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# Cytoplasmic activation of GAF, an IFN-γ-regulated DNA-binding factor

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**We have investigated events following treatment of cells with interferon-γ (IFN-γ) that lead to the immediate transcriptional activation of an inducible gene. A gamma-interferon activation factor (GAF) was activated in the cytoplasm of human fibroblasts immediately after IFN-γ treatment and bound to a newly identified target DNA sequence, the gamma-interferon activation site (GAS). The time course of activation of GAF was different in fibroblasts and HeLa cells and correlated well with IFN-γ-induced transcriptional activation in both cell types. IFN-γ-dependent activation of GAF also occurred in enucleated cells (cytoplasts), showing that an inactive cytoplasmic precursor is converted to the active factor. These findings support the concept that ligand-specific signals originating at the cell surface are transmitted through latent cytoplasmic proteins which are activated to bind specific DNA sites and then move to the nucleus to activate the transcription of specific sets of genes.**
*Key words:* γ-IFN/transcription stimulation

## Introduction

A crucial step by which polypeptide ligands exert their influence on intracellular physiology is the transcriptional activation of genes. Since different polypeptides induce different sets of genes, the specificity of the receptor–ligand interaction has to be maintained throughout the transfer of information to the nucleus so that only selected genes will be induced. It is central to our understanding of signal transduction to find and study the molecules that embody this specificity.

Interferon-γ (IFN-γ), a cytokine produced by activated T lymphocytes, is a major regulator of both the nonspecific and the specific immune response (De Maeyer and De Maeyer-Guignard, 1988). Transcriptional induction by IFN-γ can be immediate without the need for new protein synthesis, or it may be delayed and require new proteins to be made (Blanar *et al.*, 1988; Caplen and Gupta, 1988; Luster *et al.*, 1988; Amaldi *et al.*, 1989; Decker *et al.*, 1989; Lew *et al.*, 1989). The human gene encoding a cytoplasmic guanylate-binding protein (GBP, not related to G proteins of the plasma membrane; Cheng *et al.*, 1985) is induced in fibroblasts by IFN-γ within 15 min with no protein synthesis requirements (Decker *et al.*, 1989). It is this prompt transcriptional response on which we have concentrated in our work on GBP.

Interferons are subdivided into two classes, IFN-α/β and IFN-γ, which act through distinct cell surface receptors (Aguet *et al.*, 1988; Uze *et al.*, 1990). Some IFN-responsive genes are exclusively induced by one IFN class whereas others respond to both IFN classes (Luster *et al.*, 1985, 1988; Larner *et al.*, 1986; Fan *et al.*, 1988; Porter *et al.*, 1988; Decker *et al.*, 1989; Reid *et al.*, 1989). Promoters that are induced by IFN-α have a consensus DNA-binding site, the ISRE (interferon-α stimulated response element), to which a well characterized activating protein complex, ISGF-3, binds to induce IFN-α-dependent transcription (Levy *et al.*, 1988, 1989; Fu *et al.*, 1990; Kessler *et al.*, 1990). The GBP gene is also induced by IFN-α and its promoter contains an ISRE (Lew *et al.*, 1991). Analogous to genes that are exclusively induced by IFN-α, the response of the GBP gene to IFN-α is most likely mediated by ISGF-3. Deletion analysis within the GBP promoter, however, indicated a requirement for additional sequences for IFN-γ inducibility. In this report we describe the gamma-interferon-activation factor (GAF), a DNA-binding protein that is activated by IFN-γ and that binds specifically to the sequences required for the transcriptional activation of the GBP promoter by IFN-γ. Like ISGF-3, GAF also exists in a latent cytoplasmic form. It becomes rapidly activated in the cytoplasm when fibroblasts or cytoplasts (enucleated cells) are treated with IFN-γ. The formation of active GAF correlates with the cell type-specific transcriptional induction of the GBP gene. It is therefore likely to be a crucial factor in the primary transcriptional response of cells to IFN-γ.

## Results

### *The interaction of GAF with the GBP promoter*

The *GBP* gene responds transcriptionally to both IFN-α and IFN-γ. To examine its control region, we recently cloned the *GBP* promoter, introduced mutations at various sites and studied the response of these mutant constructs to both IFN classes. The promoter contains an ISRE (−130 to −116) that accounts for the IFN-α inducibility of GBP. Neighboring sequences in the promoter downstream of the ISRE were required for the IFN-γ-induced GBP transcription (Lew *et al.*, 1991). Based on this knowledge we searched for DNA-binding factors that specifically associated with this region and might therefore mediate induction by IFN-γ. Evidence for such a factor, which we termed GAF (gamma interferon activation factor), was only detected using the exonuclease III (exoIII) protection assay (Wu, 1985; Kovesdi *et al.*, 1986). It was not possible to obtain clear results using the sequences of interest (−125 to −101) as a probe in the more widely used gel mobility retardation assay. Previous

evidence, however, had shown the exoIII assay to be at least as sensitive as gel retardation assays (Kovesdi *et al.*, 1986). Moreover, the exoIII assay has the potential to detect DNA–protein complexes that are not stable to separation in an electrophoretic field.

When fragments of the GBP promoter spanning the sequence from −76 to −216 (top strand) or −195 to −76 (bottom strand) were incubated with HeLa cell extracts, a region extending from −99 to −128 was protected from digestion with exoIII specifically when the extracts were derived from IFN-γ-treated cells (Figure 1). Both exoIII stops could be observed with probes containing the authentic promoter sequence. ExoIII protection experiments with extracts of euploid fibroblasts gave precisely the same results, protecting the −99 to −128 region of the GBP promoter. (Figure 4, which will be described later, shows identical protected bands with extracts from either cell type.) To make sure, however, that the stop at G −128 did not stem from a protein binding to the ISRE, a DNA probe with the ISRE mutated at T −126 was used in an exoIII experiment. The same protection was observed when this mutant fragment was used (Figure 1). Mutation of the ISRE region abrogates the association of constitutive or IFN-α-inducible, ISRE-binding proteins (Kessler *et al.*, 1990; Lew *et al.*, 1991) and thus implies that the IFN-γ-induced protein(s) responsible for the exonuclease protection in the −99 to −128 region did not depend on the intact ISRE sequence. To define which bases were most critical in the binding site of the GAF, we used mutated promoter fragments (point mutations or deletions) as competitors during the exoIII protection assay (Figure 2a). Effective competition in the assay was evident for all fragments of DNA except those with mutations between −109 and −121. Identical results were obtained with nuclear extracts from HeLa cells treated with IFN-γ for 4 h (Figure 2a) or with nuclear extracts from FS2 fibroblasts after a 15 min IFN-γ treatment (not shown). These competition experiments defined a region which we termed the gamma interferon activation site (GAS, Figure 2a,b) as crucial for GAF binding. Based on these results, an oligonucleotide (−125 to −101) was made containing the gamma IFN activated site, GAS, but not the full ISRE. This oligonucleotide competed efficiently for GAF binding (Figure 1) but did not bind or compete with the binding of the factors known to associate with the ISRE (Levy *et al.*, 1988 and data not shown). Therefore, the GAS and the ISRE are distinct, but overlapping sites in the GBP gene.

### *In vivo activity of the GAS*

To test whether the GAS functioned as an IFN-γ-responsive element *in vivo*, we prepared plasmids containing one or four copies of the GAS oligonucleotide linked to basal elements of the HIV LTR promoter driving the CAT reporter gene. A HeLa S3 culture was transfected with these recombinant constructs and divided into two samples, one of which was treated with IFN-γ for 15 h while the other was left untreated. Extracts from the transfected cells were then assayed for CAT activity. A single copy of the GAS oligonucleotide rendered the promoter responsive to IFN-γ (Figure 3, GAS★HC). Four copies produced a higher

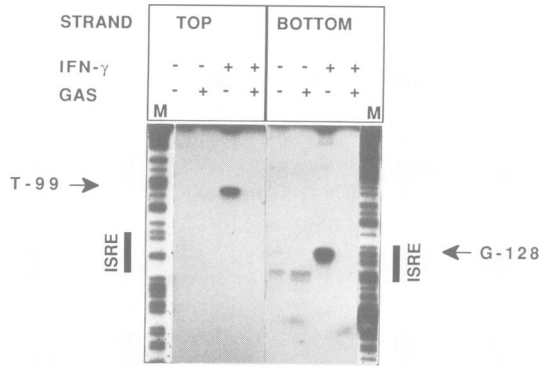


**Fig. 1.** Exonuclease III protection of the *GBP* promoter by the GAF. Nuclear extracts of HeLa cells that were either left untreated or treated with IFN-γ for 4 h were assayed with top- and bottom-strand probes as described in Materials and methods. Where indicated, 50 ng of specific oligonucleotide comprising the GAS (−125 to −101) were added.

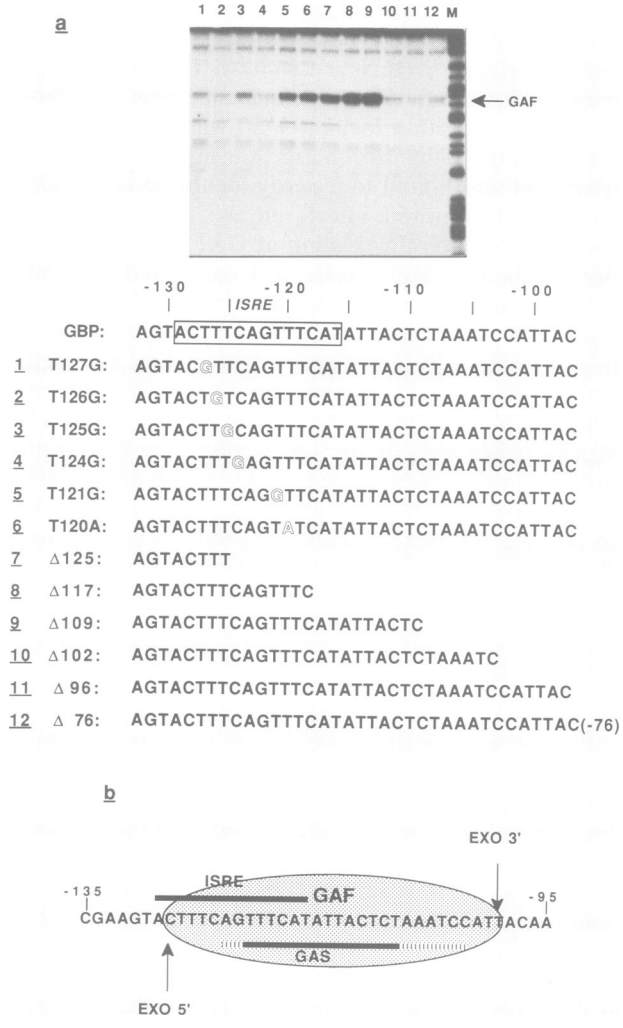


**Fig. 2.** (**a**) Definition of the GAS. Exonuclease III assays using the top strand probe (see Figure 1) were performed in the presence of a 30-fold excess of each of the mutated fragments indicated below. Mutants in lanes 5–9 failed to compete and define the GAF binding site. (**b**) A diagram of the binding sites in the GBP promoter. The ISRE is marked to coincide with the consensus bases determined for other IFN-α-inducible genes (Levy *et al.*, 1988, 1989) and by deletion from nucleotide −127 (Lew *et al.*, 1991). Bases within the GAS are represented by the black bar and possible limits of the site are within the region of the vertical line. The oval, stippled shape indicates the region protected by protein from exonuclease III digestion.

inducibility that quantitatively resembled the inducibility obtained with the active GBP promoter (Figure 3, compare 4 × GAS★HC and GBP★HC). Thus, the GAS functions to render a heterologous promoter IFN-γ-inducible *in vivo*.

### *GAF activation correlates with IFN-γ-induced transcription*

We have previously described the time course of the transcriptional response of the chromosomal GBP gene in fibroblasts and in HeLa cells (Decker *et al.*, 1989; Lew *et al.*, 1989; Figure 4a). Induction of transcription by IFN-γ in fibroblasts as tested by the nuclear 'run-on' assay occurred within half an hour and was independent of ongoing protein synthesis, while HeLa cells required many hours to reach a maximal response and active protein synthesis was needed for full induction. If GAF is the critical factor of IFN-γ-induced transcription, its time course of activation and cycloheximide sensitivity might be expected to correlate with these transcriptional patterns. Therefore, we monitored the time course and requirement for active protein synthesis of GAF activation in these two cell types (Figures 4b and 5). Like transcription, GAF activity was induced rapidly in fibroblasts and more gradually in HeLa cells. Also, induction of GAF DNA-binding activity was sensitive to cycloheximide (CHX) in HeLa cells but completely resistant in fibroblasts (Figure 5). Thus, the activation and presence of GAF closely paralleled the cell type-specific pattern of transcriptional induction of the GBP gene. This is strong evidence that GAF is a crucial positive-acting, IFN-γ-dependent gene activator.

Interestingly, there were differences in the detailed time course of GAF activation and GBP gene transcription. Mainly, the maximum GAF activity was not sustained for as long as maximal transcription rate (Figure 4b). These differences may reflect different requirements for the induction and for the maintenance of transcription (Lew *et al.*, 1991). Also, it is quite possible that other factors play a role, particularly during later stages of the transcriptional response. IFN-γ-regulated ISRE-binding factors have been described (Miyamoto *et al.*, 1988; Blanar *et al.*, 1989; Driggers *et al.*, 1990; Pine *et al.*, 1990). One of these, variously called ISGF-2 (Pine *et al.*, 1990), IRF-1 (Miyamoto *et al.*, 1988) or IBP-1 (Blanar *et al.*, 1989), has been suggested to mediate the response to IFN-γ (Blanar *et al.*, 1989). However, ISGF-2 (IRF-1) induction occurs only after a lag phase of 1 h and is inhibited in the presence of CHX in either fibroblasts or HeLa cells (Levy *et al.*, 1988; Lew *et al.*, 1991). Thus ISGF-2 (IRF-1) is not a reasonable candidate for the primary activation of transcription in response to IFN-γ and its role, if any, in the IFN-γ response remains unclear.

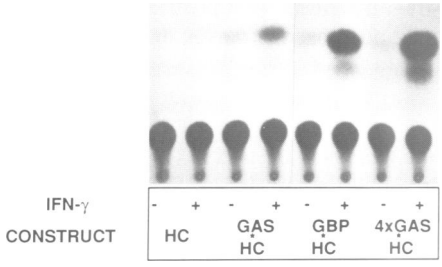


**Fig. 3.** IFN-γ-induced expression of the HIV LTR basal promoter mediated by the GAS. HeLa cells were transfected with 15 μg of the indicated constructs and either left untreated or treated with IFN-γ for 15 h. CAT activity was then determined in a standard assay.

### *GAF activation occurs in the cytoplasm of fibroblasts*

During the course of our experiments we noticed that GAF activity was readily detectable in cytoplasmic, as well as nuclear extracts from IFN-γ-treated fibroblasts. To test whether the cytoplasm was the site of initial activation we generated enucleated cells (cytoplasts) from untreated fibroblasts (Figure 6a). This preparation contained $<10\%$ intact cells. Cytoplast protein was obtained by hypotonic

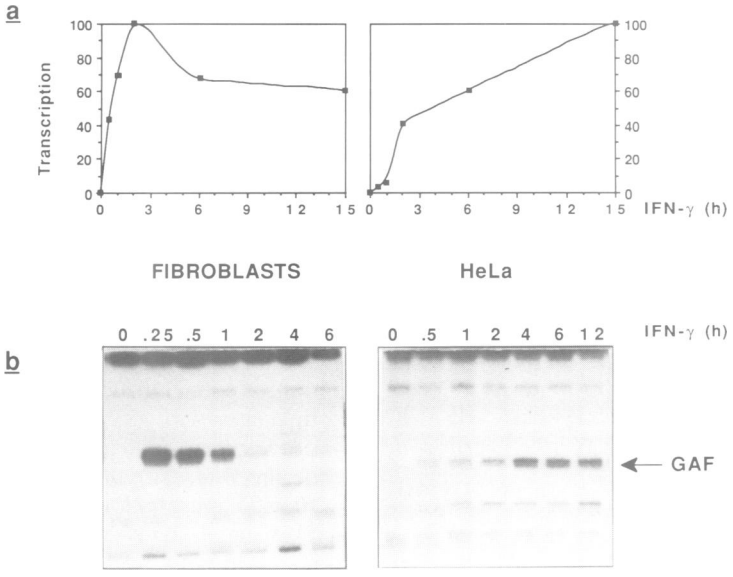


**Fig. 4.** The time course of transcription and GAF induction by IFN-γ. (**a**) IFN-γ-induced GBP gene transcription in primary diploid fibroblasts (FS2) or HeLa cells as determined in nuclear run-on assays. (**b**) GAF activity in nuclear extracts at indicated intervals after IFN-γ treatment of fibroblasts or HeLa cells. The two electrophoretic runs were for slightly different times but marker fragments allowed assignment of the GAF band to the same nucleotide with all extracts.

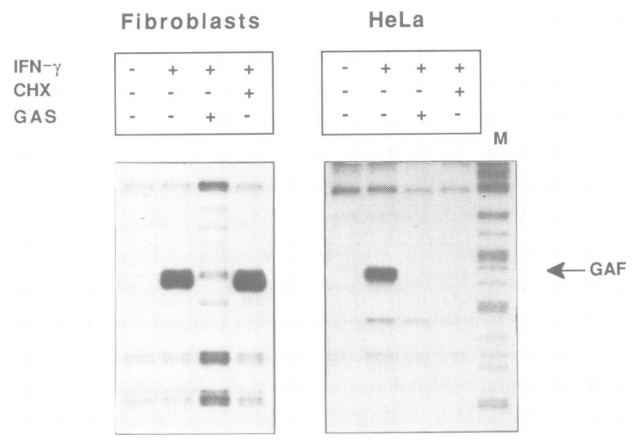


**Fig. 5.** The effect of protein synthesis inhibition on GAF induction by IFN-γ. Fibroblasts and HeLa cells were treated for 0.5 h and 4 h, respectively, with IFN-γ in the presence or absence of protein synthesis inhibitor (CHX, 50 μg/ml). Nuclear extracts were then used for exonuclease III assays (top strand probe) in the presence of either nonspecific oligonucleotide or specific GAS oligonucleotide (50 ng).

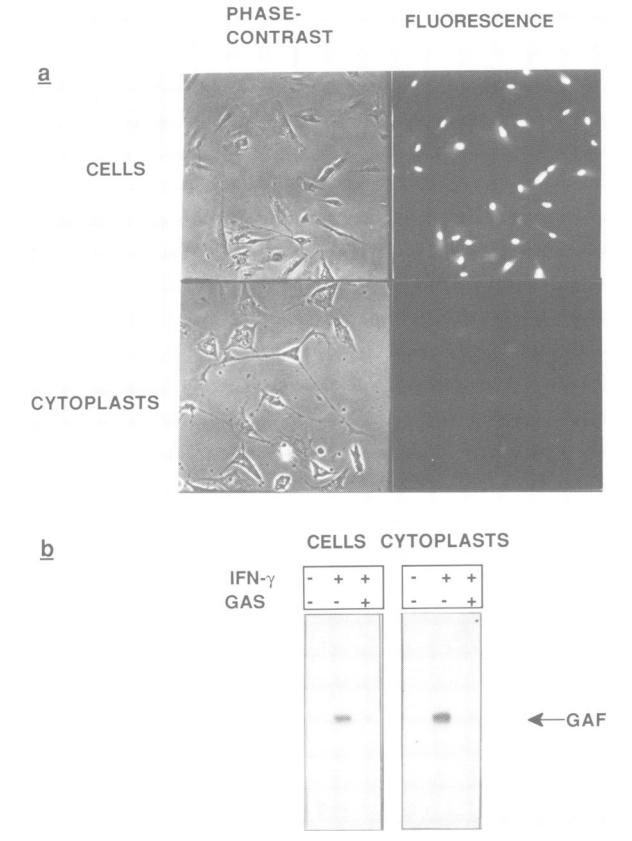


**Fig. 6.** Cytoplasmic activation of the GAF. (**a**) Cytoplasts were prepared by enucleation of cytochalasin B-treated fibroblasts and stained with ethidium bromide to indicate the presence of nuclei. (**b**) ~5 × $10^6$ intact cells or cytoplasts were left untreated or treated with IFN-γ for 15 min. Cytoplasmic or cytoplast extracts were then prepared in an identical manner and subjected to exonuclease III assays.

swelling and mechanical disruption followed by centrifugation to remove cell organelles and debris. [The same approach was used by Dale *et al.* (1989) to show IFN-α-dependent cytoplasmic activation of a factor that is probably ISGF-3.] Treatment of cytoplasts with IFN-γ led to a rapid induction of GAF activity (Figure 6b). To ensure that the GAF detected in cytoplast extracts was not the result of leakage from a few contaminating nuclei, we assayed GAF activity in an equal amount (judged by protein content) of cytoplasmic extract from intact fibroblasts that was prepared in the same way as described above for cytoplasts. If GAF actually leaked from the nucleus, then cytoplasmic extracts from nucleated cells should contain far more activity than the cytoplast preparations. However, we found that GAF activity was higher in cytoplast extracts (Figure 6b) indicating that activation was truly cytoplasmic. Possibly, the lower amount of GAF activity in the cytoplasm of the whole cells suggests a decrease due to nuclear translocation.

## Discussion

The goal of this study on the IFN-γ-regulated expression of the GBP gene was to extend our understanding of how a specific polypeptide ligand attached to a surface receptor can elicit a specific transcriptional response in cells. In fibroblasts the prompt IFN-γ-dependent induction of the GBP gene does not require new protein synthesis (Decker *et al.*, 1989) and thus the protein or proteins that form the gene activator could be expected to exist in untreated cells. From functional analyses of the GBP promoter, we knew that sequences near the RNA initiation site in the GBP gene were required for a maximal response to IFN-γ. The key to understanding this response was the discovery of GAF using an exoIII protection assay. It was then possible to define a DNA-binding site, the gamma-IFN activitation site or GAS, within the sequences required for inducibility. Furthermore, the previous information on the time course of transcription and sensitivity to CHX in HeLa cells (and insensitivity in fibroblasts) allowed us to correlate the IFN-γ-dependent presence of GAF and IFN-γ-dependent transcription of the GBP gene. Whether the GAF protein(s) participate(s) in all immediate IFN-γ-induced transcription is not clear at present. Only a few genes in which immediate transcriptional activation of IFN-γ is proven have been characterized. We have examined the available upstream sequences in MHC class I and II genes and the 9/27 gene without finding a good match with the GAS sequence.

The protein(s) required to form the GAF existed in the cytoplasm of fibroblasts before IFN-γ treatment and is (are) therefore available both to receive a signal from the occupied IFN-γ receptor and to move to the nucleus where transcription is stimulated by binding to the GAS. In addition, the transcriptional induction of the *GBP* gene by IFN-γ is not influenced by alterations in the levels of generally used second messengers (Lew *et al.*, 1989). Ligand-specific transcriptional induction might therefore not require global fluctuations in general second messengers but result from a few specific protein−protein interactions originating at the occupied receptor and leading to the rapid activation of the specific transcriptional regulator (Levy and Darnell, 1990). This suggestion seems all the more compelling in view of the earlier discovery that the individual components of latent cytoplasmic ISGF-3, the IFN-α-specific transcriptional activator, assemble within a minute specifically in response to the occupation of the IFN-α receptor (Levy *et al.*, 1989). The cytoplasmic activation of these transcriptional regulators

suggests that their participation in the signal transfer to the nucleus represents a mechanism for ensuring a limited transcriptional response to each polypeptide ligand (Levy and Darnell, 1990).

Other cytoplasmic proteins that are latent, inactive forms of a transcriptional activator are known. For example, it is thought that the cytoplasmic glucocorticoid receptor–HSP90 complex dissociates in response to the binding of ligand. Only then will the now activated receptor translocate to the nucleus and bind to specific sites (Picard and Yamamoto, 1987; Beato, 1989). Members of the rel family of proteins (dorsal, Rushlow *et al.*, 1989; p50 of NFkB, Ghosh *et al.*, 1990; Kieran *et al.*, 1990; c-rel, Bull *et al.*, 1990) are regulated through controlled nuclear translocation. The transcription factor NF$\varkappa$B is so far the best studied example. It is bound to the inhibitor IkB in the cytoplasm (Baeuerle and Baltimore, 1988) and can be activated in a variety of ways including LPS, TNF and phorbol ester treatment of cells (Sen and Baltimore, 1986). These activations are all thought to depend on a phosphorylation that releases the active factor from association with its inhibitor (Ghosh and Baltimore, 1990). NF$\varkappa$B induces the expression of a large variety of genes and could be a more widely required member of the same category of regulatory proteins we are studying after IFN treatment (Lenardo and Baltimore, 1989). The activation of ISGF-3 by IFN-$\alpha$ and now GAF by IFN-$\gamma$, however, marks the first two cases where cytoplasmic transcription factor activation depends strictly on a specific cell surface receptor–ligand interaction. Rapid communication between the intracellular receptor domain and the transcription factor without loss of specificity could most easily be achieved if the protein recognizing the liganded receptor in the cytoplasm was a part of the active transcription factor. A complete biochemical analysis of GAF and ISGF-3 will allow us to test this possibility.

## Materials and methods

### *Cell culture and treatment of cells*

HeLa S3 cells were obtained from the American Type Culture Collection (Rockville, MD). Human diploid fibroblasts (FS2) were a gift from E.Knight (Dupont, Wilmington, DE). Both cell types were grown to confluency in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% calf serum before further treatment. Recombinant IFN-$\gamma$, kindly provided by Dr D.Vapnek of Amgen, was added to a final concentration of 0.5 ng/ml (= 100 antiviral units). CHX (Sigma) was added to a final concentration of 50 $\mu$g/ml. Cells were pretreated with CHX for 5–10 min before addition of IFN.

### *Preparation of cytoplasts*

Cytochalasin B-treated FS2 cells were enucleated by centrifugation on Ficoll (Pharmacia) as recently described (Dale *et al.*, 1989). The cytoplasts were stained with ethidium bromide to monitor the loss of nuclei microscopically. Prior to IFN treatment the cytoplasts were cultured until tightly adherent in DME + 10% calf serum (2–3 h).

### *Plasmids and mutagenesis*

The cloning of genomic DNA containing the GBP gene promoter is described in detail in Lew *et al.* (1991). A *Hin*dIII–*Sma*I fragment encompassing the promoter sequences from −216 to +19 was cloned into pGem (Promega). A filled-in *Eco*RI site was subsequently used to fuse the *GBP* gene promoter to DNA containing the *CAT* gene together with the SV40 splice and polyadenylation sites. The resulting construct, GC3, was used to create the site-specific or *Bal*31 mutations shown in Figure 2. For HIV-LTR constructs an oligonucleotide containing the *GBP* gene promoter sequences from −125 to −101 or multiples thereof were cloned into the *Bam*HI site of the parental HIV-LTR construct (Lew *et al.*, 1991). The plasmid containing authentic *GBP* gene promoter sequences in the HIV-LTR context was made by cloning a *Sca*I–*Ssp*I fragment (−129 to −76) into the same parental HIV-LTR plasmid. All cloning procedures or *Bal*31 deletion mutagenesis were according to standard methods (Sambrook *et al.*, 1989). Site-specific mutagenesis was done using the gapped heteroduplex method (Inouye, 1983) or the deoxy-uracil method (Kunkel, 1985) using a BioRad kit and the manufacturer's instructions.

### *Cell extracts*

Nuclear extracts were prepared as previously published (Dignam *et al.*, 1983; Levy *et al.*, 1988). Cytoplast extracts were prepared after briefly washing cytoplasts in cold PBS. The enucleated cells were carefully removed from a 10 cm culture dish with a rubber policeman in cold PBS and collected by centrifugation. After swelling in 10 ml of hypotonic RSB buffer (10 mM Tris–HCl, pH 7.4, 10 mM KCl, 3 mM $MgCl_2$, 1 mM DTT) for 10 min on ice the cytoplasts were again collected by centrifugation and broken by Dounce homogenization in a small volume (80–100 $\mu$l) of RSB buffer containing 10% glycerol. Particulate matter was removed by centrifugation for 5 min in a microfuge. The extract was either assayed immediately or stored at −80°C. Cytoplasmic extracts were made in an identical fashion using intact fibroblasts.

### *Exonuclease III assay*

The assay has been published previously (Wu, 1985; Kovesdi *et al.*, 1986). The top strand probe was made by cloning *GBP* gene promoter sequences −216 to −76 (*Hin*dIII–*Ssp*I frgament) from GC3 (see above) into the *Hin*dIII and *Sma*I sites of pGem. From this plasmid a uniquely 5′-labeled *Hin*dIII–*Eco*RI fragment was isolated and used in the assay. The bottom strand probe was derived from either the same plasmid or a plasmid in which the same *Hin*dIII–*Ssp*I fragment from the T126G mutant (see Figure 2) was cloned into pGem. Since the GAF exo stop on the bottom strand lies on the upstream part of the ISRE (see text) we preferred the use of this mutant which retains GAF binding but abolishes the binding of ISRE-specific factors and thus allows a clear interpretation. The bottom strand probe was isolated after uniquely labeling at the pGem *Pvu*II site (+98) and subsequent digestion with *Dra*I (−195 in the *GBP* gene promoter). Routinely, between 30 and 60 $\mu$g extract protein from nuclear or cytoplast extracts were incubated with 1 ng of probe and 4 $\mu$g of nonspecific poly(dIdC)-poly(dIdC) competitor (Pharmacia) in a standard binding reaction. Digestion of the probe was then carried out with 175 U of exoIII (Boehringer) for 15 min at 32°C. Reaction products were analyzed on an 8% sequencing gel. As a marker for the reaction products a standard Maxam–Gilbert G+A reaction of the probes was used.

### *Nuclear run-on assay*

We have previously described this assay (Decker *et al.*, 1989).

### *Transfection of HeLa S3 cells and CAT assay*

A modification (Golub *et al.*, 1989) of the standard DEAE–dextran protocol (Lopata *et al.*, 1984) was chosen. One 15 cm plate of confluent cells was transfected with 15 $\mu$g of DNA and then split into two equal halves to give identically transfected cells for control and IFN treatment. The cells were cultured for 24 h and then left untreated or treated for 15 h with IFN-$\gamma$. After washing with PBS, extracts were made as described (Sambrook *et al.*, 1989). CAT assays with these extracts were done following a standard procedure (Sambrook *et al.*, 1989).

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