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► **To cite this version:**

V Gouilleux-Gruart, F Gouilleux, C Desaint, Jean-François Claisse, Jean-Claude Capiod, et al.. STAT-related transcription factors are constitutively activated in peripheral blood cells from acute leukemia patients.. Blood, 1996, 87 (5), pp.1692-1697. 10.1182/blood.V87.5.1692.1692 . hal-03410573

**HAL Id: hal-03410573**

**<https://hal.science/hal-03410573>**

Submitted on 24 Nov 2021

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## STAT-Related Transcription Factors Are Constitutively Activated in Peripheral Blood Cells From Acute Leukemia Patients

By Valérie Gouilleux-Gruart, Fabrice Gouilleux, Corinne Desaint, Jean-François Claisse, Jean-Claude Capiod, Jacques Delobel, Renate Weber-Nordt, Isabelle Dusanter-Fourt, François Dreyfus, Bernd Groner, and Lionel Prin

**A signal transduction pathway activated by many cytokines has recently been elaborated. The JAK kinases and the signal transducers and activators of transcription (STAT) factors have been found to be essential components. In this report, we describe the presence of constitutively activated STAT factors in peripheral blood cells from patients with acute leukemia. We used oligonucleotide probes from the  $\beta$ -casein and IRF-1 gene promoters and the ISRE probe to detect STAT proteins in nuclear extracts from acute leukemia cells in bandshift assays. Specific DNA protein complex formation was observed with the probes from the  $\beta$ -casein and IRF-1 gene promoters, but not with the ISRE oligonucleotide probe, when cell extracts from acute lymphoblastic leukemia**

**(ALL) and acute myeloid leukemia (AML) were investigated. We used nonradioactive oligonucleotides as competitors to show the specificity of the complex formation. Specific antibodies directed against the individual STAT proteins were used in supershift experiments. STAT5- and STAT1-related factors were detected in ALL and STAT1-, STAT3-, and STAT5-related proteins were present in nuclear cell extracts from AML. Since the cells were not treated with cytokines before the nuclear proteins were extracted, we conclude that these factors are constitutively activated in vivo. It is likely that the constitutive activation of STAT proteins is a part of the events of leukemogenesis.**

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**D**EVELOPMENT, maintenance, and functional activation of immune surveillance and effector cells are under the control of a wide spectrum of interleukins (ILs), growth factors, and hormones. The interaction of a cytokine with its receptor triggers specific events such as gene transcription, protein expression, and release leading either to differentiation or proliferation depending on the cells. The STAT proteins are a new family of transcription factors involved in cytokine, growth factor, and hormone signal transduction.<sup>1-5</sup> Cytokine binding to their receptors leads to the tyrosine phosphorylation of associated cytoplasmic protein tyrosine kinases. These kinases belong to the JAK family. JAKs phosphorylate STAT proteins, and STAT proteins dimerize and translocate to the nucleus where they bind to specific DNA sequences in promoter regions of responsive genes.<sup>1-5</sup>

Different members of the STAT factor family have been cloned and the cytokines and hormones stimulating their activation have been studied. p91-Stat1 constitutes the interferon- $\gamma$  (IFN- $\gamma$ )-activated transcription factor,<sup>6</sup> p91-STAT1, and p113-STAT2 together with the p48 subunit constitute the IFN- $\alpha/\beta$  responsive transcription factor (ISGF-3).<sup>7,8</sup> APRF-STAT3 is activated in response to IL-6, epidermal growth factor (EGF), IL-12 and granulocyte

colony-stimulating factor (G-CSF),<sup>9-12</sup> STAT4 in response to IL-12.<sup>11,13</sup> Prolactin, IL-3, IL-5, granulocyte-macrophage (GM)-CSF, growth hormone (GH), IL-2, thrombopoietin, and erythropoietin cause MGF-STAT5 activation.<sup>14-19</sup> Two highly related STAT5 proteins, STAT5a and STAT5b, have been described. Their expression is tissue specifically regulated.<sup>20-22</sup> STAT6 is mainly activated in response to IL-4 and IL-3.<sup>23,24</sup>

Because cytokines not only regulate the differentiation of the cells but also play an important role in proliferation, it is reasonable to assume that aberrations in the JAK-STAT pathway could be involved in tumor formation. Several observations support this hypothesis. It has been shown that an amino acid substitution in the *Drosophila* homolog of the JAK kinase (*Drosophila*<sup>hopTum-1</sup>) gene causes leukemia-like hematopoietic defects.<sup>25</sup> Overactivity of the mutated protein is responsible for the malignant neoplasia.<sup>26</sup> Recently, the constitutive activation of the JAK-STAT pathway has been described in T cells transformed with human T-cell lymphotropic virus-1 (HTLV-1). IL-2-independent growth acquired by cells transformed with HTLV-1 correlates to acquisition of the constitutively activated JAK-STAT pathway.<sup>27</sup>

We examined whether constitutively activated STAT proteins can be detected in cells obtained from patients with hematologic malignancies. We derived nuclear extracts from acute leukemia cells and analyzed them in bandshift assays by using specific oligonucleotide probes and antibodies.

### MATERIALS AND METHODS

**Patients.** Three patients with acute lymphoblastic leukemia (ALL), five patients with acute myeloid leukemia (AML), and three controls were investigated. Diagnosis was established according to French-American-British (FAB) classification<sup>28</sup> and associated with immunologic and genetic studies on peripheral blood (PB) and bone marrow (BM).

**Cells.** The total leukocyte population was collected after PB sample sedimentation. Plasma enriched cells were centrifuged at 400g at 4°C. Nuclear proteins were extracted. When mentioned, mononuclear cells were separated by the Ficoll (Techgen, Les Ulis, France) sedimentation procedure.

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Submitted September 18, 1995; accepted December 18, 1995.

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0006-4971/96/8705-0054\$3.00/0

**Table 1. Clinical Characteristics of the Patients 1 to 4**

| Patient No. | Age | Type   | White Blood Cell Counts 10 <sup>9</sup> /L | N  | Eo | B (%) | Lc (%) | M | Bl | Cytogenetic           |
|-------------|-----|--------|--|----|----|-------|--------|---|----|-----------------------|
| 1           | 27  | ALL    | 188  | 4  | 0  | 0     | 8      | 1 | 87 | t(9;22)               |
| 2           | 66  | M1 AML | 99   | 4  | 0  | 1     | 8      | 2 | 85 | ND                    |
| 3           | 26  | ALL    | 11,3                                       | 10 | 0  | 0     | 13     | 2 | 70 | Complex abnormalities |
| 4           | 68  | M5 AML | 23,6                                       | 14 | 1  | 0     | 8      | 0 | 77 | ND                    |

Abbreviations: N, neutrophil; Eo, eosinophil; B, basophil; Lc, lymphocyte; M, monocyte; Bl, blast; ND, not determined.

**Preparation of nuclear extracts and bandshift assays.** Cells were resuspended in lysis buffer containing 10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 1.5 mmol/L MgCl<sub>2</sub>, 0.1% NP40, 5 µg/mL leupeptin, 5 µg/mL aprotinin, 1 mmol/L EDTA, 0.5 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and left at 4°C for 8 minutes. After centrifugation at 14,000 rpm for 2 minutes, the supernatants were discarded and nuclei were extracted with 50 µL hypertonic buffer containing 20 mmol/L HEPES pH 7.9, 420 mmol/L NaCl, 1 mmol/L EDTA, 25% glycerol, 5 µg/mL leupeptin, 5 µg/mL aprotinin, 0.5 mmol/L DTT, 1 mmol/L PMSF, and 100 µmol/L vanadate. The extracts were centrifuged at 14,000 rpm for 5 minutes at 4°C and stored at -70°C. Nuclear extracts were used for bandshift experiments as previously described.<sup>15</sup> The autoradiographs were exposed for 20 hours to 4 days in AML analysis.

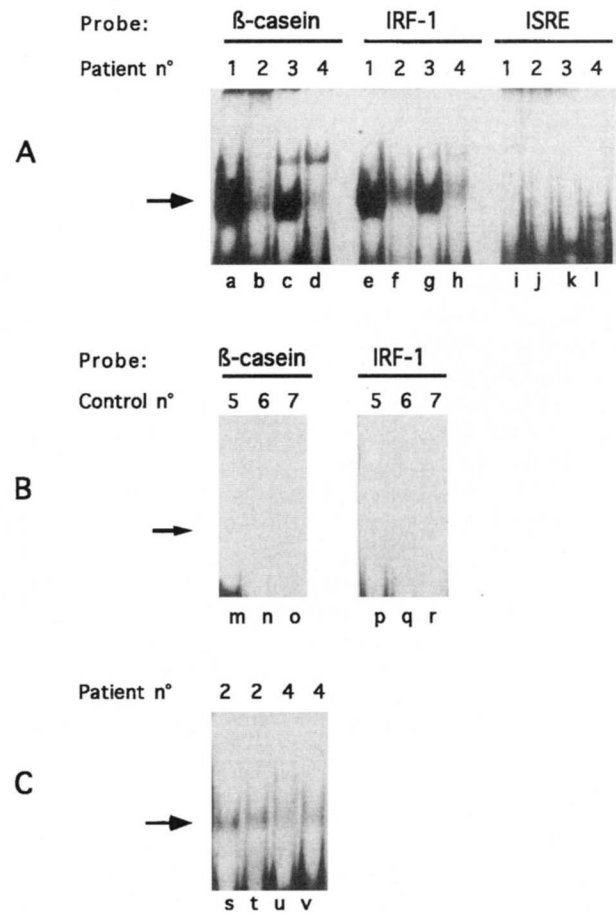
**Oligonucleotide probes.** The gamma activated site (GAS) sequence present in the IRF-1 gene promoter (5'-GATCCATTCCCCGAAATGA-3'), the MGF binding site of the bovine β-casein gene promoter (5'-AGATTCTAGGAATTCAAATC-3'), and ISRE present in ISG-15 gene promoter (5'-GATCGGGAAAGGGAAACCGAACTGAAGCC-3') were used as probes in bandshift assays. The oligonucleotides were end-labeled with polynucleotide kinase and <sup>32</sup>P adenosine triphosphate (ATP). The following oligonucleotides were used in competition experiments: GAS element present in the Ly-6E gene: 5'-CATGTTATGCATATTCCTGTAGTG-3'. GAS sequence from the FcγRI gene: 5'-GTATTTCCAGAAAAGGAAC-3'. FcεRIIb: 5'-GAATTTCTAAGAAAGGGA-3'. Iε: 5'-GTCAACTTCCCAAGAACAGAA-3'. GAS sequence in GBP: 5'-AAGTACTTTTCAGTTTCATATTACTCTAAATC-3'. APRE from the α2-macroglobulin gene: 5'-GATCCTTCTGGGAATTCCTA-3'. SIEm67 from the Fos gene promoter: 5'-CATTTCCCGTAAATC-3'.

**Antibodies.** Anti-STAT1 antibody was purchased from UBI (Washington, DC). Three anti-STAT3 antibodies were tested: the anti-STAT3(30C) provided by D. Levy (New York University Medical Center, New York), the anti-STAT3(C-20) purchased from Santa Cruz, CA, and the anti-STAT3(C) from J. Darnell (Rockefeller University, New York, NY). He also provided the anti-STAT2 and anti-STAT4 antisera. Anti-STAT5 antiserum has been produced in chickens.<sup>15</sup>

**RESULTS**

**Nuclear proteins from patients with acute leukemia bind to distinct GAS elements.** To analyze the presence of constitutively activated STATs in leukemic cells, nuclear extracts from total leukocytes were derived from patients with ALL (nos. 1 and 3), AML (nos. 2 and 4, see Table 1) and from controls. Three different probes were used in the bandshift assays: (1) the GAS sequence present in IRF-1 gene promoter and able to bind STAT1, STAT3, and STAT5; (2) the β-casein promoter oligonucleotide probe able to bind STAT5 with high affinity and STAT1, STAT3, STAT6 with a lower affinity; and (3) the ISRE sequence recognized by

ISGF3α complex. As shown in Fig 1A, a STAT DNA-binding complex was observed with the IRF-1 and the β-casein oligonucleotide probes and cell extracts from the patients with acute leukemia (lanes a through h). This complex was not formed with cell extracts from controls (Fig 1B, lanes



**Fig 1. Bandshift analysis of nuclear extracts from leukocytes isolated from PB of patients (A) with ALL (patients 1 and 3), AML (patients 2 and 4), and controls 5 to 7 (B). Ten micrograms of protein was introduced into each binding reaction and incubated with the β-casein oligonucleotide probe (lanes a through d, m through o), IRF-1 oligonucleotide probe (lanes e through h, p through r), or ISRE oligonucleotide probe (lanes i through l). (C) Comparison of DNA-protein complexes formed either with total leukocyte proteins (lanes s and u) or with mononuclear cell proteins (lanes t and v) from patients 2 (lanes s and t) and 4 (lanes u and v) in bandshift assays. The IRF-1 oligonucleotide was used as a probe.**

**Table 2. Analysis of STAT DNA-Binding Activities in Nuclear Extracts From Patients With Acute Leukemia**

| Patient No. | Type   | STAT DNA-Binding Activity* | Anti STAT1† | Anti STAT2† | Anti STAT3† | Anti STAT4† | Anti STAT5† |
|-------------|--------|----------------------------|-------------|-------------|-------------|-------------|-------------|
| 1           | ALL    | +                          | -           | -           | -           | -           | +           |
| 2           | M1 AML | +                          | -           | -           | +           | -           | -           |
| 3           | ALL    | +                          | -           | -           | -           | -           | +           |
| 4           | M5 AML | +                          | -           | -           | +           | -           | -           |
| 5           | M5 AML | +                          | -           | -           | +           | -           | -           |
| 6           | M4 AML | +                          | +           | -           | +           | -           | +           |
| 7           | M4 AML | +                          | ND          | ND          | +           | ND          | +           |
| 8           | ALL    | +                          | +           | ND          | -           | ND          | +           |

Abbreviation: ND, not determined.

\* Formation of DNA-binding complex in presence of IRF-1 oligonucleotide probe.

† Absence or presence of the STAT related factor in the DNA-binding complex incubated with the corresponding anti-STAT antibody.

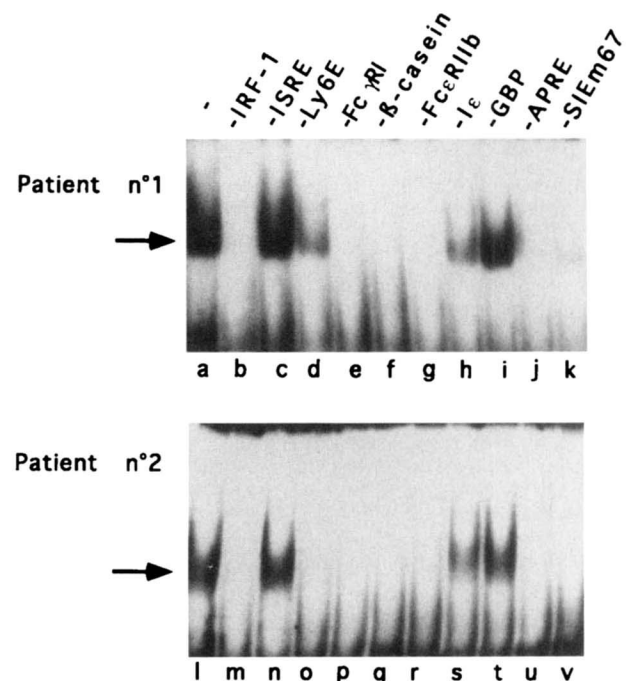
m through r). No complexes formed between the ISRE probe and cell extracts from patients (lanes i through l). The DNA-binding complexes formed by the cell extracts from the two patients with ALL (nos. 1 and 3) yielded a stronger signal when compared to those formed by the cell extracts from patients with AML (nos. 2 and 4). The same amount of protein was introduced onto the complex formation.

We separated the mononuclear cell fraction containing the blast population from polymorphonuclear cells (neutrophils, eosinophils, and basophils represent between 4% and 15% of leukocytes in patients 1 to 4, see Table 1) by Ficoll gradient centrifugation. The bandshift with the IRF-1 oligonucleotide probe and proteins from mononuclear cells from patients 2 and 4 are presented in Fig 1C. The DNA-binding complex present in total leukocytes (lanes s and u) was still detectable when nuclear proteins from purified mononuclear cells (blast population) were used (lanes t and v). Similar results were obtained before and after cell separation. This excludes the possibility that the cell manipulation is responsible for the results. A band with slower mobility is present in nuclear cell extracts from patients 3 and 4 (Fig 1A, lanes c, d, g, and h). This band is not competed by an excess of nonradioactive oligonucleotide and is not specific (data not shown). It cannot be observed after Ficoll purification of the cells (Fig 1C, compare lanes u and v).

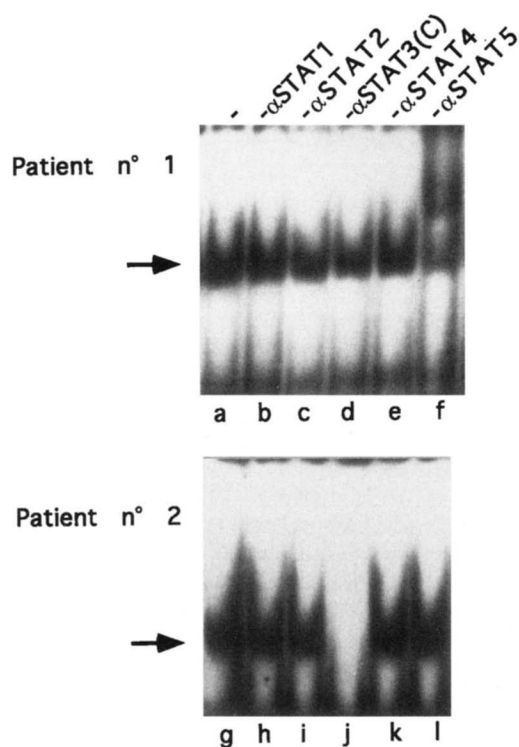
Nuclear cell extracts from patients 5 to 8 were obtained after mononuclear cell purification and tested in bandshift assays with the IRF-1 oligonucleotide probe. Specific DNA-binding complexes were detected with all cell extracts studied (Table 2).

*Analysis of the DNA-binding specificity of the complexes detected with leukemic cell extracts.* The DNA-binding specificities of the complexes were further characterized in bandshift competition assays by using the IRF-1 oligonucleotide probe and nuclear cell extracts from patients no. 1 (Fig 2, lanes a through k) and 2 (lanes l through v). Various GAS elements were introduced in the assay as competitors. The results show that the DNA-binding specificities of the complexes detected with nuclear extracts from patients 1 and 2 are different. The DNA-binding complexes observed with the nuclear extracts from patients 1 and 2 were competed by the corresponding cold probe (lanes b and m), but also by APRE (lanes j and u) and by GAS elements found in the Fc $\gamma$ RI (lanes e and p),  $\beta$ -casein (lanes f and q), and Fc $\epsilon$ RIIb

(lanes g and r) gene promoters. The Ly-6E oligonucleotide showed a partial competition for the complex formed with nuclear extracts from patient no. 1 (lane d), whereas the competition is complete for the complex detected with nuclear extracts from patient no. 2 (lane o). The same observation was made by competition with SIE m67 oligonucleotide (lanes k and v). No competition was observed with the GAS element from the guanylate binding protein gene (GBP) promoter (lanes i and t) and the ISRE element from the ISG-15 gene promoter (lanes c and n). The competition obtained with the GAS element present in the I $\epsilon$  promoter is strong but not complete. This is similar for the extracts from both



**Fig 2. Oligonucleotide competition for DNA-complex formation with distinct subset of GAS elements.** Leukocyte nuclear extracts from patients 1 (lanes a through k) and 2 (lanes l through v) were analyzed in bandshift assays with the IRF-1 oligonucleotide probe in presence of various GAS elements. The indicated oligonucleotides were used in competition assays in a 100-fold molar excess as indicated.



**Fig 3. Analysis of DNA-protein complexes by antibody supershift assays.** Nuclear cell extracts prepared from patients no. 1 and 2 were incubated without (lanes a and g) or with anti-STAT1, -STAT2, -STAT3(C), -STAT4, -STAT5 specific antisera (lanes b through f, h through l) in presence of IRF-1 radioactive oligonucleotide probe.

patients (lanes h and s). These data suggest that the STAT-related factors constitutively activated in patients 1 and 2 are not identical.

*STAT1-, STAT3-, and STAT5-related factors are present in the DNA-binding complexes.* We examined which STAT was constitutively activated in leukemic cells by using specific antibodies directed against STAT1, STAT2, STAT3, STAT4, and STAT5 (Fig 3). Bandshift experiments were performed with the IRF-1 oligonucleotide probe and nuclear extracts from cells of patients with ALL (no. 1) and with AML (no. 2) in the presence of the STAT-specific antisera. The complex formed with the nuclear proteins from the ALL (no. 1), associated with translocation t(9,22), was supershifted by STAT5 antiserum (lane f), but not by STAT1, STAT2, STAT3, and STAT4 antisera (lanes b through e). The DNA-binding complex formed with the nuclear proteins from the AML patient (no. 2) was not recognized by STAT1, STAT2, STAT4, and STAT5 antisera (lanes h, i, k, and l). However, DNA-binding complex formation was inhibited with the anti-STAT3(C) antiserum (lane j).

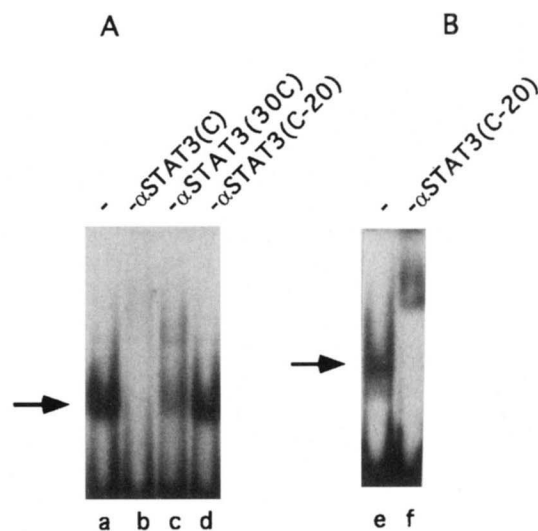
The results obtained with the nuclear extracts from patients 3 to 8 and STAT antisera are presented in Table 2. As for patient no. 1, the complexes formed with the nuclear proteins from patients with ALL (nos. 3 and 8) were supershifted by the STAT5 antiserum. This antiserum reacts also with DNA-binding complexes from patients with AML nos. 6 and 7. STAT3-related factor is part of the complexes de-

tected in all patients with AML (nos. 2 and 4-7). Supershift of the DNA-binding complex formed with ALL (no. 8) and AML (no. 6) occurred in presence of anti-STAT1 antibody.

We tested three antibodies directed against different parts of the STAT3 protein (Fig 4A). DNA-binding complexes formed with the IRF-1 oligonucleotide probe (lane a) and nuclear proteins from AML patient (no. 2) were inhibited by the anti-STAT3(C) antiserum (lane b) and supershifted by the anti-STAT3(30C) antiserum (lanes c). A similar pattern of antibody recognition was obtained with the complex formed by the proteins from AML no. 4 (data not shown). No change in the mobility of the complex was detected with the anti-STAT3(C-20) antibody (lane d). This antibody is raised against the amino acids 750-769 of the carboxy terminus of STAT3 and recognizes STAT3 factor in the T47D human mammary carcinoma cell line treated with IL-6 for 15 min (Fig 4B, lane f). The anti-STAT3(C) and the anti-STAT3(30C) antisera are respectively directed against amino acids 688 to 727 and 718 to 770 of the carboxy terminus. These results suggest that the STAT3-related factor in the AML differs in the amino acids of the C terminus from the previously described STAT3 protein.

#### DISCUSSION

We identified constitutively activated DNA-binding proteins in leukemic cells from patients with ALL and AML by bandshift analysis. The competition experiments with various GAS elements, able to bind STAT proteins with different efficiency, show the existence of distinct STAT related transcription factors in the nuclear extracts from acute leukemia.



**Fig 4. (A) Supershift assays with three different anti-STAT3 antibodies.** Nuclear extracts from AML were incubated without (lane a) or with the anti-STAT3(C) antiserum (lane b), with the anti-STAT3(30C) antiserum (lane c) and with the anti-STAT3(C-20) antibody (lane d) in presence of IRF-1 radioactive oligonucleotide probe. (B) Nuclear extracts from the T47 D human mammary carcinoma cell line treated with IL-6 for 15 minutes were incubated without (lane e) or with the anti-STAT3(C-20) antibody (lane f) in presence of IRF-1 radioactive oligonucleotide probe.

A distinct pattern of competition by different oligonucleotides has been established when recombinant STAT5 protein has been analyzed in bandshift assays.<sup>15</sup> An identical pattern of competition was found when the proteins from ALL cells were tested. This strongly suggests that STAT5 or a closely related factor is activated in these cells. This conclusion was supported by the observation that the DNA-binding complex was supershifted by the anti-STAT5 antiserum. A protein with this DNA-binding specificity is also induced by erythropoietin, thrombopoietin or GM-CSF in UT7 cell line.<sup>15,17</sup> STAT5-related factor was also detected in two of five patients with AML.

Oligonucleotide competition and recognition by anti-STAT3 antibodies indicate that in M1, M4, and M5 AML, STAT3 is part of the DNA-protein complex. The use of three slightly different antibodies demonstrates that the STAT3-related proteins present in AML differ in their C terminus from the described STAT3 transcription factor. It is possible that a highly related STAT3 factor, as in the case of STAT5a and STAT5b,<sup>20-22</sup> or splice variants of STAT3 protein, as described for STAT1 $\alpha$  and STAT1 $\beta$ ,<sup>7</sup> have been detected. Recently, a short form of STAT3, STAT3 $\beta$ , was identified by its interaction with an N-terminal segment of *c-jun* in the yeast two-hybrid screen of a mammalian cDNA library. STAT3 $\beta$  is missing the 55 C-terminal amino acid residues and has 7 additional amino acid residues at its C terminus.<sup>29</sup> The STAT3-related protein detected in AML patients might be related to STAT3 $\beta$ . However, another explanation for the results obtained with the three anti-STAT3 antibodies is that STAT3 might be involved in a complex with different proteins in the AML cells and thus the epitope recognized by the anti-STAT3(C-20) antibody is not available to be recognized by this antibody. Supershift experiments with the various anti-STAT antibodies showed that STAT1-related factor is also constitutively activated in some patients with ALL or AML.

STAT DNA-binding occurred without the *in vitro* stimulation of the cells before nuclear protein extraction. These proteins need to be phosphorylated on tyrosine before they translocate to nucleus and bind to DNA.<sup>1-5</sup> This implies that the STAT proteins detected in nuclear extracts from ALL and AML cells are tyrosine phosphorylated. The addition of antiphosphotyrosine antibody in the bandshift assays leads to a decrease of the DNA-complex formation (data not shown). Furthermore, these results might indicate that the tyrosine kinase responsible for the tyrosine phosphorylation of STAT proteins is activated. STAT proteins are thought to be the substrates of the JAK tyrosine kinases. It has been reported recently that a mutation of the *hopTum-1* gene, the *Drosophila* homolog of JAK kinase causes leukemia-like hematopoietic defects.<sup>25</sup> This mutation leads to the constitutive activity of the protein.<sup>26</sup> Identification of target genes induced by constitutively activated JAK in *Drosophila* and activated STAT proteins in human leukemic cells will be of great interest. Constitutively activated STAT proteins have also been observed in human breast tumors<sup>30</sup> and in mice with an onco-transgene.<sup>31</sup>

The mechanism by which the STAT proteins become activated in ALL and AML cells is not known. The production

of cytokines and growth factors by leukemic cells might be involved in the differential activation of STAT proteins in acute leukemia. ALL and AML are divided in various subtypes characterized by the lineage and the degree of maturation of the cells involved in the leukemia. It is possible that activation of other STAT members is implicated in the other subtypes depending on cell differentiation, maturation, and cytokine production. Another mechanism of constitutive activation of STAT proteins could be a bypass of cytokine receptors by an oncogenic pathway. Recently, an enhanced DNA-binding activity of a STAT3-related protein has been described in cells transformed by the Src oncoprotein.<sup>32</sup> The participation of the strong BCR-ABL kinase activity resulting from the translocation t(9;22)<sup>33,34</sup> can be envisaged in the constitutive activation of STAT proteins in leukemia associated with the Ph<sup>1</sup> chromosome. Jak-STAT signaling is induced by the *v-abl* oncogene.<sup>35</sup> These data might explain the strong STAT5 constitutive activation observed in nuclear extracts from the Ph<sup>1</sup> positive ALL and in the K562 chronic myelogenous leukemia cell line (data not shown).

Our findings that constitutive activation of STAT1, STAT3, and STAT5 occurs in cells from acute leukemia raises the possibility that STAT signaling pathway regulates expression of cellular genes that participate to leukemogenesis.

#### ACKNOWLEDGMENT

We are grateful to Dr D. Levy for providing the anti-STAT3(30C) antiserum. We thank Dr J. Darnell for the gift of anti-STAT2, anti-STAT3(C) and anti-STAT4 antisera.

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