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Overlapping Elements in the Guanylate-Binding Protein Gene Promoter Mediate Transcriptional Induction by Alpha and Gamma Interferons

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The gene encoding a 67-kDa cytoplasmic guanylate-binding protein (GBP) is transcriptionally induced in cells exposed to interferon of either type I (alpha interferon [IFN-α] or INF-β) or type II (IFN-γ). The promoter of the GBP gene was cloned and found to contain an IFN-α-stimulated response element, which mediated the response of the GBP gene to IFN-α. On the basis of transfection experiments with recombinant plasmids, two different elements were delineated. Both were required to obtain the maximal response of the GBP gene to IFN-γ: the IFN-α-stimulated response element and an overlapping element termed the IFN-γ activation site. Different proteins that act on each element were investigated, and their possible involvement in IFN-γ-induced transcriptional regulation is discussed.

Interferons (IFNs) are cytokines which render cells resistant to viral infection and regulate cell growth and differentiation (9). The two types of IFN, type I or α/β and type II or γ, bind to different cell surface receptors and induce the transcription of distinct but overlapping sets of genes in target cells (21, 27, 32, 37). A promoter element termed the ISRE (IFN-α-stimulated response element) is necessary and sufficient to mediate the transcriptional induction of several genes by IFN-α (5, 20, 30, 31). A DNA-binding protein termed ISGF-3 is responsible for the IFN-α-dependent activation through the ISRE. This conclusion is based on a variety of evidence, including the parallel induction of ISGF-3 and transcription even in the absence of ongoing protein synthesis (19, 20), and on in vitro transcriptional activation of an ISRE-containing promoter by ISGF-3 (13a). Other factors bind to the ISRE, but their role, if any, in IFN-regulated transcription is unknown (7, 19, 20, 34, 36). ISGF-3 is not activated by IFN-γ in either HeLa cells or euploid fibroblasts (8).

Sequences that appear closely related to the ISRE are present not only in the promoters of genes induced by IFN-α (20) but also in some genes induced by IFN-γ (23) or by both IFNs (31; this report) as well as in the promoters of the genes encoding IFN-α and IFN-β, which are induced by viruses but not by IFNs (20). Both natural (31) and artificial (12, 24) ISRE-like sequences can confer inducibility by both IFN-α and IFN-γ on transfected heterologous plasmid constructs, demonstrating that the ISRE is sufficient to mediate both responses for transfected genes and raising the following question: why don't all endogenous ISRE-containing genes respond to both IFN-α and IFN-γ?

Addressing this question, Reid et al. (31) reported differences in the response to IFN-γ of recombinant constructs containing different sequences flanking the ISRE. They showed that a 20-nucleotide segment including the ISRE from the 6-16 gene (which is inducible by IFN-α but not IFN-γ) could mediate induction by both IFNs when placed upstream of a heterologous promoter. However, under the same transfection conditions, this same ISRE sequence flanked by a larger fragment of the 6-16 promoter responded only to IFN-α. Therefore, the induction by IFN-γ of the construct containing the ISRE alone appeared to be an artifact of removing the ISRE from surrounding sequences. Thus, for chromosomal genes which are demonstrated to be transcriptionally activated by IFN-γ, we are left with this question: what role, if any, does the ISRE play in the response to IFN-γ?

Given a clearly defined DNA site that is relevant for transcriptional induction by IFN-γ, proteins must be found and defined that fulfill the requirements of transcriptional activators. Blanar et al. (2) have proposed that an IFN-γ-inducible ISRE-binding factor, which they termed IBP-1, mediates the response of the *H2-K*^{b} gene to IFN-γ. On the basis of its apparent molecular weight, ISRE-binding characteristics, and IFN-γ inducibility, IBP-1 is probably identical to the previously described ISGF-2 (also called IRF-1) (25, 28). Is ISGF-2 (IRF-1, IBP-1) the factor responsible for the response of ISRE-containing genes to IFN-γ?

In this report, we describe the cloning and characterization of the promoter of the guanylate-binding protein (GBP) gene, which is inducible by both IFN types (3, 8, 21). The GBP gene promoter contains an ISRE which in recombinant plasmids can mediate the response to IFN-α. There were two overlapping elements in the GBP gene promoter which could respond to IFN-γ: the ISRE and an element that we have termed the IFN-γ activation site (GAS). Both elements were required for the full IFN-γ response. Given the existence of these two separate binding sites and known proteins that recognize each, we discuss the possible mechanisms for the initial and prolonged transcriptional stimulation of the GBP gene by IFN-γ.

MATERIALS AND METHODS

Cells and reagents. HeLa S3 cells were obtained from the American Type Culture Collection (Rockville, Md.). Human diploid FS-2 fibroblasts were kindly provided by E. Knight.

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Both cell types were grown to confluency in monolayer cultures in Dulbecco modified Eagle medium (DME) supplemented with 10% calf serum. Human IFN-α was a gift from P. Sorter (Hoffman La Roche), and human IFN-γ was a gift from D. Vapnek (Amgen). Unless otherwise stated, IFN-α was used at 500 antiviral units per ml and IFN-γ was used at 100 antiviral units per ml (1 U of IFN-α per ml = 30 fM IFN-α; 1 U of IFN-γ per ml = 2.9 pM IFN-γ). Cycloheximide (Sigma) was used at 50 μg/ml. AmpliTaq polymerase was from Perkin Elmer Cetus. DEAE-dextran and acetyl coenzyme A were from Pharmacia. All other chemicals were from Sigma.

Plasmid and oligonucleotide DNAs. oligonucleotides are shown below with the 5′ end to the left (where double-stranded sequences are shown, this applies to the top strand). Many of the constructs shown below (including all site-directed mutagenesis products) were sequenced according to published procedures (35).

GBP oligonucleotides used in the polymerase chain reaction (PCR) and hybridization were as follows:

01 (+37 to +17):	TCCTTAGTTCACGTGCACTGG
02 (+23 to +3):	GCACTGGCTTCTAGCACTTCT
PCR-1 (+19 to +2):	TGGCTTCTAGCACTTCTG
PCR-2 (+20 to +37):	GTGCTCGTGAACTAAGGA

Two GBP gene promoter-chloramphenicol acetyltransferase (CAT) constructs were used. For GBP-CAT (=5′d216, indicating 5′ deletion to position −216), GBP gene promoter sequences from the *Hin*dIII site at −216 (retained in the construct) to the end of the PCR product (+19) were cloned into the *Hin*dIII-*Sma*I sites in pGEM-1 (Promega). A fragment containing the full CAT-coding sequence plus simian virus (SV40) splice and polyadenylation sites was cloned into the filled *Eco*RI site of this plasmid so that the GBP first exon (to +19) was fused to the 5′ end of the CAT sequence. All pGEM-1 sequences outside the polylinker were retained. This plasmid formed the basis for the constructs described below.

Construct 5′d1600 consisted of GBP-CAT with sequences from *Pst*I to *Hin*dIII (−1600 to −216) from the GBP genomic clone inserted at *Hin*dIII site. Construct 5′d2600 consisted of GBP-CAT with sequences from *Eco*RI to *Hin*dIII (−2600 to −216) from the genomic GBP clone inserted at *Hin*dIII site. These constructs were made by using standard molecular biology techniques (35).

HIV-LTR-CAT constructs. The parent human immunodeficiency virus (HIV)-CAT plasmid contains sequence from −57 to +80 from the HIV long terminal repeat (LTR) (33) fused to the same CAT and SV40 sequences used in the constructs above. This sequence was inserted into the *Sal*I and *Pvu*II sites of pGEM-1 such that the polylinker between the SP6 promoter and the *Sal*I site was upstream of the truncated HIV LTR (the *Sal*I site was destroyed). Constructs containing the GBP ISRE, GBP GAS, or GAS-plus-ISRE oligonucleotides were made by ligating the relevant oligonucleotides into the *Bam*HI site of HIV-CAT. The sequences of the GBP and GAS oligonucleotides are as follows:

GBP ISRE: GATCTAGTACTACTTTCAGTTTCATATTAGGG
ATCATGATGAAAGTCAAAGTATAATCCCCTAG

Contains GBP sequences from −135 to −112 from the GBP gene promoter

GBP GAS: GATCCGTCAGTTTCATATTACTCTAAATCCA
GCAGTCAAAGTATAATGAGATTTAGGTCTAG

Contains GBP sequences from −125 to −101 from the GBP gene promoter

The construct containing both an ISRE and a GAS oligonucleotide contained the GAS sequence downstream of the ISRE in the reverse orientation from that found in the GBP promoter. The construct containing GBP sequences upstream of HIV-CAT (see Fig. 5B) was made by cloning the *Sca*I-*Ssp*I (−129 to −76) fragment of the GBP gene promoter into the *Sma*I site of HIV-CAT.

Site-directed mutants. The deletion mutant shown in Fig. 5A and the point mutants of GBP-CAT were made by site-directed mutagenesis, using a gapped heteroduplex template according to the method of Inouye (16). The starting templates were GBP-CAT digested with *Bgl*I (which cuts within the ampicillin resistance marker) and GBP-CAT digested with *Hin*dIII and *Bam*HI (thus leaving −216 to −13 single stranded in the heteroduplex). Both were phosphatase treated to avoid background from circularization. The mutagenic oligonucleotides used are listed below. Residues in lowercase indicate differences from the wild-type sequence. The products of the mutagenesis were screened by using labeled mutant oligonucleotides, purified, and sequenced by standard techniques.

d127/119:	GAACGAAGTAC( )CATATTACTC	(Fig. 5A)
C128G:	CGAAGTAgTTTCAG	(Fig. 6)
T127A, T127G:	GAAGTACa/g/cTTCAGTTTC	(Fig. 6)
T126A:	AAGTACTaTCAGTTT	(Fig. 6)
T126G:	GAAGTACTc/gTCAGTTTC	(Fig. 6)
T125G, T125C:	AAGTACTTa/c/gCAGTTTC	(Fig. 6)
C124G:	GTACTTTgAGTTTC	(Fig. 6)
T121G:	CTTTCAGa/c/gTTCATATTAC	(Fig. 6)
T120A:	TTTCAGTaTCATATT	(Fig. 6)
mg1:	GTTTCAgAaTtCTCTAAATC	(Fig. 7)
mg2:	TTTCATCTagCgCTAAATCC	(Fig. 7)
mg3:	ATTACTCgAgATCCATTAC	(Fig. 7)

Determination of GBP-CAT transcription start sites. Total RNA was prepared by the guanidinium isothiocyanate method (4). Poly(A) mRNA selection was performed as described previously (35). For determination of the transcription start sites of the transfected GBP-CAT construct, we cloned the 490-bp *Hin*dIII-*Eco*RI fragment of GBP-CAT (containing sequences from −216 to +19 from GBP fused to the first 257 nucleotides of CAT) into the respective sites in pGEM-1. A labeled antisense RNA probe was transcribed from this template (linearized with *Hin*dIII) with T7 RNA polymerase in the presence of [α-^{32}P]UTP. This probe was hybridized to poly(A) RNA from IFN-treated or untreated cells, and the probe fragments protected from RNase T_2 digestion were separated on 8% polyacrylamide – 42% urea gels at 1,800 V (6). This probe protects 257 nucleotides of the transcript from the control plasmid RSV-CAT, used to normalize transfection efficiency.

Preparation of cell extracts for protein-DNA binding assays. Nuclear extracts were prepared by a modification of the procedure of Dignam et al. (10). Cells were harvested in phosphate-buffered saline (PBS) by scraping and centrifugation and were lysed by homogenization in hypotonic buffer (10 mM Tris hydrochloride [pH 7.4], 10 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, protease inhibitors). Protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 2 g of aprotinin per ml, 0.5 g of leupeptin per ml, 0.7 g of pepstatin per ml [final concentrations]) and dithiothreitol were added from stock just before the experiment. Nuclei were separated from cytoplasmic extracts by brief centrifugation and extracted in 2 pellet volumes of a buffer containing 0.45 M

KCl (0.3 M final concentration) and 20% glycerol in addition to the components listed above for 30 to 45 min on ice. Extracts were clarified by centrifugation at 14,000 × *g* for 10 min.

Band mobility shift assays. Band mobility shift (gel retardation) assays were performed as described elsewhere (13, 14), with modifications as described by Levy et al. (19). The probe (140-bp *Hin*dIII-*Ssp*I [−216 to −76] fragment from the PCR clone) was labeled by fill-in with Klenow fragment in the presence of α-^{32}P-labeled deoxynucleoside triphosphates; 0.5 ng of probe was used per assay.

Exonuclease III protection assays. Exonuclease III protection assays were performed as described elsewhere (8a). For the competition experiments (see Fig. 7), a fixed molar excess of mutant DNA fragments was used (yielding about 50% competition) and the competition was compared with that obtained with different amounts of wild-type fragment (supplemented to the same amount of total DNA with a nonspecific fragment). Thus, 4% binding indicates that a 4-fold excess of wild-type DNA competed for binding as efficiently as a 100-fold excess of mutant DNA.

In situ gel hybridization to genomic DNA. Genomic DNA was prepared from HeLa or FS-2 cells according to published procedures (35). A 20 μg sample of DNA was digested with restriction enzymes and loaded (per lane) on a 0.7% agarose gel. Following electrophoresis, the DNA in the gel was denatured in 0.5 M NaOH–0.15 M NaCl for 1 h, neutralized in 0.5 M Tris hydrochloride (pH 7.7) for 1 h and dried onto 3MM filter paper (Whatman) under vacuum for 2 to 3 h. Before use, the gel was removed from the paper by brief soaking in H_2O. Probes (see above) were labeled by random priming (cDNA probe) or kinase treatment (oligonucleotide probes) in the presence of [α-^{32}P]dCTP or [α-^{32}P]ATP, respectively. Hybridization was in 5 × SSPE–0.1% sodium dodecyl sulfate (SDS)–20 μg of yeast RNA per ml–20 μg of sheared denatured salmon sperm DNA per ml for approximately 18 h at 65°C (cDNA) or 37°C (oligonucleotides). Gels were washed at various stringencies, covered with Saran Wrap, and subjected to autoradiography. The same gel could be exposed and rewashed several times. Final stringencies were 0.1 × SSPE–0.1% SDS at 65°C for cDNA and for oligonucleotides 0.2 × SSPE–0.1% SDS at 50°C.

PCR and lambda cloning. Lambda genomic libraries were screened according to published procedures (35). Genomic HeLa DNA was digested with *Hin*dIII, and the fragments of around 1.5 kb were purified on a 0.7% agarose gel. These were diluted and ligated in 1 μg of DNA per ml–1 mM ATP–20 U of ligase per ml at 10°C for 16 h; 50 ng of this DNA was used as a template in reactions containing 0.5 μg of each PCR primer (see above). PCR reactions were performed according to the manufacturer's recommendations in a 100-μl final volume. The cycle used was 94°C for 2 min, ramp for 1 s; 48°C for 2 min, ramp for 3 s; 72°C for 4 min (plus 10 s per cycle automatic extension), ramp for 1 s to 94°C; this procedure was followed for 35 cycles.

Transfection and CAT assays. HeLa S3 cells were transfected by a modification of the standard DEAE-dextran procedure (15). Cells (approximately 10^7 per transfection) were trypsinized to remove them from tissue culture plates and washed in DME without serum. They were resuspended in 0.6 ml of 0.1-mg/ml DEAE-dextran in DME containing 5 μg of DNA. The mix was incubated for 30 to 45 min at 37°C in a CO_2 incubator, and then dimethyl sulfoxide was added to a 10% final concentration during shaking. After 1 to 2 min, 10 ml of DME was added, and the cells were collected by centrifugation at 1,000 × *g* for 5 min. Cells were resuspended in 1 ml of DME plus 10% calf serum and plated onto 100-mm tissue culture dishes. This procedure was scaled up appropriately for samples involving IFN treatments, and the transfected cells were separated into equal aliquots before plating. Cells were allowed to attach and grow overnight (mortality from the transfection was about 40%) and then treated with IFNs for 15 h. Cells were washed with PBS, harvested by scraping and centrifugation in 40 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–1 mM EDTA, and resuspended in 120 μl 0.25 M Tris hydrochloride (pH 8). Extracts were made by freeze-thaw, cleared by centrifugation, and inactivated at 65°C for 10 min. CAT assays were done as described previously (35). β-Galactosidase was assayed as described previously (35) on extracts prepared as described above that had not been heat inactivated.

RESULTS

The GBP gene is a member of a multigene family. Previous studies showed that an identical set of four transcription start sites used upon transcriptional induction of the GBP gene by IFN-α or IFN-γ (8). The numbering scheme for nucleotides in the GBP gene designates the first base of the shortest transcript as +1. Genomic DNA was isolated from human cells, digested with restriction enzymes, and hybridized to GBP cDNA probes to estimate the number of GBP gene-related sequences in the human genome. The GBP cDNA hybridized to around 10 fragments at high stringency (Fig. 1A) and to >50 at slightly lower stringency (data not shown), suggesting that the GBP gene is a member of a multigene family. Hybridization with the overlapping 01 (+17 to +37) and 02 (+3 to +23) antisense GBP oligonucleotides also revealed multiple bands (>20) at low stringency but detected only three to five bands at high stringency (Fig. 1B). A still higher stringency wash resulted in labeling of a single identical band (for example of 1.5 kb in the *Hin*dIII digest) by each of these probes (Fig. 1C). We interpret these results to mean that there is a single gene whose sequence matches perfectly that of the induced mRNA and a large number of genes with similar but variably diverged sequences.

Cloning of the GBP gene promoter. The observations presented above suggested that it would be difficult to identify the authentic GBP 5′ exon among a number of other homologous genes by using classical screening techniques. We therefore used the inverse PCR technique to generate a circularly permuted fragment containing the GBP 5′ exon (26).

*Hin*dIII-digested HeLa DNA was separated by electrophoresis on an agarose gel, and the fragments of about 1.5 kb were purified and ligated under dilute conditions to form circular templates (Fig. 2). This mixture presumably contained the authentic GBP 5′ exon but no other sequences that hybridized to GBP oligonucleotides 01 and 02 at high stringency (Fig. 1B and C). Two divergent oligonucleotide primers were synthesized, centered at +19 and spanning bases +2 to +37 (see Materials and Methods). By using the circularized genomic fragments as a template, PCR reactions with these two primers generated a 1.5-kb species (Fig. 2). This species was not produced in reactions that contained only one of the primers or in reactions in which linear, rather than circularized, genomic fragments were used as templates (Fig. 2). The 1.5-kb PCR products were cloned, labeled, and used to probe genomic DNA as described above (data not shown). Two bands were detected, the stronger one of which

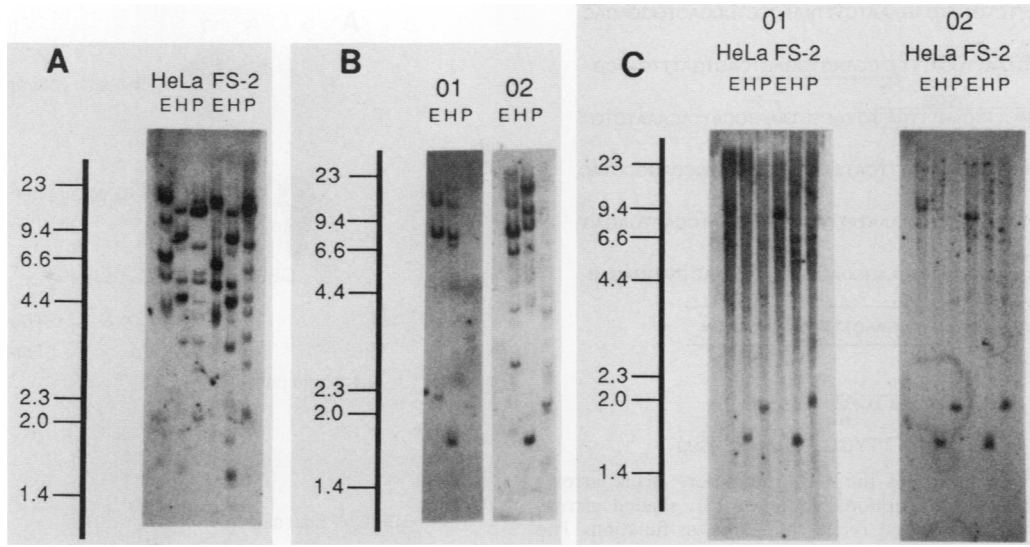


FIG. 1. Detection of multiple GBP gene-related sequences in the human genome. Genomic DNA obtained from either HeLa cells or diploid fibroblasts (FS-2) was digested with *Eco*RI (E), *Hin*dIII (H), or *Pst*I (P) and probed with GBP cDNA (A) or two oligonucleotides (01 and 02) complementary to the 5′ end of the GBP gene at high (B) or very high (C) stringency (see text). The hybridizations to all probes were performed in situ without prior transfer of the DNA to membranes (see Materials and Methods for details).

was identical to the band identified at the highest stringency with the oligonucleotide probes, confirming that the cloned PCR product represented the GBP sequence. The hybridization pattern of the second genomic DNA fragment suggested that it contained additional DNA sequences of about 200 bp in the 5′ exon region that failed to hybridize to oligonucleotides 01 and 02. This gene may correspond to a second homologous IFN-γ-inducible mRNA species (2a).

The GBP gene promoter contains an ISRE. The sequence of the PCR clone confirmed that it was a circularly permuted version of authentic GBP gene, containing the entire first exon of the GBP gene, a consensus splice donor site followed by an intron at +52, and 215 bp upstream of +1 (Fig. 3). This promoter sequence contained no consensus TATA or CCAAT elements, which may explain the multiple transcription start sites previously described for the GBP gene (8). There was an imperfect 14-bp inverted repeat at −178 to −148 and a sequence very similar to the ISRE located at

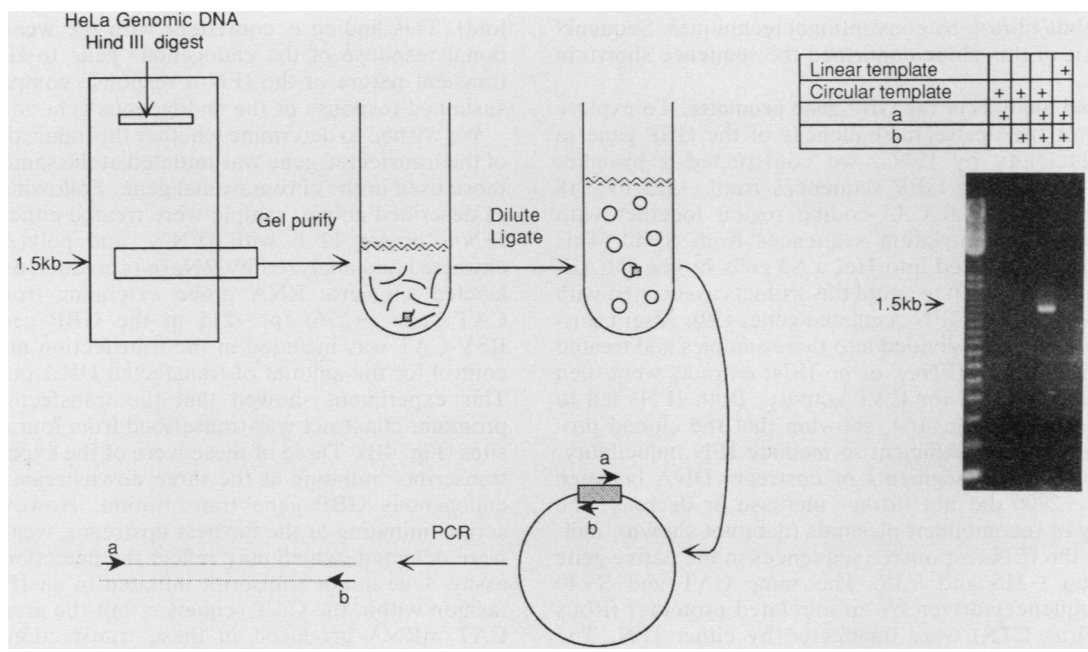


FIG. 2. Cloning of GBP gene upstream sequences. An inverse PCR reaction with oligonucleotide primers (a and b) complementary to the two DNA strands of the GBP gene 5′ end in opposing directions was performed on *Hin*dIII-digested genomic DNA enriched for fragments of about 1.5 kb. The agarose gel shows a reaction product of 1.5 kb which contained about 200 bp of 5′-flanking DNA.

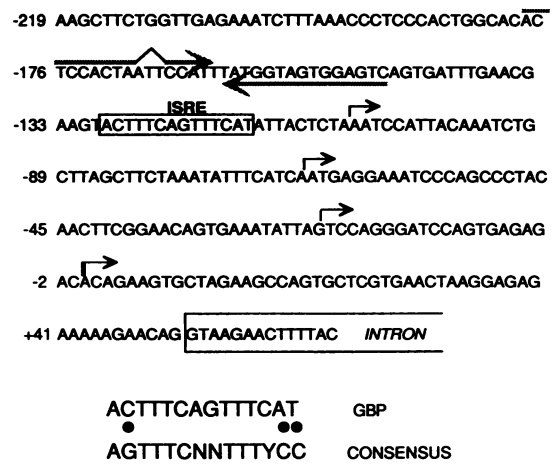


FIG. 3. DNA sequence of the GBP promoter. Black arrows indicate the sites of transcriptional initiation (8); shaded arrows indicate an imperfect inverted repeat of unknown function. The ISRE is shown in alignment with a consensus element derived previously (20).

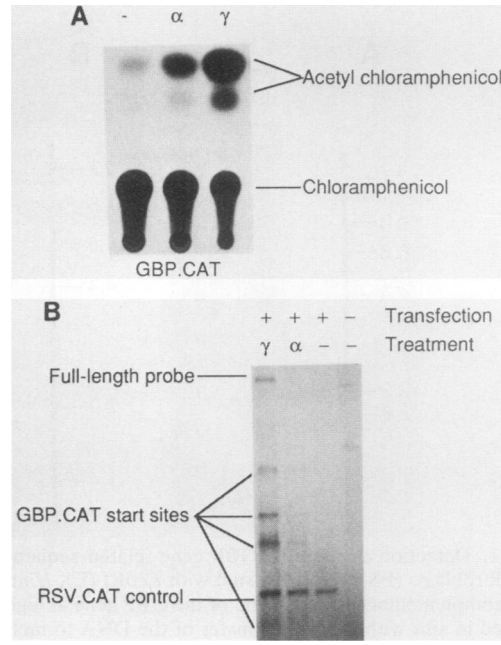


FIG. 4. Interferon inducibility of the GBP gene promoter. (A) The GBP gene promoter (−215 to +18) was linked to the CAT reporter gene, transfected into HeLa cells, and treated with IFN as indicated. CAT activity was measured after 15 h of treatment with either IFN. (B) Poly(A) RNA was extracted from HeLa cells transfected with the same GBP promoter construct after 12 h of IFN-γ or 6 h of IFN-α treatment. The RNA was analyzed for the presence of correctly initiated transcripts in an RNase T_2 protection assay. A constitutively expressed plasmid (RSV-CAT) was cotransfected to control for transfection efficiency. The three upper bands shown (GBP-CAT) represent transcripts that were correctly initiated from within the GBP gene promoter; the lowest represents initiation from within the fused CAT sequences.

−130 to −116. Since the pattern of GBP gene transcription following IFN-α treatment was identical to that of several other IFN-α-activated genes that have an ISRE (21), it was expected that the GBP gene promoter would also contain an ISRE. The GBP ISRE diverged from the consensus ISRE at three positions (−128, −117, and −116), one of which (−128) is highly conserved in other ISREs (20). Band mobility shift experiments using purified ISGF-3 (which is the positive activator of the IFN-α transcriptional response [19, 20]) showed that the GBP ISRE did bind to this factor, although the affinity of ISGF-3 for the GBP ISRE was considerably lower than its affinity for the ISG-15 ISRE (data not shown).

The PCR clone was used to isolate a genomic GBP clone from a lambda library by conventional techniques. Sequencing of a part of this clone confirmed the sequence shown in Fig. 3.

Functional analysis of the GBP gene promoter. To explore the ability of the upstream sequences of the GBP gene to confer inducibility by IFNs, we constructed a plasmid, GBP-CAT, containing GBP sequences from −215 to +18 linked to the bacterial CAT-coding region together with splicing and polyadenylation sequences from SV40. This plasmid was transfected into HeLa S3 cells by the DEAE-dextran method (15, 22) to avoid the artifacts associated with $CaPO_4$ transfection of IFN-regulated genes (29). After transfection, cultures were divided into three samples and treated for 15 h with IFN-α, IFN-γ, or no IFN; extracts were then prepared and assayed for CAT activity. Both IFNs led to induction of CAT (Fig. 4A), showing that the cloned promoter sequence was sufficient to mediate IFN inducibility. Inclusion of various segments of upstream DNA between −215 and −2900 did not further increase or decrease the inducibility of recombinant plasmids (data not shown), indicating that the IFN-responsive sequences in the native gene lay between −215 and +18. The same CAT and SV40 reporter sequences driven by an unrelated promoter (Rous sarcoma virus LTR) were unaffected by either IFN. The stimulation of CAT activity produced from transcription of plasmid GBP-CAT after IFN-γ treatment (20- to 30-fold) was considerably greater than after IFN-α treatment (5- to 10-fold). This finding is consistent with the weaker transcriptional response of the endogenous gene to IFN-α and the transient nature of the IFN-α response compared with the sustained response of the endogenous gene to IFN-γ (21).

We wished to determine whether the induced transcription of the transfected gene was initiated at the same start sites as those used in the chromosomal gene. Following transfection as described above, sample were treated either for 6 h with IFN-α or for 12 h with IFN-γ, and poly(A) RNA was extracted and analyzed by RNase protection assays, using a labeled antisense RNA probe extending from within the CAT gene (+276) to −215 in the GBP gene promoter. RSV-CAT was included in the transfection mix to act as a control for the amount of transfected DNA in each sample. This experiment showed that the transfected GBP gene promoter construct was transcribed from four different start sites (Fig. 4B). Three of these were of the expected sizes for transcripts initiating at the three downstream start sites of endogenous GBP gene transcription. However, no transcripts initiating at the farthest upstream, weakest start site were detected, which may reflect the detection limits of the assay. One minor transcript initiated in an IFN-dependent fashion within the CAT sequence, but the great majority of CAT mRNA produced in these transfections did indeed reflect correctly initiated, IFN-dependent transcription regulated by the transfected GBP gene promoter sequences.

The ISRE together with downstream sequences is required

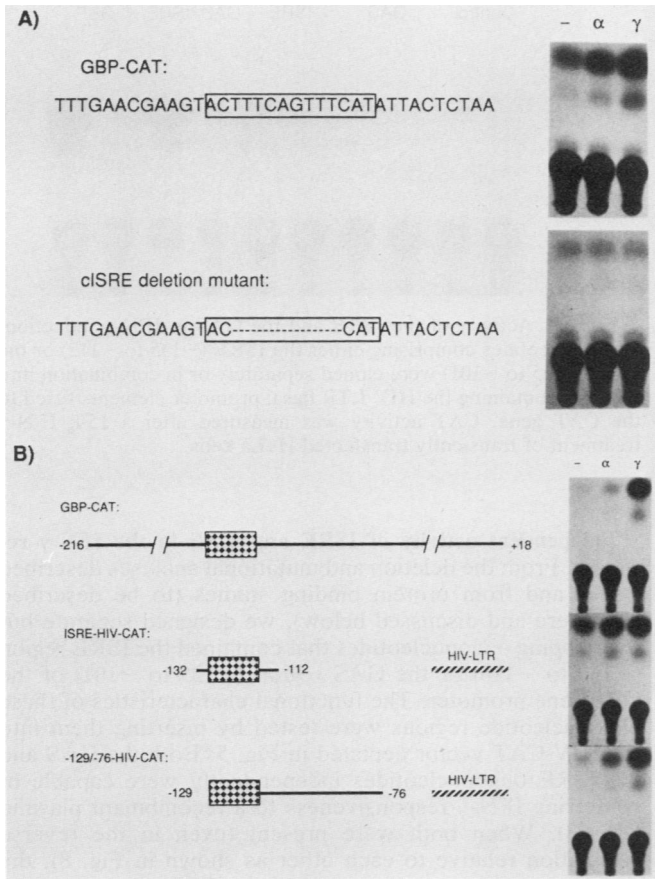


FIG. 5. Narrowing of GBP gene promoter DNA sequences that are required for IFN inducibility. HeLa cells were transfected with plasmids as shown and assayed for CAT activity after 15 h of treatment with either IFN. (A) Effect of removing the central 9 bp of the ISRE; (B) effect of removing DNA either immediately upstream (−129/−76-HIV-CAT) or downstream (ISRE-HIV-CAT) of the ISRE on IFN inducibility.

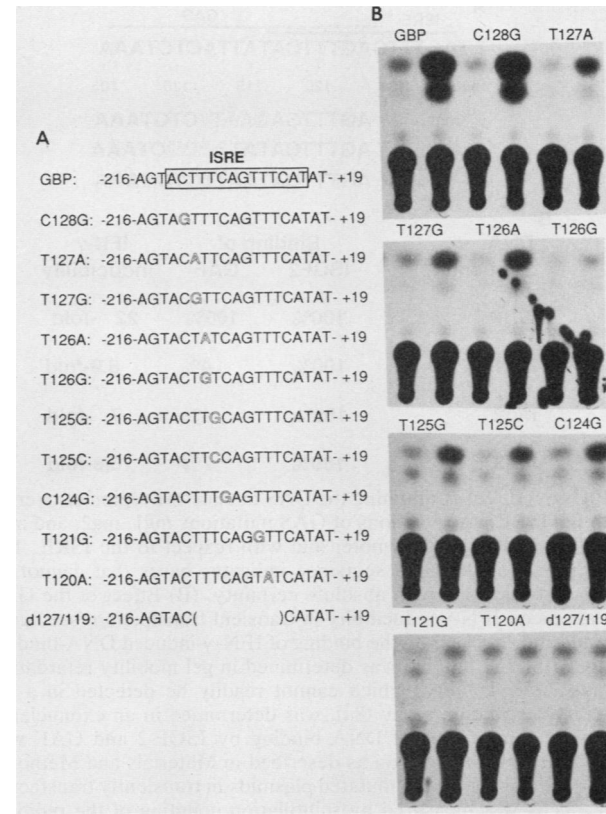


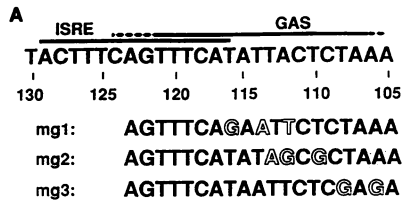
FIG. 6. Effects of point mutations within the ISRE on IFN-γ inducibility. The mutants were generated by the gapped heteroduplex method (16), using plasmid GBP-CAT and appropriate oligonucleotide primers. CAT activity, generated by the mutated plasmids during transient transfection in HeLa cells, was measured after a 15-h treatment of the transfected cells with IFN-γ.

for full IFN-γ induction. To begin to localize the sequences required for IFN-γ-dependent transcription directed by the GBP gene promoter, various deletion mutants were prepared, transfected, and tested for expression after IFN-α and IFN-γ treatment. Figure 5 illustrates major results from these experiments. First, a construct with GBP gene promoter sequences beginning at −129 and extending to −76, fused to an otherwise inactive promoter (HIV LTR), was induced well by both IFNs, narrowing the sequences of interest to this 53-bp region. Like the full GBP gene promoter, this construct responded about five times better to IFN-γ than to IFN-α. Second, deletion of nine bases within the ISRE abolished both the IFN-α and, somewhat to our surprise, also the IFN-γ responses. At this point it appeared that the ISRE might be all that was required to respond to either IFN. However, a plasmid containing the intact ISRE (−135 to −112) but lacking the region immediately downstream (−112 to −76) did not provide a full response to IFN-γ: this plasmid had a much higher uninduced base line, possibly indicating a negative-acting element in the missing region, and the plasmid was inducible about two- to five-fold by either IFN-α or IFN-γ. These findings suggested that the ISRE, a well-characterized 14-bp conserved region that was originally identified as mediating IFN-α responsiveness, might be involved in but not sufficient for IFN-γ responsiveness. Our approach to settling this issue more definitively was to introduce mutations within the highly conserved ISRE and within necessary downstream bases to try to distinguish the effects of such mutations on IFN-α and IFN-γ responsiveness.

Mutations in bases from −128 to −120 were prepared and tested (Fig. 6A). These mutations include the most highly conserved and stringently required nucleotides of other ISREs, such as the T's at −127, −126, and −125 which are part of the two sets of three T's present in every ISRE. These mutations did not drastically affect the IFN-γ response (Fig. 6B) but did drastically reduce IFN-α response (data not shown). In one experiment, the autoradiogram of the CAT assay was used to locate di- and triacetylated forms of chloramphenicol, the appropriate spots were eluted, and radioactivity was determined. In the chromatogram from the uninduced sample there was 501 cpm in the acetylated forms, in the IFN-α treated sample there was 559 cpm, and in the IFN-γ-treated sample there was 3,086 cpm. Similar results were found for mutations at −127, −126, −125, and −124 (Fig. 6). Thus, it was unambiguously clear that mutations which completely disable the ISRE as an IFN-α-responsive element leave the IFN-γ responsiveness largely intact.

In contrast to mutations on the upstream side of the ISRE,

A



B

DNA Sequence	Binding of ISGF2	Binding of GAF	IFN-γ Inducibility
GBP	100%	100%	22 -fold
mg1	100%	4%	6.9-fold
mg2	100%	4%	7 -fold
mg3	100%	7%	6.8-fold

FIG. 7. Effect of mutating the GAS outside the region of overlap with the ISRE. (A) Positions of GAS mutations mg1, mg2, and mg3 within the GBP gene promoter and with respect to the ISRE. The broken part of the GAS sequence indicates bases that cannot be assigned to this site with absolute certainty. (B) Effect of the GAS mutations on IFN-γ inducibility in transient transfection (see figure legends and text) and on the binding of IFN-γ-induced DNA-binding factors. ISGF-2 binding was determined in gel mobility retardation assays. GAF activity, which cannot readily be detected in a gel mobility retardation assay (8a), was determined in an exonuclease III assay. The extent of DNA binding by ISGF-2 and GAF was determined by competition as described in Materials and Methods. IFN-γ inducibility of the mutated plasmids in transiently transfected HeLa cells was measured by scintillation counting of the radioactivity in CAT-converted [^{14}C]chloramphenicol after separation from the unconverted form by thin-layer chromatography.

mutations at −121 and −120 had a significant effect on decreasing the IFN-γ response (Fig. 6).

Importance of the GAS sequences for the IFN-γ response. To more directly demonstrate the importance for IFN-γ inducibility of the GBP gene of the sequences on the right end of the ISRE region and of bases adjacent to the ISRE, we prepared plasmids bearing mutations or deletions in the region from −116 to −102 and, after transfection of these recombinants, tested their response to IFN-γ. Figure 7 summarizes results of these experiments with three point mutants in the region from −116 to −106 (deletion of nucleotides −102 and further downstream was without effect on IFN-γ inducibility; data not shown). First, the wild-type construct (which contains both the ISRE and downstream sequence) gave a 22-fold induction by IFN-γ. The mutation in mg1, which changed the last nucleotide in the ISRE (T to G) and two downstream nucleotides, decreased the IFN-γ response by about 70%, as did the mutations in mg2 and mg3, which are completely outside the demonstrated requirements of the ISRE region. The remaining 30% of activity in these mutants can be attributed to the ISRE that remained intact in this construct. Thus, a second region of the GBP gene promoter was clearly required for the full IFN-γ response; the sequences comprising this region interdigitated with the 3′ end of the ISRE and extended downstream of the ISRE. We have termed this second site the IFN-γ activation site, or GAS. Both the ISRE and the GAS were required for a full response to IFN-γ: mutations that damaged one or other element responded poorly to IFN-γ, and mutations in the region of overlap between the ISRE and the GAS completely abolished the response to IFN-γ.

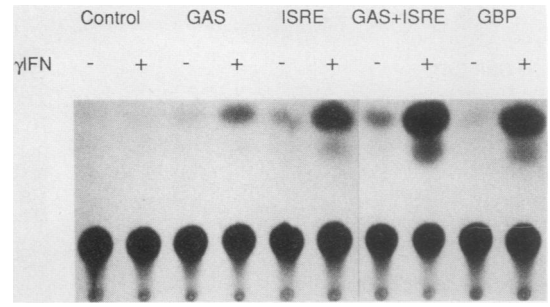


FIG. 8. Activity of the ISRE and the GAS in IFN-γ induction. Oligonucleotides comprising either the ISRE (−135 to −112) or the GAS (−125 to −101) were cloned separately or in combination into a vector containing the HIV LTR basal promoter elements fused to the CAT gene. CAT activity was measured after a 15-h IFN-γ treatment of transiently transfected HeLa cells.

Independent activity of ISRE and GAS in the IFN-γ response. From the deletion and mutational analyses described so far and from protein binding studies (to be described elsewhere and discussed below), we designed separate but overlapping oligonucleotides that contained the ISRE region (−130 to −116) or the GAS region (−125 to −101) of the GBP gene promoter. The functional characteristics of these oligonucleotide regions were tested by inserting them into the HIV-CAT vector depicted in Fig. 5. Both the GAS and the ISRE oligonucleotides independently were capable of conferring IFN-γ responsiveness to a recombinant plasmid (Fig. 8). When both were present (even in the reverse orientation relative to each other as shown in Fig. 8), the response was as strong as that of the GBP gene promoter itself. Thus, both GAS and ISRE elements can act independently in stimulating IFN-γ-dependent transcription and together give a strong response equivalent to that of the native promoter.

Proteins that may interact with the GAS and ISRE in response to IFN-γ. Since several proteins are known to interact with the ISRE, we considered their possible role in the ISRE-dependent IFN-γ induction of transcription. ISGF-3, the IFN-α-activated, positive-acting factor, is not activated by IFN-γ. By using the GBP gene promoter as a probe in gel mobility retardation experiments, two other binding activities were detected. These activities corresponded to the previously identified ISGF-1 and ISGF-2 (IRF-1) by mobility and competition criteria (Fig. 9; 19, 20): ISGF-1, a constitutive protein, and ISGF-2, which has also been called IRF-1 and IBP-1 by other investigators (2, 25). ISGF-1 does not change its affinity during IFN-α or IFN-γ activation and is therefore unlikely to play a major regulatory role. ISGF-2 (IRF-1) was induced in both HeLa cells and fibroblasts upon treatment with IFN-γ (Fig. 9). However, ISGF-2 induction required protein synthesis (Fig. 9B), whereas the IFN-γ-induced transcription of the endogenous GBP gene in fibroblasts does not (8). In HeLa cells, inhibition of protein synthesis merely delays and reduces the IFN-γ-dependent transcription of GBP but does not prevent it (21). Moreover, ISGF-2 activity is regulated by IFN-γ through transcriptional induction of its gene (28) and appeared in IFN-γ-treated HeLa cells or fibroblasts with very similar kinetics only after a lag phase of about 1 h (Fig. 9A). In contrast, the transcriptional response of the GBP gene can be observed in fibroblasts within minutes of IFN-γ treatment (8; unpublished results), and there is a considerable differ-

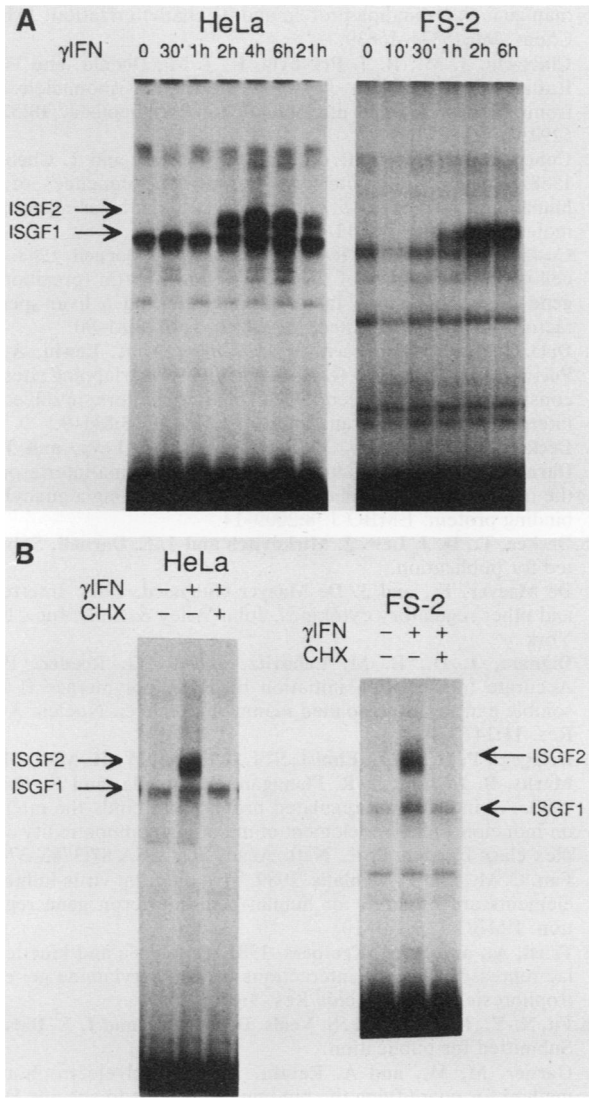


FIG. 9. Binding of ISGF-1 and ISGF-2 (IRF-1) to the GBP gene promoter. Nuclear extracts generated from HeLa or FS-2 cells were incubated with a labeled GBP promoter fragment (−216 to −76) and analyzed for the presence of ISGFs in a gel mobility retardation assay. (A) HeLa cells or FS-2 fibroblasts treated for the indicated periods with IFN-γ; (B) HeLa cells or FS-2 fibroblasts treated for 2 h with IFN-γ in the presence or absence of cycloheximide (CHX).

ence in the time course of the GBP gene response to IFN-γ in HeLa cells and fibroblasts. Thus, while it is possible that ISGF-2 (IRF-1) plays a role at a later stage of the response to IFN-γ, the lack of correlation with the initial induction of transcription suggests that a different factor is responsible for the regulation of this process.

ISGF-2 (IRF-1) has been demonstrated in cotransfection experiments to be able to transcactivate genes containing its binding site (25). Thus, the ISRE-dependent transcription in a transfection assay could be due to this factor. It is also possible that ISGF-2 (IRF-1) is used at this site to maintain transcription after IFN-γ-stimulated activation of the endogenous gene by a different factor.

We have recently characterized a new factor, GAF (IFN-γ-activating factor), that binds to the GAS region, producing an exonuclease III protection from nucleotides −128 to −99. This factor failed to bind to mutants in the GAS (see Fig. 7), in accord with the greatly decreased inducibility of mutants in this region. The appearance of GAF activity in IFN-γ-treated cells parallels the transcriptional induction of the GBP gene (8a). Thus, the GAS sequences defined functionally in this report and the cognate protein GAF probably play an important role in the IFN-γ-induced transcriptional response.

DISCUSSION

Despite the importance of IFN-γ as a regulator of the immune system and of cell growth and differentiation, relatively little progress has been reported on the immediate events in IFN-γ-stimulated gene transcription. Many of the biological effects of IFN-γ require many hours or days to observe, and some genes that do respond to IFN-γ, such as the class II major hiscompatibility genes, require many hours of IFN-γ exposure to be activated (1). Only a few genes have been shown to respond within a few minutes to transcriptional induction by IFN-γ, one of which is the GBP gene, on which we have concentrated (8). To study the immediate transcriptional response of GBP to IFN-γ, we cloned the promoter region of the gene, discovering along the way that the cDNA for this protein cross-reacts with a very large number of potentially related genes.

One important characteristic of the transcriptional response of the GBP gene to IFN-γ is its prolonged nature: in fibroblasts, transcription is maximal within 1 h and then remains elevated for many (>48) hours; in HeLa cells, there is a slower rise in transcription and again there is a prolonged period of active transcription (8, 21). It is obviously possible to envision that interacting protein factors could be responsible for different phases of the IFN-γ transcriptional response. It should be borne in mind that the standard transfection techniques for detecting *cis* elements required for a transcriptional response do not allow easy examination of the time course of transcriptional responses; rather, the determined amount of accumulated reporter mRNA or protein reflects the sum of events in the overall transcriptional response within the period of the assay.

With these caveats in mind, we dissected the promoter region of the GBP gene to search for sequences required for IFN-γ induction of recombinant reporter plasmids and found two regions of importance. Like the entire promoter, the sequence between −129 and −76 conferred on plasmids the ability to respond to both IFN-γ and IFN-α (in a ratio of 5:1), similar to the response of the chromosomal gene. Within this 53-bp region, a good match for the previously identified IFN-α-dependent ISRE consensus was found. Moreover, the ISRE-like sequence from the GBP gene promoter conferred on plasmids the ability to be induced by both IFN-α and IFN-γ, although the IFN-γ response in such constructs was only equal to and not five times stronger than the response to IFN-α. Block deletion of the ISRE sequence or removal of the central nine bases rendered plasmids uninducible by either IFN-α or IFN-γ. However, a more detailed analysis of mutants in and downstream of the ISRE region revealed an important second site, termed GAS, that was crucial for a large part of the IFN-γ inducibility. Finally, the ISRE and the GAS were independently cloned and artificially rejoined (in nonoverlapping fashion and in reverse orientation). Tests of these plasmids in transfection experiments proved that each element could direct IFN-γ-depen-

dent transcriptional responses and that the artificial assembly gave results similar to those for the wild-type promoter.

After we learned about the functional importance of the GAS region, a protein termed GAF was detected that binds this region (8a). Mutants that exhibited a greatly diminished GAS activity also failed to bind GAF. Furthermore, GAF is induced in a rapid and protein synthesis-independent manner in fibroblasts and is induced more slowly and depends on protein synthesis in HeLa cells, paralleling the IFN-γ activation of GBP gene transcription (8, 8a, 21). Thus, we conclude that the binding of GAF to the GAS is crucial in the immediate and early IFN-γ transcriptional response.

The role of the ISRE and of any proteins that bind to it in the course of IFN-γ-induced transcription is less clear. Since the ISRE and GAS overlap, it may be that they cannot be simultaneously occupied. However, the transcriptional induction of the GBP gene after IFN-γ treatment may demand more than GAF. In fibroblasts, IFN-γ-dependent transcription is still more than 50% of maximum after 15 h, and only after 10 to 15 h does stimulation reach a maximum in HeLa cells. At these late times, GAF has all but disappeared in fibroblasts and has dropped to low levels in HeLa cells (8a). The delayed appearance of ISGF-2 (IRF-1), which was shown to be a positive-acting factor in cotransfection experiments with reporter plasmids (25, 28a), would make this factor a good candidate for supporting the sustained IFN-γ-dependent transcription by binding to the ISRE, its cognate site. However, factors other than ISGF-2 (IRF-1) are likely to be involved: protein synthesis is required in both fibroblasts and HeLa cells for ISGF-2 to appear, whereas (as noted above), especially in the case of fibroblasts, the transcription rate is unaffected by inhibition of protein synthesis over at least 15 h. Recently, another IFN-γ-regulated protein, ICSBP, has been cloned and shown to bind to the ISRE (11). Further work is required to understand the role of the ISRE and its cognate proteins in the physiological IFN-γ response.

If both the GAS and ISRE do normally function to regulate the GBP gene (and others like it), it is provided with a greater and more flexible responsiveness than it would have with either site alone. Multiple response elements detected by transfection experiments have been observed in several other cases. For example, two virus-responsive elements were identified in the promoter of the IFN-β gene, one (PRD I) that is ISRE-like and another (PRD II) that is NF-κB-like (12). Both sites can be bound by virus-inducible factors (ISGF-2 and NF-κB, respectively), and both sites conferred virus responsiveness on heterologous promoters (12, 18, 24). A third virus-responsive element, the TG sequence, was identified in the promoter of the IFN-α1 gene (24). Thus, the existence of multiple target sequences for stimulation of individual genes by particular signals could be a general feature of transcriptional regulation, increasing the flexibility and redundancy of regulatory networks.

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