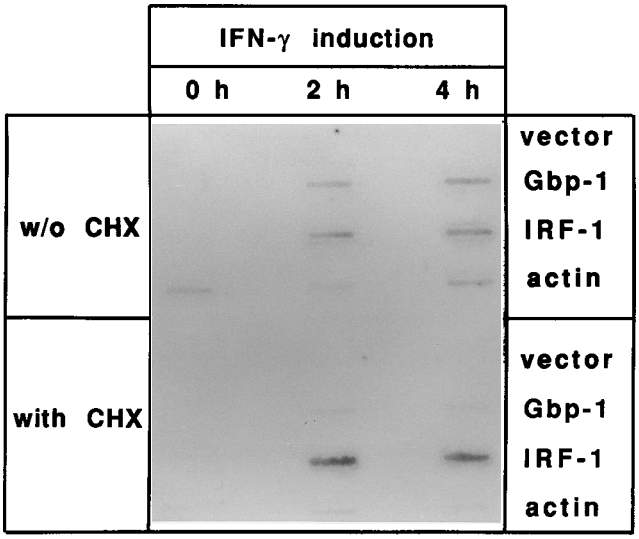

FIG. 1. Full-scale induction of the *Gbp* genes by IFN-γ in mouse embryonic fibroblasts requires protein synthesis. Cells were treated for 2 or 4 h with 1,000 U of rat IFN-γ per ml in the presence or absence of 75 μg of CHX per ml. Run-on RNA was hybridized to filters containing vector DNA and cDNA probes for mouse *Gbp-1*, mouse IRF-1, and human actin. After being washed at high stringency, the filters were exposed to X-ray film.

Cloning of the promoters of the mouse *Gbp* genes. A lambda phage library of BALB/c liver DNA was screened with a radiolabeled cDNA fragment that contained sequences from near the 5′ end of *Gbp-1* mRNA (7). Sequence analysis of the 1.4-kb *Eco*RI fragment of a positive clone revealed that it contained exon 1, parts of intron 1, and about 1 kb of upstream sequence. However, the genomic sequence of exon 1 differed from our published *Gbp-1* cDNA sequence (7) by base changes at positions 9 and 14, and by the presence of 6 extra nucleotides between cDNA positions 14 and 15. Two more base changes in exon 1 were detected upon comparing the genomic

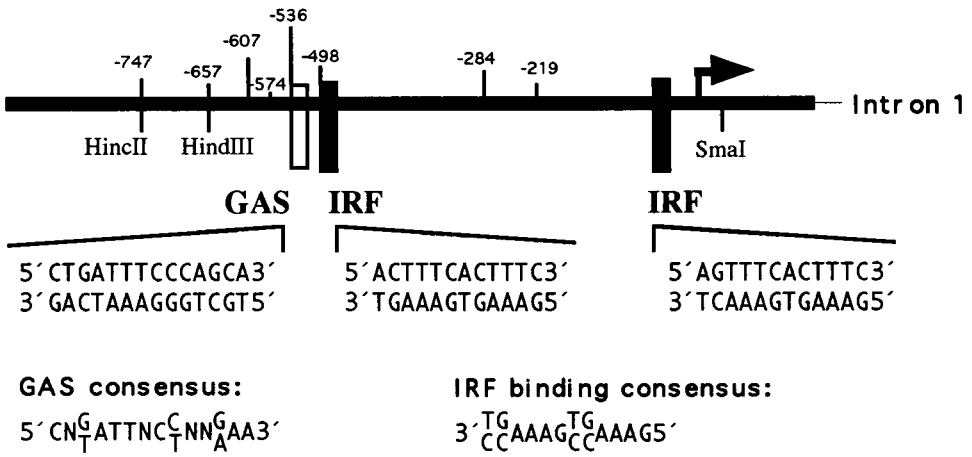

FIG. 2. Overview of the organization of the mouse *Gbp* promoter and location of potentially important regulatory sequences. The promoters of the *Gbp-1* and the *Gbp-2* genes have almost identical structures. The arrow marks the major transcription start site that was mapped for the *Gbp-1* gene (30). The *Hin*dIII-*Sma*I promoter fragments of both *Gbp-1* and *Gbp-2* promoters functioned as IFN-γ-inducible elements when cloned upstream of the promoterless luciferase gene. The minimal GAS consensus motif (52) and the IRF-binding consensus sequence (53) are shown. The 5′ end points of *Gbp-2* promoter deletion constructs are indicated.

Gbp sequence with that of a recently isolated full-length *Gbp-1* cDNA clone (3a). These differences could be due to polymorphic differences between the BALB/c sublines used for the genomic and cDNA libraries. Alternatively, these results could indicate that we cloned the 5′ end of a closely related nonallelic *Gbp* gene. To distinguish between these possibilities, we prepared PCR oligonucleotide primers corresponding to sequences around positions −1000 and +150 of the genomic *Gbp* clone (Fig. 2), amplified the intervening sequences from genomic DNA of a BALB/c mouse, and cloned the PCR products into a plasmid vector. The exon 1 sequences of 11 of these clones were determined. Seven sequences corresponded to the *Gbp-1* cDNA sequence, whereas the other four showed the 6-bp insertion and the other changes previously observed to be present in our genomic *Gbp* clone. This result strongly suggested that we were dealing with the promoters of two closely related *Gbp* genes, which we designated *Gbp-1* and *Gbp-2*. *Gbp-1* is the gene that codes for the well-characterized 3.0-kb *Gbp* mRNA (7). Accordingly, the promoter fragment isolated from our positive lambda phage must be derived from the *Gbp-2* gene. Restriction analysis and partial sequencing demonstrated that the two *Gbp* genes had virtually identical upstream sequences (data not shown). Furthermore, the *Hin*dIII-*Sma*I fragments of both promoters functioned equally well as IFN-inducible control elements (Fig. 3A). Nicolet and Paulnock (30) recently published the *Gbp-1* promoter sequence (GenBank accession number L12199), which agrees with our own sequencing data.

The GAS element is dispensable for inducibility of the mouse *Gbp* promoter by IFN-γ. The in vivo activities of the two *Gbp* promoters were measured by using Swiss 3T3 mouse fibroblasts. Various *Gbp* promoter fragments were cloned in the correct orientation in front of the promoterless luciferase gene of expression vectors pHSIIα and pGL2. Cultures transiently transfected with the resulting constructs were induced for 16 h with either mouse IFN-α or rat IFN-γ, and luciferase activities in the cell extracts were determined. The *Gbp-1* and the *Gbp-2* promoters both conferred some basal transcriptional activity to the luciferase constructs in 3T3 cells (Fig. 3A). Treatment of such cells with IFN-γ resulted in enhanced luciferase activities (typically about eightfold). Luciferase activities in cultures treated with IFN-α were not significantly different from those of the untreated control cultures (Fig. 3A).

The *Gbp* promoters contain two binding motifs for transcription factor IRF-1 and a sequence that matches the consensus of a GAS element (Fig. 2). To test whether the latter sequence might serve as a target for the corresponding transcription factor Stat1, we performed a series of band shift competition assays: various restriction fragments of the *Gbp-2* promoter and a double-stranded oligonucleotide corresponding to the putative GAS motif of the mouse *Gbp* promoters were tested for their abilities to inhibit the interaction between Stat1 and a GAS oligonucleotide of the human *GBP-1* promoter. Inhibition correlated with the presence of the putative GAS element (data not shown), suggesting that this element is functional. To verify the role of this element and to define other regulatory elements of the mouse *Gbp-2* promoter, we reduced its size by progressive 5′ deletions and tested the resulting constructs for IFN inducibility in transfected 3T3 cells. Unexpectedly, this analysis showed that the GAS element and the upstream IRF-binding site could be deleted from the *Gbp-2* promoter without significantly reducing its inducibility by IFN-γ. Trimming the *Gbp-2* promoter to 219 nucleotides of upstream sequence failed to abolish inducibility (Fig. 3B). None of the various deletion constructs was induced in response to IFN-α. Functional analysis of various mouse *Gbp-1* promoter constructs in a macrophage cell line by Nicolet and Paulnock (30) gave similar results: they trimmed the *Gbp-1* promoter to position −59 without observing dramatic effects on its inducibility by IFN-γ. Taken together, these results strongly suggested that the downstream IRF-binding site in the mouse *Gbp* promoters, rather than the GAS motif, is of critical importance for inducibility by IFN-γ.

Overexpression of IRF-1 cDNA results in constitutive high-level activation of the *Gbp-2* promoter. To further evaluate the role of the IRF-binding sites in the *Gbp-2* promoter, we tested whether it responded to overexpression of transcription factor IRF-1. 3T3 cells were cotransfected with a *Gbp-2* promoter-luciferase construct and either an expression plasmid encoding IRF-1 or the empty vector. As expected, the vector alone had no effect on *Gbp-2* promoter activity: its basal activity level was moderately high in uninduced cells and was increased about sixfold in cells treated with IFN-γ (Fig. 4). Simultaneous transfection of the *Gbp-2* promoter construct and IRF-1 cDNA resulted in a very high level of luciferase activity that could not be up-regulated any further by treatment of the transfected

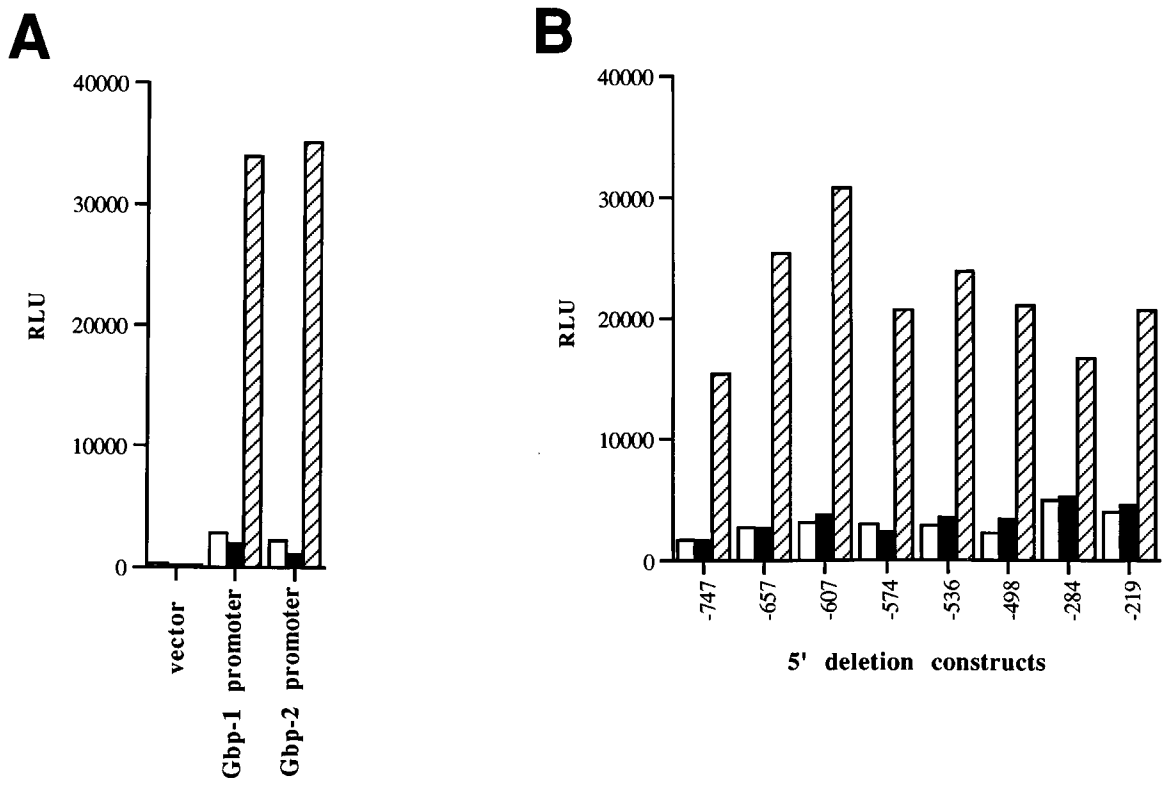

FIG. 3. Responses to IFN-α and IFN-γ of various *Gbp* promoter-luciferase constructs in mouse 3T3 cells. (A) Comparison of the activities of *Gbp-1* and *Gbp-2* promoter constructs. The *Hin*dIII-*Sma*I fragments of the two promoters were cloned upstream of the promoterless luciferase gene of the reporter plasmid pHSIIα (44). Cells were transiently transfected with the indicated DNAs and treated for 16 h with plain medium (open bars), 1,000 U of recombinant mouse IFN-α_2 (46) per ml (black bars), or 1,000 U of recombinant rat IFN-γ (43) per ml (striped bars). Luciferase activities in the cell lysates were determined as described in Materials and Methods. (B) Functional analysis of 5′ deletion constructs of the *Gbp-2* promoter. Promoter deletion fragments were cloned upstream of the promoterless luciferase gene in the reporter plasmid pGL2 (Promega). Cells were transiently transfected with the indicated DNAs and treated for 16 h as described in the legend for panel A. RLU, relative light units.

cells with either IFN-α or IFN-γ (Fig. 4). By contrast, a moderate reduction of luciferase activity was observed to occur in IFN-γ-treated cells. A clear stimulating effect of IRF-1 cDNA cotransfection (4- to 23-fold) was observed for all subsequent experiments with 3T3 and L929 cells, even without induction by IFN-γ (data not shown).

The endogenous *Gbp* promoter fails to respond to IFN-γ in ES cells with homozygous targeted disruptions of the IRF-1 gene. If regulation of the mouse *Gbp* promoter by IFN-γ was indeed dependent on IRF-1, expression of this gene should be strongly reduced in cells lacking transcription factor IRF-1. ES cells with targeted disruptions of both alleles of the IRF-1 gene (41) and wild-type control cells were treated for 6 or 12 h with plain medium, IFN-α, or IFN-γ before RNA was isolated and analyzed for *Gbp* transcripts by Northern blotting. The ES cells originated from mouse strain 129, which is of the *Gbp-1*^{a} genotype (51). Consequently, wild-type ES cells should express both *Gbp* mRNAs under appropriate induction conditions. Since expression of transcription factor IRF-1 in wild-type cells varies during differentiation in vitro (41), we performed experiments with ES cells that were grown for 15 h (undifferentiated cells) or 4 days (differentiated cells) in the absence of BRL conditioned medium before induction with IFNs.

In differentiated wild-type cells, we observed very strong *Gbp* signals irrespective of IFN treatment (Fig. 5). The stronger signal results from hybridization of the radiolabeled probe to the 3.0-kb *Gbp-1* mRNA (7), whereas the weaker signal at about 2.8 kb resulted from hybridization to mRNA derived

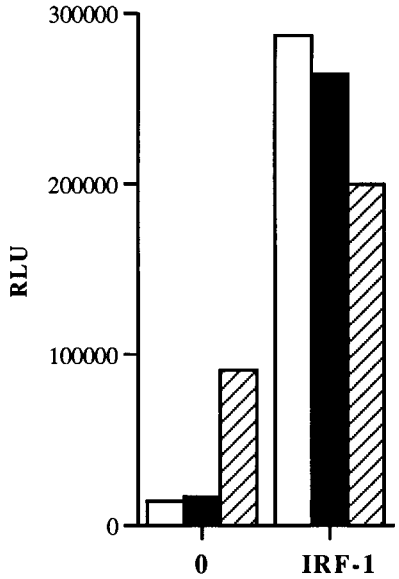

FIG. 4. Constitutive activation of a *Gbp* promoter-linked reporter gene by cotransfection with an IRF-1 cDNA expression plasmid. 3T3 cells were transiently transfected with the *Hin*cII-*Sma*I *Gbp-2* promoter-luciferase construct and either CDM8 vector DNA (0) or IRF-1 cDNA (IRF-1). Treatment with plain medium (open bars), 1,000 U of mouse IFN-α_2 per ml (black bars), or 1,000 U of rat IFN-γ per ml (striped bars) was for 16 h. Luciferase activities in the cell lysates were determined. RLU, relative light units.

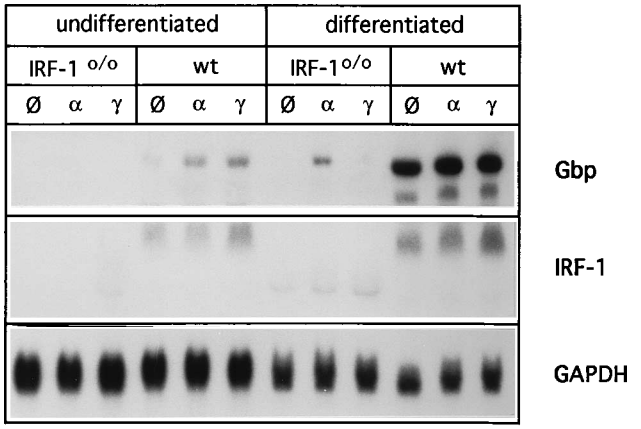

FIG. 5. Expression of the endogenous *Gbp* genes in IRF-$1^{0/0}$ and wild-type ES cells. Undifferentiated and differentiated ES cells were left untreated (ø) or were stimulated for 12 and 6 h, respectively, with 500 U of recombinant human IFN-α_2/α_1 (α) per ml (which is active on mouse cells [55]) or 500 U of mouse IFN-γ (γ) per ml. Total RNA was prepared, and 20-μg samples were subjected to Northern blot analysis. The membrane was sequentially hybridized to radiolabeled *Gbp-1*, IRF-1, and GAPDH cDNA probes. wt, wild type.

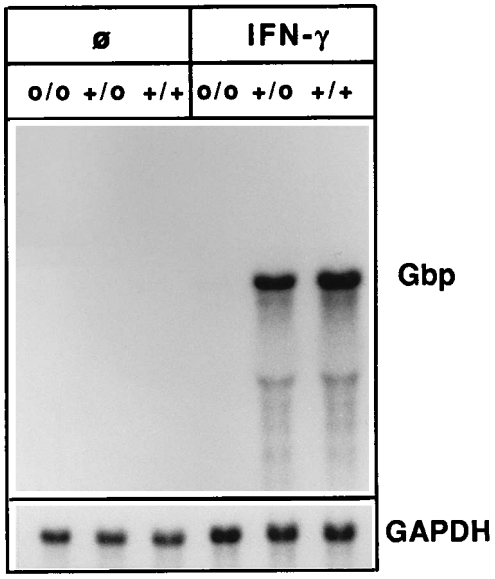

FIG. 6. Weak induction of *Gbp* genes in the spleens of IRF-$1^{0/0}$ mice treated with IFN-γ. Wild-type 129 mice (+/+) and 129 mice with homozygous (0/0) or heterozygous (+/0) disruptions of the IRF-1 locus were left untreated (ø) or were treated for 6 h with 50,000 U of rat IFN-γ by the intraperitoneal route (IFN-γ). RNA was extracted from the spleens, and 25-μg samples were analyzed for *Gbp* transcripts by Northern blotting. *Gbp-1* and *Gbp-2* transcripts were poorly resolved on this gel. Hybridization of the membrane with a probe for GAPDH demonstrated that similar amounts of RNA were loaded in each lane.

from the *Gbp-2* gene. High-level constitutive expression of the *Gbp* genes in differentiated wild-type ES cells was paralleled by constitutive expression of IRF-1 in these cells (Fig. 5). In differentiated IRF-$1^{0/0}$ ES cells, virtually no *Gbp* RNA was detected in the absence of IFN treatment. More importantly, treatment of these cells with IFN-γ had no detectable effect on *Gbp* gene expression: *Gbp* RNA levels remained barely detectable (Fig. 5), indicating that other IFN-γ-activated factors had no stimulatory effect on the *Gbp* promoters in these cells. By contrast, significant activation of the *Gbp* promoter by IFN-α was observed in differentiated IRF-$1^{0/0}$ cells (Fig. 5). Quantitation of the hybridization signals showed that differentiated IRF-$1^{0/0}$ cells treated with IFN-α contained about 17-fold-lower levels of *Gbp* RNA than did wild-type cells. The *Gbp* signals of untreated or IFN-γ-treated IRF-$1^{0/0}$ cells were too weak for accurate quantitation: both values were at least 100-fold lower than those for differentiated wild-type cells.

In the absence of IFN, undifferentiated wild-type ES cells contained 30- to 50-fold-lower levels of *Gbp* RNA than did differentiated cells. Induction with IFN-α or IFN-γ resulted in two- to threefold-increased *Gbp* RNA levels (Fig. 5). No *Gbp* RNAs were detected in untreated and IFN-γ-treated IRF-$1^{0/0}$ cells, whereas treatment of undifferentiated IRF-$1^{0/0}$ ES cells with IFN-α resulted in a small increase of the *Gbp* RNA level (hardly visible in Fig. 5).

Hybridization of the RNA blot with an IRF-1 cDNA probe showed that undifferentiated wild-type ES cells contained levels of the corresponding RNA only about three- to fivefold lower than those in differentiated cells and that this level was slightly higher in cells treated with IFN-α or IFN-γ (Fig. 5). Assuming that IRF-1 mRNA levels reflect IRF-1 protein levels, these results suggest that the mere presence of IRF-1 cannot explain the different *Gbp* RNA levels in differentiated and undifferentiated cells. As described earlier (41), IRF-$1^{0/0}$ ES cells expressed low levels of an aberrant IRF-1 mRNA (Fig. 5).

Weak induction of *Gbp* genes in organs of IRF-$1^{0/0}$ mice treated with IFN-γ. To determine whether the IRF-1 dependence of the mouse *Gbp* promoters was restricted to cultured cells, we treated mice of the *Gbp-1*^{a} strain 129 with disrupted or wild-type IRF-1 alleles for 6 h with IFN-γ before analyzing the *Gbp* transcript levels in various organs. Injection of 50,000 U of rat IFN-γ into the peritoneal cavity of IRF-$1^{+/0}$ and wild-type mice (which carry one or two functional IRF-1 alleles) resulted in very high levels of *Gbp* RNA in spleens (Fig. 6), lungs, and livers (data not shown). Untreated control animals contained about 25-fold-reduced levels of *Gbp* transcripts in the examined organs. By contrast, IRF-$1^{0/0}$ mice contained very low levels of *Gbp* RNA in spleens (Fig. 6), lungs, and livers (data not shown) irrespective of treatment. Quantitation showed that the IFN-γ-treated IRF-$1^{0/0}$ mouse contained levels of *Gbp* transcripts in the various organs 20- to 25-fold lower than those in the treated wild-type mouse.

DISCUSSION

Gene activation by IFN-γ is thought to depend on the interaction of activated transcription factor Stat1 with its target sequence GAS located in the promoter region of IFN-γ-inducible genes (11, 47, 48). This simple picture of signal transduction in response to IFN-γ is complicated by findings that other factors can bind to sequence motifs of IFN-γ-regulated promoters (19, 24) and that some of them are themselves subject to regulation by IFN-γ, like transcription factor IRF-1 (28, 35). Such DNA-binding proteins are thought to play important roles in amplifying or down-regulating the IFN-γ signal (15, 19, 28, 31), but they have previously not been recognized as principal regulators of IFN-γ-inducible genes. We have now shown that the transcriptional response of the mouse *Gbp* genes to IFN-γ is dependent on transcription factor IRF-1. For IFN-γ-mediated activation, the *Gbp* genes seem not to require the binding of transcription factor Stat1 to their promoters.

Cloning of the IFN-γ-inducible mouse *Gbp* promoter was more complicated than anticipated. The recombinant lambda phage that we initially isolated contained genomic sequences that seemed to represent exon 1 of the mouse *Gbp-1* gene.

However, this sequence differed from the cloned *Gbp-1* cDNA by a 6-bp insertion and several base changes. A more detailed analysis by PCR of genomic DNA of a locally bred BALB/c mouse revealed that it contained two variants of *Gbp* exon 1, suggesting that these variants represented two separate *Gbp* genes rather than allelic forms of the same gene. Nicolet and Paulnock (30) recently isolated a recombinant lambda phage that contains the bona fide *Gbp-1* promoter from the same commercial mouse BALB/c DNA library that we have used to isolate the *Gbp-2* promoter. Since inbred mice were analyzed, these findings showed that the mouse genome contains two *Gbp* genes, designated *Gbp-1* and *Gbp-2*. The two *Gbp* genes not only have almost identical structures but also have promoters that function equally well in response to IFN-γ induction when cloned in front of a promoterless reporter gene.

Our deletion analysis revealed that a putative GAS regulatory element could be removed from the *Gbp-2* promoter constructs without significantly affecting their IFN-γ inducibility in transiently transfected 3T3 cells. Other investigators reached a similar conclusion with deletion constructs of the *Gbp-1* promoter transfected into the murine macrophage cell line RAW 264.7 (30). They showed that the critical *cis*-acting element for IFN-γ inducibility was located some 40 to 50 nucleotides upstream of the major transcription start site and that this element is homologous to the IFN-stimulated response element (ISRE) of IFN-α/β-inducible genes. Inducibility by IFN-γ was abolished when this element was mutated (30), stressing its importance for proper regulation.

ISRE motifs have been shown to mediate gene induction in response to IFN-α/β (9, 18, 23, 38). Since the isolated mouse *Gbp-1* and *Gbp-2* promoters failed to respond to IFN-α, it seemed that the ISRE-like motif in these promoters may not serve this function. The ISRE-like motif of mouse *Gbp* promoters shows a perfect match to the IRF-binding consensus sequence (53). We therefore speculated that the response of the *Gbp* promoters to IFN-γ resulted from the action of transcription factor IRF-1. At first glance, this scenario seemed unlikely because IRF-1 had been described as a transcription factor that regulates IFN-β gene expression and influences IFN-α/β-responsive genes (12, 28, 36, 40). However, earlier studies with artificial promoters containing synthetic IRF-binding motifs showed that they responded unexpectedly well to IFN-γ (25, 38). Overexpression of IRF-1 cDNA resulted in a strong activation of the *Gbp-2* promoter that could not be explained by an indirect effect of IRF-1-induced IFN-α/β, because treatment of 3T3 cells with high doses of IFN-α/β had no significant stimulating effect on our constructs. Direct proof that *Gbp* promoter activity depends on IRF-1 came from studies with ES cells and mice with targeted disruptions of both IRF-1 alleles. IRF-$1^{0/0}$ ES cells treated with IFN-γ contained almost no *Gbp* mRNAs, whereas wild-type ES cells contained high levels of *Gbp-1* and *Gbp-2* mRNAs. Interestingly, the *Gbp* genes of IRF-$1^{0/0}$ ES cells could be induced to significant levels by IFN-α, demonstrating that the response of the *Gbp* promoter to IFN-α/β is not dependent on IRF-1 and must occur by a different mechanism. IRF-$1^{0/0}$ mice treated with IFN-γ showed 20- to 25-fold-lower levels of *Gbp* transcripts in spleens, lungs, and livers than did wild-type mice. Since these organs contain many different cell types, it seems that no alternative pathways for the induction of *Gbp* genes by IFN-γ would exist besides those that include transcription factor IRF-1. This conclusion agrees with recent findings from work with embryonic fibroblasts from IRF-$1^{0/0}$ mice of *Gbp-1*^{b} genetic background, which showed that the *Gbp-2* gene was refractory to induction by IFN-γ but not IFN-α (22).

A surprising finding of our experiments with deletion constructs was that the GAS motif in the mouse *Gbp* promoters did not mediate the IFN-γ response. This finding agreed well with the nuclear run-on experiments which showed that the mouse *Gbp* genes responded poorly to IFN-γ in cells with blocked protein synthesis. GAS motifs have been shown to be both necessary and sufficient for immediate transcriptional induction of a number of IFN-γ-responsive genes (10, 33, 35, 47). Among these, the human *GBP-1* gene represents a special case because its promoter contains an ISRE motif that overlaps the GAS element. Point mutations in the ISRE that specifically destroyed the binding sites for transcription factors ISGF-3 and IRF-1 had a strong reducing effect on IFN-γ inducibility. However, residual promoter activity remained and could be attributed to the GAS element (24). Recent experiments indicated that the protein required during an IFN-γ response is IRF-1 (51a). Thus, the IRF-binding site and IRF-1 protein are important for the induction of *Gbp* genes in both humans and mice. Nonetheless, the two species appear to differ in their utilization of and requirements for the GAS motif for the IFN-γ response. As a consequence, the human *GBP* gene is strongly induced by IFN-γ in fibroblasts with blocked protein synthesis (10), whereas the mouse *Gbp* genes require protein synthesis for full-scale induction in this cell type.

IFN-γ-responsive genes can be grouped according to their transcription factor requirements. Certain genes become activated by binding of transcription factor Stat1 to their promoters, like, for example, the IFP 53 (52), FcγRI (32, 34), ICSBP (21), and IRF-1 (35) genes. Since Stat1 is a preformed protein that is activated by tyrosine phosphorylation (33, 47), these genes respond rapidly to IFN-γ even in the presence of protein synthesis inhibitors. Other genes respond more slowly and require protein synthesis for full-scale induction by IFN-γ, indicating that de novo synthesis of other transcription factors is needed. The induced factor required for IFN-γ-triggered activation of the major histocompatibility complex class II genes was recently identified as transcription factor CIITA (4). In the cases of the mouse *Gbp* genes (reference 22 and this work) and the gene for iNOS (22, 26), the transcription factor required for induction by IFN-γ is IRF-1. It thus appears that IFN-γ-mediated activation of Stat1 induces the transcription of many genes, including those that code for new transcription factors which, in turn, stimulate the expression of other genes. These factors could simply enhance the inducing effect of Stat1: IRF-1 was proposed to maintain transcription of the human *GBP* gene after its activation by Stat1 (11). Alternatively, they might stimulate responsive promoters independently of Stat1. Evidence in favor of the latter possibility comes from the observations that expression of cDNAs for IRF-1 (this work) or CIITA (4) resulted in the activation of the mouse *Gbp* or major histocompatibility complex class II genes, respectively, even in the absence of IFN-γ.

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