# Interleukin 4 Activates a Signal Transducer and Activator of Transcription (Stat) Protein which Interacts with an Interferon-$\gamma$ Activation Site-like Sequence Upstream of the $I_\epsilon$ Exon in a Human B Cell Line

## Evidence for the Involvement of Janus Kinase 3 and Interleukin-4 Stat

**Xu Fenghao,* Andrew Saxon,*‡ Andrew Nguyen,* Zhang Ke,* David Diaz-Sanchez,* and André Nel*§**

**The Hart and Louise Lyon Laboratory, Division of Clinical Immunology and Allergy, Department of Medicine, ‡The Molecular Biology Institute, and §The Jonsson Comprehensive Cancer Center, UCLA School of Medicine, University of California, Los Angeles, California 90024-1680*

## Abstract

**Germ line $C_\epsilon$ transcripts can be induced by IL-4 in the human B cell line, BL-2. Utilizing a IFN-$\gamma$ activation site-like DNA sequence element located upstream of the $I_\epsilon$ exon, we demonstrated by gel mobility shift assays that IL-4 induced a binding activity in the cytosol and nucleus of BL-2 cells. This factor was designated IL-4 NAF (IL-4-induced nuclear-activating factors) and was identified as a tyrosine phosphoprotein, which translocates from the cytosol to the nucleus upon IL-4 treatment. Because these are the characteristics of a signal transducer and activator of transcription (Stat) protein, we determined whether antibodies to Stat proteins will interfere with gel mobility shift and found that antibodies to IL-4 Stat, also known as Stat6, but not antibodies to other Stat proteins, interfere with the formation of the IL-4 NAF complex. Congruous with the involvement of a Stat protein, IL-4 induced robust Janus kinase 3 (JAK3) activity in BL-2 cells. Cotransfection of JAK3 with IL-4 Stat into COS-7 cells produced an intracellular activity which bound the same IFN-$\gamma$ activation site-like sequence and comigrated with IL-4 NAF in electrophoretic mobility shift assay. These results show that IL-4 NAF is IL-4 Stat, which is activated by JAK3 in response to IL-4 receptor engagement. (*J. Clin. Invest.* 1995. 96:907-914.) Key words: interleukin-4-induced nuclear-activating factor • germline • Immunoglobulin E • transcription • signaling**

## Introduction

IL-4 exerts pleiotropic effects on the function of a variety of cell types including lymphocytes, macrophages, fibroblasts, and endothelial cells (1). In B cells, IL-4 is involved in cell proliferation and differentiation. Among the differentiation effects are cell surface expression of MHC-II and CD23 ($Fc_\epsilon R_{II}$) gene products, as well as Ig heavy chain class switching to IgG1 and IgE (2-5). While induction of proliferation appears to involve the phosphorylation of the substrate, 4PS/IRS-1, and recruitment of the second messengers phosphoinositide 3-kinase (PI-3 kinase) and Grb2, not much is known about the pathways by which IL-4 directs cellular differentiation (6-9).

IgE isotype switching involves the sequential steps of germline transcription followed by heavy chain gene rearrangement (4, 5, 10). IL-4 induces germline transcription (4, 5, 10), while IFN-$\gamma$ inhibits the process (11). To examine if IFN-$\gamma$ and IL-4 have either antagonistic or analogous effects on transcription, an IL-4 induced DNA binding factor which reacts with an IFN-$\gamma$ response element (also known as an IFN-$\gamma$ activation site or GAS[1]), was demonstrated to be present by electrophoretic mobility shift assays (EMSA) in the human monocytic cell line, Thp-1 (12). This factor, which interacts with an upstream sequence in the $Fc_\gamma R_I$ gene, was named IL-4-induced nuclear-activating factor (IL-4 NAF) (see Fig. 1 *A*; 12). A homologous 9-bp sequence in the promoter of the CD23 gene was described by Kohler and Rieber and designated IL-4 nuclear-activated factor (see Fig. 1 *A*; 13). In murine B cells, an IL-4-inducible factor which interacts with the initiation site of the germline $I_\epsilon$ exon, was designated signal-transducing factor of IL-4 (STF-IL4) by Schindler et al. (14). Utilizing the $Fc_\gamma R_I$ probe to purify an interactive Thp-1 cell protein, Hou et al. have recently cloned an IL-4-induced DNA binding factor which they designated the signal transducer and activator of transcription by IL-4 (IL-4 Stat) (15). Stat proteins are a novel family of transcription factors which, after phosphorylation and homo- or heterodimerization in the cytosol, shuttle to the nucleus where they interact with select promoter sequences (16, 17). Although not proven, it is likely that IL-4 NAF represents a homo- or heterodimerized version of IL-4 Stat.

In addition to IL-4 Stat, several of the Stat genes have now been cloned, including Stat1$\alpha/\beta$ (p91/p84), Stat2 (p113), Stat3 (p89, acute phase response factor, APRF), Stat4 (p88), and Stat5 (p90, mammary gland factor, MGF) (18-21). It would appear as if the connection between Stat proteins and a variety of activating receptors is based on the use of a novel family of tyrosine protein kinases also known as Janus kinase (JAK) (16, 17). Moreover, there appears to be selectivity in the use of JAK's and Stat proteins by specific receptors. For instance, IFN-$\alpha$ utilizes JAK1 and Tyk2 to activate Stat1 and

Address correspondence to André Nel, Division of Clinical Immunology and Allergy, Department of Medicine, 52-175 Center for Health Sciences, 10833 Le Conte Avenue, UCLA School of Medicine, Los Angeles, CA 90024-1680. Phone:310-825-6620; FAX:310-206-8107.

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1. *Abbreviations used in this paper:* APRE, acute phase response element; EMSA, electrophoretic mobility shift assay; GAS, IFN-$\gamma$ activation site; IL-4 NAF, IL-4-induced nuclear activating factor; JAK, Janus kinase; PTK, protein tyrosine kinase; Stat, signal transducer and activator of transcription.

Stat2, while IFN-$\gamma$ utilizes JAK1 and JAK2 to activate Stat1 (16, 17, 22). Recently, it was reported that IL-2 and IL-4 activate JAK3 in human T lymphocytes and NK cells (23, 24); the homologous use of JAK3 by these two cytokine receptors is attributed to their sharing of a common $\gamma$ ($C\gamma$) subunit (25). Moreover, it appears as if JAK3 interacts directly with $C\gamma$ and that JAK3 plays a role in signaling by the IL-4 receptor (26, 27). Taken together with the fact that IL-4 NAF and STF-IL-4 are known to be tyrosine phosphoproteins, it is possible that they are JAK3 substrates (12, 14).

We chose a GAS-like element upstream of the human $I_\epsilon$ exon as an electrophoretic mobility shift assay (EMSA) probe to demonstrate induction of a binding factor, designated IL-4 NAF, in a human B cell line which undergoes germline $C_\epsilon$ transcription. This protein is a tyrosine phosphoprotein which translocates from the cytosol to the nucleus, and which comigrates during EMSA with IL-4 Stat which has been activated in COS-7 cells by cotransfection of JAK3.

## Methods

*Materials.* Monoclonal antiphosphotyrosine antiserum (4G10) and polyclonal anti-JAK1 were from Upstate Biotechnology Inc. (Lake Placid, NY). Antibodies recognizing Stat 1 (p91) and Stat 3 (p89) were generously provided by Dr. Chris Schindler, Department of Medicine, Columbia University, New York (14). Culture media components were from HyClone Laboratories (Logan, UT) and M.A. Bioproducts (Walkerswille, MD). [$\alpha^{32}$P]dCTP were from ICN Biomedicals Inc. (Costa Mesa, CA). Double-strand poly(dI-dC) was from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Salmon sperm DNA and Klenow fragment were from Stratagene Inc. (La Jolla, CA). Human recombinant IL-4 and IL-6 were from R & D Systems (Minneapolis, MN). Plasmid Maxiprep kit was from Promega Corp. (Madison, WI). pcDNA3 expression vector was from Invitrogen (San Diego, CA). The tyrosine phosphatase, PTP-1C, was generously provided by Dr. Nick Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Anti-JAK3 antiserum and JAK3 cDNA was a gift from Dr. John J. O'Shea (Laboratory of Experimental Immunology, National Cancer Institute, Frederick, MD; 23). IL-4 Stat cDNA and rabbit antiserum to its protein product were generously provided by Dr. Steven L. McKnight (Tularik Inc., San Francisco, CA; 15). The human BL-2 cell line was generously provided by Dr. Jan de Vries (DNAX Research Institute, Palo Alto, CA; 28). This cell line produces IgM and expresses germline $C_\epsilon$ in response to IL-4 treatment. COS-7 cells were generously provided by Dr. Owen Witte (HHMI, UCLA, Los Angeles, CA). All other materials were of the highest purity grade available and were obtained from Sigma Chemical Co. (St. Louis, MO).

*Cell culture.* BL-2 cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine, 50 U/ml penicillin and 50 U/ml streptomycin (complete medium). COS-7 cells were cultured in DME, supplemented with 10% FCS, and 100 U each of penicillin and streptomycin.

*Cellular stimulation and extraction.* Whole cell extraction was performed as described by Wegenka et al. (29). Briefly, $5 \times 10^6$ cells in 500 $\mu$l complete medium were either mock-treated or treated with 20 ng/ml IL-4, 100 U/ml IL-6, 100 U/ml IL-2 or 100 ng/ml PMA for the indicated time periods at 37°C. Cells pellets were washed twice with ice-cold PBS and then lysed in 50 mM Tris/HCl, pH 8.0, 10 mM 3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propanesulfonate-(CHAPS), 2 mM EDTA, 100 $\mu$M sodium orthovanadate, 5 mM NaF, 1 mM DTT, 750 mM PMSF, 10 $\mu$g/ml each of aprotinin, pepstatin, and leupeptin. After 20 min at 4°C, insoluble material was removed at 14,000 *g* for 5 min in a microcentrifuge.

Cytosolic and nuclear extracts were performed as described previously with minor modifications (30). Briefly, $5 \times 10^7$ BL-2 cells were lysed by freeze-thawing in 100 $\mu$l hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM PMSF, and 0.5 mM DTT). After three additional freeze and thaw cycles, the lysates were neutralized with 100 $\mu$l 2× cytosolic buffer (30 mM Hepes, pH 7.9, 40% glycerol, 190 mM KCl, 0.4 mM EDTA, 0.2 mM PMSF, 0.4 mM DTT, 2 mM NaF, 0.2 mM $Na_3VO_4$, 1 $\mu$g/ml leupeptin, and 1 $\mu$g/ml aprotinin) and spun through a 40% sucrose cushion for 10 min at 3,000 *g* at 4°C. The supernatant was transferred into a fresh tube and respun at 14,000 *g* for 10 min at 4°C. The supernatant was kept as cytosolic extracts. The nuclear pellet was transferred to fresh tubes and extracted by vortexing in 25 $\mu$l nuclear extraction buffer (20 mM Hepes, pH 7.9, 20% glycerol, 240 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM PMSF, 1 mM EDTA and 0.1% NP-40). Samples were spun at 14,000 *g* for 10 min at 4°C and the supernatants were collected as nuclear extracts.

*Performance of EMSA.* Double-stranded oligonucleotide probes used in the EMSA were as follows: The GAS-like sequence upstream of the human $I_\epsilon$ exon (S1 oligonucleotide): 5′-GACTTCCCAAGA-ACAG-3′ (31); IL-6 acute phrase response element (APRE) in the $\alpha_2$-macroglobulin promoter: 5′-GATCCTTCTGGGAATTCCTA-3′ (32); IL-6 response element in the c-*jun* promoter (JRE-IL6), 5′-GCGCTT-CCGACAGTGACGCGAGCCG-3′ (33). The complementary oligonucleotide was annealed and end-labeled with [$^{32}P$]dCTP. 8 $\mu$l (5–10 $\mu$g) of cytosolic, nuclear or whole cell extract were incubated with 10 ml 2× binding buffer (40% glycerol, 40 mM Tris/HCl, pH 7.9, 100 mM KCl, 2 mM EDTA, 2 mM DTT, 0.1% NP-40, 50 $\mu$g/ml poly-[dI-dC]) and 2 $\mu$l (10,000 cpm) probe for 20 min at room temperature and then separated on 6% gels, which were dried and autoradiographed. For supershift, 6 $\mu$l cell extract was mixed with 2 $\mu$l antibody before adding binding buffer and probe.

*Immunoprecipitation and Western blot.* After cytokine stimulation, $2 \times 10^7$ cells in each sample were lysed in 200 $\mu$l lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM $Na_3VO_4$, 10 mM NaF, 2 mM PMSF, 10 $\mu$g/ml leupeptin, 20 $\mu$g/ml aprotinin, 0.5 mM EDTA) for 10 min at 4°C. After preclearing of lysates at 13,000 *g*, the supernatants were incubated with 5 $\mu$l antiserum to JAK1 or JAK3 for 1 h at 4°C. This was followed by the addition of 35 $\mu$l of a 50% suspension of protein A Sepharose beads for another hour. After washing of the beads in lysis buffer minus EDTA, the pellet was boiled with sample buffer and separated by 8% SDS-PAGE. Proteins were transferred to Immobilon-P membranes, which were overlayed with 1 $\mu$g/ml antiphosphotyrosine antibody and then developed with an ECL protocol as recommended by the manufacturer (Amersham Corp., Arlington Heights, IL).

*Plasmid construction and transient transfections.* JAK3 cDNA was subcloned into the EcoRI site of the pcDNA3 vector. IL-4 Stat was subcloned into the same expression vector via the EcoRI and XhoI sites. COS-7 cells were grown to 60% confluency in 100-mm petri dishes. Utilizing the $Ca_3(PO_4)_2$ method, these cells were transfected with either 15 $\mu$g empty vector, 5 $\mu$g pcDNA3-IL4 Stat, 10 $\mu$g pcDNA3-JAK3, or both pcDNA3-IL-4 Stat and pcDNA3-JAK3. Salmon sperm DNA was used to adjust the total amount of DNA in each dish. 9 h after transfection, unincorporated DNA was washed away with warm Hank's solution and replaced with 12 ml fresh DME for a further 40-h culture. Whole cell extracts were prepared as described above.

## Results

*BL-2 cells contain an IL-4–inducible factor which binds to a GAS sequence which appears upstream of the $I_\epsilon$ exon.* BL-2 cells were treated with IL-4 for time periods ranging between 1 and 15 min, and cellular extracts were assayed by EMSA utilizing a $^{32}$P-labeled oligonucleotide corresponding to base pairs −153 to −138 (S1 oligonucleotide) of the human $I_\epsilon$ promoter (Fig. 1 *A*). This sequence is homologous to the 9-bp palindromic motif of the $Fc_\gamma R_I$ promoter (Fig. 1 *A*), which binds IL-4 NAF (13). Whole BL-2 cell extracts showed rapid induction of an IL-4–inducible factor, which we designated IL-4 NAF according to the nomenclature of Kotanides and Reich

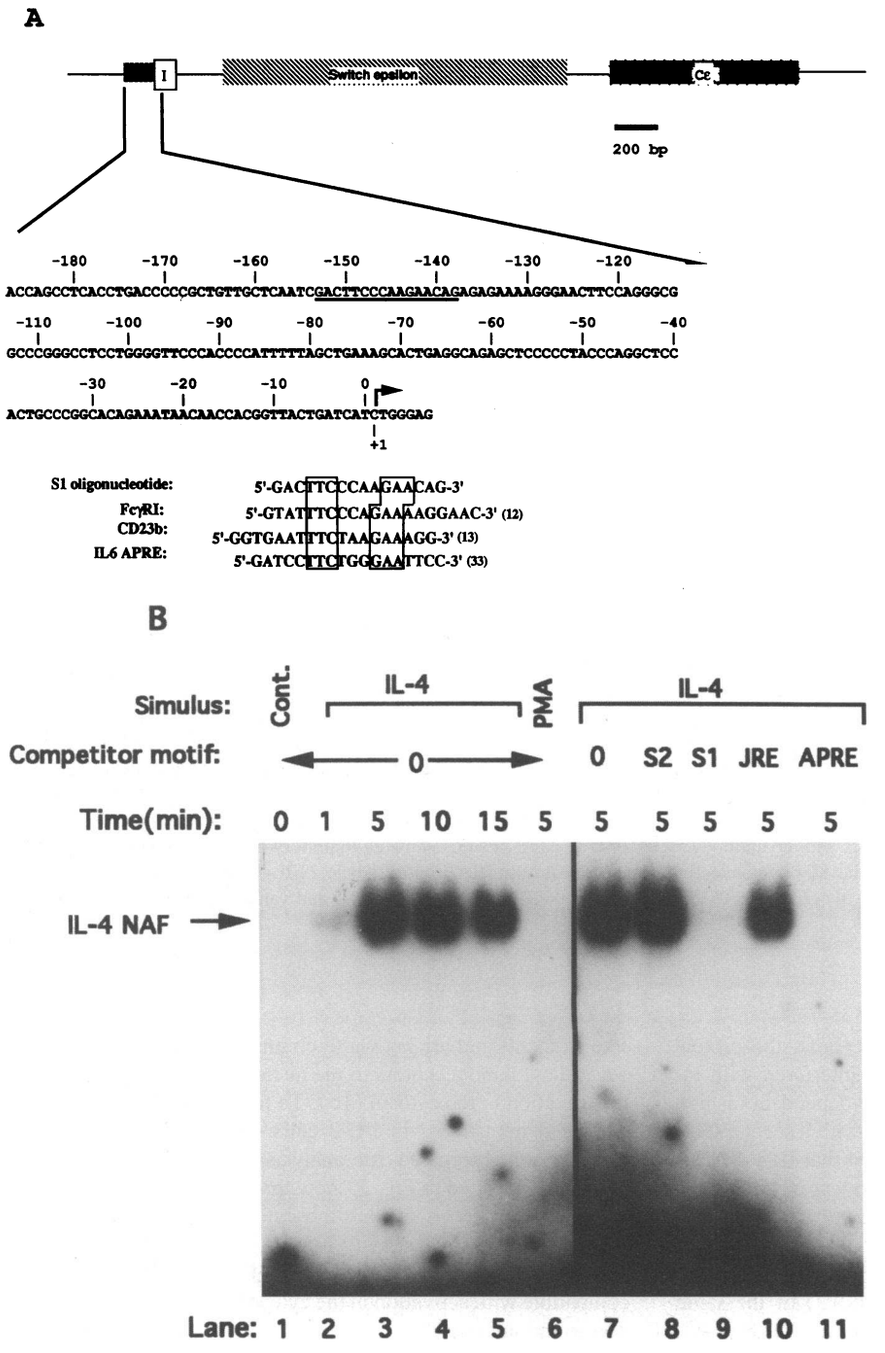


*Figure 1.* IL4 induces an activity which recognizes a GAS-like sequence upstream of the human $I_\epsilon$ promoter in BL-2 cells. (*A*) The sequence upstream of the human $I_\epsilon$ exon, including the site used for conducting EMSA (*underlined*), is shown. Base pairs were numbered from the first initiation codon backwards. Below, the sequence of the S1 oligonucleotide is compared with IL-4 response elements in the promoters of $Fc_\gamma R_I$ and CD23b, as well as the IL-6 APRE. The imperfect palindromes contained in these sequences are underlined. (*B*) Autoradiogram of EMSA conducted with the S1 oligonucleotide. $5 \times 10^6$ BL-2 cells were mock stimulated (control [*Cont.*]) or stimulated with 100 ng/ml PMA or 20 ng/ml human recombinant IL-4 for the indicated time period. Whole cell extracts were prepared and EMSA was done as described in Methods. The IL-4–induced binding factor, designated IL4 NAF (lanes *1–5*), was not induced by PMA (lane *6*). IL-4 NAF was competed for by a 100-fold excess of unlabeled S1 (lane *9*), but not unlabeled sequence 2 (*S2*), which corresponds to base pairs −174 to −152 (lane *8*). While an E-26 specific–like IL-6 response element in the *junB* promoter (*JRE*) failed to compete with IL-4 NAF (lane *10*), the GAS-like IL-6 response element, also known as the APRE, did compete for binding (lane *11*).

(Fig. 1 *B*, lanes *2–5*). No shift complex was seen during treatment with PMA (Fig. 1 *B*, lane *6*), or exposure of BL-2 cells to IL-2, IFN-γ, or insulin (not shown). The IL-4–induced complex was competed with excess unlabeled S1 oligonucleotide, but not unlabeled S2 oligonucleotide, which corresponds to the region immediately adjacent to S1 (base pairs −174 to −152) (Fig. 1 *B*, lanes *7–9*). While another GAS sequence, the IL-6 responsive APRE (Fig. 1 *A*), interfered with IL-4 NAF binding, a non-GAS like IL-6 response element in the c-*jun* promoter, known as JRE-IL-6, had no effect on IL-4 NAF binding (Fig. 1 *B*, lanes *10* and *11*). IL-6 by itself failed, however, to induce a shift complex with S1 in BL-2 or any other cell type (not shown). IL-4 NAF activity was also induced by IL-4 in EBV-transformed B cell lines which also undergo germ-line $C_\epsilon$ transcription upon IL-4 treatment (not shown). Monoclonal antibodies to CD40, a receptor known to enhance IL-4–induced germ line $C_\epsilon$ transcription, did not activate IL-4 NAF activity (not shown).

*IL-4 NAF is a tyrosine phosphoprotein which translocates from the cytosol to the nucleus of BL-2 cells.* IL-4 NAF activity was induced in BL-2 cells in the presence of cyclohexamide, which is consistent with the posttranslational modification of a preformed factor (not shown). The type of posttranslational modification was determined to be protein tyrosine phosphory-

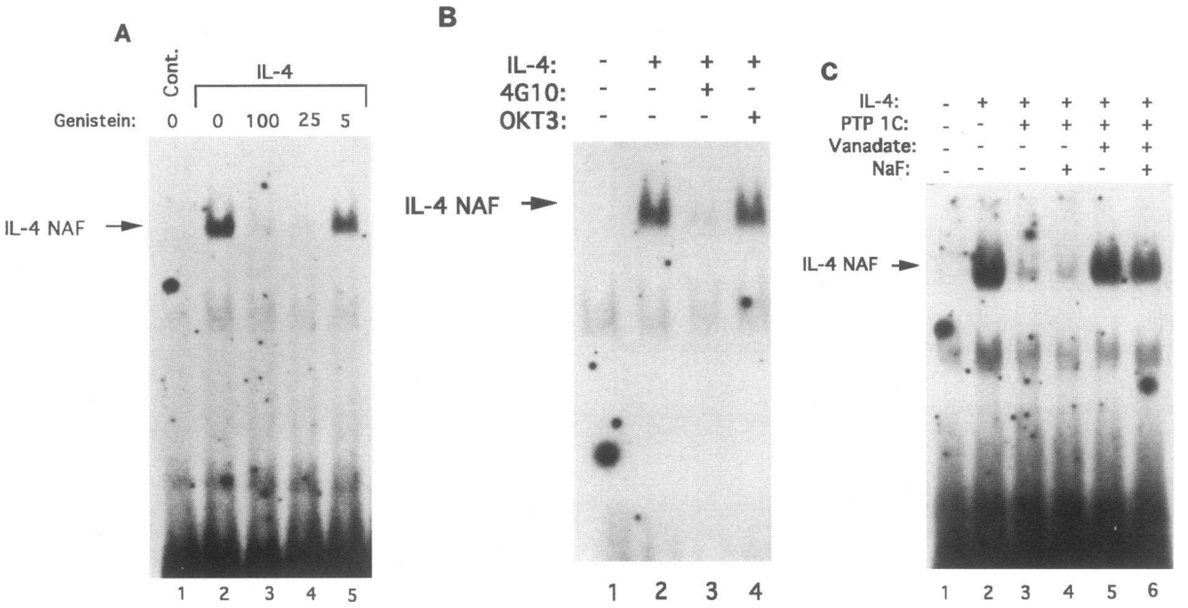


*Figure 2.* IL-4 NAF induction and binding to the S1 oligonucleotide is dependent on protein tyrosine phosphorylation. $5 \times 10^6$ BL-2 cells were precultured with varying amount genistein for 2 h at 37°C before stimulation with 20 ng/ml IL-4 for 10 min at 37°C. Control cultures were stimulated with IL-4 without prior genistein exposure. Whole cell extracts were used for conducting EMSA. In some vials, 10 $\mu$g of extract was preincubated with 1 $\mu$g 4G10 (antiphosphotyrosine), or 1 $\mu$g OKT-3 (isotype control), before addition of the probe. In another set of vials 10 $\mu$g of extract was incubated with 1 $\mu$g PTP-1C, a tyrosine phosphatase, for 30 min at 30°C in absence or presence of 1 $\mu$M $Na_3VO_4$, a tyrosine phosphatase inhibitor, or 10 $\mu$M NaF, a serine/threonine phosphatase inhibitor. (*A*) EMSA showing inhibitory effect of genistein on IL-4 NAF induction. (lane *1*) Nonstimulated control (*Cont.*); (lanes *2–5*) IL-4 stimulation after preloading with genistein at the indicated dose ($\mu$M). (*B*) EMSA showing inhibitory effect of antiphosphotyrosine mAb (*4G10*) on IL4 NAF binding to the S1 oligonucleotide. (lane *1*) Nonstimulated control; lanes *2–4*) IL-4 stimulation and pretreatment of the cell extract with no antibody, 4G10 or OKT3. (*C*) EMSA showing inhibitory effect of tyrosine phosphatase PTP-1C on the binding activity of IL-4 NAF to the S1 oligonucleotide. (lane *1*) Nonstimulated control; (lane *2*) IL-4 stimulated; (lane *3*) IL-4–stimulated whole cell extracts plus PTP-1C; (lane *4*) plus both PTP-1C and NaF; (lane *5*) plus both PTP-1C and vanadate; (lane *6*) plus PTP-1C and both vanadate and NaF.

lation based on the following observations. First, the protein tyrosine kinase (PTK) inhibitor, genistein, interfered with induction of IL-4 NAF in BL-2 cells in a dose-dependent fashion (Fig. 2 *A*). Similar observations were made with a second PTK inhibitor, erbstatin. Second, we demonstrated that IL-4 NAF is a tyrosine phosphoprotein as determined by prevention of a shift complex when cell lysates were prior incubated with antiphosphotyrosine antibodies (Fig. 2 *B*, lane *3*). In contrast to supershift induced during EMSA with 4G10, no shift was seen with an unrelated monoclonal antibody (OKT3) of the same isotype (Fig. 2 *B*, lane *4*). Third, we showed that the phosphotyrosyl residues on IL-4 NAF are required for DNA binding by determining the effect of a protein tyrosine phosphatase, PTP-1C on already activated IL-4 NAF: PTP-1C abrogated the formation of a shift complex when nuclear extracts were exposed to this enzyme before conducting EMSA (Fig. 2 *C*, lane *3*). To insure that the loss of DNA binding was a result of the action of the tyrosine phosphatase, we included sodium orthovanadate, a tyrosine phosphatase inhibitor, and NaF, a serine threonine phosphatase inhibitor, with the reactions. While sodium orthovanadate negated the effect of PTP-1C on IL-4 NAF binding, NaF did not exert any effect (Fig. 2 *C*, lanes *4–6*).

Since IL-4 NAF is a tyrosine phosphoprotein which interacts with a GAS sequence, this raised the possibility that this factor belongs to the Stat protein family. These factors are phosphoproteins which bind with varying affinity to a series of GAS-like elements and are known to communicate information from several cytokine receptors to the nucleus after homo- or heterodimerization in the cytosol (16). To test if similar translocation can be demonstrated in BL-2 cells, nuclear and cytoplasmic extracts were prepared for analysis by EMSA. The results, which are depicted in Fig. 3, show that while IL-4 NAF activity was rapidly induced in both the cytosol and the nucleus, the increase in cytosolic activity (1 min) preceded the major increase in nuclear activity (2 min) (Fig. 3). This latency is compatible with activation in the cytosol, followed by translocation to the nucleus.

*IL-4 induces robust tyrosine phosphorylation of JAK3 in BL-2 cells.* The induction of tyrosine phosphorylation of Stat proteins requires the participation of a family of PTK's, namely the JAK's (17). It would appear as if select cytokine receptors use specific combinations of JAK's to activate one or more Stat proteins (16, 17). It has recently been shown that the IL-4 receptor in T lymphocytes and NK cells activate JAK3 (23, 24). BL-2 cells were treated with IL-4 for various lengths of time, and immunoprecipitation performed for JAK1 and JAK3. These complexes were analyzed by antiphosphotyrosine immunoblotting. While IL-4 induced relatively weak tyrosine phosphorylation of JAK1 in BL-2 cells (Fig. 4 *A*, lanes *2–6*), there was a robust activation of JAK3 (Fig. 4 *B*, lanes *2–6*). IL-4–induced JAK3 phosphorylation was suppressed by genistein at doses $> 25$ $\mu$M, which is in agreement with the effect of this

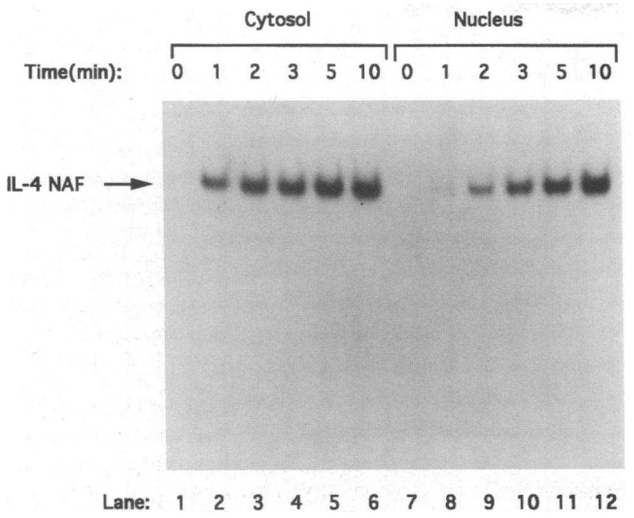


*Figure 3.* IL-4 NAF activity is induced in both the cytosol and the nucleus by IL-4. BL-2 cells were mock treated (lanes *1* and *7*) or stimulated with 20 ng/ml IL-4 for the indicated time periods (lanes *2–6* and *8–12*). The cytosolic and nuclear extracts were prepared as described (31). Lactic dehydrogenase activity was checked to show that there was no cytosolic contamination of nuclear extracts. 10 $\mu$g of each extract was examined by EMSA utilizing the S1 oligonucleotide as probe. Notice the appearance of a prominent shift band in the cytosol at 1 min (lane *2*), a point at which nuclear activity is barely noticeable (lane *8*).

inhibitor on induction of IL-4 NAF activity (not shown). PMA failed to activate JAK1 or JAK3 in BL-2 cells (Fig. 4 *A*, lane *7*; Fig. 4 *B*, lane *7*). In contrast to IL-4, IL-6 induced robust tyrosine phosphorylation of JAK1 but not JAK3 in the IL-6 responsive B cell line, AF-10 (Fig. 4,*A* and *B*, lanes *8* and *9*). IL-4 failed to induce tyrosine phosphorylation of JAK2 in BL-2 cells (data not shown).

Taken together with the data in Figs. 2 and 3, the findings in Fig. 4 strongly suggest that IL-4 NAF is a Stat protein which operates downstream of JAK3 in BL-2 cells. During conducting of EMSA, antisera to Stat1 (Fig. 5), Stat2 (not shown), and Stat3 (Fig. 5) failed to block the shift of the IL-4 NAF complex. A rabbit antiserum to Stat6 did, however, block the formation of this DNA-protein complex as shown in Fig. 5 (lane *3*). Moreover, antiphosphotyrosine immunoprecipitates from BL-2 cells, showed induction of 100- and 130-kD phosphoproteins by IL-4 on antiphosphotyrosine overlay (Fig. 6). While p130 likely represents activated JAK3, p100 is the exact molecular weight of IL-4 Stat, also known as Stat6.

*Transfection of JAK3 and IL-4 Stat into COS-7 cells induces a DNA binding activity which coelectrophoreses with IL-4 NAF.* To establish that JAK3 can activate IL-4 NAF, we attempted overexpression of JAK3 in BL-2 cells without success. We did succeed, however, in transfecting COS-7 cells with JAK3 and could demonstrate that the transfected kinase is constitutively tyrosine phosphorylated (not shown). On cotransfecting JAK3 with IL-4 Stat, we were able to show by EMSA that an S1 oligonucleotide binding activity is generated in the cytosol of COS-7 cells (Fig. 7, lane *6*). Moreover, this shift complex comigrated with IL-4 NAF from BL-2 cells (Fig. 7, lanes *1* and *2*). No shift complexes were induced in COS-7 cells transfected with empty vector, JAK3 cDNA or IL-4 Stat cDNA only (Fig.

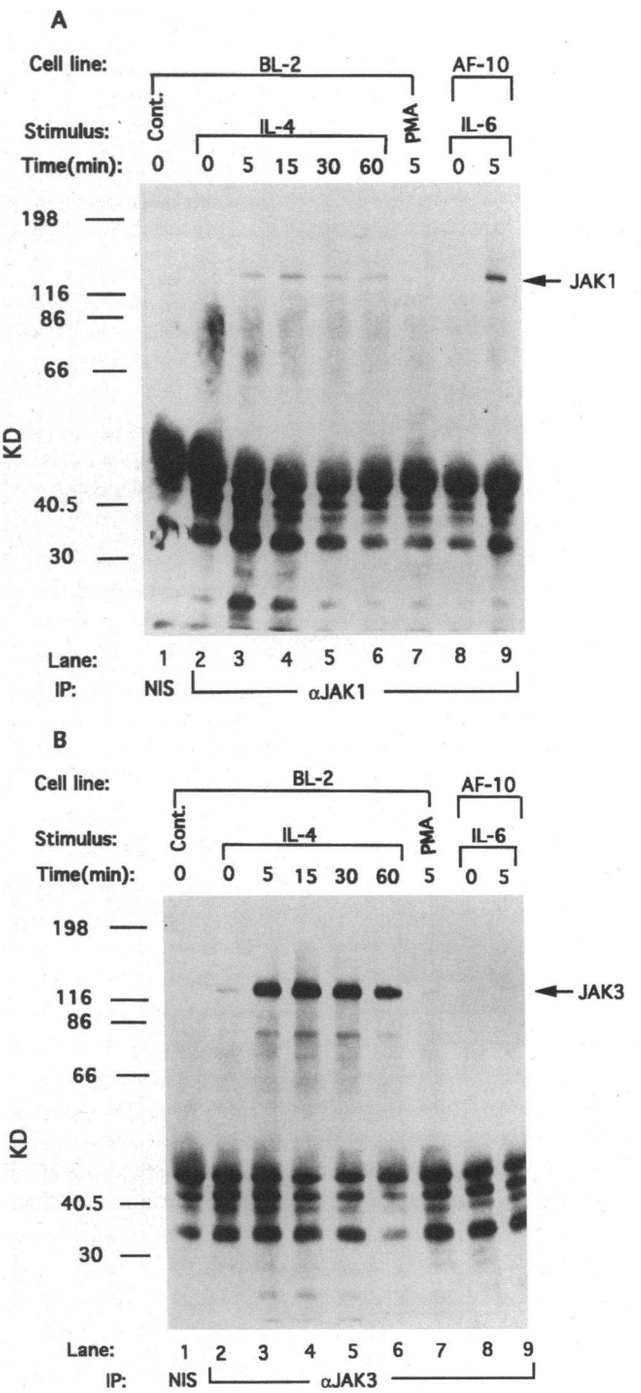


*Figure 4.* Tyrosine phosphorylation of JAK1 and JAK3 in response to IL4 treatment. 2 × $10^7$ BL-2 cells and AF-10 cells were mock treated (control [*Cont.*]) or stimulated with 20 ng/ml human recombinant IL-4, 100 ng/ml PMA or 100 U/ml human recombinant IL-6 for the indicated time period. Cell lysates were precleared by centrifugation at 13,000 *g* for 10 min and were immunoprecipitated with either 5 $\mu$l anti-JAK1 or 5 $\mu$l anti-JAK3 antibody. Proteins were separated on 8% SDS-PAGE and the phosphorylation of JAK1 and JAK3 assessed by antiphosphotyrosine immunoblotting as described in Methods. AF-10 is a human IgE producing myeloma cell line which responds to IL-6 treatment with increased proliferation. (*A*) Antiphosphotyrosine immunoblot of JAK1 immunoprecipitates. (*B*) Antiphosphotyrosine immunoblot of JAK3 immunoprecipitates. IP, immunoprecipitation; NIS, nonimmune serum.

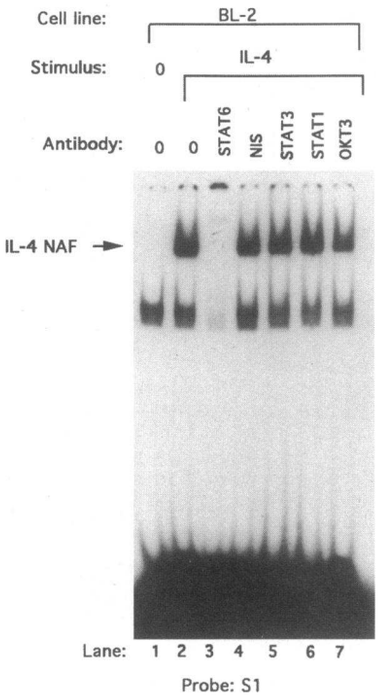


*Figure 5.* Antibodies to Stat6, but not Stat1 and Stat3, interfere with the formation of the IL-4 NAF complex in BL-2 cells. $5 \times 10^6$ BL-2 cells or AF-10 cells were either mock stimulated or stimulated with 20 ng/ml IL-4 for 10 min at 37°C. Nuclear extracts were prepared and examined by EMSA with labeled S1 as described. 2 $\mu$l of anti-Stat1 (*STAT1*), anti-Stat3 (*STAT3*), anti-Stat6 (*STAT6*), or nonimmune serum (*NIS*) were used together with 6 $\mu$l cellular extract to attempt supershift or block the protein–DNA interaction. Unstimulated cells (lane *1*); IL-4–stimulated cells, no preincubation with antiserum (lane *2*); preincubation with anti-Stat6 (lane *3*); preincubation with rabbit nonimmune serum (*NIS*; lane *4*); preincubation with anti-Stat3 (lane *5*); preincubation with anti-Stat1 (lane *6*); preincubation with control (*OKT3*) mAb (lane *7*).

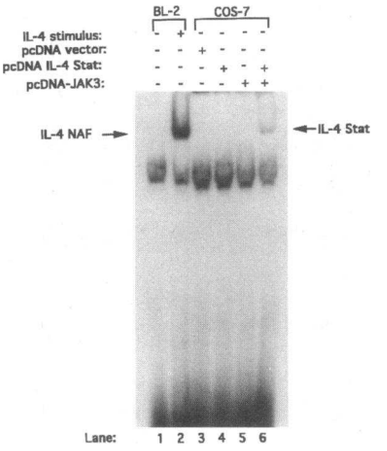


*Figure 7.* EMSA comparing induction of S1 bonding activity in JAK3/IL-4 Stat cotransfected COS-7 cells and IL-4–treated BL-2 cells. BL-2 cells were stimulated with IL-4 for 10 min and whole cell extracts were prepared as described in Fig. 1. COS-7 cells were transfected with empty pcDNA3 vector, JAK3 cDNA, IL-4 Stat cDNA, or a combination of JAK3 + IL-4 Stat. Whole cell extract was prepared as described for BL-2 cells. Both cellular extracts were used to conduct EMSA with labeled S1 oligonucleotide as described in Fig. 1 *B*. Activated IL-4 Stat and IL-4 NAF comigrated.

7, lanes *3–5*). These results strongly suggest, that IL-4 Stat is activated by JAK3, whereupon this transcription factor binds to the same site as IL-4 NAF.

## Discussion

In this paper we show that IL-4 induces a tyrosine phosphoprotein, IL-4 NAF, which interacts with a GAS sequence upstream of the human $I_\epsilon$ exon. IL-4 NAF activity increases in the cytosol before appearing in the nucleus, which is compatible with cytosol-to-nuclear translocation. IL-4 NAF is identical to IL-4 Stat, a novel member of the Stat family which recognizes the same GAS after activation by JAK3 in a cotransfected COS-7 cell line.

Our study shows that the IL-4 response element in human $I_\epsilon$ promoter is homologous to the response element characterized by Kohler and Rieber (13), Kotanides and Reich (12), and Schindler et al. (14). Kohler and Rieber identified the so-called NF-IL4 which interacts with a 9-bp motif (5′-TTCTAAGAA-3′) in the human CD23b gene promoter (13; Fig. 1 *A*). Kotanides and Reich described induction of IL-4 NAF in a human monocytic cell line, Thp-1, which recognizes a GAS in the promoter of the human $Fc_\gamma R_I$ gene (12; Fig. 1 *A*). This finding is compatible with the ability of Stat proteins to display varying affinity for a series of GAS-like elements (16, 17). It is interesting that the IL-6–induced GAS response element in the promoter of the $\alpha_2$-macroglobulin gene (32) competes for binding of IL-4 NAF to the S1 oligonucleotide (Fig. 1 *B*). IL-6 failed, however, to induce a mobility shift complex with the S1 oligonucleotide. The IL-4 NAF motif contains an inverted GAA repeat on opposite strands (12), similar to other IL-4 response elements in the $Fc_\epsilon R_{IIa}$ (34), human $Fc_\epsilon R_{IIb}$ (35), murine $C_\gamma 1$ (36), murine $C_\epsilon$ (37), murine MHCIIE$\beta$ (38), human MHCII-DR$\alpha$ (38), and human $Fc_\gamma R_I$ genes (38) (Fig. 1 *A*). Schindler et al. identified the so-called signal transducing factor for IL-4 (STF-IL4) which interacts with a GAS sequence in the IRF-1 gene promoter as well as an upstream element (R1/R2 sequence) in the murine $I_\epsilon$ promoter (14). Interestingly, the R1/R2 nucleotide does not contain the inverted GAA repeat.

IL-4 NAF requires to be phosphorylated on tyrosine residue(s) to bind to the S1 oligonucleotide (Fig. 2 *B*). Both the in vitro removal of phosphate groups from tyrosine residues (Fig. 2 *C*) as well as the in vivo interference with tyrosine phosphorylation (Fig. 2 *A*) abrogated IL-4 NAF activity. These characteristics, together with the feature that IL-4 NAF is activated in both the cytosol and nucleus (Fig. 3), strongly suggested to us that IL-4 NAF is a Stat protein (16, 17). Moreover, the delay by which nuclear activity is induced with respect to the cytosolic increase suggests intracellular translocation from the cytosol to the nucleus, which is another characteristic of Stat proteins (Fig. 3). Antibodies to Stat1, Stat2, and Stat3 failed to bind to IL-4 NAF (Fig. 5). Utilizing the identical GAS sequence which was used for identifying IL-4 NAF, Hou et al.

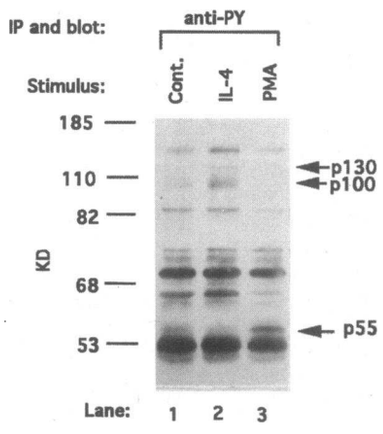


*Figure 6.* IL-4 induced protein tyrosine phosphorylation of 130- and 100-kD proteins in human BL-2 cells. Cell stimulation and immunoprecipitation procedures were as described in Fig. 4, with the exception that 5 $\mu$l 4G10 was used. Proteins were resolved by 8% SDS-PAGE and antiphosphotyrosine immunoblotting performed with 1 $\mu$g/ml 4G10. (lane *1*) nonstimulated cells; (lane *2*) IL-4–stimulated cells; (lane *3*) PMA-stimulated cells. Notice induction of p130 and p100 by IL-4 and p55 by PMA.

recently purified and cloned the gene of a new Stat protein, which was designated IL-4 Stat, also known as Stat6 (15). Utilizing an antiserum to Stat6, we were able to show that the IL-4 NAF complex could be supershifted/blocked, proving that IL-4 NAF = IL-4 Stat = Stat6 (Fig. 5). Moreover, we demonstrated IL-4–induced phosphorylation of a 100-kD protein in BL-2 cells, which is the identical molecular weight of IL-4 Stat (Fig. 6).

Stat protein activation involves the JAK family, which currently consists of four members, JAK1, JAK2, JAK3, and Tyk2 (16, 17). It would appear as if differential association of one or more of these kinases with the activating receptor may determine which Stat proteins are activated. For instance, Stat1 activation by IFN-$\gamma$ requires both JAK1 and JAK2 to activate Stat1, while Stat1 and Stat3 activation by the IL-6 receptor requires JAK1, JAK2, and Tyk2 (16, 17). Recently it was shown that IL-2, IL-4, IL-7, and IL-9 activate JAK3, and that this ability is dependent on their sharing of a common signaling subunit, $C_\gamma$, which is physically complexed to JAK3 (23–27). Moreover, IL-4 has been shown to induce the tyrosine phosphorylation of JAK3 in T cells and NK cells (23, 24). The tyrosine phosphorylation of JAK's is indicative of their activation. Similarly, in BL-2 cells we found robust induction of tyrosine phosphorylation of JAK3 by IL-4 in association with weak JAK1 phosphorylation (Fig. 4). It is possible that JAK1 associates with the ligand-binding 140-kD subunit of the IL-4 receptor and contribute to the activation of JAK3 when the former binds to $C_\gamma$. Altogether, these findings suggest that JAK3 is the PTK which is directly involved in activation of IL-4 NAF. To prove this notion more directly, we initially attempted to overexpress JAK3 in BL-2 cells, but did not succeed. As an alternative, we cotransfected JAK3 and IL-4 Stat cDNA constructs into COS-7 cells. This has resulted in activation of a cellular protein which bound the S1 oligonucleotide sequence (Fig. 7). Moreover, this complex comigrates with IL-4 NAF (Fig. 7). Because this complex was absent from cells which were transfected by JAK3 or IL-4 Stat only, it is reasonable to assume that JAK3 actually modified the cotransfected IL-4 Stat protein. The exact molecular details of the interaction between the kinase and the Stat protein and the involvement of the IL-4 receptor needs to be further studied.

Insofar as germline $C_\epsilon$ transcription is important in the subsequent event of IgE gene rearrangement, it is likely that Stat6 plays a role in IgE production in B lymphocytes. Whether binding of IL-4 NAF to the $I_\epsilon$ upstream site actually leads to transcriptional activation is unclear. Mutational alteration of critical base pairs in the 9-bp palindromic motif of the CD23 promoter disrupted IL-4–induced activation of a luciferase reporter gene construct (13). When the sequence upstream of the $I_\epsilon$ promoter shown in Fig. 1 *A* was linked to a luciferase reporter gene and transfected into BL-2 cells, we found that IL-4 alone induced weak (twofold) stimulation of luciferase activity (not shown). This is in agreement with the relatively weak activation of a related luciferase construct transfected into DG75 and BL-2 cells by Albrecht et al. (39). In the presence of anti-CD40 antibodies, however, we found that IL-4 stimulated a definitive increase (eightfold) in luciferase activity in transiently transfected BL-2 cells (not shown). CD40 is known to deliver a signal which synergizes with the IL-4 activation pathway in human B lymphocytes undergoing $C_\epsilon$ rearrangement (40, 41). CD40 ligation failed, however, to activate IL-4 NAF and did not exert additional effects on IL-4 NAF activation by IL-4 (not shown). The exact role of IL-4 NAF on germline transcription needs to be studied further.

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