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# Induction of the Ly-6A/E gene by interferon $\alpha/\beta$ and $\gamma$ requires a DNA element to which a tyrosine-phosphorylated 91-kDa protein binds

(Ly-6/interferon activation site/inducible transcription factor/tyrosine phosphorylation)

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**ABSTRACT** **The murine Ly-6A/E gene is transcriptionally induced in cells exposed to interferon $\alpha/\beta$ or $\gamma$ (IFN-$\alpha/\beta$ or IFN-$\gamma$). Analysis of the 5′ flanking sequence using reporter plasmids that contain upstream elements of the Ly-6E gene has previously identified an ≈850-base-pair IFN-responsive region that lacked an IFN-$\alpha$-stimulated response element (ISRE), the element present and required for an IFN-$\alpha$ response of a number of genes. Analysis by deletion and stable transfection of the IFN-responsive region of the Ly-6E promoter has defined an 80-base-pair region containing an IFN-$\gamma$ activation site (GAS) but no ISRE that allows IFN-$\gamma$ and IFN-$\alpha$ inducibility of the Ly-6E gene. As tested by specific antiserum, a 91-kDa protein known to be activated in IFN-$\alpha$- or IFN-$\gamma$-treated cells binds to the GAS element from the Ly-6E promoter. The 91-kDa protein exists as an inactive cytoplasmic precursor and depends on tyrosine phosphorylation for its activation. Thus the same 91-kDa protein appears to act in the signal transduction pathways of both types of IFN for the Ly-6-A/E gene.**

Interferons (IFNs) exert their pleiotropic biologic effects by stimulating the expression of a number of genes in their target cells. Some of these genes are known to be very promptly activated at the transcriptional level without new protein synthesis (1). The most extensively studied group of genes is induced by type I IFNs—e.g., Mx (2), 6-16 (3, 4), ISG-15 (5), ISG-54 (6), and ISG-56 (7). There are also a few genes that are known to be activated immediately at the transcriptional level by IFN-$\gamma$ and to a much lesser extent, if at all, by IFN-$\alpha$. These include IP-10 (8) and IP-30 (9) genes. There are genes that are immediately induced by both types of IFNs. These include the guanylate-binding protein (GBP) gene (10) and ISGF-2 (IRF-1) (11).

The transcriptional response of a number of genes to IFN-$\alpha$ requires a conserved promoter element termed ISRE (IFN-$\alpha$-stimulated response element) (5, 6, 12) to which a multiprotein DNA-binding protein complex, ISGF-3, binds and directs transcription (13, 14). The GBP gene that is transcriptionally responsive (15, 16) to IFN-$\alpha$ and IFN-$\gamma$ contains an ISRE and an overlapping sequence termed GAS that directs the IFN-$\gamma$ response. Mutations that rendered the ISRE inactive left the GAS functionally intact for IFN-$\gamma$ and surprisingly for IFN-$\alpha$ responses, implying that the GAS element might also direct an IFN-$\alpha$ response (16). The factor that binds to the GAS of the GBP gene has recently been identified and characterized (17). A 91-kDa protein that is one of the components of ISGF-3$\alpha$ complex specifically binds to GAS and not ISRE. In fibroblasts, in response to IFN-$\alpha$, the 91-kDa protein is phosphorylated on tyrosine along with other ISGF-3$\alpha$ proteins (18, 19).

The mouse gene encoding the Ly-6A/E antigen is highly responsive to IFN-$\alpha/\beta$ and IFN-$\gamma$ (20). Analysis of the 5′ flanking region of the Ly-6E gene using the chloramphenicol acetyltransferase (CAT) reporter gene in stable transfections localized the IFN-responsive element(s) for IFN-$\alpha/\beta$ and IFN-$\gamma$ to a region between −1760 to −900. Surprisingly, this region lacked any obvious sequence homology to the consensus ISRE, suggesting that the IFN-$\alpha$ response of Ly-6E was due to some factor other than ISGF-3.

Here we describe a deletion analysis of the Ly-6E promoter region that identified an IFN-$\alpha$- and IFN-$\gamma$-responsive element(s) in the 80 bp between −1270 and −1190. Within the 80 bp there is an element with significant homology to GAS and with no sequence resembling an ISRE. Extracts of human and mouse cells treated with appropriate protein that bind to the 80-bp region in response to IFN-$\alpha$ or IFN-$\gamma$ contain a 91-kDa tyrosine-phosphorylated protein. These results provide an example of an IFN-inducible promoter in which a naturally occurring GAS-like sequence clearly devoid of an ISRE homology can act in an independent fashion to mediate IFN inducibility to IFN-$\alpha/\beta$ and IFN-$\gamma$ and also suggest that immediate IFN transcriptional response through the GAS element could be used in other genes inducible by both types of IFN.

## MATERIALS AND METHODS

**Plasmid Constructions.** Plasmid pCAT-5 has the CAT gene linked to the Ly-6E promoter sequence extending up to −900 (20). There are unique *Hin*dIII and *Xba* I sites located in the polylinker immediately upstream of the −900 position in pCAT-5 and these sites were used to subclone various DNA fragments derived from the −1760 to −900 region of the Ly-6 promoter.

**Northern Blot Analysis.** Total RNA was prepared from BALB/3T3 cells (ATCC CCL 163) by the acid/guanidinium thiocyanate/phenol/chloroform extraction method (21). For Northern blots, 10 $\mu$g of total RNA was denatured with glyoxal and analyzed as described (22). The Ly-6E cDNA has been described (22) as has the IRF-2 cDNA (23).

**Gel Mobility Shift Assays.** Crude whole cell extracts were prepared with lysis buffer containing 50 mM Tris·HCl (pH 8.0), 280 mM NaCl, 0.05% Nonidet P-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mg of leupeptin per ml, 3

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Abbreviations: IFN, interferon; ISRE, IFN-$\alpha$-stimulated response element; CAT, chloramphenicol acetyltransferase; CHX, cycloheximide; GBP, guanylate-binding protein.

mg of aproteinin per ml, 1 mg of pepstatin per ml, and 0.1 mM $Na_3VO_4$.

An oligonucleotide corresponding to the Ly-6E GAS element was synthesized and used in gel mobility shift assays. The sequence of the double-stranded Ly-6E GAS oligonucleotide is as follows:

5'-CATGTTATGCATATTCCTGTAAGTG-3'
3'-AATACGTATAAGGACATTCACGTAC-5'

The oligonucleotide was labeled with all four $\alpha$-$^{32}$P-labeled dNTPs using Klenow DNA polymerase. One nanogram of labeled oligonucleotide was mixed with 2 $\mu$g of poly(dI·dC) in 11.5 $\mu$l of gel mobility shift buffer containing 20 mM Hepes (pH 7.9), 4% Ficoll, 1 mM $MgCl_2$, 40 mM KCl, 0.1 mM EGTA, and 0.5 mM dithiothreitol. One microliter of extract was added per sample and the binding reaction was carried out at room temperature for 20 min. Five microliters of the reaction mixture was analyzed on nondenaturing 4% polyacrylamide gels. For competition, a 50-fold excess of unlabeled oligonucleotide was added to the reaction mixture. Antiserum against the 91-kDa protein was added at 1:120 final dilution.

**Immunoblot Analysis for Tyrosine Phosphorylation.** Immunoprecipitation of whole cell extracts was carried out using a 91-kDa specific antiserum as described (17). The immunoprecipitates were analyzed on a 7% SDS/polyacrylamide gel and transferred onto nitrocellulose. The blot was incubated with 5% bovine serum albumin in 1× TBST (17) overnight at room temperature and then immunoblotted with anti-phosphotyrosine antibody (PY20, ICN) using an enhanced chemiluminescence detection kit (Amersham).

## RESULTS

**Time Course of IFN-Mediated Induction of Ly-6 RNA and Effect of Inhibition of Protein Synthesis.** To establish the kinetics of Ly-6 mRNA induction in BALB/3T3 fibroblasts, cultures were incubated with IFN-$\alpha/\beta$ or IFN-$\gamma$ for various times before RNA extraction and Northern blot analysis. There is a low level of detectable Ly-6 mRNA in these cells and these levels increased markedly within 2 hr and continued to rise until 8 hr after exposure to either type of IFN (Fig. 1). This is in contrast to the results obtained in YAC-1 (T-cell lymphoma) (24) and A20-2J (B-cell lymphoma) cell lines (unpublished data). There is no detectable Ly-6 RNA in these cell lines and the earliest transcripts appear after 8–16 hr of treatment with IFN.

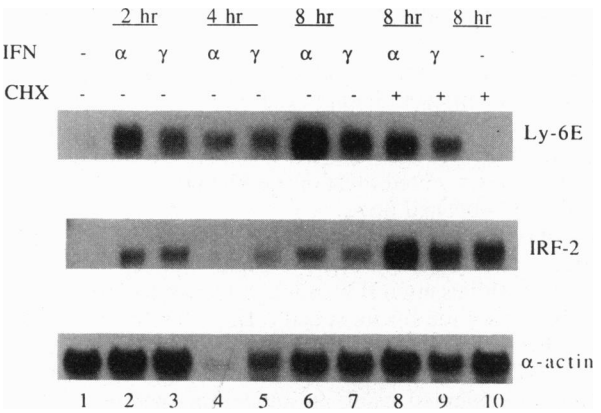


FIG. 1. Time course and effect of cycloheximide (CHX) on IFN-inducible expression of Ly-6E and IRF-2 RNA in BALB/3T3 cells. Cells were treated with IFN-$\alpha$ or IFN-$\gamma$ in the presence (+) or absence (−) of CHX for different periods as indicated. Total RNA was isolated and subjected to analysis. To standardize for the amount of RNA loaded, filters were also probed with $\alpha$-actin. All lanes had approximately the same amount of RNA except lane 4 as shown.

In fibroblasts, protein synthesis was not required for IFN-$\alpha$ or IFN-$\gamma$ induction of Ly-6E as tested by CHX addition 30 min prior to IFN-$\alpha$ or IFN-$\gamma$ treatment. CHX treatment itself did not change the constitutive mRNA levels. The rapidity of mRNA induction following IFN-$\alpha$ and IFN-$\gamma$ treatment and the concomitant lack of dependence on ongoing protein synthesis is identical to that seen with the human GBP gene in FS2 fibroblasts (10).

To determine whether the induction properties of Ly-6 mRNA were similar to other previously characterized IFN-inducible genes in this fibroblast cell line, RNA blots were rehybridized with a cDNA from the IFN-inducible mouse IRF-2 (23). The kinetics of induction for both genes are quite similar but there are two important differences: (*i*) basal levels of IRF-2 mRNA are not detectable and (*ii*) expression of IRF-2 gene can be induced by CHX treatment alone. These data suggest that, unlike Ly-6E, expression of IRF-2 is repressed by a factor that requires ongoing protein synthesis. In addition, the apparent absence of IRF-2 mRNA in uninduced cells and the time course of IFN-mediated expression, which parallels that of other IFN-inducible genes, is not consistent with the previously hypothesized role for IRF-2 as a repressor of IFN-inducible genes (23).

**Localization of an IFN-Responsive Element(s) of the Ly-6E Gene That Is Functional in Fibroblasts.** We have previously shown an IFN-responsive element(s) to reside within the −1760 to −900 region of the Ly-6E promoter (20) using chimeric constructs with progressive deletions stably transfected into BALB/3T3 mouse fibroblasts. We then wanted to determine whether this region was required for the IFN-dependent induction of the whole genomic construct. The region between −1760 and −900 was deleted from the genomic clone and used to derive stable transfectants as described (20). Seven clones were stained with monoclonal antibody 34.11.3, which is specific for the transfected Ly-6A antigen. All showed significant basal levels but none of them was inducible with IFN-$\alpha$ or IFN-$\gamma$ (data not shown). The endogenous Ly-6E gene in these transfectants was IFN inducible. These results indicate that the region between −1760 and −900 is indispensable for IFN-dependent transcription of the Ly-6A/E gene in BALB/3T3 fibroblasts.

The chimeric construct pCAT-5 contains Ly-6E 5′ flanking sequences up to −900 and is unresponsive to IFN-$\alpha/\beta$ and IFN-$\gamma$ in transient and stable transfections of BALB/3T3 fibroblasts. Since this region is sufficient for constitutive expression in CAT assays (20), this plasmid was used as a recipient for the subcloning of various DNA fragments derived from the −1760 to −900 region. All chimeric constructs were stably transfected into BALB/3T3 cells with pSV2neo (Fig. 2).

Stable cell lines were established from single colonies and analyzed for basal and IFN-inducible CAT activity as described (20). The values of IFN-stimulated CAT expression were considered significant only when they were higher than twice the basal expression. Although there was noticeable quantitative clonal variation (Fig. 3), there was generally a 3- to 7-fold induction.

Of the first six constructs tested (pG1, -2, -3, -4, -9, and -10) the shortest effective sequence that gave response to both IFNs was pG10, which contained sequences from −1480 to −1190. The lack of IFN inducibility of all the clones stably transfected with pG5 indicates that the deletion of the −1220 to −1050 sequence disrupts the IFN-responsive region. This suggested that the important region included the sequences from −1220 to −1190. Analysis of pG21 containing sequences from −1270 to −1190 demonstrated that all of the clones expressing basal levels of CAT were inducible by IFN-$\alpha/\beta$ and IFN-$\gamma$. Sequence analysis of this region of Ly-6E pro-

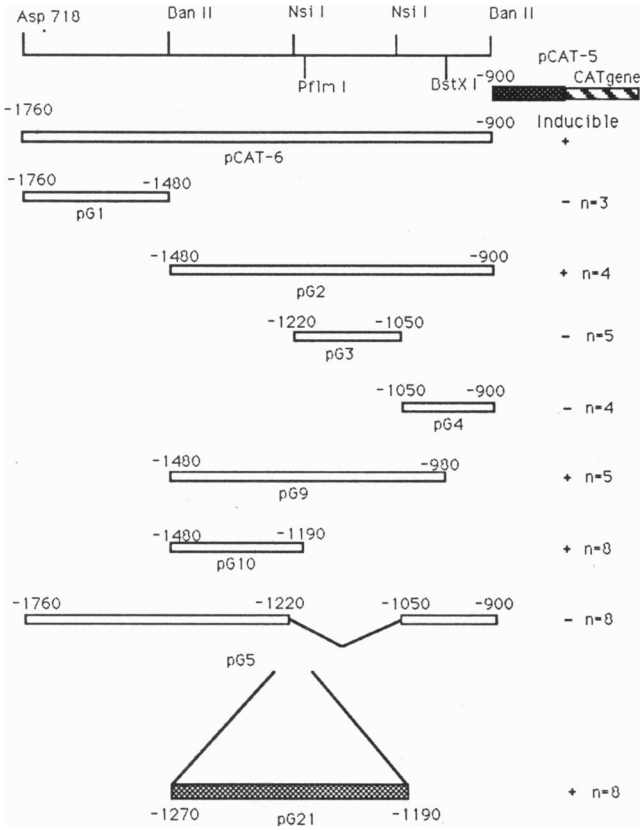


FIG. 2. Schematic representation of the deletion constructs used in stable transfections. The IFN inducibility by IFN-$\alpha/\beta$ and IFN-$\gamma$ was either observed (+) or absent (−) from a given transfectant. In this series of transfectants essentially all transfectants derived from a given DNA construct behaved very similarly. The number of independent transfectants analyzed is indicated by n.

moter revealed a region between −1223 and −1211 having the sequence CATATTCCTGTAA that is very similar to the IFN-$\gamma$ activation site (GAS) of the human GBP gene promoter (CATATTACTCTAA) (15). These results provide evidence that "GAS" can mediate responsiveness of Ly-6E promoter to IFN-$\alpha/\beta$ and IFN-$\gamma$.

**The 91-kDa Protein Binds to the GAS Site from the Ly-6E Promoter.** The factor in human nuclear extracts that binds the GBP GAS has been well characterized. A comparison of the binding of the human protein with the GBP GAS element and the 22-nucleotide Ly-6E GAS site showed that the human protein bound more strongly to the Ly-6E probe than to the GBP probe (Fig. 4*A*). The binding was specifically inhibited with the homologous probe (Fig. 4*A*, lanes 2 and 4). In addition, binding with the GBP probe can be specifically inhibited with the entire Ly-6 DNA fragment as well as the sequence from −1220 to −1190. The DNA fragment from −1220 to −1280 did not compete (data not shown).

We next wanted to identify the nature of the IFN-inducible protein bound to the GAS element from the Ly-6E promoter. Murine BALB/3T3 fibroblasts were treated with IFNs and whole cell extracts were prepared and subjected to gel mobility shift analysis. A DNA–protein complex was detected from cells treated with either IFN-$\alpha/\beta$ or IFN-$\gamma$ for 15 min and the shift activity declined after 2–4 hr (Fig. 4*B*, lanes 2–7). This DNA–protein complex was specifically inhibited with the Ly-6E GAS oligonucleotide (Fig. 4, lanes 1, 8, and 9).

Since the protein that binds to the GAS element from the GBP gene promoter has been identified as a 91-kDa protein (17), we next tested whether the same 91-kDa protein bound

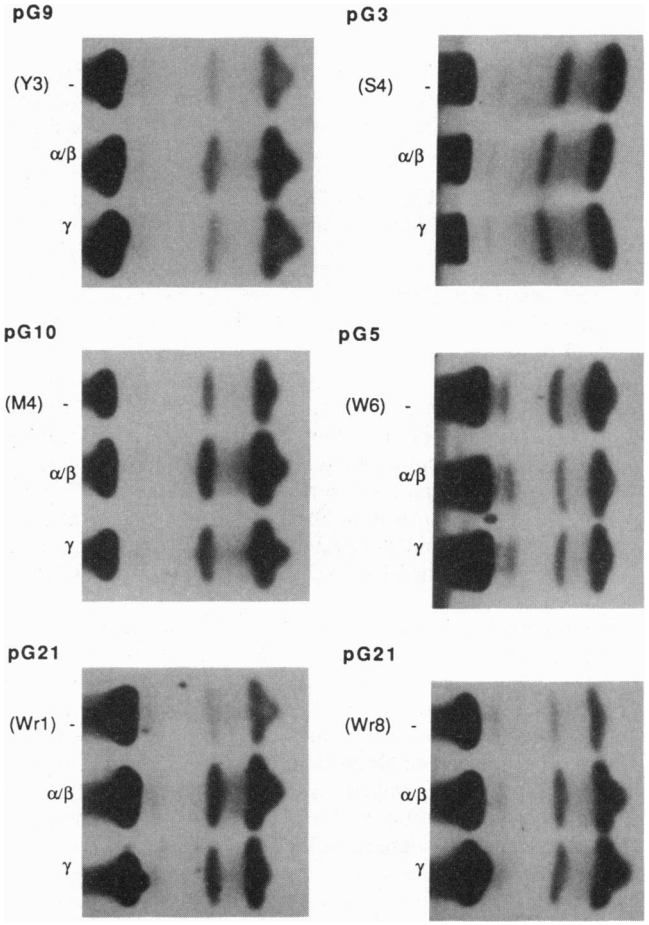


FIG. 3. IFN-inducible CAT expression from BALB/3T3 cells stably transfected with chimeric Ly-6E-CAT constructs. IFN-treated and untreated cell extracts were assayed for CAT activity. Representative clones Y3, S4, M4, and W6 were derived from the plasmids pG9, pG3, pG10, and pG5, respectively. Wr1 and Wr8 represent two independent clones derived using pG21 plasmid.

to the Ly-6E element. Antiserum referred to as 91m (prepared against amino acids 597–703 of the 91-kDa protein) specifically blocked the formation of the gel shift complex induced by either IFN-$\alpha$ or IFN-$\gamma$, whereas preimmune serum had no effect (Fig. 4, lanes 10 and 11). Thus the IFN-$\alpha$- or IFN-$\gamma$-activated 91-kDa protein from BALB/3T3 interacts with the specific antibody to the human 91-kDa protein. The gel shift complex was a doublet and both bands showed the same characteristics. Similar results were observed in HeLa cells, and two-dimensional gel mobility/SDS electrophoresis analysis indicated that the lower band may be due to the binding of a truncated form of the 91-kDa protein (K.S. and J.E.D., unpublished data).

**IFN-Dependent Tyrosine Phosphorylation of the 91-kDa Protein.** The 91-kDa protein is rapidly phosphorylated on tyrosine residues upon IFN induction and tyrosine phosphorylation is required for its specific DNA binding (17, 19). To test whether the 91-kDa protein also is phosphoryated in murine fibroblasts, extracts from BALB/3T3 cells were immunoprecipitated using an antiserum specific to the 91-kDa protein, and immunoprecipitates were separated on SDS gels, transferred to nitrocellulose, and immunoblotted with an anti-phosphotyrosine monoclonal antibody.

Tyrosine phosphorylation on the 91-kDa protein was clearly detected in cells treated with either IFN-$\alpha/\beta$ or IFN-$\gamma$ for 15 min, whereas no reactivity was observed in untreated cells (Fig. 5). The level of phosphorylation declined after 2–4

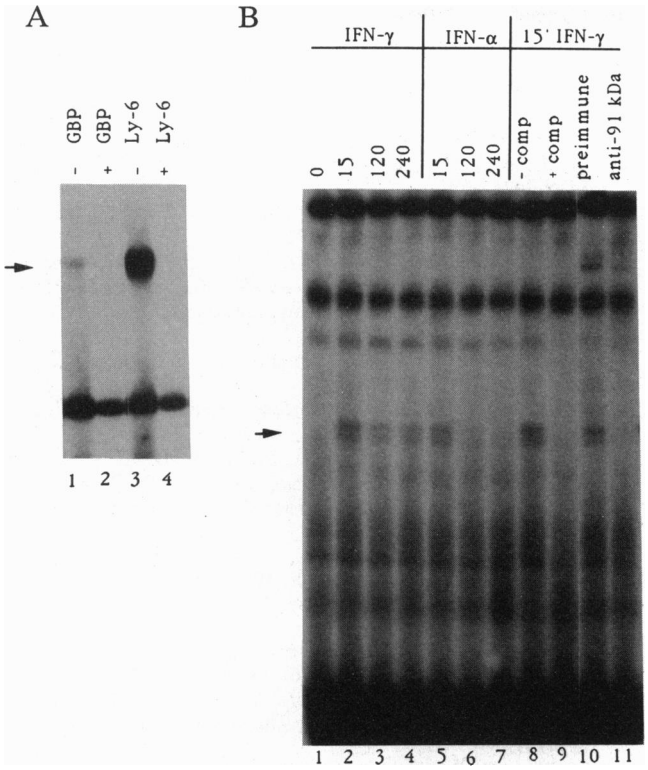


FIG. 4. Characterization of the IFN-activated factor by gel mobility shift assays. (*A*) Comparison of GBP and Ly-6E GAS binding to the 91-kDa protein in human FS2 fibroblasts. Nuclear extracts were incubated with either the GBP (lanes 1 and 2) or the Ly-6E probe (lanes 3 and 4). The absence (−) or presence (+) of a 50-fold molar excess of the same unlabeled competitor DNA fragment is indicated. (*B*) Mobility shift assays using $^{32}$P-labeled Ly-6E GAS oligonucleotide were performed with whole cell extracts from BALB/3T3 cells. The position of the IFN-inducible shift complex is indicated by an arrow. Lane 1, untreated; lanes 2–4, treated with IFN-γ for the indicated time periods; lanes 5–7, treated with IFN-α/β for the indicated time periods. Extract from cells treated with IFN-γ for 15 min was used for competition and antiserum test (lanes 8–11). Lane 8, no competitor; lane 9, a 50-fold molar excess of unlabeled GAS oligonucleotide; lane 10, preimmune serum; lane 11, antiserum against the middle portion of the 91-kDa protein (amino acids 591–703). Antiserum was added at 1:120 final dilution.

hr, which correlated well with the DNA-binding activity of the 91-kDa protein (Fig. 4). It was noted that the level of tyrosine phosphorylation on the 91-kDa protein was slightly higher in cells treated with IFN-α/β for 15 min than in cells treated with IFN-γ for 15 min, whereas the DNA-binding activities in both cases were similar as detected by gel mobility shift assays. This is probably explained by the finding that a fraction of the phosphorylated 91-kDa protein becomes a part of the ISGF3 complex (19), which binds to the ISRE and does not bind GAS.

## DISCUSSION

The experiments described in this paper aid considerably in clarifying the complicated and interrelated mechanisms of gene activation by IFN-α and IFN-γ. Some genes are activated only by IFN-α, some only by IFN-γ, and some by both ligands. The genes in the latter category could have indispensable roles in the biologic cycles stimulated by either IFN. Response of genes to either IFN-α or IFN-γ alone would be expected to depend on at least two different DNA response elements, one responsive to IFN-α-induced transcription factors and one to IFN-γ-induced factors. Genes

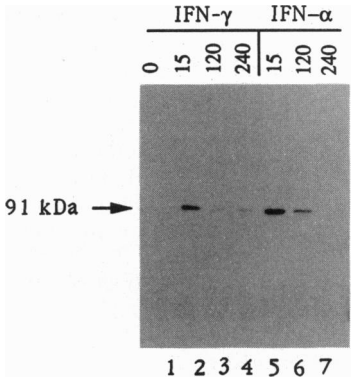


FIG. 5. Anti-phosphotyrosine blot analysis of the 91-kDa protein. The 91-kDa protein was immunoprecipitated with the antiserum against the middle portion of the 91-kDa protein from extracts described in the legend to Fig. 4. The immunoprecipitates were analyzed by 7% SDS/PAGE and transferred to a nitrocellulose filter. The nitrocellulose filter was then immunoblotted with anti-phosphotyrosine monoclonal antibody. IFN treatments were for the times (min) indicated.

that respond to either ligand could have either an IFN-α- and IFN-γ-responsive element or a single site responsive to both.

The previously defined IFN-α-responsive ISRE binds a multiprotein complex termed ISGF-3 that is induced by IFN-α but not by IFN-γ. Thus for IFN-α there is an explanation for a distinct pathway of gene activation separate from IFN-γ. However, in the only previous definition of an IFN-γ immediate response element, the GAS site of the GBP gene, two complications were noted. First, the GAS site of GBP that can be transcriptionally induced by IFN-α and IFN-γ was immediately bounded by an ISRE. However removal of sequences critical for IFN-α-dependent ISRE function left the IFN-γ response intact, so the site was defined as an IFN-γ-dependent site. Later work also showed that the GAS site alone was capable of directing a response to IFN-α (15, 16).

The present work on the Ly-6E gene clears up several questions that the study of the GBP gene had not answered completely. First of all, expression of the Ly-6E gene is activated by IFN-α and IFN-γ as is GBP. Deletion analysis located an 80-bp sequence that conferred on reporter plasmid constructs responsiveness to both ligands. This region contained a sequence very similar (11 of 13 bases) to the GAS site of GBP but the region lacked any resemblance to the well-defined ISRE sequence. This region was shown by direct analysis to produce the same gel shift in response to IFN-γ or IFN-α that is produced with the GBP GAS site. Likewise, by competition analysis the Ly-6E site interrupted the previously defined GAF DNA complex but had no effect on complex formation with the ISRE (unpublished data).

Recently, a site in the FcγRI promoter, designated GRR, that confers responsiveness to IFN-γ in macrophage cells was shown to be a binding site for the 91-kDa protein (25). The DNA complex detected using the GRR probe can be specifically inhibited using the Ly-6E GAS oligonucleotide. By comparing the GAS sites from promoters of the Ly-6E, GBP, and FcγRI genes, a consensus binding site has been recognized, TT(C/A)CNNNAA.

The formation of the gel shift complex of the Ly-6E site in response to either IFN-α or IFN-γ was inhibited by antiserum to the 91-kDa protein that binds to the GAS site of the GBP gene in response to IFN-γ. Immunoblot analysis of the cell extracts with anti-phosphotyrosine antibody confirmed the 91-kDa protein to be tyrosine phosphorylated in BALB/3T3 cells treated with IFN-α or IFN-γ. This 91-kDa molecule is one of four proteins (the others are 113 kDa, 84 kDa, and

48 kDa) found in the ISGF-3 complex. Since the antiserum to 113-kDa protein that is part of the ISGF-3 did not affect the gel shift complex formed with the Ly-6E oligonucleotide (as it does with the GBP GAS site oligonucleotide), we conclude that the 91-kDa molecule is the effective molecule in activating the Ly-6A/E gene as it is in the activation of the GBP gene (17) and the FcR$\gamma$1 gene (25). The GBP gene might be activated either by the ISGF-3 multiprotein complex binding to the ISRE or by the 91-kDa protein alone binding to the GAS site. The IFN response of the Ly-6E gene to either ligand can only utilize a single GAS site for binding to IFN-$\alpha$- or IFN-$\gamma$-activated 91-kDa protein.

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