were prepared (*19, 22*) in the absence of phosphatase inhibitors; 30 mM KCl was included to maintain equivalent osmolarity, and 1.5 mM $MgCl_2$ was added to stabilize nuclei. The protein tyrosine phosphatase (PTPase) used in this experiment was a bacterially expressed T cell PTPase [D. E. Cool *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5257 (1989)], purified to homogeneity. Briefly, portions of the nuclear extracts (3 μl, 12 μg of protein) received either 1 μl of 10 mM vanadate or 1 μl of water and then either 2 μl of PTPase buffer [25 mM imidazole (pH 7.0), 0.2% 2-mercaptoethanol, and BSA (1 mg/ml) or 2 μl of fivefold serial dilutions of the PTPase in this buffer. Reactions were incubated for 30 min at 37°C and then placed on ice. Water (1 μl) was added to reactions that had received sodium vanadate before incubation, and 1 μl of sodium vanadate was added to the others (so that all reactions contained 0.5 mM sodium vanadate before mobility-shift assay). These reactions were then subjected to mobility-shift assay with high-affinity SIE probe (*10*).

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A Single Phosphotyrosine Residue of Stat91 Required for Gene Activation by Interferon-γ

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Interferon-γ (IFN-γ) stimulates transcription of specific genes by inducing tyrosine phosphorylation of a 91-kilodalton cytoplasmic protein (termed STAT for signal transducer and activator of transcription). Stat91 was phosphorylated on a single site (Tyr^{701}), and phosphorylation of this site was required for nuclear translocation, DNA binding, and gene activation. Stat84, a differentially spliced product of the same gene that lacks the 38 carboxyl-terminal amino acids of Stat91, did not activate transcription, although it was phosphorylated and translocated to the nucleus and bound DNA. Thus, Stat91 mediates activation of transcription in response to IFN-γ.

Signaling from a ligand-bound cell surface receptor to the nucleus can be accomplished by tyrosine phosphorylation of latent cytoplasmic transcriptional factors that are transported to the nucleus (*1, 2*). In cells treated with IFN-α, a 113-kD protein and two closely related proteins of 91 and 84 kD become phosphorylated on tyrosine, whereas after IFN-γ treatment the 91- and 84-kD proteins but not the 113-kD protein become phosphorylated on tyrosine. After activation the proteins are translocated to the nucleus where they take part in transcriptional activation (*1, 2*). We refer to this group of tyrosine kinase substrates as STAT proteins.

The Stat91 phosphoprotein can, by itself, recognize the IFN-γ–activated site (GAS), a DNA element required for immediate gene induction by IFN-γ (*2–5*). We identified the single tyrosine in Stat91 that is phosphorylated in response to IFN-γ and found that this residue is required for the Stat91-dependent gene activation by cotransfection assays. The protein Stat84, a product of differential splicing that lacks the COOH-terminal 38 amino acids of Stat91 (*6*), became phosphorylated on the same site, was translocated to the nucleus, and bound to GAS, but did not activate transcription. We conclude that phosphorylated Stat91 acts as the primary positive-acting transcription factor in response to IFN-γ.

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Thermolysin digestion of ^{32}P-labeled Stat91 from IFN-γ–treated cells yielded a single peptide labeled on tyrosine (*2*). The Stat91 protein contains 19 tyrosines (6), and to determine the location of the phosphorylated residue or residues, we examined a tryptic digest of ^{32}P-labeled Stat91 from IFN-γ–treated cells (Fig. 1A). IFN-γ induced phosphorylation of a single tryptic peptide (X) on tyrosine. Peptide X was recovered and stepwise Edman degradation done. The labeled phosphotyrosine was released in the fourth degradative cycle (Fig. 1B). Computer alignment of all the potential tryptic peptides showed a single peptide [amino acids 698 to 703 (*7*)] in which tyrosine was the fourth amino acid, revealing this peptide as the major candidate for IFN-γ–stimulated tyrosine kinase action (Fig. 1C). The putative phosphorylated peptide contained a single tyrosine at residue 701.

We prepared a synthetic peptide corresponding to amino acids 693 to 707. This peptide was exposed to purified $p43^{v\text{-}abl}$

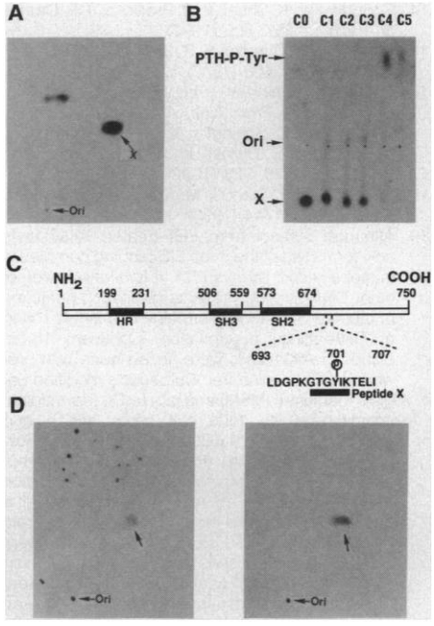

Fig. 1. Identification of the phosphotyrosine residue in Stat91. (**A**) Tryptic phosphopeptide map of ^{32}P-Stat91 from IFN-γ–treated FS2 cells (*16*). Phosphoamino acid analysis indicated that only peptide X contains phosphotyrosine (*2*). (**B**) Edman degradation of peptide X (*14*). The position of the PTH-P-Tyr marker detected by ultraviolet light is indicated. (**C**) Schematic diagram showing the site of the phosphotyrosine residue in Stat91. HR, heptapeptide repeat; SH2, *Src* homology domain 2; and SH3, *Src* homology domain 3. (**D**) The synthetic peptide LDGPKGTGYIKTELI which was phosphorylated with ^{32}P-labeled tyrosine was digested with trypsin and analyzed by two-dimensional peptide mapping either alone (left panel) or mixed with the same amount of ^{32}P-labeled peptide X (right panel). Ori, origin. The synthetic peptide (10 μg) (obtained from Genetics) was incubated with 1 U of $p43^{v\text{-}abl}$ (Oncogene Science), in 50 mM Hepes (pH 7.4), 0.1 mM EDTA, 0.015% Brij 35, 0.1 mM ATP, 10 mM $MgCl_2$, and 2 μCi of [γ-^{32}P]ATP for 30 min at 30°C. The ^{32}P-labeled peptide was subjected to electrophoresis at pH 3.5 on a thin-layer chromatography plate and purified. Tryptic digestion of ^{32}P-labeled peptide was done as described (*14, 15*). L: Leu; D: Asp; G: Gly; P: Pro; K: Lys; T: Thr; Y: Tyr; I: Ile; E: Glu.

protein kinase [Oncogene Science (8)] and [γ-^{32}P]adenosine triphosphate (ATP). Although labeling was inefficient, only tyrosine was phosphorylated. The labeled synthetic phosphopeptide was cleaved with trypsin, and the resulting peptide migrated identically with peptide X during two-dimensional peptide mapping. Thus, we conclude that Tyr^{701} is the single residue in Stat91 that is tyrosine phosphorylated in response to IFN-γ.

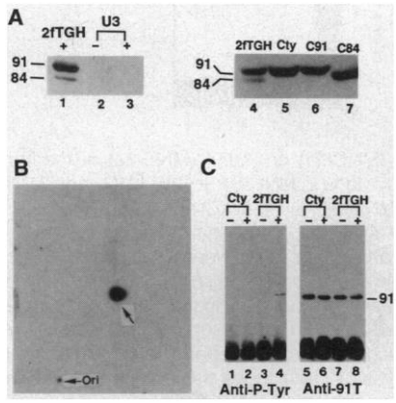

Fig. 2. Analysis of phosphorylation of Stat91 and Stat84 in established cell lines. (**A**) Protein immunoblot analysis with antiserum to Stat91 (anti-91) of whole cell extracts from parental 2fTGH cells (lanes 1 and 4); mutant U3 cells lacking Stat91 and Stat84 proteins (lanes 2 and 3); U3 cells expressing Stat91 (C91, lane 6), Stat84 (C84, lane 7), or the Tyr^{701} mutant MNCty (Cty, lane 5). (**B**) Tryptic peptide map of Stat84. C84 cells were labeled with [^{32}P] orthophosphate for 3 hours and then treated with IFN-γ for 10 min. Immunoprecipitation with anti-91 and tryptic peptide mapping of the ^{32}P-labeled 84-kD protein was done as described (Fig. 1). (**C**) Proteins in whole cell lysates from 2fTGH (lanes 3, 4, 7, and 8) and Cty (lanes 1, 2, 5, and 6) cells were immunoprecipitated with anti-91T (*2*) and separated by SDS-PAGE (7% gel). The blot was then probed with a monoclonal antibody to phosphotyrosine 4G10 (UBI, lanes 1 through 4). The blot was stripped and reprobed with anti-91T (lanes 5 through 8). U3A cells (5×10^5) (*11*) were transfected with 4 μg of expression vector and 16 μg of pBSK (Stratagene) plasmid by the calcium phosphate procedure (*17*). Cells were selected in Dulbecco's modified Eagle's medium containing G418 (0.5 mg/ml) (Gibco, BRL) 48 hours after transfection. Individual colonies were screened for the expression of appropriate proteins by protein immunoblotting. Cell lines were maintained in the presence of G418 (0.2 mg/ml). Expression vectors using the cytomegalovirus promoter and encoding Stat91 and Stat84 were constructed by insertion of the cDNA into the Not I–Bam HI cloning site of an expression vector pMNC (*17*). The TAT codon for Tyr^{701} was changed to TTT by standard mutagenesis procedure with the polymerase chain reaction (*18, 19*). The sequence was verified by DNA sequencing (U.S. Biochemical, Cleveland, Ohio). Molecular sizes are indicated to the left (A) or to the right (C) in kilodaltons.

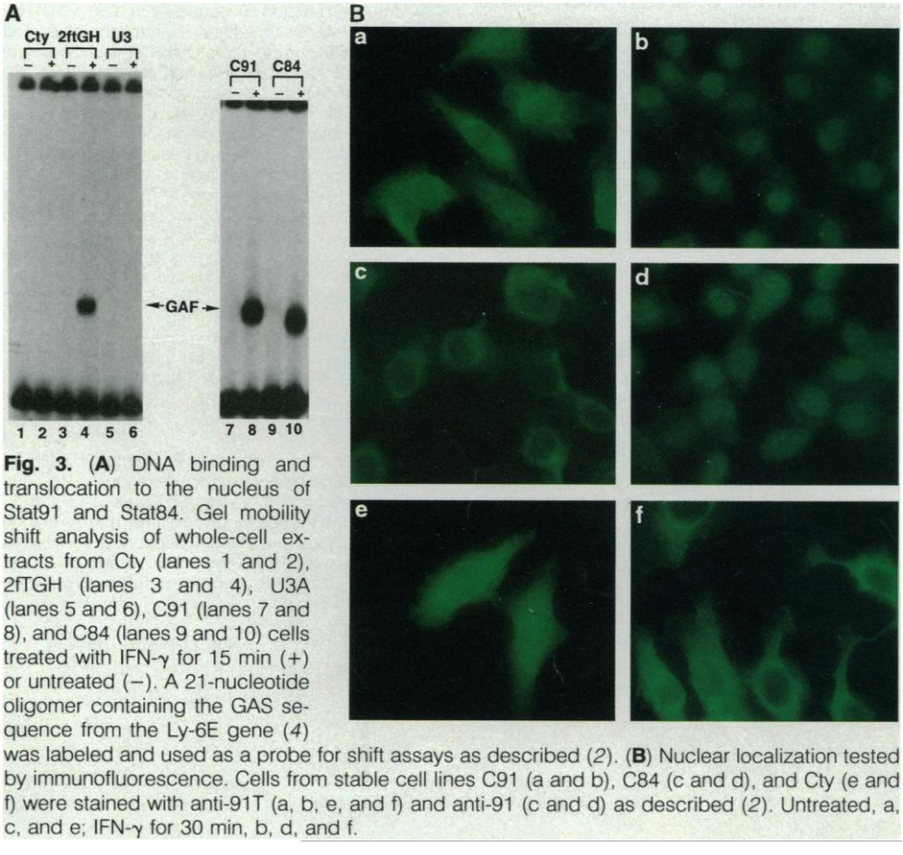

Fig. 3. (**A**) DNA binding and translocation to the nucleus of Stat91 and Stat84. Gel mobility shift analysis of whole-cell extracts from Cty (lanes 1 and 2), 2fTGH (lanes 3 and 4), U3A (lanes 5 and 6), C91 (lanes 7 and 8), and C84 (lanes 9 and 10) cells treated with IFN-γ for 15 min (+) or untreated (−). A 21-nucleotide oligomer containing the GAS sequence from the Ly-6E gene (*4*) was labeled and used as a probe for shift assays as described (*2*). (**B**) Nuclear localization tested by immunofluorescence. Cells from stable cell lines C91 (a and b), C84 (c and d), and Cty (e and f) were stained with anti-91T (a, b, e, and f) and anti-91 (c and d) as described (*2*). Untreated, a, c, and e; IFN-γ for 30 min, b, d, and f.

To test the functional importance of phosphorylation of Tyr^{701}, we changed the TAT codon for tyrosine to TTT, which encodes phenylalanine. The wild-type and mutant DNAs were inserted into an expression vector. The gene encoding Stat91 produces two mRNAs with different 3′ ends (*6*), which are translated to produce Stat91 or Stat84. An expression vector containing complementary DNA (cDNA) encoding Stat84 was also constructed.

These constructs were introduced by permanent transfection into U3A cells, which do not respond to IFN-α or IFN-γ (*9, 10*) because they do not express Stat84 or Stat91 (*11*). Full-length Stat91 restores the ability of these cells to respond to IFN-α and IFN-γ, as tested by IFN-induced accumulation of mRNA from endogenous genes. The 84-kD protein restores the accumulation of IFN-α–responsive mRNA but not IFN-γ–responsive mRNA (*11*).

We studied three cell lines: C91 (expressing Stat91), Cty (expressing Stat91 in which Tyr^{701} was changed to Phe), and C84 (expressing Stat84) (Fig. 2A). A monoclonal antibody to phosphotyrosine was used to detect IFN-γ–dependent tyrosine phosphorylation in protein immunoblots. The mutant Stat91 protein was not phosphorylated on tyrosine in response to IFN-γ, whereas Stat91 from either the wild-type parental cell (2fTGH) or the C91 cell was phosphorylated on tyrosine when treated with IFN-γ (Fig. 2C) (*12*). This experiment confirmed that residue 701 is the sole site on Stat91 that is phosphorylated on tyrosine in response to IFN-γ.

We determined whether Stat84 was phosphorylated on the same site as Stat91. We labeled C84 cells with ^{32}P and treated them with IFN-γ; the ^{32}P-labeled Stat84 was immunoprecipitated and cleaved with trypsin. The resulting tryptic phosphopeptides were analyzed by 2D phosphopeptide mapping (Fig. 2B). A major spot was identified that migrated similarly to peptide X from Stat91 (Fig. 1A). When mixed, the two peptides migrated identically (*12*). Thus, we conclude that Stat84 is also tyrosine phosphorylated on Tyr^{701} in response to IFN-γ.

We tested the function of the Stat91 and Stat84 proteins and the Tyr^{701} $\rightarrow$ Phe mutant in various steps in the signal transduction pathway that results in IFN-γ–dependent gene activation. Removal of phosphate from the Stat91 phosphoprotein by calf intestinal phosphatase or inhibition of in vivo phosphorylation with staurosporine abolishes Stat91 DNA binding activity (*2*). The IFN-γ–dependent DNA-protein complex, IFN-γ–activated factor (GAF), was detected in the wild-type parental cells (2fTGH) and

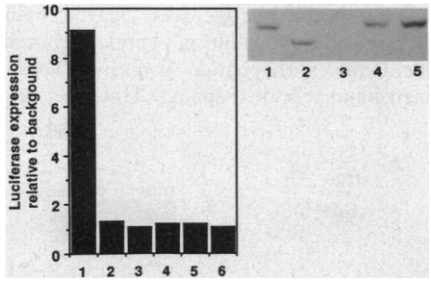

Fig. 4. Analysis of transcriptional activation. An oligonucleotide corresponding to the herpes simplex virus thymidine kinase (TK) promoter from −35 to +10 was fused to the Hind III site of pZLUC, a luciferase reporter construct (TK-LUC). One copy of the 91-kD binding site [a 21-nucleotide oligomer from the Ly-6E gene (*4*)] was inserted into the Bam HI cloning site of TK-LUC (GAS-LUC). U3 cells were transfected by the calcium phosphate method as described (Fig. 2) with 4 μg of each construct. The cells were also transfected with 4 μg of pMNC alone (*17*) (MNC) or pMNC encoding Stat91 (MNC-91) or Stat84 (MNC-84) or the Tyr^{701} mutant of Stat91 (MNC-ty). Lane 1, MNC-91 + GAS-LUC; lane 2, MNC-84 + GAS-LUC; lane 3, MNC + GAS-LUC; lane 4, MNC-ty + GAS-LUC; lane 5 MNC-91 + TK-LUC; and lane 6, GAS-LUC. Relative transfection efficiencies were monitored by inclusion of a β-galactosidase expression plasmid (pCMVβ, Promega). Then, 36 hours after transfection, cells were treated with IFN-γ (5 ng/ml) for 6 hours, collected, and assayed for luciferase activities (Promega). (**Left**) Data shown are taken from one representative experiment and represent the relative luciferase activity in cells treated with IFN-γ as compared with that from untreated cells (arbitrarily set to 1 U). (The luciferase assay was corrected for relative expression of a β-galactosidase.) Each transfection was independently repeated at least three times. (**Right**) Cell lysates from these same transfections were analyzed for the expression of proteins by protein immunoblotting with anti-91.

in C91 cells (Fig. 3A). The C84 cells also responded to IFN-γ, yielding a DNA-protein complex that migrated somewhat faster, as would be expected for a smaller protein (Fig. 3A). In contrast, cells expressing the Tyr^{701} mutant (Cty) failed to produce an IFN-γ–dependent DNA binding protein.

We also tested IFN-γ–induced translocation to the nucleus. Immunofluorescence in C91 or C84 cells detected throughout the cell before IFN-γ treatment increased in the nucleus after IFN-γ treatment (Fig. 3B). In contrast, the Tyr^{701} mutant protein did not move to the nucleus in response to IFN-γ, suggesting that phosphorylation on Tyr^{701} is required for the nuclear translocation of Stat91 (Fig. 3B).

We transiently transfected U3 cells with Stat91, Stat84, and the Tyr^{701} mutant protein and measured the transcriptional response to IFN-γ in these cells. A target gene was constructed containing luciferase as the reporter and bearing one copy of the binding site for the Stat91 phosphoprotein upstream of an RNA start site otherwise lacking promoter elements. Cells transfected with the target gene and the wild-type Stat91 expression vector showed a 5- to 10-fold stimulation of luciferase expression when treated with IFN-γ (Fig. 4). The IFN-γ–dependent transcriptional activation required the presence of Stat91; IFN-γ did not enhance transcription in U3A cells transfected with the reporter vector alone or a vector lacking the GAS site. Cells transfected with the reporter vector and the Tyr^{701} mutant did not respond to IFN-γ, suggesting a requirement for phosphorylation for gene activation. Likewise, the vector expressing Stat84 did not support an IFN-γ–induced increase in transcription. Protein immunoblot analysis indicated that Stat91, Stat84, and Tyr^{701} mutant proteins were expressed during the transient transfection (Fig. 4). Similar experiments done in human kidney 293 cells support the same conclusion (*12*). The results with transient transfections are in accord with findings that in U3A cells accumulation of mRNA from endogenous cellular genes in response to IFN-γ requires Stat91 (*11*). In those experiments, also, Stat84 failed to direct the IFN-γ response.

We have also found that endogenous genes normally induced by IFN-γ cannot be induced in U3A cells complemented with the Tyr-Phe^{701} mutant protein (*12*). However, U3A cells respond to IFN-α when transfected with either Stat91 or Stat84 (*11*). Thus, Stat84 can fulfill the required role in the multimeric ISGF-3 complex induced by IFN-γ in which either Stat84 or Stat91 joins with a Stat113 and a 48-kD DNA binding protein (*11*). Cells reconstituted with the Tyr-Phe^{701} mutant protein cannot form ISGF-3 nor do IFN-α–induced mRNAs accumulate in such cells (*13*).

After IFN-γ treatment, Stat84 acts in parallel with Stat91 up to the point of gene activation: Stat84 can be phosphorylated and translocated and binds to DNA. However, only Stat91 acts by itself as a direct DNA binding protein capable of transcriptional activation. These results suggest that the 38 COOH-terminal amino acids of Stat91 are essential for activation of transcription through a GAS site and raise the possibility that Stat84 might act negatively if it were abundant in particular cells.

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