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Stat4, a Novel Gamma Interferon Activation Site-Binding Protein Expressed in Early Myeloid Differentiation

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Interferon regulation of gene expression is dependent on the tyrosine phosphorylation and activation of the DNA-binding activity of two related proteins of 91 kDa (STAT1) and/or 113 kDa (STAT2). Recent studies have suggested that these proteins are substrates of *Janus* kinases and that proteins related to STAT1 are involved in a number of signalling pathways, including those activated in myeloid cells by erythropoietin and interleukin-3 (IL-3). To clone STAT-related proteins from myeloid cells, degenerate oligonucleotides were used in PCRs to identify novel family members expressed in myeloid cells. This approach allowed the identification and cloning of the *Stat4* gene, which is 52% identical to *STAT1*. Unlike *STAT1*, *Stat4* expression is restricted but includes myeloid cells and spermatogonia. In the erythroid lineage, *Stat4* expression is differentially regulated during differentiation. Functionally, *Stat4* has the properties of other STAT family genes. In particular, cotransfection of expression constructs for Stat4 and Jak1 or Jak2 results in the tyrosine phosphorylation of Stat4 and the acquisition of the ability to bind to the gamma interferon (IFN-γ)-activated sequence of the interferon regulatory factor 1 (IRF-1) gene. *Stat4* is located on mouse chromosome 1 and is tightly linked to the *Stat1* gene, suggesting that the genes arose by gene duplication. Unlike Stat1, neither IFN-α nor IFN-γ activates Stat4. Nor is Stat4 activated in myeloid cells by a number of cytokines, including erythropoietin, IL-3, granulocyte colony-stimulating factor, stem cell factor, colony-stimulating factor 1, hepatocyte growth factor, IL-2, IL-4, and IL-6.

Hematopoiesis is regulated by the interaction of cytokines with receptors of the cytokine receptor superfamily. These effects are mediated, in part, by inducing the expression of genes associated with growth or differentiation. The mechanisms by which cytokines regulate gene expression are largely unknown. Recent studies have shown that *Janus* (Jak) kinases associate with receptors of the cytokine receptor superfamily and are activated following ligand binding (21, 23, 28, 30, 35). The interferons (IFNs) also activate Jak kinases. Signalling through the IFN-α receptor requires Jak1 and Tyk2 (20, 25, 26, 31), while signalling through the IFN-γ receptor requires Jak1 and Jak2 (20, 34). The Jak kinases are required for phosphorylation of proteins of 91 and 113 kDa, components of the IFN-stimulated gene factor 3α complex, also termed signal transducers and activators of gene transcription (STAT) 1 and 2, respectively (24). On the basis of these observations, it was proposed (35) that hematopoietic growth factors might utilize similar signalling pathways to regulate gene transcription, either by using STAT1, STAT2, or related family members.

The potential involvement of STAT-related proteins has been examined. Recent studies have shown that multiple cytokines induce the tyrosine phosphorylation of proteins that can acquire the ability to bind to IFN-γ response elements (12). These activities were found to not be associated with STAT1 or STAT2. Interleukin-6 (IL-6) also induces a DNA-binding activity, termed acute-phase response factor, which is serologically and functionally related to STAT1 (14) and which exists in a complex containing the IL-6 signal transducer, gp130, and JAK1. Consistent with this, acute-phase response factor activity has recently been cloned and found to be 52% identical to that of STAT1 (1). IL-4 has also been shown to induce a DNA-binding activity that is not a known STAT protein but which has similar properties (9). We have observed that both erythropoietin (EPO) and IL-3 induce the tyrosine phosphorylation of proteins that are serologically related to STAT1 and which acquire the ability to bind an IFN-γ-activated sequence (30a). Together the data suggest that additional STAT-related proteins that may be substrates of the Jak kinases and which are involved in a variety of signalling pathways in myeloid cells exist.

One approach to the cloning of novel members of gene families has been the use of degenerate oligonucleotides in PCR. This approach can be applied to identify novel STAT1-related proteins on the basis of the observation that STAT1 and STAT2 are highly related (5) and might define a gene family. We have used this approach to begin to identify STAT-related proteins that might be of particular importance in hematopoietic growth or differentiation. In the results presented here, we describe the cloning and characterization of a novel STAT family gene which we have termed *Stat4*. Unlike *STAT1* or *STAT2*, the expression of *Stat4* is restricted and regulated during myeloid differentiation. Functionally, STAT4 can be tyrosine phosphorylated by Jak1 or Jak2 and acquires IFN-γ activation site-binding activity. However, STAT4 does not participate in a number of signalling path-

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ways, including EPO or IL-3, indicating that additional members of this family are yet to be identified.

MATERIALS AND METHODS

Cloning of cDNAs for *Stat4*. Regions conserved between STAT1 and STAT2 were used to design degenerate oligonucleotide primers for PCR amplification of STAT-related genes. The sense primer 5′-GCATCGATGA(A/G)(A/C)NNCA(A/G)CCNTG(T/C)ATGCC-3′ corresponded to amino acids 320 to 326 (ERQPCMP) of STAT1 and contained a *Cla*I cloning site, while the antisense primer 5′-GCGTCGACGG(G/A)TT(T/C)TCNGG(A/G/T)AT(A/G)TT(T/C)TC-3′ corresponded to amino acids 657 to 663 (ENIPENP) of STAT1 and contained a *Sal*I cloning site. Murine cDNA was derived from 1 μg of total RNA from an IL-3-dependent myeloid cell line, DA-3, by using a cDNA kit (Amersham) and was used in PCR amplification with 20 pmol of each primer through 40 cycles of amplification (94°C, 1 min; 50°C, 1 min; and 72°C, 1.5 min). Products of the correct size were digested with *Cla*I and *Sal*I and cloned into pBluescript II (Stratagene) and sequenced. One clone contained a sequence with high homology to STAT1 and was labeled by random priming and used to screen a murine testis lambda gt11 cDNA library (Clontech). Eighteen clones were isolated. To be sure that the 5′ and 3′ regions were cloned, 5′ and 3′ rapid amplification of cDNA ends was done with a kit (Life Technologies). From overlapping cDNA clones, a sequence of 3 kb was obtained by reading in both directions. This sequence contained a single large open reading frame of 2,244 bp. The first ATG had a flanking sequence that conformed to the Kozak consensus sequence for a translation initiation site. The predicted amino acid sequence for the open reading frame is shown in Fig. 1. The regions showing similarity to SH2 and SH3 domains are underlined and indicated.

Cotransfections and DNA-binding assays. For all the experiments, COS 7 cells (5×10^5) were transfected with pXM or pRK5 expression vectors containing full-length cDNAs for Jak1 or Jak2 (4 μg) or Stat4 (10 μg). The pRK5 Jak1 and Jak2 expression constructs have been previously described (27). For detection, the pXM-Stat4 construct contained an epitope tag at the carboxyl terminus which involved changing the carboxyl terminus of Stat4 from PYSAE to YPYDVPDYA by PCR. The underlined sequence is derived from the influenza virus hemagglutinin protein and is recognized by the monoclonal antibody 12CA5 (22, 36). Sixty hours after the introduction of the constructs, whole-cell extracts were prepared by incubating the cells in lysis buffer (0.5% Nonidet P-40, 50 mM Tris-Cl [pH 8.0], 10% glycerol, 0.1 mM EDTA, 250 mM NaCl, 0.1 mM Na_3VO_4, 50 mM NaF, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 3 μg of aprotinin per ml, 2 μg of leupeptin per ml, 2 μg of pepstatin A per ml) on ice for 30 min. Whole-cell extracts were used for either immunoprecipitations and Western blotting (immunoblotting) or for gel mobility shift assays. Immunoprecipitations of whole-cell extracts were done with the 12CA5 monoclonal antibody (Berkeley Antibody Company). The immunoprecipitates were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer and resolved on SDS–7% PAGE. The proteins were transferred to nitrocellulose, and the blots were probed with either the 12CA5 monoclonal antibody or with a monoclonal antibody against phosphotyrosine (4G10; Upstate Biologicals Inc.). Extracts from transfected COS cells were assayed for the presence of complexes capable of binding to the interferon regulatory factor 1 (IRF-1) GAS probe. Whole-cell extracts containing 17 μg of total protein were initially incubated for 30 min on ice with or without the 12CA5 monoclonal antibody in 1× lysis buffer made 167 mM in NaCl. Each reaction mixture was then incubated, on ice, with 0.5 μg of poly(dI-dC) for 15 min and then for 20 min with 1 ng of Klenow-labeled IRF-1 GAS probe. The reactions were electrophoresed through a 4.5% nondenaturing polyacrylamide gel. The GAS probe sequence was 5′-CTAGAGCCTGATTTCCCCGAAATGATGAGCTAG-3′, containing the murine IRF-1 sequence (underlined, −132 to −108), including the two inverted GAANN repeats (18, 29).

Gene mapping of *Stat4*. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as previously described (3). A total of 205 N_2 mice were used to map the *Stat4* locus. DNA manipulations were as previously described (7), and blots were prepared with Zetabind nylon membranes (AMF-Cuno). The *Stat4* probe, a 1-kb cDNA fragment, was labeled with [α-^{32}P]dCTP with a random primed labeling kit (Amersham); washing was done to a final stringency of 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C. Major fragments of 9.0, 4.8, 2.6, and 2.2 kb were detected in *Pvu*II-digested C57BL/6J DNA, and major fragments of 13.0, 4.8, and 2.2 kb were detected in *Pvu*II-digested *M. spretus* DNA. The presence or absence of the 13.0-kb *M. spretus*-specific *Pvu*II fragment was monitored in backcross mice. A description of the probes and restriction fragment length polymorphisms for IL-1 receptor type 1 (*Il1r1*) and cytotoxic T-lymphocyte-associated protein 4 (*Ctla4*) has been reported previously (4). The glutaminase (*Gls*) probe has not been previously reported and consisted of an ~590-bp *Eco*RI fragment of rat cDNA that detected major *Bam*HI fragments of 9.0 kb (C57BL/6J) and 7.0 kb (*M. spretus*). Recombination distances were calculated as previously described (6) by using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns. References for the human map positions of loci mapped in this study can be obtained from Genome Data Base, a computerized data base of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, Md.).

Nucleotide sequence accession number. The sequence for STAT4 has been deposited in GenBank under accession number U09351.

RESULTS

Comparison of the amino acid sequences of STAT1 and STAT2 indicated regions of sequence identity (5). To clone related genes, degenerate oligonucleotides were designed to regions of identity and used in PCR with cDNA from a murine IL-3-dependent myeloid cell line. Primers to a conserved region in the SH2 domain and a region in the middle of the gene amplified fragments of the correct size (~1 kb) which were cloned and sequenced. While the majority of the clones contained the murine homologs of *STAT1* and *STAT2*, one of the cloned fragments encoded a *STAT1*-related gene which we have termed *Stat4*. By using the PCR-amplified fragment, cDNAs clones were isolated and sequenced.

The deduced amino acid sequence in which Stat4 is aligned with the human STAT1 amino acid sequence is shown in Fig. 1. The *Stat4* gene would encode a protein that contains 748 amino acids with a predicted molecular mass of 86 kDa. Consistent with this, the in vitro-translated protein is 85 kDa (data not shown) and COS cells transfected with a eucaryotic expression construct produce a protein of 85 kDa (data shown

FIG. 1. Amino sequence alignment of murine *Stat4* with human *STAT1*. From overlapping cDNA clones a sequence of 3 kb was obtained from reading in both directions. This sequence contained a single large open reading frame of 2,244 bp. The first ATG had a flanking sequence that conformed to the Kozak consensus sequence for a translation initiation site. The predicted amino acid sequence for the open reading frame is shown. The regions showing similarity to SH2 and SH3 domains are underlined and indicated.

below). The predicted amino acid sequence of Stat4 is 52% identical to the human Stat1 sequence. Within the SH2 domain, the two sequences are 69% identical and there is complete conservation of the sequence GTFLLRFSE which includes the Arg residue corresponding to the critical phosphotyrosine binding site (32, 33). In contrast, there is 51% identity in the region showing similarity to SH3 domains. STAT1 and STAT2 proteins contain an alpha helical region in the amino terminus containing a heptad leucine repeat (L L V L L). In the predicted Stat4 protein, there is also an alpha helical region and the comparable positions are L L A V L. Lastly, a single site of tyrosine phosphorylation occurs in STAT1, in the peptide GPKGTGY^{701}IKTEL (24). This region is quite different in Stat4, GDKGY^{693}VPSVFIP, although there is a Tyr close to the site of the critical Y-701 in STAT1.

The tissue expression of *Stat4* is shown in Fig. 2A and B. A single transcript of approximately 3.3 kb is seen in a limited number of tissues, including spleen, lung, skeletal muscle, and bone marrow. In contrast, transcripts were not detected in a number of tissues, suggesting that unlike *STAT1*, *Stat4* expression is restricted. The highest levels of expression were detected in testes, where two transcripts of 3.3 and 3.6 kb were detected. Expression in testes was limited to developing spermatogonia as determined by cellular fractionation and the time of appearance of the transcripts during development (data not shown).

The expression of *Stat4* in cell lines was also examined (Fig. 2C). No expression was detected in the fibroblastic, T-cell, or B-cell lines examined. However, a single transcript of 3.3 kb was highly expressed in a variety of myeloid cells. These included both IL-3-dependent and -independent myeloid cell lines and mast cell lines. Importantly, a few myeloid cell lines did not contain *Stat4* transcripts, including the M1 cell line and MEL cell lines (data not shown), which have relatively mature phenotypes and the ability to be induced to terminally differentiate to macrophages or erythrocytes, respectively (13, 15). When examined, the levels of transcripts correlated to the levels of protein (data not shown). This suggested that expression of *Stat4* was lost during terminal differentiation of some myeloid cells.

To assess expression during differentiation, clonal variants of an IL-3-dependent cell line, 32Dc13, were examined. 32Dc13 cells differentiate to granulocytes over a period of 10 days when cultured in granulocyte colony-stimulating factor (CSF). During differentiation, *Stat4* transcripts gradually declined (data not shown). 32DEpo1 cells were derived from a clonal variant of 32Dc13 that expressed the endogenous EPO receptor. When cultured in IL-3, the cells do not express β-globin. However, following culture in EPO for 2 to 4 days, β-globin (Fig. 3A) as well as a number of other markers of erythroid lineage differentiation is expressed (data not shown). In these cells *Stat4* expression is regulated by IL-3. Removal of IL-3 causes a loss of transcripts, and readdition of IL-3 restores the transcript levels (Fig. 3B). However, addition of EPO does not induce the reappearance of transcripts (Fig. 3B), and no transcripts are detected in cells following continued culture in

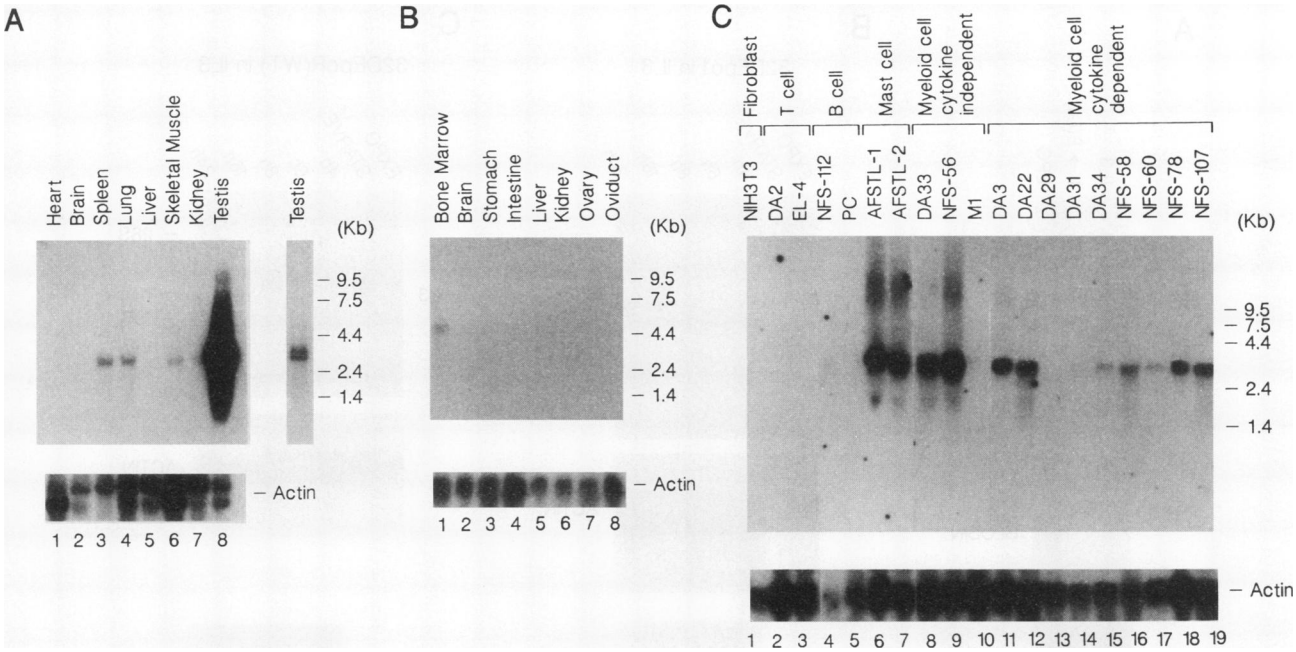

FIG. 2. *Stat4* is expressed in limited tissues and cell linages. RNAs from the tissues indicated in panel A [poly(A)$^+$ RNA, 2 μg] and panel B (total RNA, 20 μg) were resolved by electrophoresis and transferred to membranes. The filters were subsequently probed with a 1-kb cDNA probe for *Stat4* or with an actin probe (lower panels). (C) Total RNAs (20 μg) from the indicated cell lines were resolved by electrophoresis and transferred to membranes, and filters were probed as described above. The lineages of the cell lines are indicated above. The myeloid cell cytokine-dependent cells require IL-3 for maintenance of viability and proliferation.

EPO (Fig. 3A). In contrast to *Stat4*, *Stat1* was constitutively expressed in all the above cell lines, independently of the culture conditions (data not shown).

To determine if the regulation of expression of *Stat4* changed with differentiation, we also examined 32D(EpoRWT) cells. This cell line was derived from a clone of 32Dc13 cells transfected with the receptor for EPO (17). The cells grow in EPO but do not express erythroid lineage markers such as β-globin. In these cells, *Stat4* is constitutively expressed, and removal of IL-3 or culturing of the cells in EPO has no effect on transcript levels (Fig. 3C). Together the data demonstrate that *Stat4* expression is differentially regulated during myeloid differentiation.

STAT1 requires Jak kinases for tyrosine phosphorylation and, following phosphorylation, acquires the ability to participate in binding to IFN-γ activation sites (GAS). To determine whether Stat4 had these properties, an epitope-tagged derivative was expressed in COS cells in the presence or absence of Jak kinases. Transfection of a *Stat4* expression construct into COS cells alone resulted in the appearance of an 85-kDa protein that was immunoprecipitated with a monoclonal antibody against the epitope tag (Fig. 4A). Immunoprecipitation was inhibited by the peptide to which the monoclonal antibody was directed (data not shown). Under these conditions, Stat4 was not detectably tyrosine phosphorylated as assessed by Western blotting with a monoclonal antibody against phosphotyrosine. Overexpression of Jak1 or Jak2 in COS cells has been shown to result in their tyrosine phosphorylation, activation of in vitro kinase activity, and the phosphorylation and activation of the endogenous Stat1 (26). Therefore, we cotransfected the *Stat4* expression construct with expression constructs for either *Jak1*, *Jak2*, or both *Jak1* and *Jak2*. Cotransfections resulted in the tyrosine phosphorylation of Stat4 as determined by the appearance of reactivity with a monoclonal antibody against phosphotyrosine (Fig. 4A).

The abilities of extracts from the transfected COS cells to bind the GAS element of the IRF-1 gene are shown in Fig. 4B. Extracts from COS cells transfected with the *Stat4* expression construct gave a series of GAS binding complexes that were comparable to those seen in cells transfected with the parental expression construct (data not shown). Treatment of these extracts with the monoclonal antibody against the epitope tag resulted in the nonspecific reduction in one of the complexes with all the extracts (complex C). The C complex is similarly reduced by several different antisera. Extracts from cells transfected with *Jak1* or *Jak2* expression constructs had a similar pattern of GAS binding activity as well as a novel complex (complex B) which is due to activation of the endogenous STAT1 (26). This complex was not affected by the monoclonal antibody against the epitope tag but could be supershifted with an antibody to STAT1 (data not shown). Extracts from cells cotransfected with the *Stat4* expression construct, and constructs for *Jak1*, *Jak2*, or *Jak1* and *Jak2* contained an additional novel complex (complex A). This complex was inhibited with an unlabeled IRF1 probe (data not shown) and was depleted and supershifted by treatment of the extracts with the monoclonal antibody against the epitope tag. The depletion of the complex by the monoclonal antibody was competed for by the peptide to which the monoclonal was directed (data not shown). Lastly, the Stat4 complex was not supershifted with antibodies against Stat1, indicating that Stat4 does not detectably dimerize with activated Stat1 under the conditions of the experiment. Together the data demonstrate that Stat4 has GAS binding activity following tyrosine phosphorylation.

The murine chromosomal location of *Stat4* was determined by interspecific backcross analysis with progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 1,400 loci that are well distributed among all the auto-

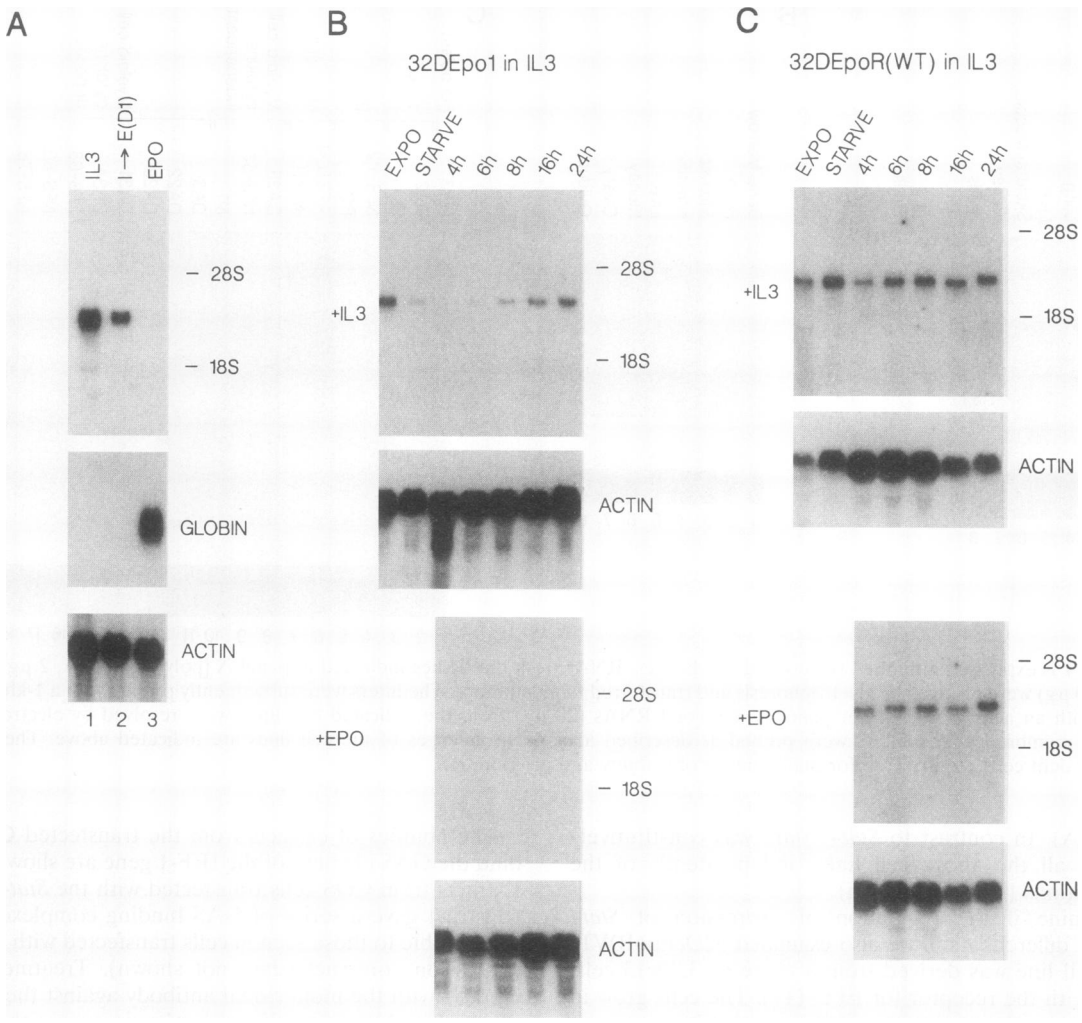

FIG. 3. Expression of *Stat4* mRNA in 32DEpo1 and 32EpoR(WT) cells grown in IL-3 or EPO. (A) 32DEpo1 cells were maintained as previously described (10) in IL-3 (IL3), transferred from IL-3 to EPO for one day [I→E(D1)], or continuously maintained in EPO (EPO). Total RNA was isolated and subjected to Northern (RNA) blot analysis as previously described (11), and the blots were probed with a 1-kb *Stat4* cDNA fragment or with a probe for β-globin (16). The blots were also probed with an actin probe (8) for control of loading. (B) 32DEpo1 cells were grown continuously, in exponential growth, in IL-3 (EXPO) or were removed from IL-3 for 16 h (STARVE). Either IL-3 (top) or EPO (bottom) was added to cultures of starved cells, and aliquots of cells were collected at the indicated times. RNA was prepared and analyzed as for panel A. (C) 32Epo(WT) cells were treated and RNA was isolated as described for 32DEpo1 cells in panel B.

somes as well as the X chromosome (3). The mapping results indicated that *Stat4* is located in the proximal region of mouse chromosome 1 linked to *Il1r1*, *Gls*, and *Ctla4* (Table 1). Strikingly, we also found that *Stat4* cosegregated with *Stat1* in 91 animals typed in common (4a), suggesting that the two genes are within 3.2 centimorgans of each other (upper 95% confidence limit) and may have arisen by gene duplication.

DISCUSSION

A variety of recent observations have suggested that there exist additional DNA-binding proteins that are related to STAT1 and STAT2. In hematopoietic cell lines, related proteins have been implicated in the responses to IL-6 (14), IL-4 (9), and IL-3 (12). Consistent with these data, we have identified IL-3- and EPO-inducible GAS binding activities which differ with differentiation and some of which are serologically related to STAT1. In each case the cytokines induce the tyrosine phosphorylation of a latent cytoplasmic protein of approximately 85 to 95 kDa which acquires the ability to bind to a GAS element. Consistent with a potential relationship to STAT1 and STAT2, the IL-6-induced activity has recently been cloned and found to be 52% identical in amino acid sequence to STAT1 (1, 37). Our data describe another member of the STAT family. Structurally, Stat4 is 52% identical to STAT1 and 47% identical to APRF/Stat3. Functionally, Stat4 is similar to the other Stat proteins on the basis of the requirement for tyrosine phosphorylation for acquisition of its GAS binding activity.

The expression of Jak1 or Jak2 in COS cells results in their tyrosine phosphorylation and activation. The basis for this activation is not known. Cotransfection studies with Jak1 or Jak2 expression constructs suggest that Stat4 is a substrate for these kinases. It should also be noted that the endogenous STAT1 is also tyrosine phosphorylated and acquires GAS binding activity in these experiments. Whether Stat1 or Stat4 is the immediate substrate of the Jak kinases cannot be deter-

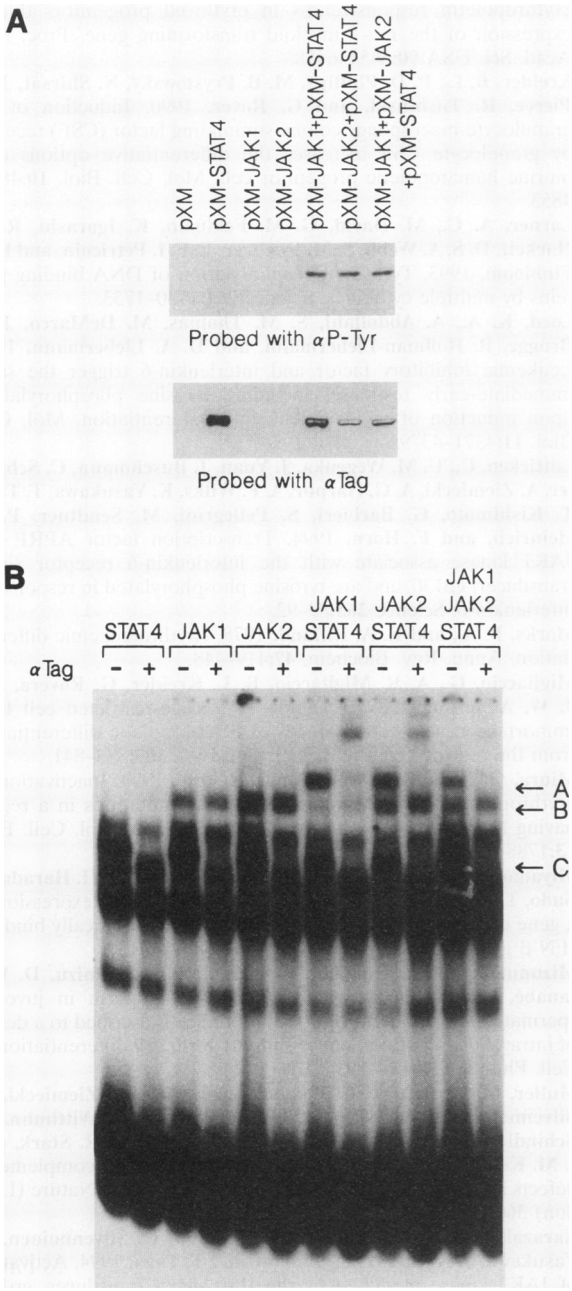

FIG. 4. Tyrosine phosphorylation of Stat4 reveals its GAS binding activity. COS 7 cells (5×10^5) were transfected with pXM or pRK5 expression vectors containing full-length cDNAs for Jak1 or Jak2 (4 μg) or Stat4 (10 μg). For detection, the pXM-Stat4 construct contained an epitope tag. Sixty hours after the introduction of the constructs, whole-cell extracts were prepared as described in Materials and Methods and were used for either immunoprecipitations and Western blotting or for gel mobility shift assays. (A) Immunoprecipitations of whole-cell extracts were done with the 12CA5 monoclonal antibody (Berkeley Antibody Company). The immunoprecipitates were dissolved in SDS-PAGE buffer and resolved on SDS–7% PAGE. The proteins were transferred to nitrocellulose, and the blots were probed with either the 12CA5 monoclonal antibody or with a monoclonal antibody against phosphotyrosine (4G10, Upstate Biologicals Inc.). (B) Extracts from transfected COS cells were assayed for the presence of complexes capable of binding to the IRF-1 GAS probe. Complexes A, B, and C are described in the text. The reaction mixtures were electrophoresed through a 4.5% nondenaturing polyacrylamide gel.

TABLE 1. *Stat4* maps in the proximal region of mouse chromosome 1

Locus interval	No. of recombinants/ no. typed	Recombination distance[a]
Il1r1 to *Gls*	12/136	8.8 ± 2.4
Gls to *Stat4*	0/112	2.6[b]
Stat4 to *Ctla4*	4/101	4.0 ± 2.0

[a] Recombination distances are in centimorgans ± one standard error.
[b] Upper 95% confidence limit.

mined in these experiments, however, since cotransfection of Jak2 results in the activation of the endogenous Jak1 and vice versa and could possibly activate other kinases.

The tyrosine phosphorylation of STAT1 occurs on Y-701 and is required for acquisition of GAS binding activity (24). We have not examined the number and sites of tyrosine phosphorylation of Stat4. However, we have mutated Y-693 to R to determine if mutation of the most likely equivalent of STAT1 Y-701 would have a comparable effect. This mutation dramatically reduces tyrosine phosphorylation and eliminates the acquisition of GAS binding activity in cotransfection experiments in COS cells with Jak1 or Jak2 kinase (data not shown).

Stat4 maps to the proximal region of chromosome 1 and is tightly linked to *Stat1*. By pulsed-field analysis, *Stat1* and *Stat4* are on distinct *Bss*HII (250- and 770-kb) and *Sfi*I (100- and 50-kb) fragments; thus, additional studies will be required to physically link the genes. It will also be important to determine whether other *Stat* genes map to this region. *Stat4* maps near the murine mutation juvenile spermatogonial depletion (*jsd*). Homozygous *jsd* mice produce type A spermatogonia which fail to differentiate (2, 19). Experiments are currently in progress to assess the potential role of the *Stat4* gene in the *jsd* mutation. *Il1r1*, *Gls*, and *Ctla4* have been mapped to human chromosome 2q12, 2q32-34, and 2q33, respectively. The mapping of *Stat4* within this region of 2q homology and the close linkage of *Stat4* to *Gls* strongly suggest that the human homolog of *Stat4* will also map in this region of human 2q homology.

Stat4, unlike *Stat1*, is not widely expressed and, within the myeloid lineage, expression is differentially regulated during differentiation in the cell lines examined. Although it is not currently known which cytokines induce the tyrosine phosphorylation and activation of Stat4, the differential regulation of *Stat4* expression provides another potential basis for the pleotropic effects that cytokines have on cells of various lineages and stages of differentiation. This is illustrated in the results presented here by differential ability of EPO to induce β-globin expression in 32DEpo1 cells but not in 32DEpo(WT) cells. Whether the differential expression of *Stat4* contributes to this difference is currently being examined.

The studies presented here have focused on the expression of *Stat4* in myeloid lineage cells; however, *Stat4* is expressed as highly in testes as it is in myeloid cell lines. In rat testes, the transcripts for *Stat4* become detectable approximately 3 weeks after birth (data not shown) with the onset of sexual maturity suggesting that *Stat4* is expressed in differentiating spermatogonia. Consistent with this, fractionation of cells from testes indicated that *Stat4* is expressed in spermatocytes and spermatides but not in Leydig cells (data not shown). *Stat4* transcripts are also not found in the testes of mice homozygous for the *jsd* mutation, although transcripts are detected in the bone marrow. These mice have type A spermatogonia, but these fail to continue differentiation. As noted above, the *Stat4* gene maps

very near the *jsd* mutation, and therefore experiments are currently in progress to determine whether the *Stat4* gene is affected in these mice.

The ability to cotransfect Jak1 or Jak2 with Stat4 and induce its tyrosine phosphorylation and DNA-binding activity suggests that Stat4 will be involved in a signalling pathway. To examine this possibility, we have assessed the ability of a number of cytokines to activate Stat4. To date, we have not observed Stat4 activation in response to EPO, IL-3, granulocyte-macrophage CSF, granulocyte CSF, IL-2, IL-4, stem cell factor, and CSF-1 (data not shown). Thus, identification of the signalling pathways that might utilize this STAT family member remains. In addition, the results indicate that more STAT-related proteins must exist to account for the STAT-related activities that are seen in the responses to the cytokines such as EPO, IL-3, IL-4, and granulocyte CSF. In this regard, the recently cloned gene encoding the APRF and Stat3 activities is tyrosine phosphorylated and activated in response to epidermal growth factor or IL-6 (1, 37). However, Stat3 and APRF are not activated by EPO, IL-3, or IL-4. Irrespective of that, the approaches used to clone *Stat4* should allow the cloning of additional STAT family members.

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