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J Immunol 2003; 170:6057-6064; doi: 10.4049/jimmunol.170.12.6057
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Characterization and Analysis of the Proximal Janus Kinase 3 Promoter

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Janus kinase 3 (Jak3) is a nonreceptor tyrosine kinase essential for signaling via cytokine receptors that comprise the common γ-chain (γc), i.e., the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Jak3 is preferentially expressed in hemopoietic cells and is up-regulated upon cell differentiation and activation. Despite the importance of Jak3 in lymphoid development and immune function, the mechanisms that govern its expression have not been defined. To gain insight into this issue, we set out to characterize the Jak3 promoter. The 5′-untranslated region of the Jak3 gene is interrupted by a 3515-bp intron. Upstream of this intron and the transcription initiation site, we identified an ~1-kb segment that exhibited lymphoid-specific promoter activity and was responsive to TCR signals. Truncation of this fragment revealed that core promoter activity resided in a 267-bp fragment that contains putative Sp-1, AP-1, Ets, Stat, and other binding sites. Mutation of the AP-1 sites significantly diminished, whereas mutation of the Ets sites abolished, the inducibility of the promoter construct. Chromatin immunoprecipitation assays showed that histone acetylation correlates with mRNA expression and that Ets-1/2 binds this region. Thus, transcription factors that bind these sites, especially Ets family members, are likely to be important regulators of Jak3 expression.

that despite the high level expression of Jak3 in monocytes, no significant defects of this cell lineage have been found in Jak3 SCID patients (33). While it is clear that Jak3 is highly expressed in hemopoietic cells, its relative expression and physiologic or pathologic functional relevance in nonhemopoietic tissues remain to be determined.

Another interesting aspect of the expression of Jak3 is that it is highly regulated by cell activation and cell differentiation. That is, in T cells, leucins or phorbol esters induce Jak3 up-regulation (26, 34). Similarly, B cell receptor cross-linking or CD40 cross-linking in B lymphocytes up-regulates Jak3 expression (35), and LPS, IFN-γ, and IL-2 induce its expression in monocytes (36). In addition, Jak3 was induced during the terminal differentiation of myeloid cell lines (37), whereas, in contrast to B cells, mature plasma cells express only very low levels of Jak3 (35). In thymus, Jak3 is highly expressed at all stages, as it is in adult, double-negative cells express only very low levels of Jak3 (35). In thymus, Jak3 is highly expressed at all stages, as it is in adult, double-negative cells express only very low levels of Jak3 (35). In thymus, Jak3 is highly expressed at all stages, as it is in adult, double-negative cells express only very low levels of Jak3 (35).

Despite this wealth of interesting findings, the promoter elements of Jak3 have not yet been characterized. Because of the pivotal role of Jak3 in lymphoid development and because of the interesting aspects of its regulated expression, we therefore thought it important to identify the Jak3 promoter and to begin defining the mechanisms involved in its regulation. In particular, we wanted to find elements that could explain the hematopoietic and activation-dependent expression.

Materials and Methods

**Cells, cell lines, and stimulation**

PBMC were prepared from venous blood anticoagulated with preservative-free heparin sedimented over Ficoll-Paque Plus (Amersham Pharmacia Biotech, Piscataway, NJ) gradients. PBMC, Jurkat cells, JCAM1.1 (Lck negative TCR−/− Jurkat), and NK3.3 cells were grown in RPMI 1640 containing 10% FCS, glutamine, and antibiotics (Biofluids, Rockville, MD). Jurkat cells that express large T Ag (Jurkat T) were also used in these studies; these cells constitutively express more Jak3 than parental Jurkat cells (data not shown). Medium for NK3.3 was enriched with Lymphotoxin (human T cell Nucleofector Kit, Amaxa Biosystems, Cologne, Germany) according to the commercial protocol, using 5 μg of purified plasmid DNA together with 0.3 μg of pEFI/Hislacz plasmid (β-galactosidase vector, Invitrogen) using Superfect transfection reagent (Qiagen, Valencia, CA). As positive control vector we used a CMV-promoter driven pG3 vector. Alternatively, human PBMC and NK3.3 were transfected by electroporation using the human T cell Nucleofector Kit (Ammax Biosystems, Cologne, Germany) according to the commercial protocol, using 5 μg of purified plasmid DNA together with 0.3 μg of pEFI/Hislacz plasmid. The transfection efficiency of electroporation, determined by flow cytometry after transfecting a green fluorescence protein vector, was 30–35%. The following day the cells were activated as described and harvested after another 30–36 h of incubation. The cells were lysed in ice-cold Reporter Lysis Buffer (Promega, Madison, WI), and the lysates were consecutively analyzed for luciferase and β-galactosidase activities with the Dual Light Kit (Tropix, Bedford, MA) on a Monolight 3010 luminometer (Analytical Luminescence Laboratory, Sparks, MD); luciferase expression is represented as normalized by β-galactosidase activity (expressed as a percentage).

**Site-directed mutagenesis**

The −74/+27 and/or the −122/+27 truncated luciferase promoter constructs were used as a template for mutations disrupting the binding consensus of several sites, using the Transformizer site-directed mutagenesis kit (Clontech, Palo Alto, CA) as previously described (38). The following primers (mutated base pairs are underlined) were used for the construct lacking the Stat binding site and both AP-1 binding sites, respectively: 5′-CTCATATCTCTGTTTTATGACGTCATGAGAA-3′ and 5′-CA GCCTCCGAGGGCCCTGCCTTTCTGGAATATCCTGAGTCGAC CAGG-3′. To create the three Ets-1 mutations, we used the following primers: 5′-CGCTGATGACGGGTTCTCTTTTCTTCTCAGG AGGGG TAGCAGTCTTCTGCCTCTTCTGAGCC-3′, 5′-GA AACCCAGGCGGGACGCCCACTACAGTGAGACCGA TGAGGCTAATTTCTCCCTGTC-3′, and 5′-GGGGCGGTATGGACAGGCC TACGGCTATTTCCTGCCCTTTGC-3′. All mutations were verified by sequencing. Transfections and luciferase assays were performed as described above.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed according to protocol using a commercially available kit (Upstate Biotechnology, Lake Placid, NY) and polyclonal Abs against Ets-1/2 and Stat-6 (Santa Cruz Biotechnology, Santa Cruz, CA) as well as anti-acetylated histone H3 and rabbit IgG (negative control; Upstate Biotechnology, Lake Placid, NY). Jurkat, HeLa, and COS7 cells were cross-linked with formaldehyde, lysed, and sonicated using an XL1020 sonicator (Heat Systems Ultrasonics, Farmingdale, NY); an aliquot of the sonicated cells was amplified by PCR for the Jak3 gene (35 cycles; 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C). The PCR products were then excised and subcloned into the pG3 vector. Alternatively, human PBMC and NK3.3 were transfectected by electroporation using the human T cell Nucleofector Kit (Ammax Biosystems, Cologne, Germany) according to the commercial protocol, using 5 μg of purified plasmid DNA together with 0.3 μg of pEFI/Hislacz plasmid. The transfection efficiency of electroporation, determined by flow cytometry after transfecting a green fluorescence protein vector, was 30–35%. The following day the cells were activated as described and harvested after another 30–36 h of incubation. The cells were lysed in ice-cold Reporter Lysis Buffer (Promega, Madison, WI), and the lysates were consecutively analyzed for luciferase and β-galactosidase activities with the Dual Light Kit (Tropix, Bedford, MA) on a Monolight 3010 luminometer (Analytical Luminescence Laboratory, Sparks, MD); luciferase expression is represented as normalized by β-galactosidase activity (expressed as a percentage).

**Results**

**Identification of the Jak3 transcriptional start site and organization of the 5′ region of the Jak3 gene**

To identify the transcriptional start site in the Jak3 gene, we employed 5′ RACE using mRNA from Jurkat T or NK3.3 cells. We identified multiple products corresponding to 5′-untranslated sequence, the largest segment containing 111 bp of untranslated mRNA (Fig. 1). The first base of this largest product was therefore assigned position +1 and is identical with base 27,800 of the human chromosome 19 cosmId R34383 as deposited in GenBank by Lamerdin et al. (AC007201). Lai et al. (41) identified the same 5′-untranslated segment of the Jak3 mRNA, but it was 20 bp shorter than the longest product found by us. These differences in length may be due to alternative transcriptional start sites.

Comparison of the obtained mRNA with genomic DNA showed a 3,515-bp intron with a splice acceptor site 13 bp upstream of the
ATG codon (Fig. 1), again consistent with the reported R34383 sequence (intron from base 27,711 to base 24,197). The intron ends with a typical pyrimidine-rich stretch and AG. After identifying the region of the bona fide transcriptional start site, we sequenced roughly 1,000 bp 5' to H11032 to identify potential promoter/enhancer elements. Fig. 1 shows 1,050 bp of genomic DNA upstream of the transcriptional start site containing the putative promoter region. With the exception of 1 bp (g instead of t at H11002), this sequence is identical with the R34383 sequence (28,850 through 27,801 bp) as well as with bp 314,420 through 313,371 of the Homo sapiens 19p13.1 sequence (Hs19_11445) (42), in accordance with the known chromosomal localization of Jak3.

The upstream JAK3 region contains tissue-specific promoter activity

We next examined whether the DNA upstream of the transcriptional start site contained promoter activity. To this end we cloned a 1,039-bp fragment of upstream DNA (~1013 to +27) into the pGL3 vector. Transfection of this construct into Jurkat T cells resulted in considerable basal promoter activity, >20-fold higher than that in the empty vector (Fig. 2A). Since promoters typically only function in the correct orientation, we also transfected a promoter construct that contained the same 1,039 bp but in reverse orientation. This reverse construct had only minimal promoter activity (Fig. 2A). These findings led us to conclude that the roughly 1,000 bp upstream of the Jak3 transcriptional start site do indeed contain promoter activity.

Previous data have indicated two features of Jak3 expression. First, Jak3 is abundantly expressed in lymphoid and hemopoietic cells, but is not highly expressed in other cell types. Second, Jak3 expression is activation dependent. Therefore, we next wanted to address whether the putative Jak3 promoter region could be responsible for such regulation.

To test whether our promoter fragment also contained elements responsible for Jak3 tissue specificity, we transfected the identical construct into nonlymphoid cells. COS7, an embryonic kidney cell

FIGURE 1. The Jak3 mRNA start sites and the upstream region containing the putative promoter. The Jak3 mRNA is printed in bold letters interrupted by a 3515-bp intron; the initiation codon is heavily underlined. The first base pairs of all mRNAs found in this study are denoted +1. The first base pair of the study by Lai (41) is marked by a double underline; the common piece of all published mRNA sequences (26, 41) upstream of the large intron is marked by a dotted underline.

FIGURE 2. The putative Jak3 promoter is active in T, but not in nonlymphoid, cells and is induced by lectin and phorbol esters. A, Jurkat T cells were transfected with empty vector, vector containing the putative Jak3 promoter region, or vector containing the putative promoter in the reverse direction. Bars show the mean ± SD of arbitrary light units normalized for β-Galactosidase (β-Gal) light units (samples were measured as triplets). B, COS7 cells were cotransfected with a β-galactosidase vector plus empty vector, vector containing the putative promoter, or a positive control vector (CMV-promoter driven pGL3 vector). Numbers given are luciferase light units normalized for β-Gal light units. C, The acetylation status of histone H3 in the nucleosomes associated with the Jak3 core promoter region was assessed by ChIP assay in Jurkat and COS7 cells. Cells were lysed, and proteins were cross-linked with formaldehyde and immunoprecipitated with Ab to acetyl histone H3 (anti-acetyl H3) or control Ab (rabbit IgG). After reversing the cross-linking, PCR for the Jak3 gene was performed. Input represents PCR amplification of the total sample. Results are representative of two independent experiments. D, Jurkat T cells were cotransfected with a β-galactosidase vector plus empty vector, vector containing the putative Jak3 promoter, or vector containing the putative promoter in the reverse orientation and were cultured without stimulation or with PHA or PMA. Results are expressed as luciferase light units normalized for β-Gal light units. Results in A–D are representative of two independent experiments.
The Jak3 promoter region is induced by T cell activation

Having demonstrated that the promoter construct was active in lymphocytes, we next investigated whether it was also regulated in an activation-dependent manner. We and others have shown that lectins and phorbol ester up-regulate Jak3 expression (26, 34). We therefore tested whether the candidate promoter segment we isolated could also be induced by these stimuli. As shown in Fig. 2D, in the absence of stimulation the promoter construct is expressed in Jurkat cells. Additionally, treatment of the cells with either PHA or PMA amplified expression (>4- and 6-fold, respectively). In contrast, the promoter construct in the reverse orientation was not activatable. Taken together, these experiments are consistent with the following conclusions. Jak3 gene promoter activity 1) resides in the region upstream of the Jak3 transcriptional start site and is orientation dependent; 2) is present in lymphoid, but not in COS7 or HeLa cells; and 3) is both basally active and inducible. Based on these properties we will therefore refer to the segment isolated as the proximal Jak3 promoter.

Signaling via the TCR activates the Jak3 promoter

PHA and PMA are nonspecific stimuli used experimentally to mimic signaling by the TCR. We next examined whether TCR cross-linking by an anti-CD3ε Ab bound to tissue culture plates also trans-activated the Jak3 reporter construct. As shown in Fig. 3A, TCR cross-linking was almost as efficient as PMA in activating the Jak3 promoter construct (>70% of the PMA-induced activation). Co-occupancy of the TCR and CD28 is a very potent stimulus for activating T cells. Some, but not all, T cell activation genes are synergistically induced by this combination. We therefore next investigated whether the cross-linking of CD28, either alone or in conjunction with TCR cross-linking, had an effect on the Jak3 promoter. As shown in Fig. 3A, CD28-dependent signals appeared to have little effect on the expression of the reporter construct.

Activity of the Src family kinase, Lck, is an early and essential step in TCR signal transduction. We therefore next wanted to investigate whether promoter activation followed the usual pathway downstream of TCR cross-linking, which is dependent on Lck. To this end we compared wild-type Jurkat T cells with Lck-negative JCAM1.1 cells (Fig. 3B). Both cell lines had equivalent high levels of CD3 surface expression as determined by flow cytometry (not shown). Whereas both PMA and anti-CD3 stimulation activated the promoter construct in wild-type Jurkat T cells, stimulation with PMA, but not with anti-CD3, led to promoter activity in JCAM1.1 cells (Fig. 3B). In conclusion, these findings indicated that TCR-dependent signals were capable of inducing this Jak3 promoter construct.

The major promoter activity resides within 240 bp upstream of the mRNA start site

We next tried to delimit the essential regions of the Jak3 promoter in terms of both basal and inducible activities. For this purpose a series of 5’ and 3’ truncation constructs were generated and transfected into Jurkat T cells. As shown in Fig. 4A, the promoter activity was similar after reducing the fragment to a 267-bp (~240/+27) segment, leaving the region upstream of the transcriptional start site intact. In contrast, a 938-bp construct lacking 75 bp upstream of the transcriptional start was completely inactive, indicating that this deletion disrupted the core promoter region. This indicated that the Jak3 promoter was located within 240 bp upstream of the longest obtained cDNA and contained essential elements within 75 bp 5’ of position +1.
The 267 bp of DNA containing the promoter activity are shown in Fig. 4B. This sequence was analyzed using the MatInspector program (43) and revealed a number of potential transcription factor binding sites. Using 5'/H11032-truncated mutants (Fig. 4C), the relative importance of the various elements was analyzed (Fig. 5A). Loss of the putative AP4 and AP1 sites between −240 and −172 had essentially no effect, whereas removal of the GATA and AP4 sites between −172 and −122 led to a mild increase in promoter activity. Removing the segment between −122 and −101, containing potential binding sites for Myb, Sp1, and AP4, did not appear to affect promoter activity. Further truncation (beyond −101), however, considerably reduced basal promoter activity. Specifically, removal of a putative double Ets-1 binding site overlapping a C/EBP site diminished the baseline activity by almost two-thirds, although the relative induction remained similar to that of the complete construct. Removal of another 18 bp containing NF-AT, C/EBP, Ets-1, and Ikaros binding sites (−240/+16 construct) reduced the remaining baseline activity by another 50%, but the relative induction stayed the same. Removing the AP1/Stat compound binding site and a putative Oct-1 site (−240/+40 construct; Fig. 5B) completely abolished both the baseline activity and the inducibility, arguing that this region contained part of the Jak3 core promoter.

**Specific mutations confirm the roles of AP-1 sites and Ets-1 binding enhancers**

To more specifically investigate the consensus AP-1/Stat/AP-1 compound site, point mutations were made in both AP-1 sites in the context of the active −240/+27 construct (Fig. 5B). Compared with the whole 267-bp construct, the baseline activity was reduced by 80% when the 3′ Ets-1 binding site was lost (−240/+2 construct), also removing a distal Ikaros site, although the relative induction remained similar to that of the complete construct. Removal of another 18 bp containing NF-AT, C/EBP, Ets-1, and Ikaros binding sites (−240/+16 construct) reduced the remaining baseline activity by another 50%, but the relative induction stayed the same. Removing the AP1/Stat compound binding site and a putative Oct-1 site (−240/+40 construct; Fig. 5B) completely abolished both the baseline activity and the inducibility, arguing that this region contained part of the JAK3 core promoter.
the larger −122/+27 construct significantly reduced luciferase activity (Fig. 6A), but did not affect inducibility. In contrast, mutation of the consensus Stat binding site did not diminish promoter activity; however, the cytokine inducibility of this construct was not assessed.

We also mutated the Ets-1 binding sites in the context of the −122/+27 fragment. Interestingly, the disruption of these sites led to a total loss in promoter activity (Fig. 6B). Moreover, it was sufficient to mutate either the 5′ (−95/−91) or the 3′ (+9) Ets-1 binding site to completely block promoter activity. These results confirm that the Ets-1 binding regions have an essential role in the regulation of the Jak3 promoter. Similar results were obtained using primary human lymphocytes. The basal Jak3 promoter activity in these cells, which shows about a 5-fold increase compared with the empty vector, is abrogated when the Ets sites are mutated (Fig. 6C). Comparable data were obtained using NK3.3 cells (not shown).

FIGURE 5. Truncations and specific mutations confirm the important role of AP-1 and Ets-1 sites. A, Jurkat T cells were cotransfected with a β-galactosidase (β-Gal) vector and equimolar amounts of empty luciferase vector or luciferase vector containing 5′ truncation constructs of the Jak3 promoter region and cultured in the presence or the absence of immobilized CD3 Abs. Results are the mean ± SD of luciferase light units normalized for β-Gal light units and are representative of three independent experiments. B, The same approach as in A was used to test 3′ truncation constructs. C, Using the same approach in the context of the −74/+27 construct, a 5′/3′ truncation mutant lacking Ets-1 binding sites, and constructs with both AP-1 sites or the Stat site mutated were transfected and compared with the −74/+27 construct and the empty vector. Results in B and C are representative of two independent experiments.

FIGURE 6. AP-1 and Ets-1 sites are critical for expression of the Jak3 promoter. A, Jurkat T cells were cotransfected with a β-galactosidase (β-Gal) vector and equimolar amounts of empty luciferase vector or luciferase vectors containing the −122/+27 or the −74/+27 5′ truncation constructs of the Jak3 promoter region with the AP-1 sites intact or mutated (ΔAP-1) and cultured in the presence or the absence of immobilized CD3 Abs. Results are the mean ± SD of luciferase light units normalized for β-galactosidase light units. B, The same approach as in A was used in the context of the −122/+27 construct to test constructs where Ets-1 binding sites were mutated. C, Primary human T lymphocytes were transfected by electroporation with a β-Gal vector and equimolar amounts of empty luciferase vector or luciferase vectors containing the whole promoter region or the −122/+27 construct with the 5′ and 3′ Ets-1 binding sites mutated and tested in the same way. D, ChIP assay with Jurkat cells and anti-Ets or control Ab. Cells were lysed, and proteins were cross-linked with formaldehyde and immunoprecipitated with Ab to Ets-1/2, Stat6 (anti-Stat6), or control Ab (rabbit IgG). After reversing the cross-linking, PCR for the Jak3 gene was performed. Input represents PCR amplification of the total sample. Results are representative of two independent experiments.
To confirm that Ets-1/2 binds the Jak3 promoter, we employed chromatin immunoprecipitation assays, which involve cross-linking of proteins to DNA in vivo, followed by immunoprecipitation of a specific protein and identification of its associated promoter DNA by PCR. By using Abs that are specific for individual Ets domain proteins, in vivo targets can be unambiguously identified. As shown in Fig. 6D, the Jak3 promoter was amplified when the immunoprecipitation was performed using a polyclonal Ab specific for Ets-1/2 (lane 4), but not with a control Ab.

Discussion

In this study we report the identification and characterization of the promoter region of the Jak3 gene, hoping to gain insight into two properties of Jak3: its propensity to be expressed in hemopoietic cells and its induction following cell activation.

All Jak3 mRNAs found in Jurkat T and NK cells begin 5′ of a large 3,515 base-pair intron with a splice-acceptor site in the 5′ untranslated region, 13 bp upstream of the ATG codon. This is similar to the putative murine Jak3 promoter, which also contains an intron in the 5′ untranslated mRNA. All published human Jak3 mRNA sequences also contain sequence 5′ of this large intron and start within the same region (26, 41). We therefore also expected to find promoter activity close to the transcriptional start sites.

Indeed, the 1-kbp region upstream of the transcriptional start site contained considerable promoter activity when cloned into a luciferase vector and transfected into Jurkat T cells. In contrast, no promoter activity was found in nonhemopoietic cells, such as COS7 or HeLa cells. Moreover, the promoter region was acetylated in lymphoid but not nonlymphoid cells, fitting the known tissue specificity of Jak3 (26–29). Consistent with the expectation of finding the core promoter near the transcriptional start site, a 267-bp fragment (~240/+27) surrounding the mRNA start site contained most of the promoter function, suggesting that this region contains the major tissue-specific and TCR-inducible promoter elements. The described promoter region exhibited considerable baseline activity and was further induced by PMA, PHA, and direct TCR cross-linking. Moreover, the induction by TCR cross-linking was absent in the Lck-negative Jurkat cell line JCAM1.1. Therefore, the Jak3 promoter is responsive to TCR-mediated signals, and stimulation via the TCR clearly represents a functional Ets-1/2 site, which family members are present in the greatest abundance.

The other motif present in the Jak3 promoter found to have significant effects were AP-1 sites. AP-1 complexes are heterodimers of Fos and Jun proteins, are ubiquitously expressed and downstream of mitogen-activated protein kinase cascades, and play an important role in the signaling response. The induction of Fos and the activation of Jun necessary to form the complex are well-known consequences of TCR signaling. Interestingly, Ets and AP-1 binding sites occur in a large number of promoter/enhancer elements, and functional cooperation between Ets and AP-1 is critical for controlled expression of many genes, including cytokines (47, 51, 52). It is notable, though, that AP-1 is not only activated in T cells following TCR cross-linking, but can be a consequence of a broad spectrum of stimuli in a variety of cells (53). Thus, it is still possible that under the appropriate circumstances activation of this pathway could lead to the expression of Jak3 in nonlymphoid cells.

In contrast to Ets-1 and AP-1 binding sites, the consensus NF-AT site also found 3′ of the core promoter appeared to be of minor importance, given the modest loss in activity after removal of this region (Fig. 5B) and the minimal effect of cyclosporin A (data not shown). This is again consistent with data on the murine Jak3 promoter, where no NF-AT site was detected (44).

While the present studies were directed toward improving the understanding of Jak3 transcription, it should be noted that regulation of transcription alone is likely to be an incomplete explanation of the regulation of Jak3 expression. In addition, determinants such as the propensity to undergo ubiquitination and proteosomal degradation (54, 55) are likely to also affect the amount of Jak3 present in a given cell at a given time. It is also notable in this regard that the Jak3 gene has an AT-rich motif in its 3′ untranslated region, similar to cytokines; so message stability may also be an important, but unexplored, aspect of regulation. In view of the essential function of Jak3, it will definitely be of interest to define all the mechanisms involved in its regulation.

Taken together, the present data indicate that the Jak3 promoter is found 60 bp upstream of a large (roughly 3500 bp) intron in the nontranslated 5′ portion of Jak3 mRNA. The promoter is tissue specific in that it is active in primary lymphocytes, Jurkat T cells, and NK3.3 cells, but not in COS or HeLa cells, consistent with the pattern of Jak3 expression. The core promoter region extends over roughly 60 bp and is surrounded by elements containing essential binding sites for Ets-1. These Ets-1 binding sites are of particular importance, as mutations of these sites lead to complete abrogation of promoter activity. Using this information, it will be important to define exactly which transcription factors regulate Jak3 expression in vivo in normal and pathologic states, in lymphoid and perhaps nonlymphoid cells.
References


