little STAT binding activity was detected in unstimulated N1186 cells, whereas N1186-94 cells did contain constitutive binding activity (Fig. 4A). Treatment of both cell populations with IL-2 augmented STAT binding activity. Corresponding to the acquisition of constitutive STAT protein activation in N1186-94 cells, these cells exhibited constitutive tyrosine phosphorylation of Jak3 (Fig. 4B), although less so than that shown by long-term HTLV-I–transformed cell lines such as MT-2 cells (Fig. 2B). In response to IL-2, Jak3 tyrosine phosphorylation was more inducible in N1186-94 cells (Fig. 4B) than in MT-2 cells (Fig. 2B). Like MT-2 cells, N1186-94 cells exhibited constitutive association of γ_c with Jak3 (Fig. 4C) and of IL-2Rβ with both Jak3 and γ_c (*15*).

Although it is conceivable that the HTLV-I transactivator protein Tax (*12*, *19*) might modulate expression of genes that contribute to constitutive activation of the Jak-STAT pathway, it appears not to be a sufficient signal. A T cell line denoted Tax I, in which a herpes saimiri–based vector drives Tax gene expression (*15*, *20*), did not exhibit constitutive STAT binding activity (*15*). Of other recently recognized HTLV-I proteins (p12, p13, and p30) encoded by the pX region (*21*), p12 is weakly oncogenic (*22*) and can associate with both IL-2Rβ and γ_c (*23*), but it remains unknown whether p12 participates in activation of the Jak-STAT pathway. Although the mechanisms by which HTLV-I transformation induces activation of the Jak-STAT pathway are not completely understood, our correlation of the constitutive Jak-STAT pathway with the acquisition of IL-2 independence in HTLV-I–transformed cells provides an additional insight into the mechanisms underlying the molecular basis of HTLV-I–mediated leukemogenesis. Because constitutive Jak-STAT activation also occurs in B cells transformed with Abelson virus (*24*), it is possible that activation of Jaks and STATs may be a strategy used by a number of viruses to mediate cell growth.

Note added in proof: With additional time in continuous cell culture, N1186-94 cells have become completely independent of IL-2. Correspondingly, in these cells the amount of constitutive STAT binding activity has increased as compared with that of the N1186-94 cells that are still partially dependent on IL-2.

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Enhanced DNA-Binding Activity of a Stat3-Related Protein in Cells Transformed by the Src Oncoprotein

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Cytokines and growth factors induce tyrosine phosphorylation of signal transducers and activators of transcription (STATs) that directly activate gene expression. Cells stably transformed by the Src oncogene tyrosine kinase were examined for STAT protein activation. Assays of electrophoretic mobility, DNA-binding specificity, and antigenicity indicated that Stat3 or a closely related STAT family member was constitutively activated by the Src oncoprotein. Induction of this DNA-binding activity was accompanied by tyrosine phosphorylation of Stat3 and correlated with Src transformation. These findings demonstrate that Src can activate STAT signaling pathways and raise the possibility that Stat3 contributes to oncogenesis by Src.

The STATs are latent cytoplasmic transcription factors that, upon activation by tyrosine phosphorylation, translocate to the nucleus and bind to specific regulatory elements that control gene expression (*1*). Members of the cytokine receptor superfamily that lack intrinsic kinase activity recruit Janus kinase (Jak) family tyrosine kinases, which are required for cytokine activation of STAT proteins (*2*). Whether other nonreceptor kinases, such as Src family tyrosine kinases, might also activate STAT proteins has not been explored. Six STAT family members have been molecularly cloned (*3*). Although the roles of STAT proteins in mediating responses to cytokines and growth factors in normal cells are being delineated (*4*), the regulation of STAT signaling pathways by oncoproteins has not been described.

We prepared nuclear extracts from normal 3Y1, NIH 3T3, or BALB/c 3T3 rodent fibroblasts or those cells transformed with the v-Src oncoprotein (*5*). The extracts were incubated with a labeled high-affinity sis-inducible element (SIE) probe (*6*), and the resulting DNA-protein complexes were analyzed by an electrophoretic mobility shift assay (EMSA) for the STAT-related DNA-binding factors sis-inducible factor (SIF)–A, SIF-B,

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and SIF-C (*7*). For comparison, nuclear extracts were also prepared from 3T3-F442A cells stimulated with leukemia inhibitory factor (LIF), which induces formation of the SIF-A and SIF-B complexes predominantly (*8*), and from 3Y1 cells stimulated with interferon γ (IFN-γ), which induces formation of the SIF-C complex (*7*, *9*). A DNA-binding factor was constitutively induced in all of the v-Src–transformed cell lines (Fig. 1) as compared with their normal counterparts. This DNA-binding factor comigrated primarily with SIF-A and to a lesser extent with SIF-B. No DNA binding was detected in the presence of excess unlabeled SIE oligonucleotide, but binding was not completed by an unrelated DNA sequence, indicating that the binding was specific.

Because the DNA-binding factor in v-Src–transformed cells migrated similarly to SIF-A, which contains homodimers of Stat3 [also referred to as acute-phase response factor (APRF)] (*10*), the ability of this factor to bind the APRF recognition sequence, acute-phase response element (APRE) (*11*), was examined. The v-Src–transformed cells contained APRE-binding factor (Fig. 2A) that was specifically competed by unlabeled APRE and SIE oligonucleotides but not by an unrelated DNA sequence. Multiple bands were observed with the APRE probe (*8*, *12*). To determine whether the Src-induced DNA-binding complex contained Stat3-APRF protein, we performed a gel supershift assay with antiserum to Stat3 (anti-Stat3) (*12*). The SIE-binding complex in v-Src–transformed cells was supershifted by anti-Stat3 (Fig. 2B), whereas anti-Stat1 and nonimmune serum had no effect on the mobility. Similar results were obtained with a different antiserum to Stat3 or when APRE was used as a probe in this supershift assay (*13*). Anti-Stat1 supershifted SIF-C in normal 3Y1 cells stimulated with IFN-γ, and anti-Stat3 supershifted SIF-A in LIF-treated 3T3-F442A cells as expected. Thus, the DNA-binding activity induced in v-Src–transformed cells is attributable to either Stat3-APRF or a closely related protein.

Tyrosine phosphorylation of STATs is essential for their translocation from the cytoplasm to the nucleus and their DNA-binding activity (*7*, *14*). To determine whether Stat3 is phosphorylated in cells transformed by the v-Src tyrosine kinase, we prepared

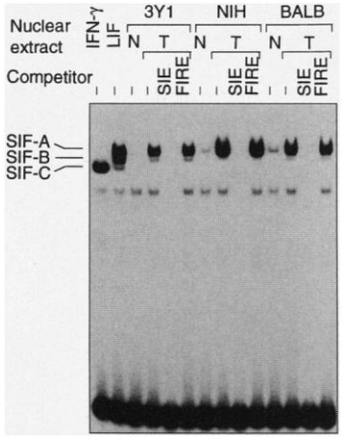

Fig. 1. Induction of SIE-binding activity in cells stably transformed by the viral Src oncoprotein. Nuclear extracts were prepared from exponentially growing normal (N) 3Y1, NIH 3T3 (NIH), BALB/c 3T3 (BALB) fibroblasts, their v-Src–transformed (T) counterparts, and cells stimulated with IFN-γ (10 ng/ml) or LIF (25 ng/ml) for 30 min (*9*). Nuclear extracts containing the same amounts of total protein were subjected to EMSA with 10 fmol of ^{32}P-labeled high-affinity SIE (m67) (*6*) probe (5′-AGCTTCATTTCCCGTAAATCCCTAAAGCT-3′) (*8*, *9*). Competitions were done in the presence of 1 pmol of unlabeled high-affinity SIE oligonucleotide or unlabeled oligonucleotide containing the *fos* intragenic regulatory element (FIRE, 5′-GTCCCCGGCCGGGGAGGCGCT-3′) (*18*). The positions of three SIF complexes (*7*, *10*) are indicated at left.

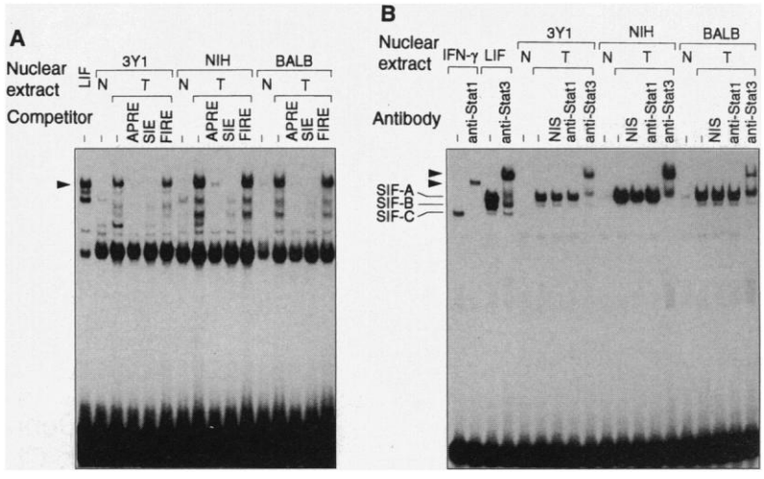

Fig. 2. Presence of Stat3-related protein in SIF complexes induced by v-Src transformation. (**A**) Nuclear extracts were prepared and then subjected to EMSA with a ^{32}P-labeled APRE probe (5′-GATCCTTCTGGGAATTCCTAGATC-3′) (*9*) in the absence or in the presence of unlabeled APRE, high-affinity SIE, or FIRE oligonucleotides as described (Fig. 1). The arrowhead on the left marks the position of specific APRE-binding activity which can be supershifted by anti-Stat3 antiserum (*13*). (**B**) Nuclear extracts were prepared and portions were incubated with 1:40 dilution of nonimmune serum (NIS), antiserum to Stat1 (anti-Stat1) (*19*), or antiserum to Stat3 (anti-Stat3) (AbC) (*12*) at room temperature for 20 min. Reaction mixtures were analyzed by EMSA with a ^{32}P-labeled high-affinity SIE probe. The positions of three SIF complexes are indicated at left; supershifted SIF-A and SIF-C complexes are denoted by the top and bottom arrowheads, respectively.

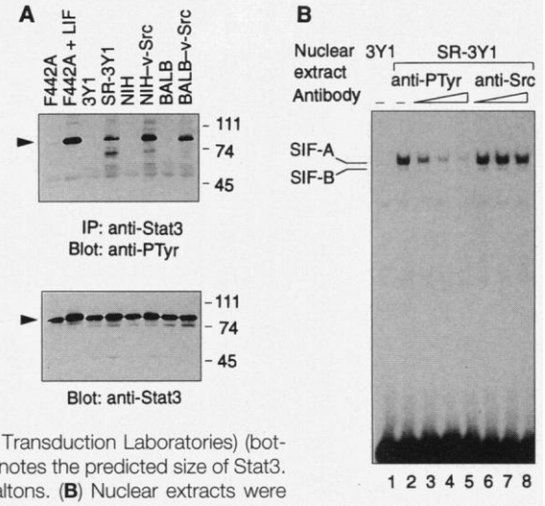

Fig. 3. Tyrosine phosphorylation of Stat3-related DNA-binding proteins in cells transformed with v-Src. (**A**) Cell lysates were prepared from exponentially growing normal 3Y1, NIH 3T3, BALB/c 3T3 fibroblasts, and their v-Src–transformed counterparts (*9*). Cell lysates also were prepared from 3T3-F442A cells either untreated or treated with LIF as described (Fig. 1). Proteins were immunoprecipitated (IP) with anti-Stat3 (1:125) (*12*) and resolved by SDS–polyacrylamide gel electrophoresis (3 to 10% gel) (*9*). The blot was first probed with monoclonal antibody to phosphotyrosine 4G10 (anti-PTyr, 0.2 μg/ml, Upstate Biotechnology) (top panel) and was then stripped and reprobed with monoclonal antibody to Stat3 (1 μg/ml, Transduction Laboratories) (bottom panel). The arrowhead on the left denotes the predicted size of Stat3. Sizes are indicated to the right in kilodaltons. (**B**) Nuclear extracts were prepared from normal 3Y1 and v-Src–transformed SR-3Y1 cells as described (Fig. 1). SR-3Y1 extracts were incubated with 1 μg (lanes 3 and 6), 2.5 μg (lanes 4 and 7), or 5 μg (lanes 5 and 8) of monoclonal antibody to either PTyr or Src (*20*), as indicated. Reaction mixtures were subjected to EMSA with a ^{32}P-labeled high-affinity SIE probe.

anti-Stat3 immunoprecipitates from cell lysates and analyzed them by protein immunoblotting with anti-Stat3 or antibody to phosphotyrosine. Although similar amounts of Stat3 were immunoprecipitated from both normal and v-Src–transformed cells (Fig. 3A), only the protein from v-Src–transformed cells contained increased amounts of phosphotyrosine. Stat3 was phosphorylated in 3T3-F442A cells stimulated with LIF (*8*). We did a gel shift assay after incubation of nuclear extracts with various amounts of monoclonal antibody to phosphotyrosine. The SIE-binding activity in v-Src–transformed cells was disrupted by the antibody to phosphotyrosine (Fig. 3B) but not by an irrelevant monoclonal antibody. Together, these results indicate that the DNA-binding complex induced in v-Src–transformed cells contains a tyrosine-phosphorylated protein.

We compared the SIE-binding factors from NIH 3T3 cells stably transformed by (i) normal cellular Src (c-Src) expressed in very high amounts, (ii) c-Src activated by a mutation of the critical regulatory Tyr^{527} to Phe, and (iii) Rous sarcoma virus v-Src (Fig. 4A). The amount of constitutive DNA-binding complex increased with increasing oncogenic potency of the Src proteins (normal c-Src being the least potent, the activated Tyr^{527} mutant being intermediate in potency, and v-Src the most potent) (*15*). Increasing amounts of DNA-binding complex also coincided with elevated levels of tyrosyl phosphorylation of Stat3 (Fig. 4B).

Our results establish that Stat3 or a closely related STAT family member is constitutively activated by the Src tyrosine kinase. Activation of Stat3 by Src may be mediated through direct or indirect mechanisms. Consistent with the possibility that Src kinase directly phosphorylates Stat3, v-Src activates Stat3 in a yeast genetic system (*16*). Alternatively, an indirect mechanism is suggested by the observation that Jak family kinases are activated in Src-transformed cells (*17*). It is possible that STAT proteins may contribute to transformation by diverse oncoproteins. Our finding that constitutive activation of Stat3 correlates with Src transformation raises the possibility that this STAT signaling pathway regulates expression of cellular genes that participate in oncogenesis.

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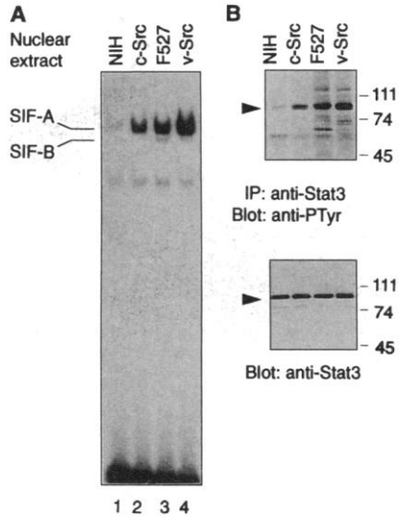

Fig. 4. Comparison of Stat3-related DNA-binding complexes in cells transformed by various Src proteins. (**A**) Nuclear extracts were prepared from normal NIH 3T3 cells (NIH), and NIH 3T3 cells transformed by overexpressed normal c-Src, an activated c-Src mutant with a $Tyr^{527} \rightarrow Phe^{527}$ substitution (Y527F), or Rous sarcoma virus v-Src (*5*) and then analyzed by EMSA with a ^{32}P-labeled high-affinity SIE probe (Fig. 1). (**B**) Cell lysates were prepared from the same cell lines, and proteins were immunoprecipitated with anti-Stat3 (1:125). Immunoprecipitates were subjected to immunoblotting with anti-PTyr or monoclonal antibody to Stat3 as described (Fig. 3). Sizes are indicated to the right in kilodaltons.

Interaction of a Tyrosine Kinase from Human Sperm with the Zona Pellucida at Fertilization

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A 95-kilodalton mouse sperm protein with characteristics of a protein tyrosine kinase has been identified as a receptor for ZP3, a glycoprotein in the egg's extracellular matrix. The structure of the human homolog was determined by screening an expression library from human testis; a testis-specific complementary DNA was isolated that encodes a protein similar to receptor tyrosine kinases and appears to be expressed only in testicular germ cells. Antibodies against a synthetic peptide from the intracellular domain recognized a 95-kilodalton human sperm protein that contains phosphotyrosine; human ZP3 stimulates the kinase activity of this sperm protein. Synthetic peptides corresponding to regions of the predicted extracellular domain inhibited sperm binding to human zona pellucida. Availability of the primary sequence of a receptor for ZP3 provides a rational starting point for sperm-targeted contraceptive development.

To fertilize an egg, mammalian sperm must first bind to the egg's extracellular matrix, the zona pellucida (ZP). Once bound, sperm undergo the acrosome reaction, an essential exocytotic event that permits a sperm to digest the ZP matrix and contact the egg. In mice, the glycoprotein ZP3 serves as both a ligand for sperm binding and a trigger for acrosomal exocytosis (*1*). Tyrosine phosphorylation in sperm appears to be important for these steps in fertilization, because inhibition of protein tyrosine kinase (PTK) activity prevents acrosomal exocytosis (*2*) and blocks fertilization. Several lines of evidence indicate that the 95-kD ZP3 receptor in mouse sperm is a mem-

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