5′ end in the R region (bp +41 to +59) with an Xho I site at the 3′ end. The amplified fragment was gel purified and cloned into the lacZ-containing vector at the Kpn I and Xho I sites upstream of the *lacZ* gene. HIV-1_{JR-CSF} LTR was digested with Hind III to remove it from the vector (mp18-1-1-JRCSF), and the 0.95-kb fragment (including the 0.4-kb 5′ flanking sequences) was cloned into the plasmid pSAFYre upstream of the *lacZ* gene. The HIV-1_{JR-CSF} LTR β-gal construct was digested with Cla I and Bam HI, and the HIV-1_{JR-FL} LTR β-gal construct was digested with Kpn I and Bam HI and LTR-*lacZ* fragments purified by electroelution and CsCl centrifugation [as in B. Hogan, F. Constantine, E. Lacy, in *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986)]. DNA was quantitated by fluorometry and diluted in microinjection buffer [10 mM tris (pH 7.4) and 0.1 mM EDTA] to a final concentration of 2 μg/ml. We made transgeni mice by standard microinjection techniques, using CD-1 donor females and (C57BL/6 × DBA/2) G1 donor males. A total of 45 HIV-1_{JRCSF} β-gal and 15 HIV-1_{JR-FL} β-gal potential transgenic founders were screened by Southern blot analysis of which two and four respectively, were transgenic.
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Activation of Transcription by IFN-γ: Tyrosine Phosphorylation of a 91-kD DNA Binding Protein

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Interferon-γ (IFN-γ) induces the transcription of the gene encoding a guanylate binding protein by activating a latent cytoplasmic factor, GAF (gamma-activated factor). GAF is translocated to the nucleus and binds a DNA element, the gamma-activated site. Through cross-linking and the use of specific antibodies GAF was found to be a 91-kilodalton DNA binding protein that was previously identified as one of four proteins in interferon-stimulated gene factor–3 (ISGF-3), a transcription complex activated by IFN-α. The IFN-γ–dependent activation of the 91-kilodalton DNA binding protein required cytoplasmic phosphorylation of the protein on tyrosine. The 113-kilodalton ISGF-3 protein that is phosphorylated in response to IFN-α was not phosphorylated nor translocated to the nucleus in response to IFN-γ. Thus the two different ligands result in tyrosine phosphorylation of different combinations of latent cytoplasmic transcription factors that then act at different DNA binding sites.

How different polypeptide ligands elicit transcriptional induction of different sets of genes is not known. However, the pathway through which IFN-α stimulates gene transcription has recently been clarified. Receptor-specific ligand binding at the cell surface results in the intracellular phosphorylation of latent cytoplasmic proteins of 113, 91, and 84 kD (*1–5*); these proteins then translocate to the nucleus to join in a multiprotein complex termed ISGF-3 (*1*) that binds to the IFN-α–responsive DNA element, the interferon-stimulated response element (ISRE). The protein that contacts DNA in the complex is a 48-kD molecule (*2, 6*) that is itself not phosphorylated in response to IFN-α. One kinase that may associate with proteins in the plasma membrane and participate in the phosphorylation of the 113-, 91- and 84-kD proteins is Tyk-2; mutant cells resistant to IFN-α can be restored to responsiveness by transfection of a cDNA encoding Tyk-2 (*7–9*).

IFN-γ causes transcriptional activation of a gene encoding a guanylate binding protein (GBP) (*10, 11*). The DNA site required for activation of transcription by IFN-γ, the GAS (γ-activated site), has been defined (*12*) and binds a protein, the γ-activated factor (GAF), that is activated in the cytoplasm (*13*). IFN-γ stimulates the phosphorylation on tyrosine of a protein of approximately 91 kD (*5*). In this report, we describe the protein composition of GAF and changes in its state of phosphorylation. Although the 91-kD protein in ISGF-3 does not contact DNA, the same 91-kD protein bound specifically to DNA at the GAS element in cells treated with IFN-γ. IFN-γ–dependent cytoplasmic tyrosine phosphorylation of the 91-kD protein was required for this binding. Phosphorylation of the 91-kD protein occurs on the same peptide after treatment of cells with IFN-γ or IFN-α, but the specificity of signaling is maintained because the 113-kD protein of the ISGF-3 protein complex is not phosphorylated in response to IFN-γ as it is in response to IFN-α.

We used an electrophoretic mobility shift assay (*14*) to identify, in extracts of human fibroblasts, a factor that formed an electrophoretically stable DNA-protein complex and that had the same properties as GAF, which was originally identified by an exonuclease III protection assay (*12, 13*; Fig. 1). A complex was formed with the GAS oligonucleotide in extracts from cells treated with IFN-γ but not that from cells treated with IFN-α (Fig. 1A) and was specifically competed by the GAS oligonucleotide but not by the ISRE (*1*). The IFN-γ–dependent activation of this DNA binding factor occurred without new protein synthesis (Fig. 1B), was maximal between 15 and 30 min after treatment of the cells with IFN-γ, and disappeared within 2 to 3 hours (Fig. 1C). This pattern correlates with the time course of IFN-γ induction of the GBP gene in fibroblasts (*10*). The Ly6E gene of mice, which encodes a surface protein, is also activated by IFN-γ through a DNA element very similar to the GAS of GBP (*15*). Just upstream of the GAS in the GBP gene there is an ISRE sequence (*12*). However, no ISRE exists in the Ly6E gene and activation of that gene by IFN-γ clearly operates through the GAS-like element that binds GAF. These results support the conclusion that the GAS site has a general

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role in the induction of transcription by IFN-γ.

The size of the protein that contacts DNA in the GAF-GAS complex was about 90 kD as determined by cross-linking with ultraviolet (UV) radiation to ^{32}P-labeled azido-deoxyuridine–substituted probe (Fig. 1D). The DNA protein is therefore similar in size to one of the ISGF-3 proteins (*2–5*).

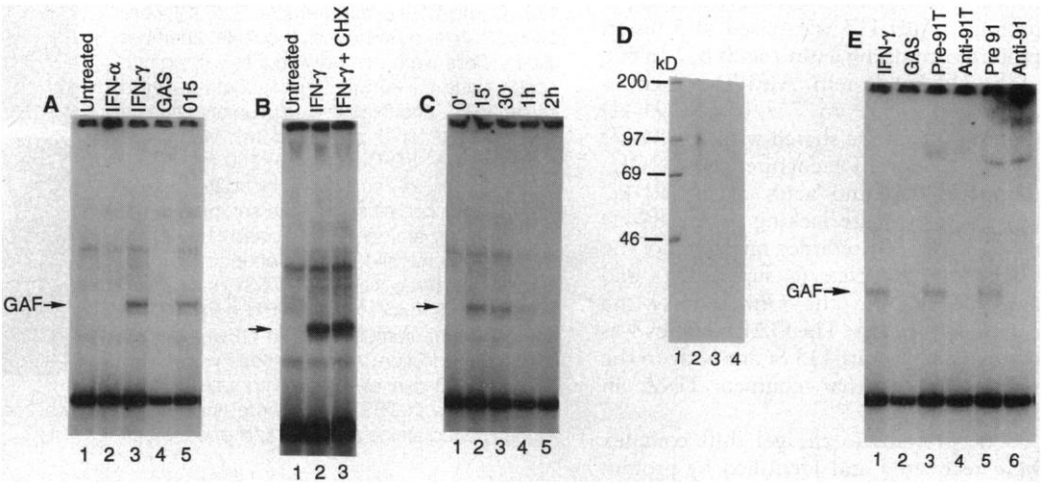

Fig. 1. Characterization of GAF by gel mobility shift assays. (**A**) Specific binding of GAF to oligonucleotide in response to IFN-γ. Mobility shift assays (*14, 25*) were done with nuclear extracts from untreated FS2 fibroblasts (lane 1) or cells treated for 15 min with IFN-α (500 U/ml) (lane 2) or IFN-γ (5 ng/ml) (lanes 3 to 5). An excess (fifty times the amount of labeled oligonucleotide) of unlabeled GAS oligonucleotide (lane 4) or unlabeled oligonucleotide representing the ISRE (015, lane 5) (*2*) were used for competition. (**B**) Effects of cycloheximide. Mobility shift assays were done as in (A) except that cells were treated with cycloheximide (50 μg/ml) for 10 min (lane 3). (**C**) Time course of GAF activation. (**D**) Analysis of UV cross-linked GAF-GAS complex. N_3dUTP substituted ^{32}P-labeled–GAS oligonucleotide was used for a mobility shift assay and detected by autoradiography (*23, 26*). The gel was then exposed to UV radiation for 5 min in a Stratagene UV linker, and the gel slice corresponding to the GAF complex was cut out and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). Lane 1, ^{14}C-labeled protein marker (Amersham); lane 2, GAF-DNA complex; lane 3, excess (100 times the concentration of labeled oligonucleotide) cold GAS oligonucleotide was included in shift reaction; lane 4, no proteins were included in shift reaction mixture. (**E**) Effect of antibodies to the 91-kD protein. Lane 1, no addition; lane 2, an excess (50 times the amount of labeled oligonucleotide) of unlabeled GAS oligonucleotide; lanes 3 and 5 preimmune sera; lane 4, anti-91T (3); lane 6, anti-91 (3). All sera were added at 1/120 final dilution. The following double-stranded GAS oligonucleotide from the GBP gene promoter (*12*) was used in gel mobility shift assays:

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5′ CATGAGTTTCATATTACTCTAAATC 3′
3′      TCAAAGTATAATGAGATTTAGGTAC 5′
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UV cross-linking analysis with N_3dUTP-substituted oligonucleotide was described (*2, 26*). The oligonucleotide

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5′ AGTTTCATATTACTCTAAA 3′
3′ TCAAAGTATAATGAGATTTAGGTAC 5′
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was labeled with 5-N_3dUTP and [^{32}P]dATP, dGTP, dCTP and the Klenow DNA polymerase.

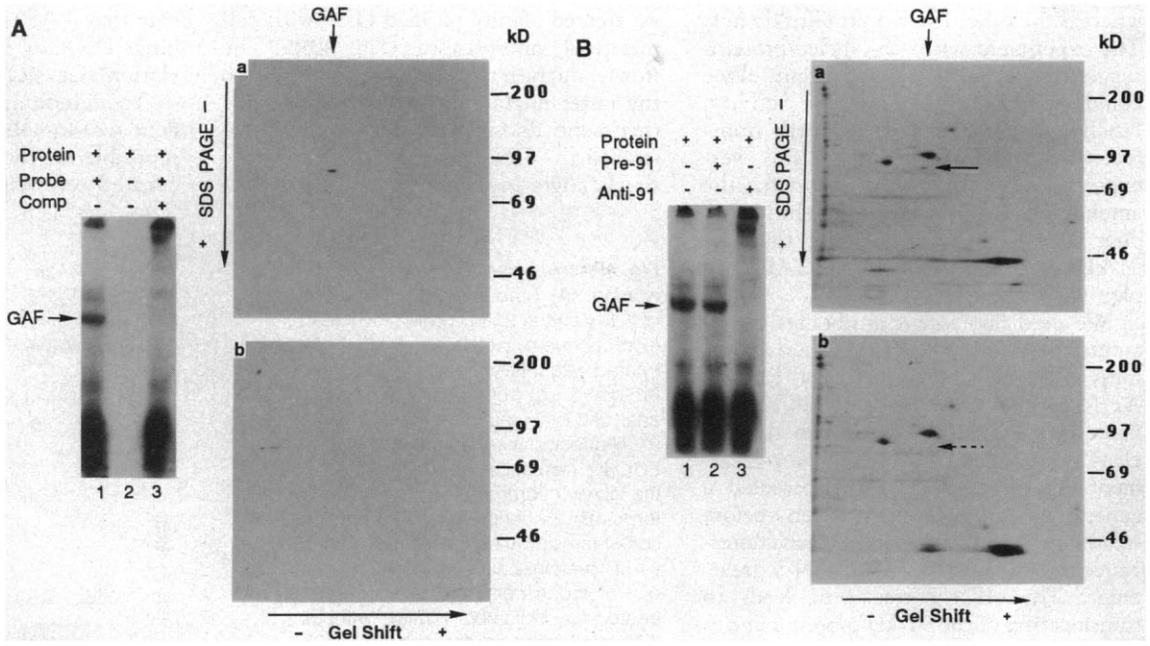

Fig. 2. Two-dimensional gel mobility shift–SDS-PAGE analysis of the GAF complex. (**A**) Detection of the 91-kD protein in the GAF gel shift complex. Partially purified GAF (see below) was used in a gel mobility shift assay (left panel, lanes 1 to 3) with ^{32}P-labeled GAS oligonucleotide. Lane 2, no probe was added; lane 3, competition (comp) with excess (50 times the concentration of labeled oligonucleotide) of cold GAS oligonucleotide. After electrophoresis and autoradiography, lanes 1 and 2 were cut out, and applied to the top of a 7% gel for SDS-PAGE analysis (a and b, respectively). The gel was then electroblotted to nitrocellulose and proteins were detected with anti-91 and an enhanced chemiluminescence (ECL) kit (Amersham). The position in the second gel that corresponds to that of the GAF complex in the first gel is indicated by an arrow. (**B**) Analysis of [^{35}S]-labeled GAF. Fibroblasts (strain FS2) were labeled for 14 hours with [^{35}S]methionine and treated with IFN-γ for 15 min. Nuclear extracts were prepared and ^{35}S-labeled proteins were collected on biotinylated GAS oligonucleotide bound to beads (*27*). After elution, proteins in the affinity-purified sample were analyzed as described in (A). Left panel, gel mobility shift (lanes 1 to 3). Preimmune serum (pre-91) (lane 2) or immune serum (anti-91) (lane 3) was added. Lanes 2 (a) and 3 (b) were cut out and further analyzed by SDS-PAGE and autoradiography. The position of the 91-kD protein is indicated by a dashed arrow.

After completing the cloning and sequencing of cDNAs encoding the four proteins (113, 91, 84, and 48 kD) of ISGF-3 (*3*, *4*, *6*), we prepared antibodies to various segments (*3*) of the ISGF-3 proteins. Anti-113 was raised to a fusion protein containing amino acids 672 to 806 of the 113-kD protein. Anti-91 recognizes amino acids 598 to 705 of the 91-kD protein, which are shared with the 84-kD protein. Anti-91T recognizes the COOH-terminal 36 amino acids of the 91-kD protein, which are lacking in the 84-kD protein. Anti-91 retarded migration of the GAF-GAS complex (a supershift), and anti-91T blocked the formation of the complex (Fig. 1E). The GAF complex was not affected by anti-113 or antibody to the 48-kD protein that contacts DNA in ISGF-3 (*16*).

The proteins in the gel shift complex were recovered and identified by protein immunoblotting; the 91-kD protein was present but the 113-kD and 48-kD proteins were not (Fig. 2A) (*16*). (Because fibroblasts contain relatively little of the 84-kD protein, we cannot be certain whether or not it binds to DNA.) Extracts of cells labeled for 14 hours with [^{35}S]methionine were used to form the GAF-oligonucleotide complex. The only labeled protein in the complex specifically removed by anti-91T was the 91-kD protein (Fig. 2B). These experiments show that the 91-kD protein contacts DNA and participates in the GAF gel-shift complex whereas the other ISGF-3 proteins do not. The experiment with ^{35}S-labeled proteins suggests that the 91-kD protein alone could be responsible for GAF activity. Finally, in extracts from 293 cells transfected with a construct that leads to overexpression of the 91-kD protein, the amount of IFN-γ–induced gel shift complex was increased, indicating that the 91-kD of ISGF-3 can form the GAF complex (*16*).

We used fluorescent antibodies to examine the localization of the reactive proteins in cells treated with IFN-γ (Fig. 3). Anti-113 yielded a generalized cellular fluorescence with no reaction in the nucleus and no change after IFN-γ treatment. In contrast, anti-91T revealed a generalized antigen distribution before treatment and an intense nuclear fluorescence within minutes after IFN-γ treatment. Thus IFN-γ treatment leads to translocation of the 91-kD protein but not the 113-kD protein whereas after IFN-α treatment both are translocated.

Protein immunoblotting with anti-91 revealed an additional, more slowly migrating protein band in extracts of cells treated with IFN-γ, the presence of which coincided in time with the presence of a GAF DNA binding activity. The amount of this protein was maximal 15 min after treatment and the protein was not detected after 2 hours (Fig. 4A). When GAF was partially purified by selecting the protein that bound to the GAS oligonucleotide fixed to beads, only the more slowly migrating form bound (Fig. 4B). In the supernatant not bound to beads, only the faster migrating 91-kD protein was found (*16*).

Phosphorylation can produce slower electrophoretic migration, and therefore we treated affinity purified GAF with calf intestinal phosphatase (Fig. 4B). The slowly migrating form was converted to the faster migrating form by phosphatase treatment. Phosphatase treatment also destroyed the DNA binding activity of the slowly migrating form that had been purified by oligonucleotide binding (Fig. 4C). Finally, staurosporine, an inhibitor of protein kinases that blocks the IFN-α–dependent formation of ISGF3 (*17*) and the IFN-α–dependent phosphorylation of the 91-kD protein (*5*) inhibited the appearance of the slowly migrating form of the 91-kD protein in cells treated with IFN-γ (Fig. 4B). Staurosporine also prevented the appearance of the GAF DNA binding activity (Fig. 4C) and blocked the IFN-γ–dependent transcription of the GBP gene in isolated nuclei (*16*). These results indicate that GAF is converted to a form that binds DNA by IFN-γ–induced phosphorylation (Fig. 4C).

To determine whether IFN-γ–dependent tyrosine phosphorylation (*5*) was responsible for the GAF activation, we incubated cells with ^{32}P and examined im-

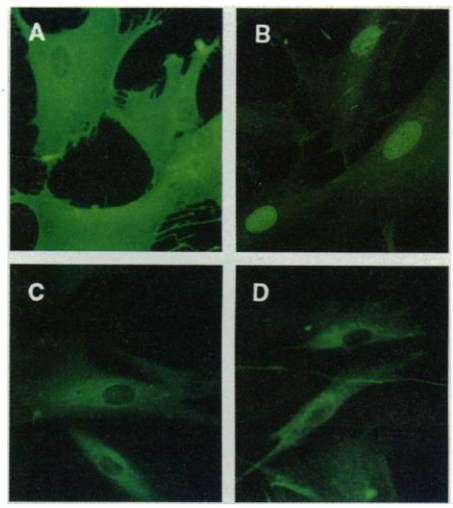

Fig. 3. Nuclear localization of the 91-kD protein but not the 113-kD protein in human fibroblast FS2 cells treated with IFN-γ. Untreated (**A** and **C**) and IFN-γ treated cells (**B** and **D**) were stained with either anti-91T (A and B) or anti-113 (C and D). Human fibroblasts (FS2) were cultured in eight-well tissue culture chamber slides. Cells were treated with IFN-γ (5 ng/ml), rinsed twice for 20 min in phosphate-buffered saline (PBS), and fixed in a solution of methanol and acetone for 2 min. After two washes in TBST [10 mM tris-Cl (pH 8.0), 100 mM NaCl, 0.02% Tween 20] cells were incubated for 40 min in TBST containing bovine serum albumin (3%). Primary antibody was added (1/100 final dilution) and the slides were incubated for 2.5 hours. After three washes in TBST, secondary antibody (fluorescein-conjugated donkey anti-body to rabbit immunoglobulin G) was added (1/200 final dilution) and the slides were incubated for 70 min at room temperature. After three washes in TBST, cells were rinsed in a solution containing 10% PBS, 90% glycerol, 0.1% *P*-phenylenediamine (pH 8.0) and dried.

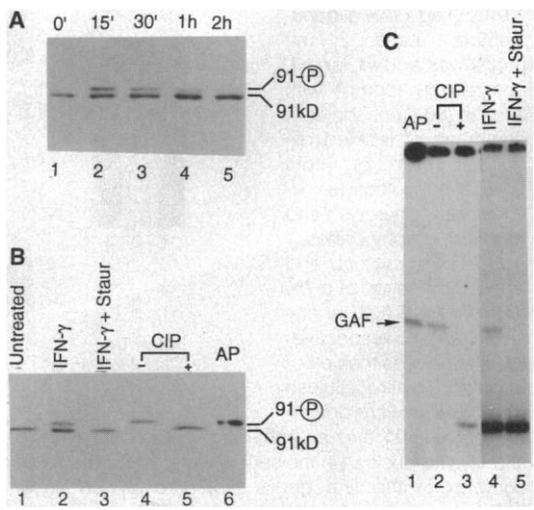

Fig. 4. Activation of GAF by phosphorylation. (**A**) Time course of the effect of IFN-γ on the 91-kD protein. Nuclear extracts were prepared from cells treated with IFN-γ for indicated times. Protein (5 μg) from each sample was analyzed by immunoblotting with anti-91. Antibody was detected with an ECL kit (Amersham). 91-P indicates the slowly migrating (phosphorylated) form. (**B**) Evidence for phosphorylation of the slowly migrating form of the 91-kD protein. Lanes 1 to 3: Immunoblot of crude cell extracts from cells treated as indicated with or without IFN-γ (15 min) or staurosporine (0.5 μM). Lanes 4 to 6: Immunoblot of affinity-purified GAF (lane 6) that was treated with (lane 5) or without (lane 4) calf intestinal phosphatase (CIP; 1.8 unit/ml; for 30 min at 30°C). (**C**) Effects of staurosporine treatment of cells and phosphatase treatment of affinity-purified GAF on DNA binding. Samples from (B) were used in the gel shift assay.

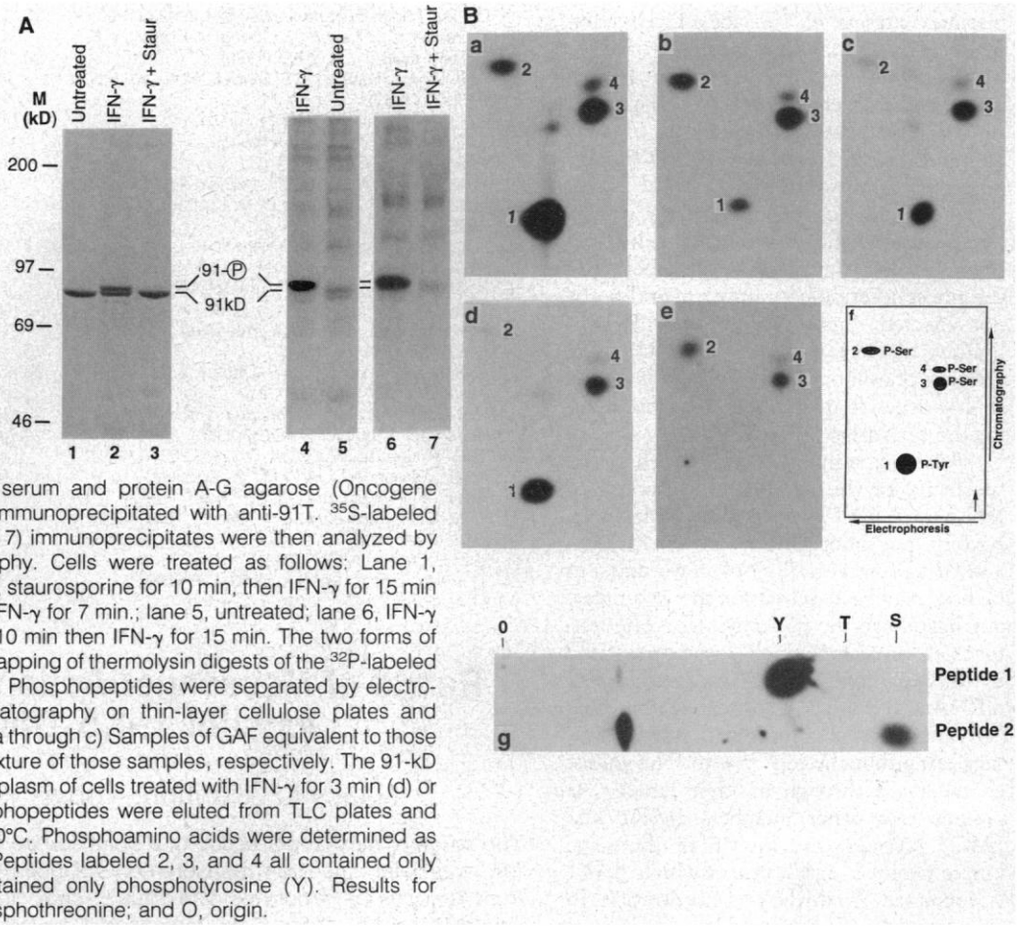

Fig. 5. IFN-γ–dependent tyrosine phosphorylation of the 91-kD protein. (**A**) Immunoprecipitation of ^{35}S- and ^{32}P-labeled 91-kD protein. Cells were labeled with [^{35}S]methionine for 14 hours (0.1 mCi/ml, in medium lacking L-methionine and cysteine) or with ^{32}P for 1.5 hours (0.5 mCi/ml [^{32}P]orthophosphate, Amersham, in medium lacking phosphate) and then treated with IFN-γ. Labeling medium was removed and the cells were washed twice with PBS and then extracted in lysis buffer [50 mM tris (pH 8.0), 280 mM NaCl, 0.5% NP-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM dithiothrietol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin, 3 mg/ml aprotinin, 1 mg/ml pepstatin, 0.1 mM Na_3VO_4]. The extract was incubated with preimmune serum and protein A-G agarose (Oncogene Science). The 91-kD protein was then immunoprecipitated with anti-91T. ^{35}S-labeled (lanes 1 to 3) and ^{32}P-labeled (lanes 4 to 7) immunoprecipitates were then analyzed by SDS-PAGE (7% gels) and autoradiography. Cells were treated as follows: Lane 1, untreated; lane 2, IFN-γ for 15 min.; lane 3, staurosporine for 10 min, then IFN-γ for 15 min in the presence of staurosporine; lane 4, IFN-γ for 7 min.; lane 5, untreated; lane 6, IFN-γ for 15 min; and lane 7, staurosporine for 10 min then IFN-γ for 15 min. The two forms of GAF are indicated. (**B**) Phosphopeptide mapping of thermolysin digests of the ^{32}P-labeled 91-kD protein recovered after PAGE (*28*). Phosphopeptides were separated by electrophoresis at pH 3.5 and then by chromatography on thin-layer cellulose plates and visualized by autoradiography at −70°C. (a through c) Samples of GAF equivalent to those shown in lane 6 and lane 7 of (A) and a mixture of those samples, respectively. The 91-kD protein was immunoprecipitated from cytoplasm of cells treated with IFN-γ for 3 min (d) or from untreated cells (e). Individual phosphopeptides were eluted from TLC plates and digested with 6N HCl for 1.5 hours at 110°C. Phosphoamino acids were determined as described (*28*) and are indicated in (f). Peptides labeled 2, 3, and 4 all contained only phosphoserine (S) while peptide 1 contained only phosphotyrosine (Y). Results for peptide 1 and 2 are shown in (g). T, phosphothreonine; and O, origin.

munoprecipitated phosphoproteins from IFN-γ–treated and untreated cells. There was ^{32}P in anti-91T immunoprecipitates from untreated cells, but at least five times more ^{32}P was incorporated into the 91-kD protein immunoprecipitated from IFN-γ–treated cells (Fig. 5A). No enhanced ^{32}P-labeling of the 113-kD protein occurred in the IFN-γ–treated cells (*16*). The electrophoretic migration of the ^{32}P-labeled protein from the cells treated with IFN-γ corresponded to the more slowly migrating form of the 91-kD protein. Only the faster moving 91-kD band was present in untreated cells (Fig. 5A). The ^{32}P-labeled proteins from both IFN-γ–treated and untreated samples were recovered and cleaved with thermolysin to yield small peptides. Phosphopeptide 1 was detected only in IFN-γ–treated cells and three less intensely labeled phosphopeptides, 2, 3, and 4, were detected in both treated and untreated cells (Fig. 5B). Only phosphotyrosine could be detected in peptide 1 whereas peptides 2, 3, and 4 contained phosphoserine (Fig. 5D) (*16*). Thus only the slowly migrating, DNA binding form of the 91-kD protein is phosphorylated on tyrosine in cells treated with IFN-γ. Protein immunoblotting with antibodies to phosphotyrosine supported the same conclusion (*16*).

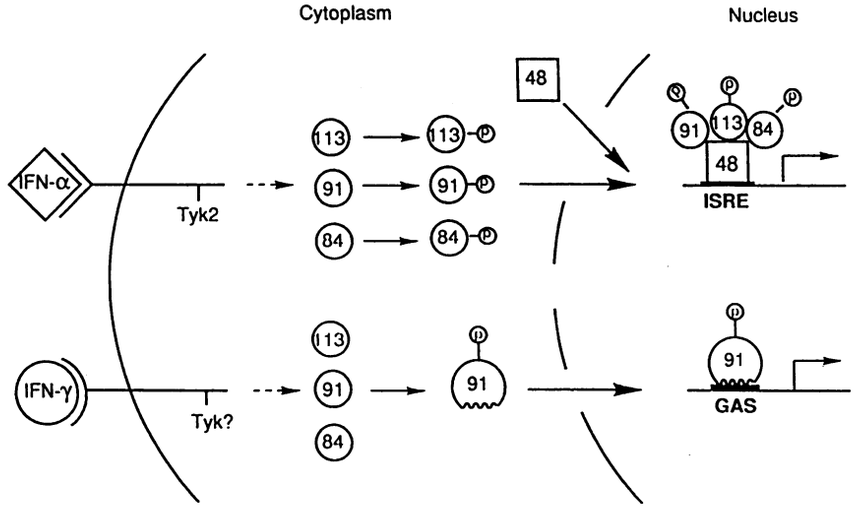

Fig. 6. Pathways of signal transduction and transcriptional activation in cells treated with IFN-α or IFN-γ.

Phosphopeptide 1 was recovered also as the major phosphopeptide from the 91-kD protein after treatment of cells with IFN-α (*5*). Equal amounts of ^{32}P-labeled peptide 1 from the two sources were mixed and two-dimensional separation showed that they migrated identically (*18*).

Because latent GAF exists in the cytoplasm rather than the nucleus (*13*), we tested whether tyrosine phosphorylation also occurred in the cytoplasm. The 91-kD protein was immunoprecipitated from cyto-

plasmic extracts of ^{32}P-labeled cells that had been treated with IFN-γ for 3 min. A peptide corresponding to peptide 1 from whole cell extracts was detected (Fig. 5B). Immunoprecipitates of nuclear extracts contained little ^{32}P-labeled 91-kD protein after only 3 min of treatment (*16*).

^{32}P-labeling of peptide 1 was greatly diminished by the treatment of cells with staurosporine (Fig. 5B) whereas labeling of the phosphoserine-containing peptides was not affected. Staurosporine also blocked-binding of GAF to DNA (Fig. 4C); thus tyrosine phosphorylation appears to be the IFN-γ–dependent modification necessary for the activation of GAF.

These experiments indicate that the specificity of the cytoplasmic response to IFN-α and IFN-γ results from differential tyrosine phosphorylation of the 113-, 91- and 84-kD proteins (Fig. 6). Two different kinases may be required for the two different ligand-specific pathways (*7*). The mutant cell line 11.1 which does not respond to IFN-α, completely lacks the Tyk-2 mRNA and the Tyk-2 protein (*7*) but does activate genes in response to IFN-γ (*19*), suggesting that the response to IFN-γ must be mediated through another kinase. At present two other members (JAK1 and JAK2) (*20, 21*) are known of the same kinase family as Tyk2, one of which, JAK1 is increased in mRNA concentration in cells treated with IFN-γ (*22*).

The 91-kD protein can function in transcriptional activation in two different ways. In cells treated with IFN-α, the 91-kD protein participates in a high-affinity DNA binding complex for the ISRE (*23*) but does not itself contact DNA (*4*). Moreover, in cells treated with IFN-α most of the phosphorylated 91-kD protein is used in forming ISGF-3. In cells treated with IFN-γ the tyrosine phosphorylated 91-kD protein does bind to DNA, but at a different DNA element, the GAS. Other proteins have been described that participate in DNA-protein interaction either alone or in combination with other proteins (*24*) but those proteins have not been shown to be phosphorylated on tyrosine.

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Range of Messenger Action of Calcium Ion and Inositol 1,4,5-Trisphosphate

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The range of messenger action of a point source of Ca^{2+} or inositol 1,4,5-trisphosphate (IP_3) was determined from measurements of their diffusion coefficients in a cytosolic extract from *Xenopus laevis* oocytes. The diffusion coefficient (*D*) of [^{3}H]IP_3 injected into an extract was 283 μm^2/s. *D* for Ca^{2+} increased from 13 to 65 μm^2/s when the free calcium concentration was raised from about 90 nM to 1 μM. The slow diffusion of Ca^{2+} in the physiologic concentration range results from its binding to slowly mobile or immobile buffers. The calculated effective ranges of free Ca^{2+} before it is buffered, buffered Ca^{2+}, and IP_3 determined from their diffusion coefficients and lifetimes were 0.1 μm, 5 μm, and 24 μm, respectively. Thus, for a transient point source of messenger in cells smaller than 20 μm, IP_3 is a global messenger, whereas Ca^{2+} acts in restricted domains.

The transduction of many hormonal and sensory stimuli is mediated by transient increases in the concentration of intracellular free calcium ($[Ca^{2+}]_i$). Ca^{2+} influx into the cytosol can be induced by (i) opening of voltage-gated and receptor-operated Ca^{2+} channels in the plasma membrane; (ii) binding of receptors that activate the phosphoinositide cascade, which leads to the production of inositol 1,4,5-trisphosphate (IP_3) and the consequent opening of channels on internal Ca^{2+} stores; and (iii) the activation of ryanodine-receptor channels (*1*). The resulting increase in $[Ca^{2+}]_i$ is then detected by Ca^{2+} sensors that alter the activities of enzymes, pumps, and other targets. Many activated cells display repeated Ca^{2+} spikes or oscillations and Ca^{2+} waves (*1–3*). These macroscopic responses are produced by the spreading, amplification, and deactivation of localized increases in $[Ca^{2+}]_i$. Knowing the range of action of spatially localized impulses of Ca^{2+} and IP_3 is therefore fundamental to understanding Ca^{2+} signaling. Previous measurements of the diffusion constants of these messengers did not eliminate interfering processes such as sequestration, degradation, and messenger amplification; this was appreciated by the investigators (*4*).

We measured the diffusion coefficients of Ca^{2+} and IP_3 in a cytosolic extract from *Xenopus* oocytes (*5*). Measurement of IP_3 diffusion requires inhibition of its degradation, which typically occurs in ~1 s (*6*). Degradation was blocked by chelating divalent cations, which are required for activity by the 5′-phosphomonoesterase and IP_3-kinase (Fig. 1A) (*7*). To avoid sequestration of Ca^{2+} by internal stores, which would affect the measured diffusion coefficient for Ca^{2+}, we added thapsigargin to inhibit Ca^{2+} pumps; hexoki-

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