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Immunology

Interferon γ-induced transcription of the high-affinity Fc receptor for IgG requires assembly of a complex that includes the 91-kDa subunit of transcription factor ISGF3

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ABSTRACT A 39-nt DNA sequence, the interferon γ (IFN-γ) response region (GRR), is necessary for the IFN-γ-induced transcription of the high-affinity Fc receptor for IgG (FcγRI) and sufficient for the IFN-γ-induced transcription of transfected plasmids. By using extracts from IFN-γ-treated cells, three protein complexes will assemble *in vitro* on a 9-nt core region in the 3′ domain of the GRR. The sequence of this core resembles the IFN-γ-activated sequence (GAS) described for the *GBP* gene. Mutations in this GAS core region prevent complex assembly and result in the loss of IFN-γ induction of reporter constructs containing the mutation. In addition to the GAS core region, a 5′ region of the GRR is necessary for optimal IFN-γ induction and for formation of one of the DNA–protein complexes. By antibody reactivity, we show that a 91-kDa protein, first identified as a component of ISGF3, the IFN-α-induced transcription complex, is present in at least two of the DNA–protein complexes. IFN-α can induce the formation of the faster-migrating 91-kDa protein–GAS complex but not the slower-migrating complex. Furthermore, IFN-α does not result in appreciable transcriptional activation of FcγRI or constructs containing the GRR. Thus, these data demonstrate that the IFN-γ-activated 91-kDa protein is required for IFN-γ induction of FcγRI and suggest that an additional complex may be required for optimal expression and specificity.

Although first identified by its shared antiviral activity, the class II interferon (IFN) IFN-γ possesses biological activities that distinguish it from the class I IFNs, IFN-α and -β (for review, see ref. 1). For example, IFN-γ is a potent immunomodulatory agent responsible for macrophage activation and enhanced antigen presentation (2). These IFN-γ-induced effects are mediated through the immediate transcriptional induction of some genes, such as the high-affinity Fc receptor for IgG (FcγRI) (ref. 3 and unpublished observation), and the delayed induction of others such as the major histocompatibility complex class II genes (4). In addition to this set of genes specifically induced by IFN-γ, classes of genes have been described that respond to IFN-α alone or to both IFN-α and IFN-γ (5–15).

Recent experiments have clarified the mechanism by which IFN-α and -γ are able to trigger transcription of specific genes. Binding of IFN-α to its cell surface receptor results in the phosphorylation on tyrosine and translocation to the nucleus of three proteins (113, 91, and 84 kDa in size) that with a 48-kDa DNA binding protein assemble on a DNA site referred to as the IFN-stimulated response element (ISRE) (16–21). This multiprotein transcription complex, termed ISGF3, is not activated by IFN-γ. Instead, IFN-γ induces the phosphorylation of the 91 (or 84)-kDa protein but not the 113-kDa protein and binds to a DNA sequence element, the IFN-γ-activated sequence (GAS) that was initially identified in the *GBP* gene (refs. 8, 9, 22, 24 and K. D. Khan, K.S., G. Lindwall, S. E. Maher, J.E.D., and A. L. M. Bothwell, unpublished results). Since the *GBP* gene is induced by both IFN-γ and IFN-α, interaction of the 91-kDa protein with the GAS may provide a common pathway for transcriptional activation by IFN-α and IFN-γ.

In previous work we have focused (25) on the mechanism by which IFN-γ specifically induces transcription of target genes. FcγRI, in contrast to the *GBP*, is induced only by IFN-γ. A 39-nt sequence, called the IFN-γ response region (GRR), has been identified in the promoter of this gene that is both necessary and sufficient for transcriptional activation by IFN-γ (25) and does not respond to IFN-α (unpublished data). These transfection results correlate with the transcriptional induction of FcγRI in macrophages in response to IFN-γ that is robust, rapid, and cycloheximide-resistant. In contrast, IFN-α treatment of macrophages results in insignificant FcγRI transcriptional induction (ref. 3 and unpublished observation). Induction of FcγRI accounts for some of the responses attributed to the activated macrophage, such as increased phagocytosis and enhanced antibody-dependent cellular cytotoxicity (26).

We now present evidence for the rapid induction of specific protein complexes that assemble on the GRR in IFN-γ-stimulated cells. Mutations that prevent assembly of these complexes *in vitro* also prevent response to IFN-γ induction in transfection experiments with reporter constructs. These complexes interact with a 9-nt core sequence in the GRR that is homologous to the GAS sites found in other IFN-γ-induced genes. The 91-kDa DNA binding protein that interacts with the *GBP* promoter also binds to the GRR of the FcγRI gene. Surprisingly, IFN-α also induces the formation of similar complexes on the GRR yet fails to induce significant transcriptional activation of FcγRI or the GRR. We have begun to reconcile these results by the identification of a second domain within the 5′ region of the GRR that is required for optimal IFN-γ induction and may afford a site for additional protein interactions. Our results demonstrate that interaction of the 91-kDa DNA binding protein with the core sequence is necessary but may not be sufficient for transcriptional activation of FcγRI and that additional interactions may be required to account for the IFN specificity exhibited by this promoter.

MATERIALS AND METHODS

Cell Culture. U937 cells (American Type Culture Collection) were maintained in suspension culture in RPMI 1640

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Abbreviations: IFN, interferon; GRR, IFN-γ response region; FcγRI, high-affinity Fc receptor for IgG; GAS, IFN-γ-activated sequence; ISRE, IFN-stimulated response element; EMSA, electrophoretic-mobility-shift assay; CAT, chloramphenicol acetyltransferase.

medium, 10% (vol/vol) fetal calf serum, and 5 μM L-glutamine. Cultures were treated with IFN-α2a (1000 units/ml) or human IFN-γ (200 units/ml), both provided by Hoffman–La Roche. Cycloheximide was used at 50 μg/ml.

Nuclear Extracts. Nuclear extracts were prepared as described (27).

Electrophoretic-Mobility-Shift Assays (EMSAs). Assays were performed as described (28, 29) using 8 μg of nuclear extract, 0.2 ng of probe (20,000 cpm), 3 μg of poly(dI·dC) (Pharmacia), and 0.2 μg of pUC19. For probe, the 39-bp GRR was isolated from p(GRR)TK (18) by using *Sal* I, and the resulting overhangs were filled using all four [^{32}P]dNTPs. Supershifts were performed using antisera to the 48-, 91-, and 113-kDa components of ISGF3 at dilutions of 1:250 (30–32).

Plasmid Constructions. p(GRR)TK and mutants of p(GRR)TK were made by cloning synthetic double-stranded oligonucleotides into the *Sal* I site of pTKCAT (33).

DNA Transfection. Chloramphenicol acetyltransferase (CAT) reporter constructs were cotransfected with 2 μg of pGL (Promega) into U937 cells as described (25, 34). Cells were then allowed to recover for 48 hr, treated with IFN for 8 hr, and harvested. An aliquot of cells from each transfection was lysed in Triton X-100 and analyzed for luciferase activity (Promega Technical Bulletin 101) by using a Turner TD-20e luminometer. The remaining cells were lysed by repeated freeze–thaw cycles (35). CAT assays were then performed (36) on extracts equalized by luciferase activity. Quantitation of CAT values was determined by densitometric scanning of the resulting autoradiograms.

Affinity Purification of the GRR Binding Complex. The GRR (25) was labeled using biotin-14-dATP (GIBCO/BRL) (37) and used as probe in standard EMSA binding reactions. The resulting biotinylated DNA–nuclear protein complexes were precipitated using streptavidin-agarose (GIBCO/BRL), washed, boiled in SDS loading buffer, and analyzed by SDS/PAGE, followed by Western blot analysis using ECL (Amersham) with either preimmune serum or antiserum to the 91-kDa component of ISGF3 (31) at a dilution of 1:4000.

RESULTS

Trans-acting protein factors that interact with the GRR to mediate IFN-γ-induced transcription were identified by an EMSA. The 39-bp GRR fragment was incubated with nuclear extracts from untreated or IFN-γ-stimulated U937 cells (Fig. 1). Three distinct complexes assembled on the intact 39-bp GRR. They were induced as early as 5 min after stimulation with lymphokine and were insensitive to the protein synthesis inhibitor cycloheximide (data not shown). Excess unlabeled GRR competed with complex formation as did a DNA

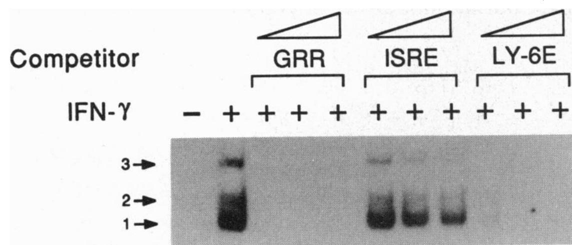

FIG. 1. GRR–protein complexes induced by IFN-γ. EMSA using nuclear extracts prepared from U937 cells uninduced (−) or induced (+) by IFN-γ for 1 hr. Competitors: double-stranded oligonucleotides encoding the GRR (5′-GCATGTTTCAAGGATTTGAGATGTATTTCCCAGAAAAGG-3′), the ISRE from the ISG15 promoter (5′-GATCCTCGGGAAAGGGAAACCGAAACTGA-3′), and the Ly-6E enhancer (5′-CATGTTATGCATATTCCTGTAAGTGCATG-3′). Concentrations of competitors were at 50, 100, and 200 molar excess above probe. Arrows indicate three distinct GRR–protein complexes.

Table 1. Sequence comparison of GAS core sites in IFN-γ-inducible genes

Site	Sequence
GRR	TTCCCAGAA
GBP	TTACTCTAA
Ly-6E	TTCCTGTAA
MIG	TTACTATAA
Consensus	TTC/ACNNNAA

MIG, macrophage-induced gene (34).

fragment that includes the GAS sites found in the IFN-γ-responsive gene *Ly-6E*. This murine gene has recently been found (Khan *et al.*, unpublished results) to be activated by an element that contains the necessary sequence shown in Table 1. In contrast, excess unlabeled ISRE, the IFN-α DNA response element, which does not contain the consensus sequence shown in Table 1, did not block the DNA–protein complex with the labeled GRR. The rapid induction, cycloheximide insensitivity, and specificity for IFN-γ response elements are consistent with the IFN-γ transcriptional response of FcγRI and suggest that these complexes represent functional components of the transcriptional activation by IFN-γ.

To establish the functional role of these complexes in IFN-γ-induced transcription, a series of GRR mutants were synthesized and tested for their ability to confer IFN-γ inducibility upon a promoter–reporter construct (Fig. 2). These same GRR mutants were analyzed by EMSA to correlate the appearance of the IFN-γ-induced GRR complex with transcriptional activation. Clusters of 4 nt were simultaneously substituted, and the remaining 35 nt of the GRR were left wild type. Two regions of the GRR were found to contribute to IFN-γ-induced transcription: a 5′ region of 12 nt (blocks 2–4), where mutations result in a 70% reduction in IFN-γ-induced CAT activity, and a 3′ region of 12 nt (blocks 7–9), where mutations destroy all IFN-γ induction. GRR mutants that contained substitutions in the 3′ region were

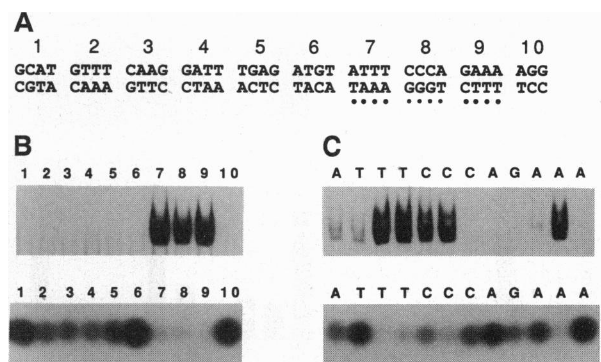

FIG. 2. Mutational analysis of the GRR. (*A*) Wild-type GRR is shown divided into 10 blocks. Each block was sequentially mutated, leaving the other 35 bp wild type. Mutations were transversions made by exchanging G with T and C with A. (*B*) For competitive gel shifts (*Upper*), each of the resulting GRR 4-base mutants was assayed for the ability to compete with wild-type GRR in a standard EMSA. The unlabeled mutants were used at a concentration 100-fold greater than that of the labeled wild-type GRR. Nuclear extracts were prepared from U937 cells induced by IFN-γ for 1 hr. For CAT activities (*Lower*), each of the GRR 4-base mutants was cloned into pTKCAT, transfected into U937 cells, and assayed for promoter activity after IFN-γ induction. (*C*) Point mutations were made in the GRR at sites spanning blocks 7–9 (marked by dots). For competitive gel shifts (*Upper*), each of the GRR point mutants was assayed for the ability to compete with wild-type GRR in a standard EMSA, as above. For CAT activities (*Lower*), each of the GRR point mutants was cloned into pTKCAT, transfected into U937 cells, and assayed for promoter activity after IFN-γ induction.

unable to compete for the induced GRR complexes, suggesting that this site interacts with induced proteins directly and is autonomous of other sites. Single nucleotide substitutions in this 3′ region revealed a discontinuous domain spanning 9 nt where alterations in any of 5 nt substantially reduced or eliminated both transcriptional activity from the GRR and the ability to compete with the formation of EMSA complexes (Fig. 2C). The sites of interaction of the induced complex with the 3′ GRR sequences defined a core binding site for a putative transcription factor induced in response to IFN-γ. This sequence, TTCCNNNAA, was conserved in several IFN-γ responsive elements, as shown in Table 1. It was contained within the GAS site described for IFN-γ-induced genes *GBP* and *Ly-6E*.

In contrast to the 3′ core site, the 5′-site mutants retained the ability to compete for the induced GRR complexes. These results predict that interactions at the 5′ site depend upon the 3′ core sequence and, therefore, separation of the GRR into 5′ and 3′ fragments would result in a diminished IFN-γ response. To test this prediction, two fragments of 24 and 23 nt were synthesized (corresponding to blocks 1–6 and 5–10) and their abilities to mediate IFN-γ induction were compared to that of the intact 39-nt GRR. As seen in Fig. 3, the 3′ fragment, which contains the GAS site, was only 25% as effective as the intact GRR, and the 5′ fragment was essentially inactive. These fragments were also tested for their ability to form EMSA complexes. Only the 3′ fragment was able to assemble EMSA complexes; no complexes were apparent when the 5′ fragment was used. However, the complexes observed with the 3′ fragment were qualitatively different from complexes observed with the intact GRR. The slowest-migrating upper complex was seen only when the intact GRR was used, suggesting that assembly of this complex depends upon both 5′ and 3′ sites in the GRR and supporting the prediction of the competition studies that interactions at the 5′ site depend upon the 3′ core sequence.

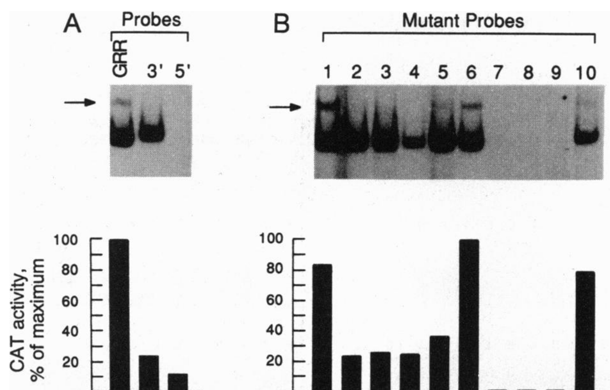

FIG. 3. Analysis of a GRR-specific complex involving interactions between 5′ and 3′ sites. (*A*) For EMSA (*Upper*), probes were the intact GRR, the 3′ domain of the GRR (5′-TGAGATGTATTTCCCAGAAAAGG-3′), and the 5′ domain of the GRR (5′-GCATGTTTCAAGGATTTGAGATGT-3′). The complex requiring the 5′ domain is indicated by an arrow. Nuclear extracts were prepared from U937 cells induced by IFN-γ for 1 hr. For CAT activities (*Lower*), the 3′ and 5′ domains of the GRR were cloned into pTKCAT, transfected into U937 cells, and assayed for promoter activity after IFN-γ induction. The resultant CAT activities are shown as a percentage of the induction exhibited by intact GRR after subtraction of pTKCAT background values. (*B*) For EMSA (*Upper*), probes were the 4-base GRR mutants described in Fig. 2. The complex specific to the 5′ domain is indicated by an arrow. Nuclear extracts were prepared from U937 cells induced by IFN-γ for 1 hr. For CAT activities (*Lower*), the GRR 4-base mutants were cloned into pTKCAT, transfected into U937 cells, and assayed for promoter activity after IFN-γ induction. The resultant CAT activities are shown as a percentage of the induction exhibited by intact GRR.

To confirm this interpretation, the 4-base mutants described in Fig. 2 were used as probes in direct EMSAs (Fig. 3*B*). Mutants in blocks 2–4 were unable to assemble the upper complex, whereas the lower complexes were intact. Mutants in blocks 7–9 were unable to assemble any of the three complexes. These results indicate that two types of complexes are forming on the GRR: a 3′ complex interacting with the core GAS sequence and a complex requiring both the 5′ and 3′ GRR sites.

Having established the functional role of these EMSA complexes in GRR-mediated IFN-γ-induced transcription, we next sought to determine the protein components of these complexes. The recent purification and cloning of the ISGF3 complex provided us with specific antibody reagents to determine whether any of the GRR complexes are related to these IFN-α-induced complexes. Antibodies to the 48-, 91-, and 113-kDa components of ISGF3 (30–32) were incubated with the induced GRR complexes defined above (Fig. 4). Antibody to the 91-kDa protein efficiently bound to the GRR complexes, resulting in their further retardation in the gel. Of the three complexes induced in response to IFN-γ, only the middle band remained unshifted by the anti-91-kDa protein antibody. Preimmune sera or antibodies to the 48- and 113-kDa proteins did not react with the GRR complexes. Antibodies raised to the N-terminal, central, and C-terminal domains of the 91-kDa protein all reacted with the GRR complexes in an identical manner (data not shown).

To determine the relationship of the immunoreactive 91-kDa species with the 91-kDa ISGF3 protein, the GRR complexes were isolated, resolved by SDS/PAGE, and analyzed on an immunoblot with antibodies to the 91-kDa protein (Fig. 5). The GRR complexes induced by IFN-γ migrated with an apparent molecular mass of 91 kDa and comigrated with the 91-kDa ISGF3 protein induced by IFN-α. Based on their immunological cross-reactivity and comigration, the 91-kDa species induced by IFN-γ on the GRR and by IFN-α on the ISRE appear to be identical.

Since IFN-α induces the formation of the ISGF3 complex, which includes the 91-kDa protein, we sought to determine whether IFN-α could induce the GRR–91-kDa protein complex in U937 cells. Despite the fact that IFN-α does not induce significant transcription of FcγRI in U937 cells or GRR-mediated induction of heterologous promoter–reporter constructs, IFN-α induced the formation of GRR–91-kDa protein complexes (Fig. 4). In contrast to the three complexes induced by IFN-γ on the GRR, IFN-α induced two complexes, comigrating with the lower two bands. As we found for IFN-γ, antibodies to the 91-kDa protein shifted the lower complex but did not affect migration of the middle band.

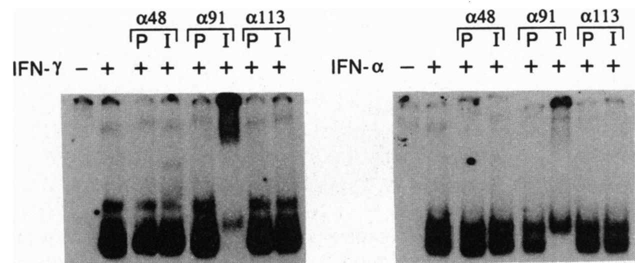

FIG. 4. Antibody specific for the 91-kDa protein of ISGF3 recognizes GRR–protein complexes induced by both IFN-γ and IFN-α. EMSA was performed using nuclear extracts from U937 cells uninduced (−) or induced (+) by either IFN-γ or IFN-α for 1 hr. Where marked, antiserum (α) specific for the 48-, 91-, or 113-kDa component of ISGF3 was included in the EMSA binding reaction. P, preimmune sera; I, immune sera.

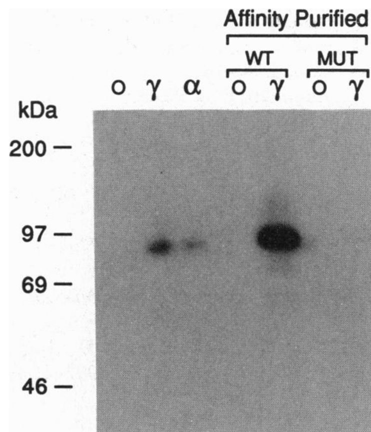

FIG. 5. A 91-kDa protein binds to the GRR and is recognized by antibody specific for the 91-kDa protein of ISGF3. A Western blot of crude and affinity-purified nuclear extracts probed with anti-91c is shown. Anti-91c does not recognize crude extracts prepared from uninduced U937 cells (lanes O) but does recognize crude extracts from cells induced by either IFN-γ (lanes γ) or IFN-α (lane α). Affinity purification of the IFN-γ-induced nuclear extract using the GRR (WT) as affinity ligand enriches for this 91-kDa protein, but affinity purification using an unrelated affinity ligand (MUT) does not.

DISCUSSION

Rapid transcriptional activation in response to extracellular stimuli is a common theme among mammalian cells. The IFN response has been an attractive model in which to address mechanistic aspects of this question, since it displays multiple levels of specificity. IFNs induce overlapping biological responses as well as specific nonoverlapping responses. Consistent with this observation, genes that respond selectively to IFN-α or IFN-γ have been identified as well as those that respond to both IFN-α and -γ. To clarify the mechanism by which these responses can be achieved, efforts have focused on characterizing the molecular basis for induced transcription for each class of genes.

The molecular basis for receptor-activated transcriptional induction by IFN-α has been clarified. IFN-α-responsive genes contain an ISRE upon which the ISGF3 proteins assemble in response to IFN-α (6–13, 18, 19). The situation for genes that respond to both IFN-α and -γ has now been reported and is different in several key aspects. Those genes contain a joint IFN-activated sequence (JIAS, which includes the GAS site) upon which a 91-kDa protein, originally identified as a subunit of ISGF3, assembles in response to either IFN (Khan *et al.*, unpublished results). In this study, we have dissected the DNA sequences and transcription factors that govern the induction of a gene that responds preferentially to IFN-γ. This gene contains a GRR that is both necessary and sufficient for IFN-γ induction (25). Within the GRR is a 9-nt core sequence that is crucial to the IFN-γ response and resembles the GAS described for the *GBP* gene (8, 9). This core sequence also assembles a complex after lymphokine stimulation that includes the 91-kDa ISGF3 protein. In this respect, IFN-γ-responsive genes appear quite similar. However, as neither FcγRI nor GRR promoter–reporter construct responds appreciably to IFN-α (ref. 3 and unpublished observations), it is reasonable to expect that other sequence elements within the GRR differentiate between IFN-γ- and IFN-α-initiated signals.

Two lines of evidence suggest that the GAS site may not be sufficient to account for the magnitude or specificity of the FcγRI response to IFN-γ. (*i*) The complete 39-bp GRR is necessary for optimal IFN-γ induction: the 3′ half of the GRR, which contains the GAS site, mediates a response to IFN-γ that is only 25% of that found when the intact GRR is present (Fig. 3*A*), and mutations made at a 5′ site in the GRR reduce IFN-γ-induced expression by 70% (Figs. 2 and 3). (*ii*) The EMSA complexes that assemble on the intact GRR in response to IFN-γ are qualitatively different from the complexes that assemble on the GAS site alone. An additional slowly migrating complex is seen only when the intact GRR is used and mutations made at the 5′ site specifically abrogate this complex (Fig. 3). This complex is shifted with antibodies to the 91-kDa protein and is not induced by IFN-α. Thus, the GRR contains two sites involved in protein–DNA interactions: a GAS site that interacts with the 91-kDa protein and a 5′ site that is required for optimal IFN-γ response and contributes to the assembly of an EMSA complex that, though it includes the 91-kDa protein, is IFN-γ-specific.

Our data suggest that these sites are not independent. As shown in Fig. 3*A*, the GAS site alone is able to assemble EMSA complexes but the 5′ site is not. It thus appears that protein interactions with the 5′ site are unstable in the detection assay used and may require additional stabilization provided by the GAS–91-kDa protein complex. Two models can be envisioned to account for the interaction between these sites. The GAS–91-kDa protein complex may be involved in stabilizing an additional complex involving the 5′ site with a second protein. Alternatively, the 91-kDa protein may interact directly with both the GAS and 5′ sites to generate the more slowly migrating complex seen in Figs. 1–4. Specificity of the GRR for IFN-γ would then result from either the induction of an additional protein–DNA interaction at the 5′ site or the induction of a conformation of the 91-kDa protein capable of interacting with the 5′ site.

The 91-kDa ISGF3 protein participates in transcriptional activation in at least two ways. In response to IFN-α, the 91-kDa protein is phosphorylated on tyrosine and assembled with the 113- and 84-kDa proteins into a complex that does not make significant contacts with ISRE, the DNA response element (18, 19). In contrast, in response to IFN-γ, the 91-kDa protein is also tyrosine phosphorylated but now assembles directly on the GAS site (ref. 24 and Khan *et al.*, unpublished results), the core sequence of which is TTA/CCNNNAA. This interaction of the 91-kDa protein with the GAS site is crucial, since mutations in the core site that abrogate the 91-kDa protein binding result in an unresponsive gene. However, for FcγRI and presumably other genes that respond preferentially to IFN-γ, optimal induction appears to require other DNA sites and possibly other protein factors. Whether these other protein factors act as positive regulators or function to dislodge preexisting negative regulators, as has been suggested for other IFN-responsive genes (23, 38), remains to be determined.

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