Interleukin-3, Erythropoietin, and Prolactin Activate a STAT5-like Factor in Lymphoid Cells

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Interleukin-3, Erythropoietin, and Prolactin Activate a STAT5-like Factor in Lymphoid Cells*

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Interleukin-3 (IL-3)-, erythropoietin (EPO)-, and prolactin (PRL)-induced signal transduction via the JAK/STAT pathway was studied in the IL-3-dependent BAF-3 lymphoid cell line. Transfected cells expressing either the long form of the PRL receptor or the EPO receptor were used. We demonstrated that IL-3, EPO, and PRL activated a transcription factor related to the mammary transcription factor STAT5 but not to STAT1, -2, -3, or -4 as opposed to interferon γ (IFNγ) which activated STAT1 in the same cells. Similarly, PRL and EPO activated a STAT5-like factor (STAT5-L) in the rat Nb2 and the human UT7 cells expressing endogenous PRL and EPO receptors, respectively. The hematopoietic STAT5-L activated by IL-3, EPO, or PRL was identified as a 97-kDa tyrosine-phosphorylated protein. These results confer to STAT5 a much broader role than previously suggested.

Signal transduction of interferons (IFNs)[1] has served as a model system for studies on activation of the JAK kinases and downstream-located latent cytoplasmic transcriptional factors known as STATs (for review see Refs. 1 and 2). Six members of the STAT family are presently known. These factors are characterized by their rapid activation through tyrosine phosphorylation (1). STAT1 and STAT2, both activated by IFNα, associate with a 48-kDa DNA-binding protein and translocate to the nucleus where they bind interferon-stimulated response element (ISRE), while only STAT1 is activated by IFNγ and binds γ-activated sequences (GAS). STAT5-MGF was first isolated as a mammary transcriptional factor (MGF) specifically activated by the hormone prolactin (PRL) in mammary epithelial cells, inducing the expression of milk proteins (3). However, the expression of STAT5 was not restricted to the mammary gland and was also detected in hematopoietic organs (3).

PRL also plays an immunomodulatory role, lymphocytes being its main target (4). PRL receptors (PRL-R) belong to the cytokine receptor superfamily (5) which includes multiple receptors expressed in hematopoietic cells such as receptors for interleukin (IL-) 2, 3, 4, 5, 6, 7, 9, 11, and 12, erythropoietin (EPO), thrombopoietin, and others (6). PRL signal transduction begins to be elucidated in a limited number of hematopoietic cells expressing either endogenous PRL-R (Nb2 rat T lymphoma cell line) or transfected PRL-R (the BAF-3 IL-3-dependent lymphoid cell line and the FDCP1 IL-3-dependent myeloid cell line) (7–10). In these cells, PRL activates the rapid and transient tyrosine phosphorylation of various proteins including its receptor and kinases of the JAK family, either JAK2 in Nb2, or JAK2 and weakly JAK1 in BAF-3 (9, 11–13). Furthermore, in the Nb2 lymphoma cells, PRL induces the expression of an immediate early gene, the interferon (IFN) regulatory factor 1 (IRF1) (7, 8), which regulates the expression of both IFNs and IFN-induced genes (14, 15). Recent studies on IRF1 indicated that its promoter contains one critical GAS element suggesting that the PRL-induced IRF1 expression could be induced by a JAK/STAT pathway in hematopoietic cells (1, 16, 17).

Two recent studies indicated that in Nb2 lymphoma cells, PRL activated two members of the STAT family, one related to STAT1 and another unknown protein of 97 kDa (12, 18). Because of the expression of STAT5 in the spleen and thymus, we first wondered whether STAT5 could be activated by PRL in hematopoietic cells. Moreover, since the mammary STAT5 was shown to be a substrate of kinase JAK2 (3), we investigated whether cytokines known to activate JAK2 such as IL-3 or EPO (19, 20) also triggered the activation of STAT5.

The BAF-3 cells express endogenous IL-3 receptors and are strictly dependent on the presence of IL-3 for growth. Transfection with either the mammary PRL-R or the EPO-R confers to these cells the ability to grow in the presence of PRL or EPO, respectively. Here we describe that in transfected BAF-3 cells, PRL, IL-3, and EPO activate a tyrosine-phosphorylated DNA binding factor, of 97 kDa, highly related to STAT5 but not to STAT1, -2, -3, or -4, as opposed to IFNγ which activates STAT1. This STAT5-like factor is also activated by PRL or EPO in hematopoietic cells expressing endogenous PRL- or EPO-R, respectively.

EXPERIMENTAL PROCEDURES

Hormones and Antibodies—Ovine prolactin (NIDDK6-PRL-19, 31 IU/mg) was a gift from the National Hormone and Pituitary Program (Baltimore, MD). Recombinant EPO, IFNγ, and IL-3 were from Boehringer and Pepro Tech. Inc., respectively.

The anti-STAT antibodies used were: anti-STAT1 N-terminal domain (anti-ISGF3 p91/84 monoclonal antibody, Transduction Laboratories, UK), anti-STAT2 peptide (gift from C. Schindler (30)), anti-STAT3 C-terminal peptide (30C, gift from D. Levy, New York University Medical Center (21)), anti-STAT4 C-terminal peptide (C-20, Santa Cruz Biotechnology, Inc.), anti-STAT5 N-terminal fusion protein containing amino acids 6 to 160 of sheep mammary STAT5 (31), and an anti-STAT raised against a STAT3 N-terminal peptide which recognizes all STAT members (21) (30N gift from D. Levy, named thereafter PANSTAT). Antiphosphotyrosine antibody 4G10 was kindly provided by Dr. B. Drucker (Portland).

Cell Culture—BAF-3 cells were transfected with cDNA coding for either the long form of the rabbit mammary PRL-R or the murine EPO-R, both cloned in the pBabeNeo expression vector, as described (9). BAF-3 cells were cultured in RPMI 1640 medium supplemented with 7% fetal calf serum (Dutscher) and either 5% culture supernatants from the IL-3-producing Wehi-3B cell line (Wehi-CM) or 10 ng/ml PRL or 2

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[1] The abbreviations used are: IFN, interferon; IL, interleukin; EPO, erythropoietin; PRL, prolactin; GAS, γ-activated sequence; IRF1, interferon regulatory factor 1; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay.

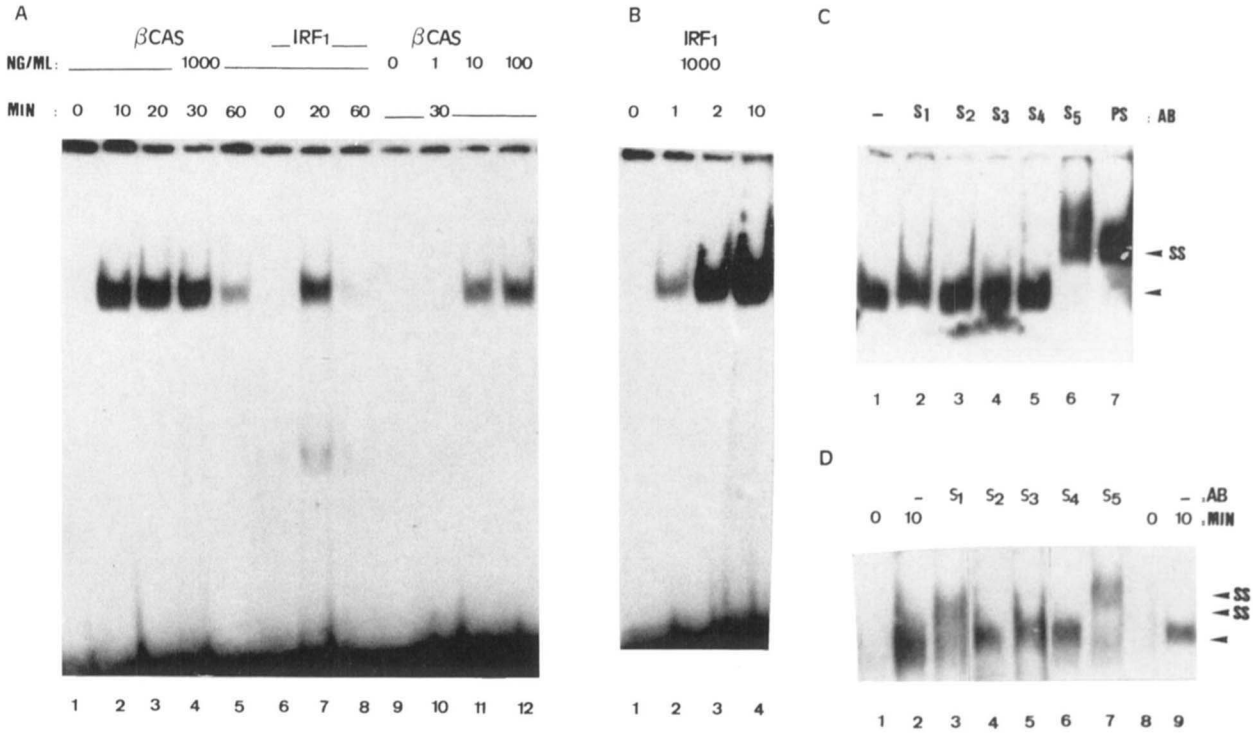

FIG. 1. **PRL activates a STAT5 DNA binding activity in BAF-3 and in Nb2 cells.** *A* and *B*, nuclear extracts from BAF-3 cells expressing PRL-R were stimulated with various concentrations of PRL (ng/ml) for the indicated times (min). Nuclear extracts were analyzed in EMSAs using either β-casein or IRF1 oligonucleotide as probes. *C*, nuclear extracts from PRL-R-transfected BAF-3 cells stimulated for 20 min with 1 μg/ml PRL were incubated with various antisera against either STAT1, STAT2, STAT3, STAT4, or STAT5 (S_1, S_2, S_3, S_4, S_5) or PANSTAT (*PS*) and with the β-casein probe. Shifted and supershifted complexes (*SS*) are pointed out. *D*, nuclear extracts from Nb2 cells (*lanes 1–7*) or PRL-R-transfected BAF-3 cells (*lanes 8* and *9*) treated for O or 10 min with 1 μg/ml PRL were incubated with the indicated antisera against either STAT1, -2, -3, -4, or -5 (S_1, S_2, S_3, S_4, S_5) and with the IRF1 probe. EMSAs were performed. Shifted and supershifted (*SS*) complexes are pointed out.

units/ml EPO (9). Nb2-11C cells (22) were maintained in RPMI 1640 medium supplemented with 7% fetal calf serum. UT7 cells (23) were cultured in α-medium supplemented with 10% fetal calf serum and 2.5 ng/ml granulocyte macrophage-colony stimulating factor (5×10^7 units/mg) as described (24).

Nuclear Extracts—Cells were first deprived of cytokines and of fetal calf serum by incubation for 4 h (for BAF-3 and Nb2 cells) or 18 h (for UT7 cells) in Iscove's medium supplemented with 0.4% detoxified bovine serum albumin (Fraction V, Sigma) and 75 μg/ml iron-saturated transferrin (Sigma) (9) and then incubated in the presence of either 1–1000 ng/ml PRL or 10 ng/ml IL-3, 10 units/ml EPO, or 1000 units/ml IFNγ for the indicated time. Nuclear extracts were prepared by first lysing the cells in a hypotonic buffer (20 mM Hepes, pH 7.9, 10 mM KCl, 1 mM EDTA, 0.2% Nonidet P-40, 10% glycerol) supplemented with 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM DTT and extracting the pelleted nuclei in a hypertonic buffer (0.35 M NaCl, 20% glycerol, 20 mM Hepes, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM DTT, with the same protease and phosphatase inhibitors being added). After 30 min at 4 °C, extracts were centrifuged for 5 min at 4 °C at 20,000 × *g*, and supernatants were immediately frozen in liquid nitrogen and stored at −80 °C.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts (~10^5 cells/point) were incubated with 16 fmol of ^{32}P-labeled oligonucleotide containing STAT5 binding site from either the bovine β-casein promoter (5′-AGATTTCTAGGAATTCAAATC-3′) or the GAS element of the IRF1 promoter (5′-GATCCATTTCCCCGAAATGA-3′). These oligonucleotides were end-labeled with polynucleotide kinase to a specific activity of 8000 cpm/fmol as described (3).

For supershift assays, an excess of antibodies was added to the nuclear extracts just prior to the addition of the probe.

Purification of the DNA Binding Activity—Nuclear extracts (10^8 cells) in the binding buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, 5% glycerol) containing 100 mM NaCl were incubated with Sepharose beads coupled to multimerized STAT5 binding sites from the β-casein gene promoter (3) for 45 min at 4 °C in the presence of 20 μg of poly(dI-dC) and 20 μg of poly(dA-dT). After washing, proteins bound to the Sepharose beads were eluted with the SDS loading buffer, separated by SDS-polyacrylamide gel electrophoresis (8%), and transferred to nitrocellulose membrane (Schleicher and Schuell, BA85). Blots were incubated first with a STAT5 antibody (1/1000), then stripped and further incubated with the phosphotyrosine antibody (1/5000).

RESULTS AND DISCUSSION

Nuclear extracts from BAF-3 cells transfected with the PRL-R (9) and treated with PRL were prepared. Band shift assays were carried out with a labeled probe from the β-casein promoter containing the STAT5 binding site. As shown in Fig. 1*A* (*lanes 1–5*), PRL induced the activation of a DNA binding complex that appeared in a very rapid and transient manner, becoming almost undetectable after 60 min of incubation. This DNA binding activity was induced in the presence of concentrations of PRL (10 ng/ml) required for proliferation of these BAF-3 cells (Fig. 1*A*, *lanes 9–12*) (9). To assess further the induction of a STAT5-related DNA binding activity, EMSAs were extended to another STAT5-DNA binding sequence, the GAS of the IRF1 promoter. This motif contains a palindromic motif 5′-TTC*XXX*GAA-3′ which is strictly required for STAT5 DNA binding (25, 31). As shown in Fig. 1*A*, *lanes 6–8*, PRL-treated cells possessed a DNA binding activity with the GAS-IRF1 oligonucleotide. This activity was induced as early as 1 min after PRL addition (Fig. 1*B*, *lanes 1–4*). Similar results were obtained with the β-casein probe.

The identity of the activated STAT factor was investigated by incubating nuclear extracts from PRL-stimulated cells with various STAT-specific antisera and analyzing them by EMSA (Fig. 1*C*). DNA binding complexes with the IRF1 probe were supershifted only with STAT5 (*lane 6*) and PANSTAT (*lane 7*) antisera. Neither STAT1 nor STAT2, -3, or -4 antisera were able to recognize the complexes, indicating that STAT5 or a STAT5-like (STAT5-L) factor, was specifically activated by PRL in the PRL-R-transfected BAF-3 cells. To strengthen this

result, we prepared nuclear extracts from another murine lymphoid cell line, the Nb2 cells, which express large amounts of endogenous PRL-R (26). As shown in Fig. 1*D*, PRL induced the rapid and transient activation of a DNA binding complex (*lane 2*). Supershift experiments (*lanes 3–7*) indicated that this complex contained both a STAT5-L protein (*lane 7*) and STAT1 (*lane 3*). It is worth noting that STAT1 activation was only detectable at high PRL concentrations.

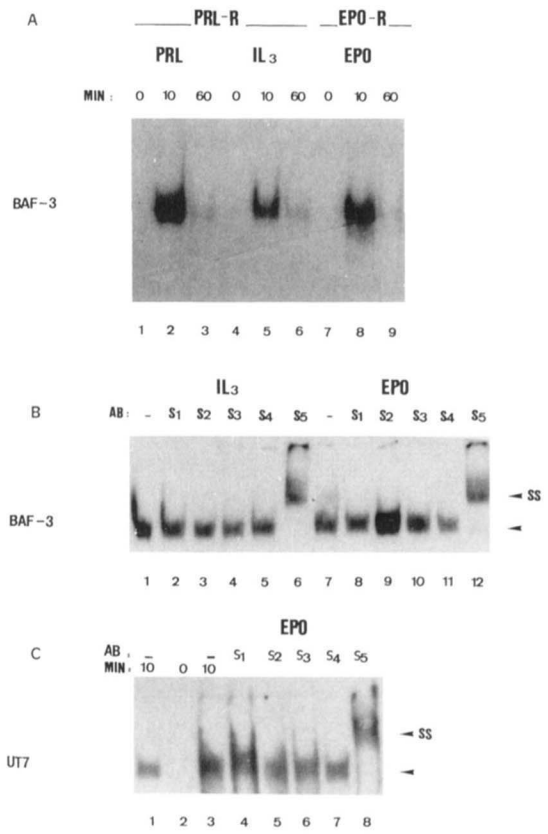

FIG. 2. **A STAT5 DNA binding activity is activated by EPO and IL-3.** *A*, BAF-3 cells expressing either PRL-R (*lanes 1–6*) or EPO-R (*lanes 7–9*) were stimulated with 1 μg/ml PRL, 10 ng/ml IL-3, or 10 units/ml EPO. Nuclear extracts were analyzed by EMSA with the β-casein probe. *B*, nuclear extracts from IL-3- (10 ng/ml, 10 min) or EPO- (10 units/ml, 10 min) stimulated BAF-3 cells expressing the EPO-R were incubated with various antisera against either STAT1, STAT2, STAT3, STAT4, or STAT5 (S_1, S_2, S_3, S_4, S_5) and with the IRF1 probe. EMSAs were performed. Shifted and supershifted (*SS*) complexes are pointed out. *C*, nuclear extracts from UT7 cells (*lanes 3–8*) or EPO-R-transfected BAF-3 cells (*lanes 1* and *2*) treated with 10 units/ml EPO for O or 10 min were prepared and analyzed by EMSA.

We next asked whether the activation of STAT5-L was restricted to PRL. Like the parental BAF-3 cells, BAF-3 cells transfected with the PRL-R were responsive to IL-3 (9). Also, BAF-3 cells expressing a transfected murine EPO-R that proliferated in the presence of EPO had been established. Nuclear extracts from either IL-3-treated PRL-R-transfected or EPO-treated EPO-R-transfected cells were analyzed by EMSA using either the IRF1 probe or β-casein probe. As shown in Fig. 2*A*, IL-3 (*lanes 4–6*) and EPO (*lanes 7–9*) induced binding activity to the β-casein probe. Complexes formed after PRL stimulation (Fig. 2*A*, *lanes 1–3*) migrated the same as the IL-3- or EPO-stimulated complexes. The PRL-, IL-3-, or EPO-induced complexes were all supershifted with STAT5 antiserum, but not with antisera directed against STAT1, STAT2, STAT3, or STAT4 (Fig. 2*B*). We next checked the activation of STAT5-L factors by EPO in the human UT7 cell line which expressed high amounts of endogenous EPO-R (27). As shown in Fig. 2*C*, in the UT7 cell line, EPO activated a transcriptional factor which migrated the same as the one induced by EPO in BAF-3 cells (*lane 1*). Supershift experiments confirmed that this factor is highly related to STAT5 (*lane 8*) and not to STAT1, -2, -3, or -4.

BAF-3 cells also expressed endogenous IFNγ-R. EMSA of IFNγ-treated cells indicated that IFNγ rapidly and transiently induced a DNA binding complex with the IRF1 probe (Fig. 3*A*, *lane 2*, and Fig. 3*B*, *lanes 1–5*) or β-casein probe (not shown) which migrated faster than the complex induced by IL-3 (Fig. 3*A*, *lanes 1* and *2*)) or PRL (not shown). Supershift assays indicated that this IFNγ complex contained STAT1 but not STAT3 or -5 (Fig. 3*B*, *lanes 6–10*). STAT5 was not activated by IL-6 in the U937 monocytic cell line, although in those cells, IL-6 induced the activation of STAT3 which bound the IRF1 probe and which was recognized by the anti-STAT3 (31). These data showed that STAT5-L proteins are specifically involved in PRL, EPO, and IL-3 signaling.

To better characterize STAT5-L proteins, nuclear extracts from PRL-, IL-3-, or EPO-treated or from untreated BAF-3 cells were incubated with Sepharose beads coupled to a multimerized STAT5 binding site from the β-casein promoter. Bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting first with a STAT5 antiserum and then with an antiphosphotyrosine antibody. Both antibodies recognized one band of 97 kDa, in PRL-, IL-3-, or EPO-activated extracts (Fig. 4).

Our data indicate that in a variety of hematopoietic cells expressing endogenous or transfected receptors (murine BAF-3, rat Nb2, and human UT7) PRL, IL-3, and EPO rapidly activate a transcriptional factor immunologically closely

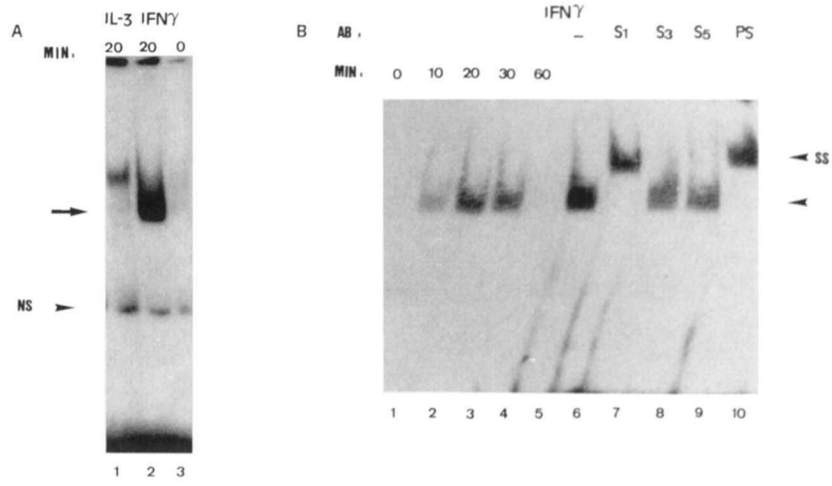

FIG. 3. **IFN$_\gamma$ activates STAT1 in BAF-3 cells.** PRL-R-transfected BAF-3 cells were incubated with IL-3 (10 ng/ml, *A*, *lane 1*) or IFNγ (1000 units/ml, *A*, *lane 2*, and *B*) for the indicated time. Nuclear extracts were analyzed by EMSA with the IRF1 probe. Supershift assays were carried out with nuclear extracts from cells treated for 20 min with IFNγ in the presence of the indicated anti-STAT antibodies: anti-STAT1, -3, -5 (S_1, S_3, S_5) and PANSTAT antibody (*PS*). Supershifted complexes are indicated (*SS*).

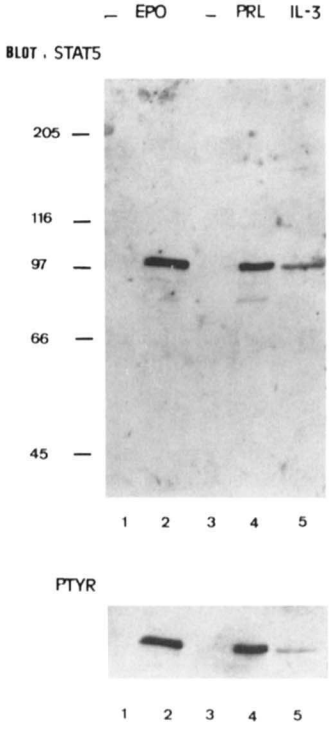

FIG. 4. **Identification of the STAT5-L DNA binding activity induced by PRL or IL-3 or EPO.** EPO-R-transfected BAF-3 cells (*lanes 1* and *2*) or PRL-R-transfected BAF-3 cells (*lanes 3–5*) were incubated without (*lanes 1* and *3*) or with 10 units/ml EPO (*lane 2*), 1 μg/ml PRL (*lane 4*), or 10 ng/ml IL-3 (*lane 5*) for 20 min. Nuclear extracts were incubated with multimerized β-casein oligonucleotides coupled to Sepharose beads. Bound proteins were analyzed by immunoblotting with STAT5 specific antiserum (*top*) and then reprobed with antiphosphotyrosine antibody (*bottom*).

related to STAT5, but not to STAT1, -2, -3, or -4. Moreover, this factor becomes tyrosine-phosphorylated following ligand action. Whether this hematopoietic factor is totally identical with the mammary STAT5 protein recently isolated or corresponds to a new highly related STAT is under investigation. It is worth noting that preliminary data[2] suggest the existence of two genes for STAT5. Whether one encodes the mammary STAT5 form and the other the hematopoietic STAT5 is currently unknown. Our findings are in agreement with a number of reports on the activation of unknown STATs by either EPO, granulocyte macrophage-colony stimulating factor, or PRL in hematopoietic cells (8, 12, 13, 21, 28, 29). In agreement with our data, these unidentified STATs may be the hematopoietic STAT5 protein, suggesting a wide activation of STAT5 in hematopoietic cells. Such results pointed out a general role of STAT5 in cell activation. One main target should be the tumor suppressor IRF1 gene whose expression was reported to be induced by a wide variety of cytokines in various cells, including BAF-3 cells (7, 8, 14, 15). Alternatively, it could indicate that STAT5 participates in a variety of specific transcriptional complexes.

[2] I. Kerr, personal communication.

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Note Added in Proof—Two murine $STAT_5$ homologs have now been cloned (32, 33) and designated $STAT_{5a}$ and $STAT_{5b}$.

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