Signal transduction of cytokine receptors is mediated by the JAK family of tyrosine kinases. Recently, the kinase partners for the interleukin (IL)-2 receptor have been identified as JAK1 and JAK3. In this study, we report the identification of splice variants that may modulate JAK3 signaling. Three splice variants were isolated from different mRNA sources: breast (B), spleen (S), and activated monocytes (M). Sequence analysis revealed that the splice variants contain identical NH2-terminal regions but diverge at the COOH termini. Analyses of expression of the JAK3 splice isoforms by reverse transcriptase-polymerase chain reaction on a panel of cell lines show splice preferences in different cell lines: the S-form is more commonly seen in hematopoietic lines, whereas the B- and M-forms are detected in cells both of hematopoietic and epithelial origins. Antibodies raised against peptides to the B-form splice variant confirmed that the 125-kDa JAK3B protein product is found abundantly in hematopoietic as well as epithelial cells, including primary breast cancers. The lack of subdomain XI in the tyrosine kinase core of the B-form JAK3 protein suggests that it is a defective kinase. This is supported by the lack of detectable autokinase activity of the B-form JAK3. Intriguingly, both the S and B splice isoforms of JAK3 appear to co-immunoprecipitate with the IL-2 receptor from HUT-78 lysate cells. This and the presence of multiple COOH-terminal splice variants co-expressed in the same cells suggest that the JAK3 splice isoforms are functional in JAK3 signaling and may enrich the complexity of the intracellular responses functional in IL-2 or cytokine signaling.

The JAKs\(^1\) are cytoplasmic tyrosine kinases with a unique structure consisting of two kinase domains lacking SH2 or SH3 motifs (1–5). Recently, members of this kinase family have been implicated in the signaling of a number of cytokine receptor superfamily members. For instance, JAK2 associates with the erythropoietin receptor (6), JAK1 interacts with TYK2 to signal in the interferon-\(\gamma\) pathway, and JAK1 associates with JAK2 to signal in the interferon-\(\gamma\) pathway (7, 8). These scenarios suggest that following ligand binding, receptor dimerization or oligomerization brings two JAK molecules into close proximity, resulting in their activation by tyrosine phosphorylation. Thus, JAK kinases may associate in a homologous manner with another JAK of the same kind, or heterologously with other family members. Once activated, the JAKs phosphorylate their associated receptors and cellular substrates, including a novel class of transcriptional activators called STAT proteins which transduce JAK signals after translocation to the nucleus. There appear to be specific STAT proteins that interact with the receptors that bind different JAK kinases. Taken together, the combinatorial interactions between the cytokine receptors and members of the JAK family appear to enhance the complexity of intracellular responses to related ligands.

Recently, we and others have cloned a new member of the JAK family, JAK3, and found that it is the IL-2 and IL-4 receptor-associated tyrosine kinase (9–11). This 120-kDa mouse JAK3 is highly homologous to the other JAK kinases, binds to the IL-2 receptor, and undergoes tyrosine phosphorylation upon IL-2 stimulation. Thus, the JAK3 kinase is predicted to be a signaling molecule central to immune function.

We now report the cloning and characterization of the human JAK3. We find that JAK3 exists as three splice variants resulting in proteins with different carboxyl termini. One variant was found to lack intrinsic tyrosine kinase activity, and may function to modulate JAK3 downstream signaling. Although JAK3 transcripts are mainly found in normal hematopoietic tissues, its expression is present in epithelial cell lines and primary cancers. Thus, JAK3 may have a role in epithelial cell biology in addition to its importance for lymphoid function.

EXPERIMENTAL PROCEDURES

Isolation of Human Jak3 cDNA—Based on sequences obtained from the 210-bp cDNA sequence of Jak3 (previously named TK5, Ref. 12), 5’ kinase-specific primers were synthesized and used in the 3’-RACE (Rapid Amplification of 3’ cDNA Ends, Life Technologies, Inc.) procedure as performed according to the manufacturer’s specifications. This resulted in the isolation of a 645-bp cDNA clone of human Jak3 gene from SKBR-3 cells, pR7. A 461-bp SKBR-3 human breast adenocarcinoma cDNA library (Clontech), a 198-bp human spleen cDNA library (Clontech), and a lambda-ZAP activated human monocyte cDNA library (kindly provided by Dr. H. Shelton Earp) were screened with the pR7 cDNA probe, labeled by random priming according to the published procedure (13). Approximately 1 \(\times\) 10\(^6\) recombinants from each library were screened and multiple overlapping cDNA clones were either subcloned into the pBluescript II vector (Stratagene) or excised with R408 helper
Human Jak3 Splice Variants

Preparation of Peripheral Blood Cell Populations and Bone Marrow Mononuclear Cells—Mononuclear cells were isolated from peripheral blood of two healthy donors using Histopaque 1077 (Sigma). Following Histopaque treatment, the red blood cell/granulocyte pellet was washed several times with a red blood cell lysing solution (0.1555 M NaCl, 0.01 M KCl, pH 7.0, and 0.1 mM NaN3/EDTA) to yield purified granulocytes. The bone marrow mononuclear cells from a healthy transplant donor were prepared by Ficoll-Paque (Pharmacia) treatment. Monocytes were separated from lymphocytes using two different methods: (i) adherence to plastic for 40 min or (ii) flow cytometry using an EPICS V system. Monocytes were reisolated by Ficoll-Paque (Pharmacia) treatment. Monocytes were confirmed by sequencing both strands of DNA with different primers and compressions were resolved by using 7-deaza-dGTP. Sequences were analyzed using the GCG program from the University of Wisconsin.

RNA Isolation and RT-PCR Amplification—Total cellular RNA was isolated by the RNAzol method (Cinna/Biotecx Laboratories, Inc.) according to the manufacturer's instructions. Approximately 10 μg of total RNA was first incubated with DNase enzyme at 37 °C for 1 h, extracted with phenol-chloroform, and ethanol precipitated. The DNase-treated RNA was then used as a template for cDNA synthesis. cDNA synthesis was performed as described (12, 14, 17) using SuperScript reverse transcriptase (Life Technologies, Inc.). For PCR amplification, the sense oligonucleotide primer used was 5'-GAGAGCGGAGCACTGCAA-3' corresponding to nucleotides 2967–2986 which is common among all splice variants of Jak3. The antisense oligonucleotide used in the blood and bone marrow expression experiments was 3'-NIF primer: 5'-CTGGCGGAGAGATGGTGGTC-3', corresponding to nucleotides 3096 to 3116 (a region common among the cell lines used in the RT-PCR, immunoblot analysis, and histoenzyme assays were obtained from the American Type Culture Collection and maintained according to the ATCC recommended medium. The lines of cell used are summarized as follows: BT-20, BT-474, MCF-7, SKBR-3, T-47D, and 600PEI are breast carcinoma cell lines; HeLa is a cervical carcinoma cell line; SW480 is a colon carcinoma cell line; Jurkat, MOLT-4, and HUT-78 are T lymphocyte leukemia cell lines, K562 is a chronic myelogenous leukemia cell line, and COS-7 is a SV-40 transformed monkey kidney cell line.

Immunoprecipitation and Western Blot Analysis—Cells (approximately 107 cells) were lysed in Nonidet P-40 buffer containing 50 μM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 25 μg/ml leupeptin, 25 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysates were shaken in the cold room for 10 min and clarified by centrifugation. The postnuclear supernatants were collected and incubated at a 1:100 dilution with JAK antibodies (UNC 36, o-mouse Jak3 and α-JAK families), a 1:1000 dilution with anti-phosphotyrosine antibodies (4G10, UBI), and a 1:250 dilution with monoclonal anti-human IL-2 receptor antibodies (UBI) at 4 °C for 4 h. The immunoprecipitates were then mixed with protein A-Sepharose beads at 4 °C for 1 h, washed, resolved on SDS-PAGE, and electrophoretically transferred to Immobilon-P PVDF membranes (Millipore). The blots were then hybridized with the appropriate antibodies. For Western blot, the proteins were prepared in a similar way, except that 50 μg of total protein from the lyses were resolved by 8% SDS-PAGE. For peptide competition, the membrane was incubated with antibody against human B-form Jak3 peptides in the presence of excess antigenic peptides (10 μg/ml) from the peptides used for competition. The immunoblot was subsequently incubated with the appropriate horseradish peroxidase-coupled sheep anti-rabbit IgG or anti-mouse IgG for detection with enhanced chemiluminescence reagents (ECL; Amersham).

Human primary breast cancers and their matched normal tissue counterparts were obtained through the Tissue Procurement Facility of the UNC SPORE in breast cancer. Tissues were homogenized and disrupted in lysis buffer as described above. 50 μg of the total cell lysate were electrophoresed and subjected to Western blot analysis using the a-β-JAK3B antibody (UNC 36) at a dilution of 1:3,000.

For IL-2 stimulation studies intended for immunoprecipitation and immunoblot analysis, stimulation was done as described previously (9, 10). After 10 min at 37°C, a 1:50 dilution of the anti-Jak3 antibody was added to create pcDNA-moJak3. A Novo-Sol partial restriction digest of mouse Jak3 yielded a 3-kb fragment (encoding regions homologous to human Jak3 from amino acids 1–1018), which was ligated to the different β-Jak3 isoforms: Snc1-XbI fragments from amino acid 1019 to 1124, amino acid 1019 to 1109 corresponding to the hJak3B and hJak3S isoforms, respectively, and a Soc1-XbsI fragment from amino acid 1019 to 1056 corresponding to the hJak3M isoform. The ligation products were then cloned into the appropriate sites of pcDNA3 (Novl-XbI for hJak3B and hJak3S, and Novl-XpsI for Jak3M), resulting in chimERIC expression plasmids—ApcDNA3 eukaryotic expression vector (Invitrogen) for mouse Jak3 and mouse Jak3K. The expression vector was then transfected into 293T cells. The various oligonucleotide primer pairs were sensitive in RT-PCR analyses but did not PCR amplify using genomic DNA because of the presence of introns between the 5′-Jak3 and the different antisense primers. PCR was run for 33 cycles (freshly isolated hematopoietic populations), 35 cycles (FACS sorted T and B lymphocytes), and 36 cycles (different transformed cell lines). An annealing temperature of 60 °C was used for the reaction. To determine the expression pattern of Jak3 splice variants, 68 °C was used to analyze the expression pattern of Jak3 splice variants. Each PCR reaction contained a reverse transcriptase negative control to rule out any genomic amplification, and a no-template control. All PCR results were repeated a minimum of three times. To normalize the RNA levels of RT-PCR, α-actin primers (12, 14, 17) were used for 23 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The PCR products were analyzed by electrophoresis on a 1.0 or 1.5% agarose gel containing ethidium bromide. In Northern Blot Analysis—Human multiple tissue Northern blots (Clonetch) were probed with the EcoRI-restricted DNA fragment from pR7 labeled by random priming. The pR7 probe was identical to the cDNA and genomic libraries probe which spanned the COOH-terminal kinase domain. Hybridization was performed in 50% formamide, 5 × SSPE at 42 °C. Filters were given final washes at 68 °C in 0.5 × SSPE and 0.5% SDS.

Antibodies—Synthetic peptides corresponding to the carboxy-terminal amino acid sequence of human B-form Jak3 variant (SVSQRolis GAGYSQPAPA) were keyhole limpet hemocyanin by gluthalionylated and used for immunization into New Zealand female rabbits. Antibodies (UNC36) were purified using the ImmunoPure(A/G) IgG purification kit from Pierce according to the manufacturer's instructions. Antibodies to mouse Jak3 and Jak3K were purchased from Dr. James Ihle (9), antibodies to human S-form Jak3 was purchased from Santa Cruz Biotechnology, Inc. (CA), whereas anti-phosphotyrosine (4G10) and monoclonal anti-human IL-2 receptor antibodies were purchased from UBI (New York). The α-murine Jak3 antibodies were raised against amino acids 966–982 (kinase subdomain VII, Ref. 9), the α-JAK antibodies were generated against amino acids 838 (9), and the human S-form Jak3 antibody were raised against the COOH terminus of the Jak3B protein (residues 1105–1124, Ref. 10). Anti-HA monoclonal antibody (clone 12C5A) were purchased from Boehringer Mannheim, which recognizes the HA peptide sequence (YPYDVPDYA).
clones of mouse Jak3 and human Jak3 isoforms designated pc3HA-Jak3B, pc3HA-Jak3S, and pc3HA-Jak3M (Fig. 6A). All chimeras were sequenced through the junctions to confirm that no introduced deletions or mutations were introduced during the subcloning steps.

**Transient Transfection of COS-7 Cells—**3 μg of plasmid DNA was introduced into COS-7 cells by transient transfection with Lipofectamine reagent as described by the manufacturer (Life Technologies, Inc.). After 60 h, cells were harvested and lysed in Nonidet P-40 buffer. Protein concentrations were determined by the Bradford (35) (Bio-Rad) method. Approximately 300 μg of total cellular protein was isolated for immunoprecipitation with anti-HA monoclonal antibodies (Boehringer Mannheim) at a dilution of 1:5000. Immunoprecipitates were isolated using protein A-Sepharose, and immunoblots were probed as described by the manufacturer. In vitro kinase assays were performed as described by Witthuhn et al. (6).

**RESULTS**

**Cloning and Characterization of Jak3 cDNA—**In order to isolate a full-length human Jak3 cDNA sequence, PCR amplification and cDNA library screening approaches were pursued. Using the 3′-RACE method, a cDNA clone called p3R7 extending from the Jak3 kinase domain to the poly(A) tail was isolated and both nucleotide and predicted protein sequence revealed a high homology with the published JAK kinase family sequences. Then a 645-bp cDNA clone (p3R7) was used as a probe to screen two other libraries, one from human activated T-lymphocytes (CD3+ and CD19), but at low levels in B lymphocytes (Fig. 2A), and the other from lymphoid tissues and cell lines (Fig. 2B). These discrepancies are not sequencing ambiguities since all clones were sequenced from both directions, and some were confirmed by multiple cDNA clones.

**Analysis of Putative Jak3 Protein Structure—**Comparison of the predicted amino acid sequence of the JAK family members revealed that the human Jak3 gene is most closely related to murine Jak2, showing 69% amino acid sequence identity followed by Jak1, showing 62% amino acid sequence identity, and Tyk2, showing 60% amino acid sequence identity. The three isoforms encode a 1094-, 1124-, and 1131-amino acid polypeptide with calculated molecular weights of 121,434, 124,764, and 125,541 corresponding to B-form, S-form, and M-form Jak3 proteins, respectively. The deduced primary protein structures reveal features typical for the JAK kinase family: a 518-amino acid NH2 terminus followed by two kinase domains with the COOH-terminal domain possessing catalytic function. In addition, a pair of tyrosine residues at amino acid positions 980-981 as well as the FWYAPE motif in subdomain VIII at amino acid positions 992-997, which so far have been only found in the JAK kinase family, were also observed (12). Subdomain VI, HRDLAA at amino acid positions 947 to subdomain IX, DVW, are tyrosine kinase specific motifs originally used in our design of the targeted degenerate oligonucleotides for identification of the Jak3 sequence (12). Analysis of the COOH-terminal kinase domain showed that the M and S splice isoforms harbor the 11 major conserved kinase subdomains (I-XI); however, the B-form Jak3 variant lacks a recognizable subdomain XI characterized by the arginine at position 1085 for the S-form and position 1092 for the M-form that is invariant among the tyrosine kinases (Fig. 1B). Highly conserved individual amino acids within the catalytic domain are expected to play important roles in kinase function since deletions within subdomain XI of the EGF receptor abolish kinase activity (21, 22). Thus, the absence of an intact subdomain XI in the B-form Jak3 suggests that this isoform lacks kinase activity.

**Expression of Human Jak3 mRNA—**Tissue expression of human Jak3 was determined by probing human multiple tissue Northern blots using p3R7 random-primed radiolabeled cDNA. Fig. 2A shows that two major transcripts of sizes 7.6 and 5.8 kb were detected in normal blood, spleen, and thymus, but a 4.8-kb transcript was detected at low levels in the spleen. These results confirm that Jak3 is normally expressed predominantly in hematopoietic tissues. By RT-PCR, we sought to determine the expression of Jak3 in primary hematopoietic cells fractionated by FACs. Fig. 2C indicates that Jak3 is expressed in the bone marrow and in all hematopoietic populations derived from the peripheral blood including monocytes, granulocytes, and T-lymphocytes (CD3+CD19-), but at low levels in B lymphocytes (CD3-CD19+).

In transformed cell lines, however, Northern blot analysis (Fig. 2B) revealed that Jak3 is expressed more widely in lymphoblastic leukemia MOLT-4, Burkitt’s lymphoma Raji, colorectal adenocarcinoma SW480, lung carcinoma A549, and melanoma G361 cell lines. This confirms our earlier expression data that Jak3 is expressed in epithelial cell lines. In addition, the Jak3 transcripts in epithelial cancer cell lines are smaller than those in lymphoid tissues and cell lines (Fig. 2, A and B). These data suggest that complex splicing of the Jak3 gene is involved which may have significance in epithelial cell biology. All RNA samples were normalized by either hybridization with a β-actin probe (data not shown) or RT-PCR with α-actin primers to ensure that comparisons were made according to the same amount of input RNA.

**Identification of Jak3 Splice Variants—**We noticed that our original Jak3 cDNA clone had COOH-terminal sequences that dramatically differed from the published human and mouse sequences (9, 10), suggesting rearrangements or splice variants. Our subsequent screening of breast (B), spleen (S), and activated monocytes (M) cDNA libraries identified a total of three distinct cDNA clones with different 3′ termini: S-form, M-form, and B-form. The fact that all clones were identical...
**Human Jak3 Splice Variants**

**A**

| Sequence of the human Jak3 variants. A, complete nucleotide sequence and predicted amino acid sequence of the human Jak3 variants. The deduced amino acid sequence is shown below with the nucleotide sequence. The ATG found in the most 5' end of the open reading frame was assigned as an initiation site for translation. The nucleotide and amino acid differences from the published L-Jak cDNA sequences are indicated (11). Splice variants diverge at amino acid position 1070. > represents the stop codon and two AATAAA polyadenylation signals are underlined. B, comparison of the COOH-terminal sequence motifs for subdomains X and XI among all known JAK3 kinases with EGF receptor. Boxed residues represent the consensus sequences in subdomains X and XI. |
|---|

**B-HJAK3** represents human B-form Jak3, **M-HJAK3** represents human M-form Jak3, **S-HJAK3** represents human S-form Jak3, and **EGFR** represents epidermal growth factor receptor. Sequences used to make the comparisons were derived from the following sources: L-JAK (11), mouse JAK3 (1MJAK3, Ref. 9), rat JAK3 (RJAK3, Ref. 19), second mouse JAK3 (2MJAK3, Ref. 20), and EGFR (18). Boxed residues represent the consensus sequences in subdomains X and XI.
until amino acid 1070 where the sequences diverged (Fig. 1) suggested that these represented splice variants. As shown in Fig. 1B, the S-form Jak3 (S-HJAK3) is identical to the published human Jak3 (L-JAK, Ref. 11) sequences. The M-form (M-HJAK3) is related to the S-form only by the presence of the Trp and Arg residues common to subdomain XI of the tyrosine kinases. However, as mentioned above, the B-form Jak3 (B-HJAK3) lacks subdomain XI of the COOH-terminal catalytic domain.

To confirm that these different forms represent splice site variants, we isolated a genomic clone of Jak3. This clone

FIG. 3. RT-PCR analysis of Jak3 splice variant expression in cell lines. The ethidium bromide-stained agarose gels show Jak3 variants and Jak3 kinase domains amplified with a common 5′-Jak3 primer, but with 3′-primers specific for each splice variant: 3′-bJak3 primer specific for B-form Jak3 splice variant was used to detect Jak3B transcripts; 3′-sJak3 primer specific for S-form Jak3 splice variant was used to detect Jak3S transcripts; and 3′-mJak3 primer specific for M-form Jak3 splice variant was used to detect Jak3M transcripts. Whereas, the common 3′-Jak3 primer was used for Jak3 kinase domain to detect the expression of all three Jak3 isoforms. The fragment sizes are 404 bp (B-form), 588 bp (S-form), 388 bp (M-form), and 240 bp (common Jak3 kinase domain). A 201-bp α-actin band was amplified in parallel from the same template as a reference band.

FIG. 4. Immunoblot analysis of human B-form JAK3. A, to test antibody specificity, total cell lysates from K562, Jurkat, BT-474, and T-47D cell lines were triple loaded and resolved on a 8% SDS-PAGE. They were electroblotted onto PVDF membrane and immunoblotted with preimmune serum, UNC36, anti-peptide antibody to B-form JAK3 (amino acids 1,077–1,094), or UNC36 in the presence of the immunogenic peptide (10 μg/ml) to which the antiserum was raised. JAK3B is seen as a single 125-kDa band. B, expression of JAK3B in paired-samples of breast tumors. 50 μg of total cell lysates from 5 primary human breast cancers (T) and their match normal tissue (N) counterparts were used. Equal amounts of BT-474 and T-47D total cell lysates were also run in parallel for the purpose of serving as control samples. In three of five samples, JAK3B is overexpressed in tumor tissues.

FIG. 2. Expression of human Jak3 transcript. Northern blot analysis of Jak3 mRNA expression in: A, normal human adult tissues and, B, human transformed cell lines. The two Northern blots were obtained from Clontech Labs. Each lane contains 2 μg of poly(A)+ RNA. The cell lines represented are: MOLT-4, lymphoblastic leukemia cell line; Raji, Burkitt’s lymphoma cell line; SW480, colorectal adenocarcinoma cell line; A549, lung carcinoma cell line; and C361, melanoma cell line. C, RT-PCR analysis of Jak3 expression in normal bone marrow. The ethidium bromide-stained agarose gel indicates the Jak3 fragment and a 201-bp α-actin band as a reference band for amount of template in each reaction. PBMC, peripheral blood mononuclear cells; CD3+CD19+ and CD3+CD19− lanes are from sorted lymphocytes (see “Experimental Procedures”).

Trp and Arg residues common to subdomain XI of the tyrosine kinases. However, as mentioned above, the B-form Jak3 (B-HJAK3) lacks subdomain XI of the COOH-terminal catalytic domain.

To confirm that these different forms represent splice site variants, we isolated a genomic clone of Jak3. This clone
showed the splice donor to be GCTGAG that encodes for amino acids 1068 and 1069 (alanine and glutamine) and that the splice donor is GCTGAG that encodes for alanine and glutamine. 1068 and 1069 (alanine and glutamine) and that the splice donor is GCTGAG that encodes for alanine and glutamine.

Fig. 5. Immunoprecipitation and immunoblot analysis of JAK3. A, a human JAK3 protein was immunoprecipitated from BT-474 total cell lysates with different sources of JAK3 antisera, resolved by 8% SDS-PAGE, electrophorseted, and probed with anti-phosphotyrosine (G410) antibody. B, JAK3B is linked to IL-2R. Total cell lysates from untreated HUT-78 T-cells were immunoprecipitated with normal preimmune rabbit serum or a monoclonal anti-JAK3 antibody (UNC36). The results indicate that JAK3B is co-immunoprecipitated with the IL-2R.

JAK3B Is a Kinase-deficient Form of the JAK Kinase That Complexes with JAK3S—The structure of the JAK3B kinase isoform shows that, unlike the S- and M-forms, this splice variant removes the kinase subdomain XI (Fig. 1B) characterized by a consensus motif CW(X6)RP (the underlined residues being invariant; Ref. 18) and substitutes in sequences of unknown significance. Deletions in the EGFR receptor within this domain are demarcated by residues 920–951 have been shown to eliminate kinase activity (data not shown). Because of its significant expression in breast cancer cell lines, we asked whether JAK3B could be detected in primary breast cancers. In five matched pairs of normal breast and breast cancers, we found augmented JAK3B expression in three tumors with absent expression in the normal breast epithelium (Fig. 4B). In two tumors, the level of JAK3B expression was equivalent to that found in BT-474 known to be a high expressor of JAK3B. Thus, the JAK3B isoform of JAK3 is expressed at significant levels in both epithelial cell lines and in primary breast carcinomas.

Characterization of the B-form JAK3 Protein—By homology with the mouse JAK3, the S-form of the human JAK3 is predicted to directly interact with the IL-2 and IL-4 receptors and is involved in the IL-2/IL-4 signaling response. In order to determine whether the other splice forms are translated and whether they are involved in IL-2 signaling as well, a monoclonal antiserum was raised against the B-form COOH-terminal peptide at amino acid positions 1077 to 1094. The B-form was chosen for study because it represented the most divergent sequence from the JAK consensus. As shown in Fig. 4A, the anti-B-form JAK3 antiserum readily recognized a 125-kDa protein in the K562, BT-474, and Jurkat cell lines that also express Jak3 transcripts. Conversely, T-47D, which has no detectable Jak3 mRNA by Northern blot analysis (data not shown), has no detectable JAK3B protein. Antibody binding by UNC36 (a-hJAK3 B form antibody) could be competed by the immunogenic peptide, attesting to the specificity of this antiserum (Fig. 4A).

To confirm that UNC36 recognized a JAK protein, the a-mouse JAK3 and a-JAK family antibodies (9) were used to immunoprecipitate BT-474 cell lysates and immunoblotted with either anti-phosphotyrosine (4G10) or B-form JAK3 antibody (UNC 36). The results show that the anti-B-form antiserum, UNC36, specifically recognizes a JAK3 protein immunoprecipitated by these two anti-JAK antibodies. In addition, the 125-kDa JAK3 protein in unstimulated HUT-78 cells immunoprecipitated by an anti-IL-2 receptor monoclonal antibody was recognized by anti-B-form JAK3 antibodies confirming that JAK3B is bound to the IL-2 receptor (Fig. 5B).

Using the various JAK3-specific antibodies, we noticed that the JAK3B protein migrated more quickly than the JAK3B isoform: JAK3S at 116 kDa and JAK3B at 125 kDa despite the calculated molecular mass of the S-form (125 kDa) being slightly larger than the B-form (121 kDa). These differences may account for the mobility shift.

Western blot and found JAK3B to be expressed in SKBR-3, HeLa, SW480, A549, HUT-78, and MOLT-4 cell lines (data not shown). Because of its significant expression in breast cancer cell lines, we asked whether JAK3B could be detected in primary breast cancers. In five matched pairs of normal breast and breast cancers, we found augmented JAK3B expression in three tumors with absent expression in the normal breast epithelium (Fig. 4B). In two tumors, the level of JAK3B expression was equivalent to that found in BT-474 known to be a high expressor of JAK3B. Thus, the JAK3B isoform of JAK3 is expressed at significant levels in both epithelial cell lines and in primary breast carcinomas.

2 K. S. Lai and E. T. Liu, unpublished data.
above), raising the possibility that any of the cloned human Jak3 cDNAs may have altered enzymatic function. Furthermore, immunoprecipitations directed against the HA epitope were used to eliminate the possibility that antibody binding to the COOH-terminal sequences would alter kinase activity. Lysates of COS-7 cells transfected with the JAK3S, -M, and -B chimeras were immunoprecipitated with anti-HA antibodies and subjected to in vitro kinase assays and immunoblot analysis. In vitro kinase activity detected the autophosphorylation of JAK3 isoforms and mouse JAK3 by autoradiography. The level of protein expression was determined by probing with α-HA.

**DISCUSSION**

Cytokines function through receptors of the cytokine receptor superfamily which include receptors for the interferons, IL-3, IL-6, erythropoietin, growth hormone, prolactin, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor. These ligand-receptor associations are further related by their use of a novel subfamily of cytoplasmic tyrosine kinases, called the Janus kinases (JAKs) to affect their intracellular signaling. Structurally, the JAK kinases are unique because of the presence of two kinase domains, an NH2-terminal (JH2) domain which has unknown enzymatic function followed by a COOH-terminal kinase domain (JH1) with catalytic activity. In addition, the JAKs harbor no SH2 or SH3 domains and contain a signature FWY-AP(E) motif which is not found in other tyrosine kinases. Recently, we and others have reported a new member of the JAK family, murine JAK3, as the kinase involved in IL-2 and the same protein complex.

By contrast, however, we found that the immunoprecipitated JAK3B was noticeably tyrosine phosphorylated in the breast cancer cell lines BT-474 as determined by anti-phosphotyrosine Western blot analysis (Fig. 5A, lane 3). BT-474 cells do not express JAK3S as assessed by RT-PCR and Western blot (Fig. 3, and data not shown), putatively lack IL-2 receptors, but express JAK3M by RT-PCR. This suggests that JAK3B may be phosphorylated by another kinase, potentially JAK3M, which may be activated by yet an unknown receptor.
IL-4 signaling (9, 10). To further examine the biology of JAK3 in human cells, we sought to clone human Jak3 cDNAs.

During this effort, we uncovered three splice variants that result in distinct COOH termini all starting at amino acid 1070. The S-, M-, and B-forms appear to be expressed in most cell lines as assessed by RT-PCR but at differing ratios from tissue to tissue. The S-form is the Jak3 sequence previously published as the signaling component of the IL-2 receptor in lymphoid cells, and in our analysis appears to be expressed predominantly in hematopoietic cell lines. The B- and M-forms, however, have a wider expression profile, being detected in cell lines derived from hematopoietic, and epithelial tissues (Fig. 3). Some cell lines express only the B- and M-forms (e.g. BT-474), or only the B-form Jak3 transcripts (e.g. SKBR-3).

Predicted protein sequences of the COOH-terminal kinase domains of the Jak3 splice isoforms reveal that they represent alterations at subdomain XI of the Jak3 kinase. The B-form Jak3 is predicted to lack a recognizable subdomain XI. A protein sequence data base search indicated that the amino acid sequence at the COOH terminus of the B-form Jak3 shows no homology with any other known proteins. We have identified the genetic basis for the generation of the Jak3 splice variants by analyzing Jak3 genomic clones; whereas the S-form and M-form Jak3 are the result of differential splicing using GCTGAG, encoding for amino acids 1068 and 1069 (alanine and glutamine), as the splice donor, the B-form Jak3 does not use this splice donor and generates a read-through transcript.

Although transcribed, it is possible that these splice variants would not be translated. To prove that a functional protein product is generated, we raised antibodies specific to the COOH terminus of the most divergent of the splice isoforms, Jak3B, and found that in cell lines known to express the Jak3B transcript, a 125-kDa protein could be detected by Western blot analysis which can be competed by excess antigenic peptides (Fig. 4A). Immunoprecipitations using Jak3 antibodies raised against non-COOH-terminal residues recognized the Jak3B-specific isoform (Fig. 5A), suggesting that the 125-kDa immunoreactive band is indeed a Jak3 protein. In addition, Jak3B co-precipitates with the IL-2 receptor in unstimulated cells much like the previously described associations between Jak3S/L-Jak and the IL-2 receptor (23). Thus, the Jak3 3′-splice variants can be translated into functional proteins.

Previous studies have demonstrated the importance of the protein tyrosine kinase COOH terminus in substrate selection and in the control of kinase activity (24–28). COOH-terminal sequences can act intramoleculately to regulate intrinsic kinase activity. 3′-Truncations of c-src sequences through retroviral transduction or sequestration of the src COOH-terminal peptides by polymya middle T result in kinase activation and induction of src’s transforming potential. In addition, the COOH-terminal tail domain of the EGF receptor is the site of substrate recruitment. Phospholipase C-γ and Grb2 have been shown to interact via their SH2 domains with the autophosphorylated COOH-terminal tail of activated EGF receptor (29–31). Similarly, the COOH terminus of nerve growth factor/Trk receptor tyrosine kinase appears to be involved in receptor-substrate interaction. Deletion of the 15 COOH-terminal amino acids abrogated Trk receptor and phospholipase C-γ substrate phosphorylation activities (32). Since the level of expression in different cell types and the changes in an important kinase structural subdomain are seen for Jak3 splice variants, we suggest that expression of these isoforms may have significant functional consequences.

The B-form of Jak3 is of particular interest because its sequence shows an absence of a recognizable kinase subdomain XI consensus. Earlier structure-function analyses of the EGF receptor showed that COOH-terminal deletions to amino acid residue 944 completely abolished its kinase activity (21, 22). The EGF receptor subdomain XI has been described to reside between amino acids 920 and 951 with its subdomain XI consensus motif at residues 927–934. Since Jak3B represents a more drastic change in this region, we suspected that Jak3B would lack kinase activity. Our results confirm this hypothesis in that neither immunoprecipitated Jak3B nor a recombinant Jak3B expressed in COS-7 cells show any autokinase activity. Furthermore, the Jak3B protein that co-precipitates with the ligand-stimulated IL-2 receptor is not significantly tyrosine phosphorylated as compared to the Jak3S isoform (Fig. 7, A and C). These data raise the possibility that Jak3B may function as a transdominant negative in Jak3 signaling. The current model of IL-2 receptor and Jak interactions shows the IL-2R β-chain recruits Jak1 and the IL-2R γ-chain binds Jak3 (23, 33), and that the co-activation of Jak1 and Jak3 in the IL-2 receptor αβγ complex is necessary to transduce IL-2 signals. The competition between a kinase active (Jak3S) and a kinase-defective (Jak3B) Jak3 may attenuate the IL-2 responses downstream of Jak3.

Alternatively, the three Jak3 isoforms may function to enrich the complexity of IL-2 signaling by recruiting different intracellular proteins and substrates. The function of a kinase-deficient dimerization partner is still unclear, but has precedence in the EGF receptor family of receptor tyrosine kinases. c-erbB3 is homologous to c-erbB4 but is unique among the kinases in that its wild-type sequence in the catalytic domain predicts for an enzymatically deficient tyrosine kinase. Confirming this prediction is the finding that although baculovirus expressed p180c(erbB3) was able to bind its ligand, neu differentiation factor, it showed no autophosphorylation and kinase activities (34). Despite its deficient enzymatic function, p180c(erbB3) is involved in heterodimerization with EGF receptor, p180c(erbB4), or p185c(erbB) and is tyrosine phosphorylated in these complexes. In cells co-expressing the EGF receptor and p180c(erbB3), the signaling protein phosphatidylinositol 3-kinase is recruited to the heterodimeric complex only by the p180c(erbB3) component. Thus, it is possible that Jak3B may function in an analogous fashion.

Despite the evidence for the absence of kinase activity in the Jak3B isoform, several intriguing possibilities are raised by our results. Immunoprecipitations of Jak3B from HUT-78 cell lysates (which contain all Jak3 isoforms) show no tyrosine phosphorylation even after IL-2 treatment; however, in the breast cancer cell line, BT-474 (which expresses only the B- and M-forms), the Jak3B appears constitutively tyrosine phosphorylated. This suggests that other kinases or other receptors may phosphorylate Jak3B. In addition, our co-precipitation data shows that the S- and B-forms of Jak3 can potentially form a ternary complex with the IL-2 receptor in HUT-78 cells (Figs. 5B and 7, B and C). If verified, this finding changes the current model of IL-2 signaling that describes only binary Jak1 and Jak3S interactions.

In this paper, we describe the cloning of human Jak3 cDNAs. Sequence comparison between our human Jak3S sequence and the recently published human Jak3 cDNA (previously called L-Jak) revealed a number of nucleotide discrepancies resulting in eight non-conservative amino acid changes. Since both cDNA sequences were derived from multiple cDNA libraries and from transformed (HUT-78, YT, Jurkat, and SKBR-3 cell lines) and non-transformed cells (spleen, activated monocyte, and phytohemagglutinin-activated T cells), these discrepancies may be due to somatic mutations, polymorphisms, and mutational artifacts engendered during the reverse transcriptase
transfecting Jak3 cDNAs should be interpreted with caution, these sequence variants are elucidated, biological studies step, or a combination of all three. Until the significance of preparation.

thank Edward Baptist for helpful assistance in the manuscript preparation.

REFERENCES

1. Firmbach-Kraft, I., Byers, M., Shows, T., Dalla-Favera, R., and Krolewski, J. (1990) Oncogene S, 1529–1536
2. Wilks, A. F., Harpur, A. G., Kuran, R. R., Ralph, S. J., Zurcher, G., and Ziemiecki, A. (1991) Mol. Cell. Biol. 11, 2057–2065
3. Harpur, A. G., Andres, A-C., Ziemiecki, A., Aston, R. R., and Wilks, A. F. (1992) Oncogene 7, 1347–1353
4. Silvennoinen, O., Wittuhnu, B. A., Quelle, F. W., Cleveland, J. L., Yi, T., and Ihle, J. N. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 8429–8433
5. Ihle, J. N., Wittuhnu, B. A., Quelle, F. W., Yamamoto, K., Thierfelder, W. E., Kreider, B., and Silvennoinen, O. (1993) Trends Biochem. Sci. 19, 222–227
6. Wittuhnu, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tung, B., Miura, O., and Ihle, J. N. (1993) Cell 74, 227–236
7. Watling, D., Guschin, D., Muller, M., Silvennoinen, O., Wittuhnu, B. A., Quelle, F., Rogers, N. C., Schindler, C., Stark, G. R., Ihle, J. N., and Kerr, I. M. (1993) Nature 366, 129–135
8. Muller, M., Bricse, J., Laxton, C., Guschin, D., Ziemiecki, A., Silvennoinen, O., Harpur, A. G., Barbieri, G., Wittuhnu, B. A., Schindler, C., Pellegrini, S., Wilks, A. F., Ihle, J. N., Stark, G. R., and Kerr, I. M. (1993) Nature 366, 166–170
9. Hara, R., Shinagawa, Y., Sugihara, K., Oishi, I., Silvennoinen, O., Witthuhn, B. A., Silvennoinen, O., Wilks, A. F., Ihle, J. N., Stark, G. R., and Kerr, I. M. (1993) Nature 366, 129–135
10. Johnston, J. A., Kawamura, M., Kirken, R. A., Chen, Y-Q., Blake, T. B., Shibuya, K., Ornato, J. R., McVicar, D. W., and O'Shea, J. J. (1993) Nature 370, 151–153
11. Kawamura, M., McVicar, D. W., Johnston, J. A., Blake, T. B., Chen, Y-Q., Lal, B. K., Lloyd, A. R., Kelin, D. J., Staples, J. E., Ornato, J. R., and O'Shea, J. J. (1993) Proc. Natl. Acad. Sci. U.S.A. 91, 6374–6378
12. Canoe, W. G., Craven, R. J., Weiner, T. M., and Liu, E. T. (1993) Int. J. Cancer 54, 571–577
13. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13
14. Graham, D. K., Dawson, T. L., Mullaney, D. L., Snodgrass, H. R., and Earp, H. S. (1984) Cell Growth & Differ. 5, 647–657
15. Streuli, M., Hall, L. R., Sag, Y., Schlossman, S. F., and Saito, H. (1987) J. Exp. Med. 166, 1548–66
16. Thomas, M. L. (1989) Annu. Rev. Immunol. 7, 339–369
17. O'Bryan, J. P., Fyre, R. A., Cosgale, P. C., Neubauer, A., Kitch, B., Prokop, C., Kipps, R., III, Le Beau, M. M., Earp, H. S., and Liu, E. T. (1991) Mol. Cell. Biol. 11, 5016–5031
18. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241, 42–52
19. Takahashi, T., and Shirasawa, T. (1994) FEMS Lett 542, 124–128
20. Rane, S. G., and Reddy, E. P. (1994) Oncogene 9, 2418–2423
21. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241, 42–52
22. Russell, S. M., Johnston, J. A., Neguchi, M., Kawamura, M., Bacon, C. M., Friedmann, M., Berg, M., McVicar, D. W., Wittuhnu, B. A., Silvennoinen, O., Goldman, A. S., Schmalstieg, F. C., Ihle, J. N., O'Shea, J. J., and Leonard, W. J. (1994) Science 266, 1042–1045
23. Ulrich, A., and Schlessinger, J. (1990) Cell 51, 203–212
24. Schlessinger, J., and Ulrich, A. (1994) Neuron 9, 383–391
25. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltos, S. (1991) Cell 64, 281–302
26. Glass, D. J., and Yancopoulos, G. D. (1993) Trends Cell Biol. 3, 262–268
27. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pavson, T. (1991) Science 252, 668–674
28. Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D. B., Zilberstein, A., Ulrich, A., Pavson, T., and Schlessinger, J. (1990) EMBO J. 9, 4355–4360
29. Rotin, D., Margolis, B., Mohammadi, M., Dai, Y., Gaia, G., Li, N., Fischer, E. H., Burgess, W. H., Ulrich, A., and Schlessinger, J. (1992) EMBO J. 11, 559–567
30. Lemmon, M. A., Ladbury, E. J., Mandiyan, V., Zhou, M., and Schlessinger, J. (1994) J. Biol. Chem. 269, 31653–31658
31. Obermeier, A., Halfter, H., Wiesmuller, K-H., Wiesmuller, H., Schlessinger, J., and Ulrich, A. (1990) EMBO J. 9, 933–941
32. Miyazaki, T., Kawahara, A., Fujii, H., Nakagawa, Y., Minami, Y., Liu, Z-J., Oishi, I., Silvennoinen, O., Wittuhnu, B. A., Ihle, J. N., and Taniguchi, T. (1993) Cell Growth & Differ. 4, 339–369
33. O'Bryan, J. P., Fyre, R. A., Cosgale, P. C., Neubauer, A., Kitch, B., Prokop, C., Kipps, R., III, Le Beau, M. M., Earp, H. S., and Liu, E. T. (1991) Mol. Cell. Biol. 11, 5016–5031
34. Carraway, K. L., III, and Cantley, L. C. (1994) Cell 78, 5–8
35. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254