The Ets Protein Spi-B Is Expressed Exclusively in B Cells and T Cells during Development

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Summary

Spi-B and PU.1 are hematopoietic-specific transcription factors that constitute a subfamily of the Ets family of DNA-binding proteins. Here we show that contrary to previous reports, PU.1 and Spi-B have very different expression patterns. PU.1 is expressed at high levels in B cells, mast cells, megakaryocytes, macrophages, neutrophils, and immature erythroid cells and at lower levels in mature erythrocytes. PU.1 is completely absent from peripheral T cells and most T cell lines based on sensitive RT-PCR assays. In contrast, Spi-B is expressed exclusively in lymphoid cells and can be detected in early fetal thymus and spleen. In situ hybridizations of adult murine tissues demonstrate Spi-B mRNA in the medulla of the thymus, the white pulp of the spleen, and the germinal centers of lymph nodes. Spi-B expression is very abundant in B cells and both Spi-B mRNA and protein are detected in some T cells. In situ hybridization and Northern blot analysis suggest that Spi-B gene expression increases during B cell maturation and decreases during T cell maturation. Gel-retardation experiments show that Spi-B can bind to all putative PU.1 binding sites, but do not reveal any preferred Spi-B binding site. Finally, both PU.1 and Spi-B function as transcriptional activators of the immunoglobulin light-chain enhancer $E_{\lambda 2.4}$ when coexpressed with Pip (PU.1-interaction partner) in NIH-3T3 cells. Taken together, these data suggest that differences in patterns of expression between Spi-B and PU.1 distinguish the function of each protein during development of the immune system.

Hematopoiesis generates eight distinct lineages from self-renewing pluripotential stem cells, which then differentiate into mature blood cells. The molecular mechanisms underlying self-renewal and lineage commitment of stem cells have not been resolved. However, the process is regulated in part by hematopoietic-specific transcription factors. For example, stem cell leukemia (SCL)[1] is required for yolk sac hematopoiesis (1, 2), GATA-1 regulates red cell maturation (3, 4); Ikaros is important for the development of lymphocytes, natural killer cells, and neutrophils (5, 6); and Oct-2 is essential for T cell–independent B cell activation and B cell maturation into immunoglobulin secreting cells (7, 8). The function of a transcription factor in hematopoiesis is somewhat correlated to its expression pattern. PU.1 is expressed at high levels in B cells, granulocytes, monocytes, and immature erythroid cells (9, 10, 11, 12). We have previously determined that PU.1$^{-/-}$-deficient embryos die between days 16 and 18 of gestation and mutant embryos do not produce T and B lymphocytes, granulocytes, and monocytes (13). In addition, some day 16.5 embryos are also anemic (13).

Transcription factors of the Ets family are widely studied as key regulators of genes involved in the immune response and cellular proliferation. Spi-B and PU.1 are members of the Ets family, which now includes ~20 members. Spi-B was cloned from a Burkitt lymphoma cDNA library using the human PU.1 Ets DNA-binding domain as a probe (12), and it shares a 67% amino acid Ets-domain identity and 43% overall amino acid sequence identity to PU.1 (12). Like most of the Ets family members, Spi-B has the DNA-binding domain at its basic carboxy terminus and an acidic amino-terminal domain that corresponds to the transactivational domain in PU.1. PU.1 and Spi-B are the most distantly related genes to other Ets family members, as their Ets domains present only 40% identity to Ets-1 (12). Spi-B can bind specifically to the putative PU.1 binding site in the SV40 promoter, and can also transactivate a reporter construct containing multiple PU boxes. Ray et al. reported that Spi-B has an identical pattern of expression to

[1] *Abbreviations used in this paper:* CAT, chloramphenical acetyl transferase; DN, double negative; DP, double positive; EMSA, electrophoretic mobility shift assays; FcγR, Fc fragments; IVT, in vitro-translated; RT, reverse transcribed; SCL, stem cell leukemia; SP, single positive.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/07/203/12 $2.00
Volume 184 July 1996 203–214

PU.1 and is expressed in all hematopoietic lineages except T cells (12).

Here we report that significant amounts of Spi-B mRNA are detected in B and T cells, but not in monocytic cells. On the contrary, PU.1 mRNA can not be detected by the most sensitive means, such as RT-PCR, in purified human peripheral T cells or any T cell lines examined. Spi-B is not present in any other non-lymphoid cells based on in situ hybridization analyses. Spi-B is first expressed in the fetal thymus and spleen, and Spi-B mRNA is localized to the medulla of the thymus, white pulp of the spleen, and the germinal centers of the lymph nodes of adult mice. Spi-B is able to bind to all predicted PU.1-binding sites in vitro, including myeloid target genes. Both Spi-B and PU.1 in Clone 13 B cells bind to the functional PU.1-binding site in the Igκ 3′ enhancer. Both Pu.1 and Spi-B function as transcriptional activators of the Igλ 2-4 enhancer. We postulate that Spi-B plays an important role in lymphocytic development based on its pattern of expression.

Materials and Methods

Northern Analysis and RT-PCR. Total RNAs were prepared from cells or tissues using Trizol (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Poly-A^+ RNAs were then prepared from total cellular RNAs using the Oligotex poly-A^+ mRNA purification protocol (QIAGEN, Chatsworth, CA). Poly-A^+ RNA samples (2 μg each) were separated by electrophoresis on 1.2% agarose gels containing formaldehyde and blotted onto Hybond-N+ membranes. Hybridizations were carried out at 42°C in 50% formamide, 50 mM hepes (pH 7.0), 5× Denhardt's solution, 3× SSC, 0.16 mg/ml salmon sperm DNA, 0.1% SDS, and 5% dextran sulfate. Membranes were washed twice in 2× SSC/0.1% SDS at room temperature for 10 min, and twice in 0.1× SSC/0.1% SDS at 55°C for 30 min. The human Spi-B probe (3′-UTR, from the EcoRV site in Spi-B to the EcoRI site in pBluescript, ~300 bp long) and murine Spi-B probe (3′-UTR, NdeI to BamHI, 600 bp long) were labeled by random hexapriming.

For RT-PCR analysis, total RNA was reverse transcribed (RT) in a 20-μl volume with reagents from Perkin-Elmer (Norwalk, CT): Moloney murine Leukemia virus reverse transcriptase (50 U), RNase inhibitor (20 U), dNTPs (1 mM final concentration), Oligo $d(t)_{16}$ (2.5 mM final concentration), $MgCl_2$ (5 mM final concentration), KCl (50 mM final concentration), and Tris-HCl, pH 8.3 (10 mM final concentration). Samples were incubated at 42°C for 15 min 99°C for 5 min, and 5°C for 5 min. The salt concentration for PCR was optimized using the Optiprime kit (Stratagene, La Jolla, CA) for each set of primers according to the manufacturer's instruction. The entire RT reaction was subjected to 30 cycles of denaturation at 94°C for 1 min, primer annealing at 65°C, and primer extension at 72°C for 2 min.

Antibodies. The coding sequence of human Spi-B cDNA was subcloned into the EcoRI site of the bacterial expression vector pGEX-4T3 (Pharmacia, Piscataway, NJ). Spi-B-GST fusion protein was induced at 1.25 mM IPTG and subsequently purified using the Bulk GST Purification Module (Pharmacia). Polyclonal anti-Spi-B antisera were obtained from rabbits immunized with the Spi-B-GST fusion protein (Pocono Rabbit Farm & Laboratory, Canadensis, PA). Monoclonal anti-PU.1 antibody was purchased from Santa Cruz Biotechnology, Inc.

In Vitro Translation and Western Blot Analysis. IVT proteins were synthesized from human Spi-B and murine PU.1 cDNA linked to the T7 promoter in pCDNA3 vectors using Promega TNT T7 Coupled Reticulocyte Lysate System. IVT proteins were quantitated by their [^{35}S]methionine incorporation, to determine that equal amounts of Spi-B and PU.1 proteins were loaded for each assay. IVT proteins and total cell lysates obtained from 2×10^6 cells each were subjected to 12% SDS–polyacrylamide gel electrophoresis followed by electrotransfer onto nitrocellulose. The membranes were probed with the polyclonal antiserum to Spi-B in 10 mM Tris, pH 7.5, 150 mM NaCl, and 5% nonfat milk. The immune complexes were revealed by the ECL Western blotting detection system (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

Cell Purification. To purify splenic B cells, ammonium chloride-lysed spleen cells were subjected to anti-Thy1.2/complement depletion. Splenic T cells were purified from red cell-depleted spleens using a Mouse T Cell Enrichment Column (R&D Systems, Minneapolis, MN) and anti-J11D/complement lysis. The purity of final cell populations was determined by flow cytometry using B220 (B cell) and CD3 (T cell) surface antigens. Purified B cells were determined to be 98% pure, and T cells were 85% pure. For T cell sorting, adult BL/6 thymi were processed into single cell suspensions and resuspended at 5×10^6 cells per ml in 5% complete media (5% fetal bovine serum, 1× non-essential amino acids, 1× L-glutamine, 1× Pen-Strep, 50 μM β-mercaptoethanol in DMEM). Thymocytes were incubated with anti-CD4 and anti-CD8 antibodies (PharMingen, San Diego, CA) at 1 μg/10^6 cells for 30 min at 4°C. The incubation was followed by two washes with complete media. Cells were resuspended at 4×10^6 cells/ml in complete media for sorting by flow cytometry.

In Situ Hybridization. Adult murine tissues (thymus, lymph nodes, heart, lung, spleen, and liver) were sectioned and mounted for in situ hybridization. Sense and anti-sense riboprobes were made from the 3′-UTR of murine Spi-B (NdeI to BamHI) linked to the T7 promoter in pBluescript. The protocol utilized was essentially as described by Eichele and coworkers (14). Tissues were fixed in 4% paraformaldehyde/PBS and embedded in paraffin. The sections were rehydrated and treated with 20 mg/ml proteinase K, and 0.1 M triethanolamide-HCl, pH 8. Pretreated slides were hybridized overnight at 60°C. The hybridization mix contained 50% formamide, 20 mM Tris-HCl, pH 8, 0.3 mM NaCl, 5 mM EDTA, 10% dextran sulfate, 0.02% BSA, 0.5 mg/ml tRNA, and 10 mM DTT. ^{35}S-labeled riboprobe was mixed with the hybridization mix at 140,000 cpm/l (90 ml/slide). A 1/100th volume of 25 mM 5′-alpha-thio-ATP was added to the probe mix, followed by 2 min of boiling. After hybridization, slides were washed with 50% formamide, 2× SSC, and 20 mM DTT for 60 min at 65°C (high stringency wash). Slide were then washed with STE (4× SSC, 20 mM Tris-HCl, pH 7.6, 1 mM EDTA) for 10 min twice at 37°C, followed by RNase A treatment (10 mg/ml in STE) for 30 min at 37°C. This high stringency wash was repeated and then the slides were washed in 2× SSC at 37°C for 10 min.

Electrophoretic Mobility Shift Assays (EMSAs). In vitro-translated (IVT) proteins (1.3 μl to 1.5 μl) or nuclear extracts (10 μg) were preincubated on ice with 1 μg of double-stranded poly(dI-dC) and 0.4 μg of salmon sperm DNA in 5× Ficoll binding buffer in a final volume of 19 μl. For competition experiments, cold competitor oligonucleotide (200–300 ng per reaction) and pre-immune or antiserum were also added at this step. Radiolabeled probes were diluted to 30,000 cpm/μl and 1 μl was added to the binding reaction for a 10-min incubation at room temperature. Oli-

gonucleotides were synthesized according to the published putative PU.1 binding sites:

c-fes (15)
GATCAAACCGCGGGAGGAGGAAGCGCGGAATCAGGA
c-fes mutant (15)
GATCAAACCGCGGGAGCACCGGGCGCGGAATCAGGA
SV40 (12)
GATCTCGGGCTCGAGTCTGAAAGAGGAACTTGGTTA
SV40 mutant (12)
GATCTCGGGCTCGAGTCTTGAAAGACCAACTTGGTT
Igκ-3′ enhancer (16)
GATCCCTTTGAGGAACTGAAAACAGAACCTAGATC
Ig J chain (17)
CTAGATTTTAAGAAAGCAGAAGCAGCAT
M-CSF receptor (18)
TCGACCTAGCTAAAAGGGGAAGAAGAGGATCAGC
Fcγ receptor (19)
CTAGGCAATTTCCCTTCCTCTTTTCTAA
β-globin (11)
GATCACCTTCCTATCAGAAAAAAAGGGGAAGCGATTAT

Transient Transfection and CAT Assays. The CAT reporter construct (B_4TKCAT) containing multiple copies of the PU.1/Pip binding site of the lambda chain enhancer $E_{\lambda 2\text{-}4}$, has been described previously (20). To create Pip/CMV the Pip cDNA was subcloned as a HindIII-XbaI fragment into the HindIII and XbaI sites in the polylinker of pRc/CMV (Invitrogen), which places the cDNA insert under the control of the CMV promoter (21). NIH-3T3 cells were transfected using Lipofectin (GIBCO BRL) and a total of 25 μg of DNA. Transfection involved 5 μg of reporter construct and 10 μg of the expression constructs, with the balance made up with empty expression vector. After 48 h at 37°C, cell lysates were prepared and CAT assays performed using 30 μl of lysate as described previously (20). TLC plates were analyzed on a Molecular Dynamics PhosphorImager; the results shown here represent an average of at least four independent transfections.

Results

Spi-B mRNAs Are Present Exclusively in Murine Thymus, Spleen, and Lymph Nodes. To determine the tissue-specificity of Spi-B mRNA expression, tissues from day 18.5 mouse embryos and adult mice were analyzed on poly-A^+ Northern blots. To distinguish the expression of Spi-B mRNA compared to PU.1 mRNA, a 3′ untranslated region (3′ UTR) probe for the mouse Spi-B cDNA was used. Spi-B mRNA has been previously detected in B cells (12), so splenocytes were used as a positive control. As shown in Fig. 1 *A*, high levels of Spi-B mRNA were detected in adult splenocytes (see lane *1*). The predominant mRNA species was 3.0 kb, in contrast to the 1.4-kb species found in human cells (see Fig. 6). However, a faint 1.5-kb species was detected. Surprisingly, Spi-B mRNA was detected at low levels in adult thymocytes as well (Fig. 1 *A*, lane *2*). In contrast, Spi-B mRNAs were completely absent in all fetal tissues examined including heart, kidney, brain, and liver (Fig. 1 *A*, lanes *3–6*). Adult murine tissues were also examined for Spi-B expression, including heart, liver, lungs, lymph nodes, small intestine, spleen, and testes. Besides the spleen, Spi-B mRNAs were also detected in axillary and mesenteric lymph nodes (Fig. 1 *B*, lanes *4* and *6*). No other organs from adult mice exhibited significant Spi-B expression.

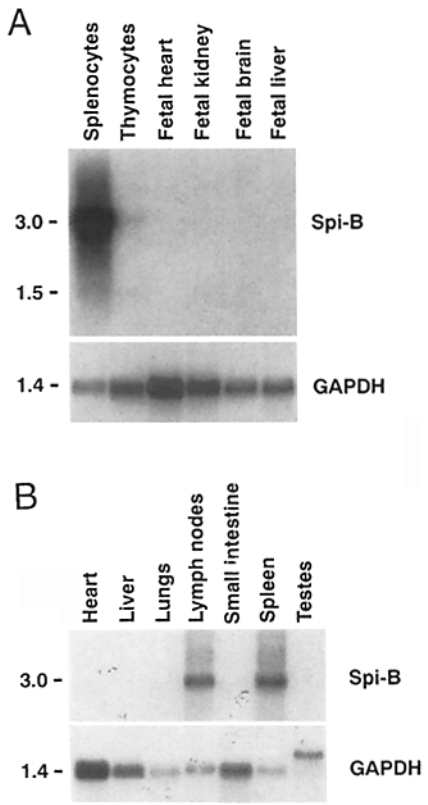

Figure 1. The tissue distribution of Spi-B expression. (*A*) The top panel shows a Northern blot analysis of mRNA samples isolated from fetal murine tissues, adult splenocytes, and adult thymocytes hybridized to a radiolabeled Spi-B 3′ UTR probe (16-h exposure). The Spi-B probe hybridized primarily to a 3.0-kb species and a smaller 1.5-kb species present in purified splenocytes and thymocytes. The bottom panel shows hybridization to the 1.4-kb glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe used as a control for the amount of RNA in each lane. (*B*) The top panel shows a Northern blot analysis of RNA samples obtained from the indicated adult murine tissues (20-h exposure). A 3.0-kb transcript was detected in the spleen and lymph nodes; however, no 1.5-kb species was apparent in this experiment. GAPDH hybridization is shown in the bottom panel. A larger GAPDH transcript was observed in the testes sample.

To further localize Spi-B mRNA expression, in situ hybridization was performed on adult mouse tissues. In adult tissues, Spi-B mRNA was observed exclusively in the thymus, spleen, and lymph nodes (Figs. 2 and 3). Only background signals were detected in heart, intestines, kidney, stomach, lung, and liver (data not shown). To assess positive in situ hybridization, sense and antisense 3′ UTR Spi-B probes were incubated with tissue sections. As shown in Fig. 2, *a*, *d*, and *g*, the sense probe gave weak hybridization signals as compared to the antisense probe (*b*, *e*, and *h*). Hematoxylin- and eosin-stained sections of thymus, spleen, and lymph nodes are shown for comparison (Fig. 2, *c*, *f*, and *i*). In the thymus, Spi-B gene expression was restricted to the medulla region (Fig. 2, *b*). As T cells mature, thymocytes

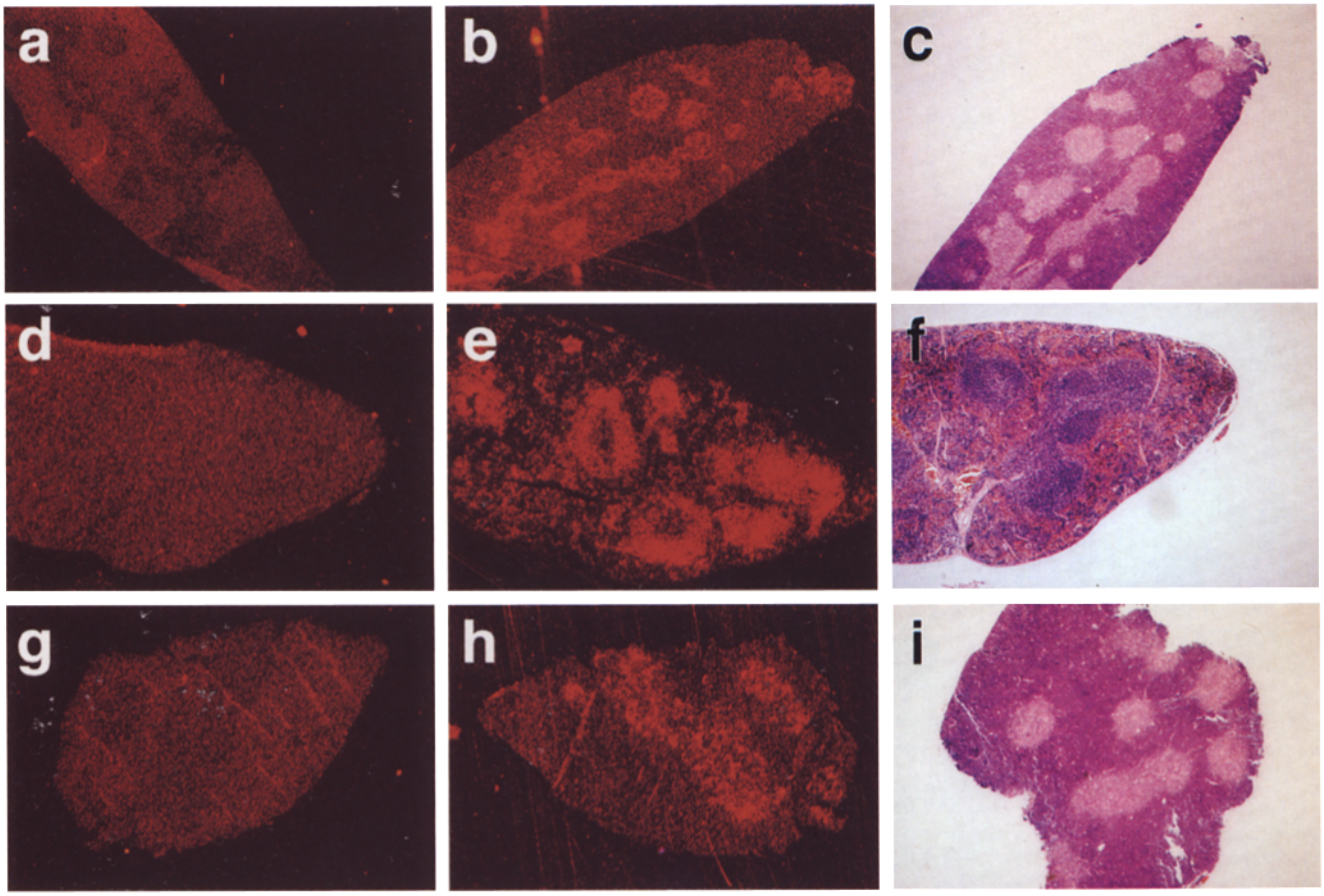

Figure 2. The spatial pattern of expression of Spi-B in the thymus, spleen and lymph nodes. In situ hybridization analyses were performed using a radiolabeled sense 3′ UTR Spi-B riboprobe (*a*, *d*, and *g*) and an antisense Spi-B riboprobe (*b*, *e*, and *h*). Specific hybridization was detected in the thymus medulla (*b*), white pulp of the spleen (*e*), and follicles and germinal centers of the lymph nodes (*h*). *c*, *f*, and *i* display hematoxylin- and eosin-stained sections of adjacent regions of the thymus, spleen, and lymph node for comparison. Magnification, 5×.

migrate from the darkly staining thymic cortex to the pale staining medulla where single positive T cells undergo negative selection. In the spleen, Spi-B mRNA was abundantly expressed throughout the white pulp, except the center portion which consists of arterioles and periarteriolar lymphocyte sheaths (PALS) (Fig. 2, *e*). The PALS contains a network of antigen-presenting cells, such as interdigitating dendritic cells, and plasma B cells. B cells congregate peripherally to the PALS in primary follicles and germinal centers. The outer most layer of the white pulp is the marginal zone, which contains T and B lymphocytes. Spi-B mRNA is present in cells surrounding the arterioles and the PALS in the white pulp of the spleen, where T and B lymphocytes reside. In lymph nodes, Spi-B is present in the primary follicles (Fig. 2, *h* and *i*). Fig. 3, shows in situ hybridization analyses with the thymus (*b*), spleen (*e*), and lymph nodes (*h*) at a higher magnification. Spi-B mRNA was present in the periphery of the thymic medulla and the germinal centers of the spleen (*b* and *e*). Fig. 3 *i*, shows a resting lymph node stained with hematoxylin and eosin; the primary follicles reside at the edge. Resting B cells accumulate in the follicles which clearly express Spi-B (Fig. 3 *h*).

Spi-B Expression Is Restricted to the Thymus and Spleen during Murine Embryogenesis. To determine the temporal and spatial patterns of expression during mammalian development, in situ hybridization experiments were performed on tissue sections obtained from mouse embryos at various gestational stages. Sense and antisense 3′ UTR Spi-B probes were hybridized to sections of day 7.5, 8.5, 10.5, 14.5, 16.5, and 19.5 mouse embryos. The results are summarized in Fig. 4 and show that Spi-B mRNAs were detected exclusively in fetal thymus and spleen. Spi-B expression was first observed in the day 14.5 thymus (Fig. 4, *a* and *c*). All tissues in day 7.5, 8.5, and 10.5 embryos failed to react with the antisense Spi-B probe and gave background signals similar to that observed with the control sense probe shown in Fig. 4 *b*. Furthermore, all other tissues in the day 14.5 embryo were negative for Spi-B mRNA production (see Fig. 4 *a*). It is interesting to note that neither the day 14.5 or day 16.5 spleen exhibited Spi-B gene expression. However, when sections of day 19.5 embryos were hybridized to the Spi-B probe, the spleen displayed strong signal intensity (Fig. 4 *e*). The control sense probe gave the expected background level of hybridization (Fig. 4 *d*). In

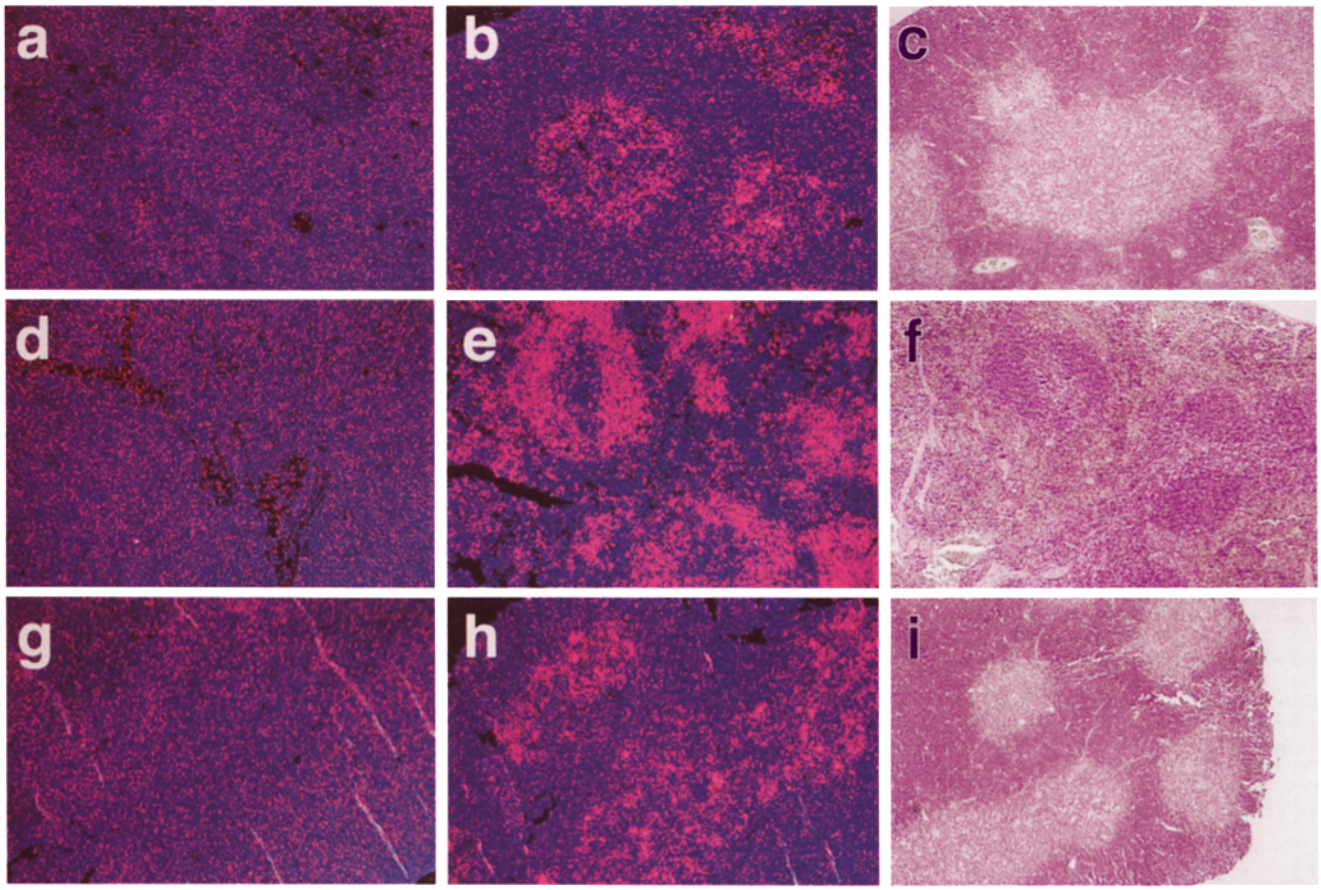

Figure 3. The spatial pattern of expression of Spi-B in adult thymus, spleen, and lymph nodes. In situ hybridization was performed using radiolabeled sense Spi-B riboprobe (*a*, *d*, and *g*) and antisense Spi-B riboprobe (*b*, *e*, and *h*). *c*, *f*, and *i* depict hematoxylin- and eosin-stained adjacent sections of the thymus, spleen, and lymph node, respectively. Magnification, 10×.

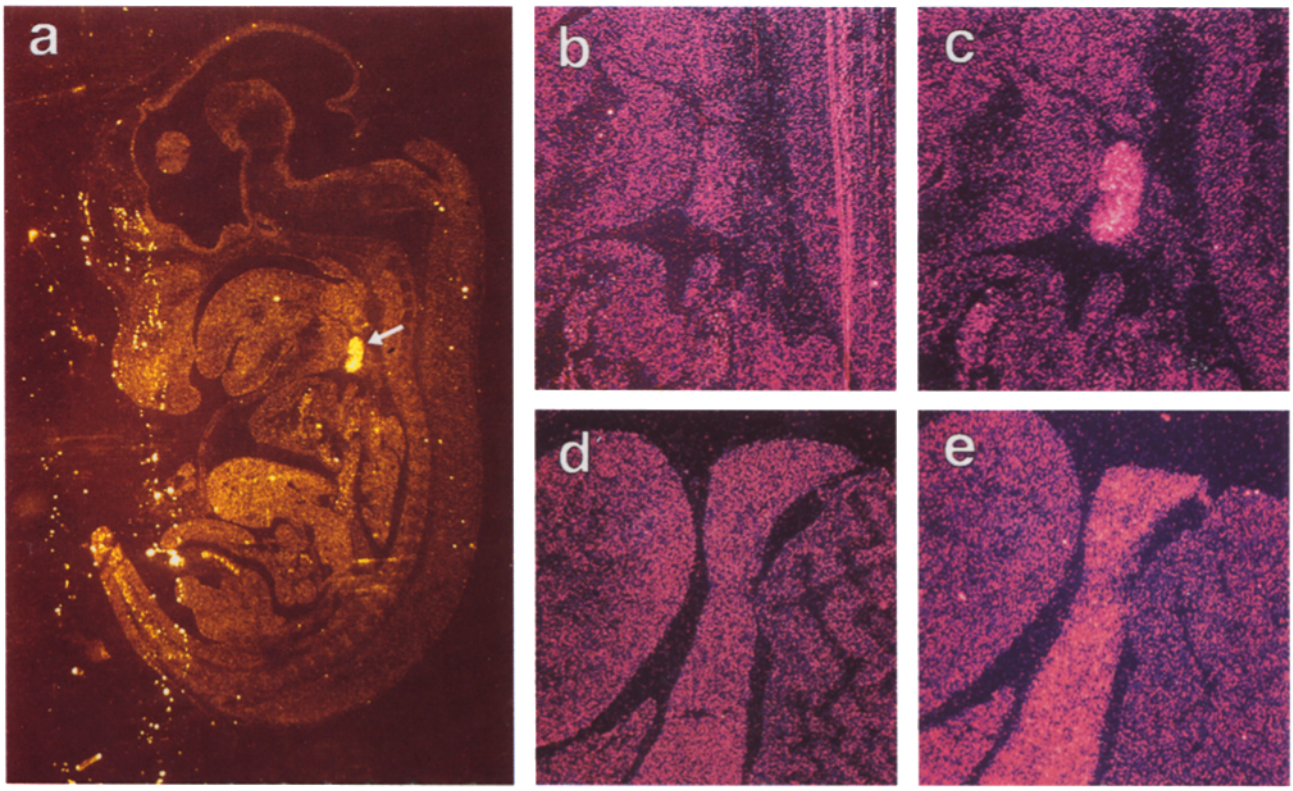

Figure 4. The temporal and spacial patterns of Spi-B expression during murine embryonic development. In situ hybridization assays were performed using the 3′ UTR sense and antisense Spi-B riboprobes on day 14.5 and 19.5 mouse embryo tissue sections; *a* shows hybridization to the antisense probe. Magnification, 12.5×. Sense and antisense Spi-B riboprobes were hybridized to day 14.5 embryonic tissues (*b* and *c*, respectively). Magnification, 25×. *d* and *e* show hybridization to day 19.5 mouse embryo tissue sections with sense and antisense Spi-B riboprobes, respectively. Magnification, 25×. Only the thymus and spleen exhibited Spi-B mRNA expression during development.

the day 19.5 embryo, Spi-B hybridization was detected only in the thymus and spleen. We conclude that Spi-B gene expression is highly tissue restricted and can only be detected in the developing lymphoid organs of the mouse embryo.

Spi-B Expression Is Developmentally Regulated during T Cell and B Cell Development. To further explore Spi-B expression during B cell maturation, immortalized B cell lines representing different developmental stages were studied. Low level Spi-B expression was detected in pro-B cells (NFS 70/c10 cells; Fig. 5 *A*, lane *1*), higher levels in one pre-B cell line (38 B9 cells, lane *2*), and dramatically increased levels in mature B cells such as A20.1 and WEHI (lanes *4* and *5*). Purified splenic B cells exhibited high levels of Spi-B as well (lane *8*). Low levels of Spi-B mRNAs were detected in the immature T cell line EL-4 (lane *7*) and column enriched, anti-J11D/complement depleted splenic T cells. To determine if Spi-B transcription changes during T cell maturation, T cells were sorted into the $CD4^-$ $CD8^-$ (double negative:DN), $CD4^+$ $CD8^+$ (double positive:DP), and $CD4^+$ or $CD8^+$ single positive (SP) populations. Interestingly, Spi-B mRNA was most abundant in the $CD4^-$ $CD8^-$ "double negative" population (Fig. 5 *C*). Furthermore, $Rag2^{-/-}$ thymi express higher levels than those obtained from C57BL/6 mice (lanes *5* and *6*). Rag $2^{-/-}$ thymi have previously been shown to contain no DP or SP T cells and to contain essentially 98% DN cells (22). These results are consistent with Spi-B expression being highest in DN T cell precursors. Furthermore, Spi-B expression was higher in the immature T cell line, EL-4, than the mature T cell line, RMA (Fig. 5 *A*, lanes 7 and *8*). Therefore, Spi-B gene expression increases during B cell maturation and decreases during T cell maturation. Surprisingly, some PU.1 expression was also detected in the $CD4^-$ $CD8^-$ sorted T cells (Fig. 5 *C*), although it was completely absent in a number of human T cell lines examined by RT-PCR analyses (Jurkat, H9, Molt 3) and purified human peripheral T cells (data not shown). RT-PCR assays for M-CSF receptor expression indicated that the RNA samples contained no contaminating monocytic cells (data not shown). Finally, it should be noted that Spi-B was found in EL-4 T cells, thymocytes, and the thymus when these samples were run separately (Fig. 5 *B*).

Spi-B Is Highly Expressed in Human B Cells and Some T Cells at Both the RNA and Protein Levels. Several human cell lines were examined for Spi-B expression by poly-A^+ RNA Northern blot analysis as shown in Fig. 6. Spi-B RNA was detected in clone 13 B cells (lane *1*), but was not present in monocytic cells such as U937 and K562 and Jurkat T cells (lanes *3–5*). Furthermore, TPA treatment of U937 cells (which converts them from monoblasts to monocytes) did not induce Spi-B (lane *2*). Spi-B expression was not apparent in the non-hematopoietic epithelial cell line HeLa or Hep G2 hepatic cells (lanes *6* and *7*).

To determine the tissue specificity of Spi-B protein expression and to confirm that the level of gene expression correlated with the level of protein expression, Western blot analyses were performed. Polyclonal antiserum raised

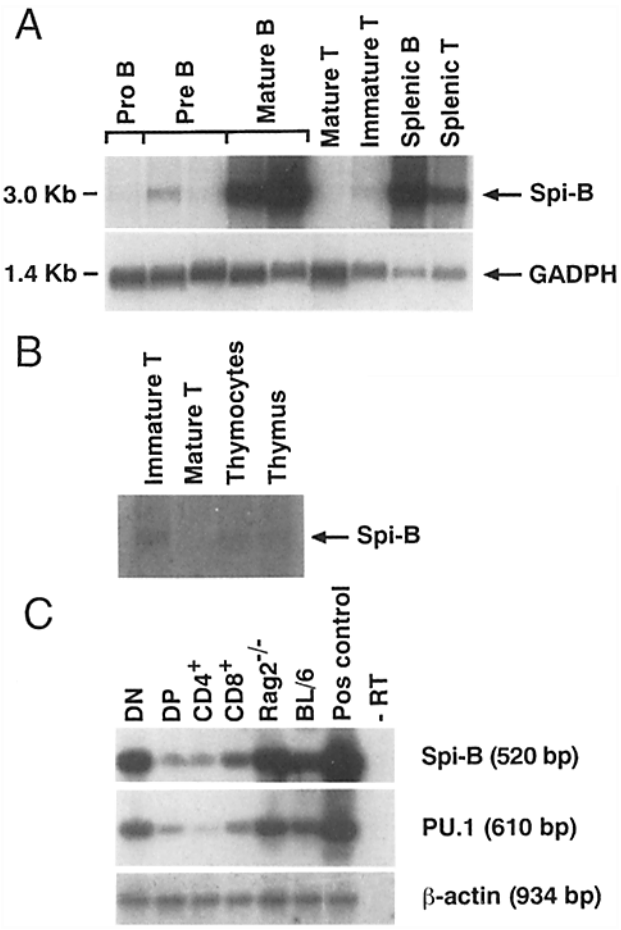

Figure 5. Spi-B mRNA expression during B cell T cell development. (*A*) The top panel shows a Northern blot analysis of RNA samples obtained from murine B and T cell lines and purified splenic B and T cells. B cell lines analyzed include NFS 70/c10 cells (Pro B), 38 B9 cells and 70Z/3 cells (Pre B), and A20.1 and WEHI cells (mature B). T cell lines examined include mature RMA T cells (isolated from a lymphoma) and immature EL-4 T cells (isolated from a thymoma). The bottom panel displays hybridization to the control GAPDH probe. (*B*) RNA samples from *A* run on a separate Northern analysis to demonstrate that weak hybridization detected in T cells was not obtained by contamination from adjacent lanes exhibiting strong Spi-B expression. (*C*) The top panel shows RT-PCR assays for Spi-B expression in FACS®-sorted $CD4^-$ $CD8^-$ (DN) T cells, $CD4^+$ $CD8^+$ (DP) T cells, and $CD4^+$ and $CD8^+$ single positive T cells. Also shown are RT-PCR assays performed on RNA samples from $RAG2^{-/-}$ and C57BL/6 thymi and A20.1 mature B cells (positive control). The final lane shows a negative control reaction containing A20.1 B cell RNA with no reverse transcriptase (RT). The middle and bottom panels depict RT-PCR assays performed on the same samples for PU.1 and mouse b-actin expression. RT-PCR products generated for Spi-B, PU.1, and β-actin mRNAs were 520, 610, and 934 bp, respectively.

against human Spi-B-GST fusion protein was specific for in vitro-translated (IVT) human Spi-B in immunoblotting assays (Fig. 7). Importantly, pre-immune sera failed to bind IVT Spi-B, PU.1, and JunB (Fig. 7 *A*, lanes *1–3*), while polyclonal antisera for Spi-B-GST specifically recognized IVT Spi-B (lane *4*). Furthermore, it did not cross-react with IVT PU.1, *c-Fos*, or JunB (lanes *5–7*). Surprisingly,

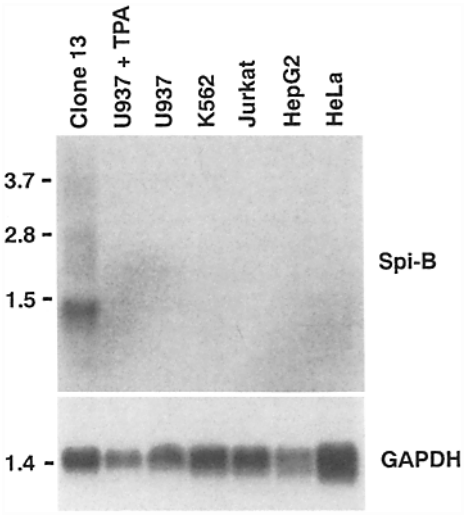

Figure 6. A Poly-A$^+$ Northern blot analysis of Spi-B expression in human cell lines. The top panel shows hybridization to a radiolabeled 3′ UTR probe of the human Spi-B cDNA of clone 13 B cell, U937 monoblast, K562 erythroid/myeloid cells, Jurkat T cell, HepG2 hepatoma, and HeLa cervical carcinoma cell RNA samples. 3.7, 2.8, and 1.5-kb RNA species are indicated. The bottom panel shows hybridization to the control GAPDH radiolabeled probe.

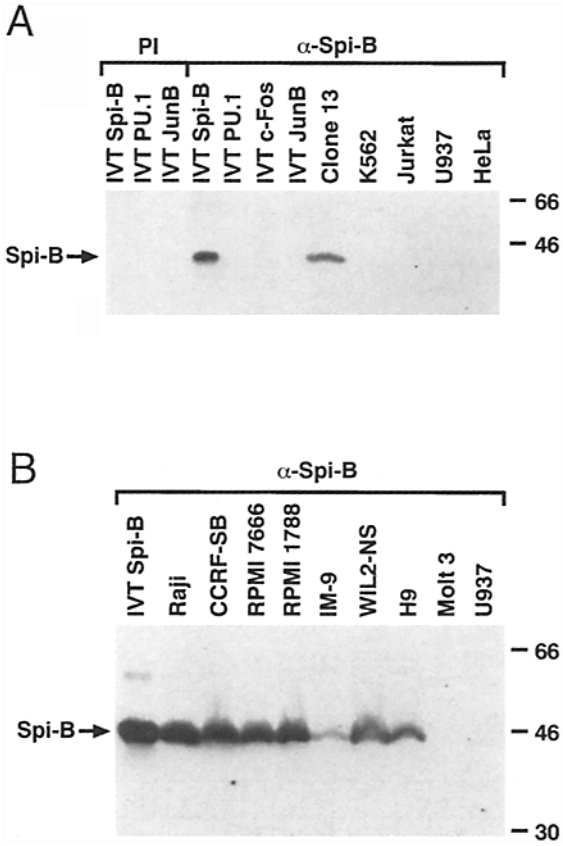

Figure 7. Western blot analysis of Spi-B protein in human cell lines. (*A*) Pre-immune sera and anti-Spi-B antiserum was reacted with IVT human Spi-B, PU.1, and Jun B proteins or IVT human Spi-B, PU.1, and c-Fos proteins. Total cell lysates from clone 13, K562, Jurkat, U937, and HeLa cells are also shown. (*B*) Anti-Spi-B antiserum was reacted with a Western blot of lysates obtained from Raji, CCRF-SB, RPMI 1666, RPMI 1788, IM-9, WIL2-NS B cells, and H9 and Molt3 T cells (IVT human Spi-B was used as a positive control). The size of molecular mass standards are indicated at the left.

the antisera did not cross react with murine Spi-B (data not shown). The 46-kd Spi-B protein was detected in all seven B cell lines examined (Clone 13, Raji, CCFR-SB, RPMI 7666, RPMI 1788, IM-9, and WIL2-NS), and one of the three T cell lines (H9) (Fig. 7, *A* and *B*). No Spi-B protein was detected in the monocytic cell lines K562 and U937 (Fig. 7 *A*, lanes *9* and *11*) or HeLa epithelial cells (lane *12*). The level of protein expression correlated with the abundance of Spi-B mRNAs. It is not surprising that Clone 13 and H9 express Spi-B proteins, since they both contain high levels of Spi-B mRNAs (Fig. 6 and data not shown). It is interesting to note that H9 T cells are less mature than either Jurkat or Molt3 cells (23). Furthermore, IM-9 cells are less mature than Raji, CCRF-SB, RPM1 7666, RPM1 1788 cells, and WIL2-NSB cells. Therefore, Spi-B expression appears to decrease as human T cells mature and increase as human B cells mature, as was noted in murine lymphocytes. Finally, no Spi-B protein was detected in peripheral human T cells (data not shown).

Spi-B Specifically Binds to Putative PU.1 Binding Sites in Monocytic and B Cell Target Genes. A growing number of PU.1 target genes have been identified in monocytic and B cell lines, including *c-fes*, the M-CSF receptor, and the high affinity receptor for Fc fragments (FcγR) in monocytes and J chain and κ and λ immunoglobulin light chains in B cells. To assess PU.1 versus Spi-B DNA-binding specificity, electrophoretic mobility shift assays (EMSAs) were performed with IVT PU.1 and Spi-B proteins. EMSAs were performed so that equal amounts of IVT PU.1 and Spi-B proteins were loaded in each reaction to compare the approximate binding affinity of these two proteins to different PU.1 binding sites. Under our experimental conditions, no proteins other than PU.1 and Spi-B in the TNT reticulocyte lysate bound to the labeled oligonucleotides (data not shown). The binding of both IVT PU.1 and Spi-B (which yielded a slower mobility EMSA complex) was shown to be specific in a series of competition assays (Fig. 8 *A*). The wild-type cold competitor oligonucleotides competed with PU.1 and Spi-B binding activities (lanes *2*, *5*, *8*, and *11*), but not competitor oligonucleotides harboring mutations in the core 5′-GGAA-3′ sequence of each PU.1 binding site (lanes *3*, *6*, *9*, and *12*). The binding activity of PU.1 and Spi-B was compared for oligonucleotides derived from the *c-fes* promoter and the SV40 enhancer. The binding activity of Spi-B to the PU.1-binding site in SV40 was similar to that of PU.1, however Spi-B appeared to bind more weakly to the *c-fes* promoter (see Fig. 8 *A*, lanes *1*, *4*, *7*, and *10*). Spi-B bound specifically and with similar affinity to PU.1 to the sites in the M-CSFR and FCγRI promoters (Fig. 8 *B*, lanes *1–8*). However, Spi-B bound to the PU sites in J chain promoter and the 3′ enhancer of Igκ with

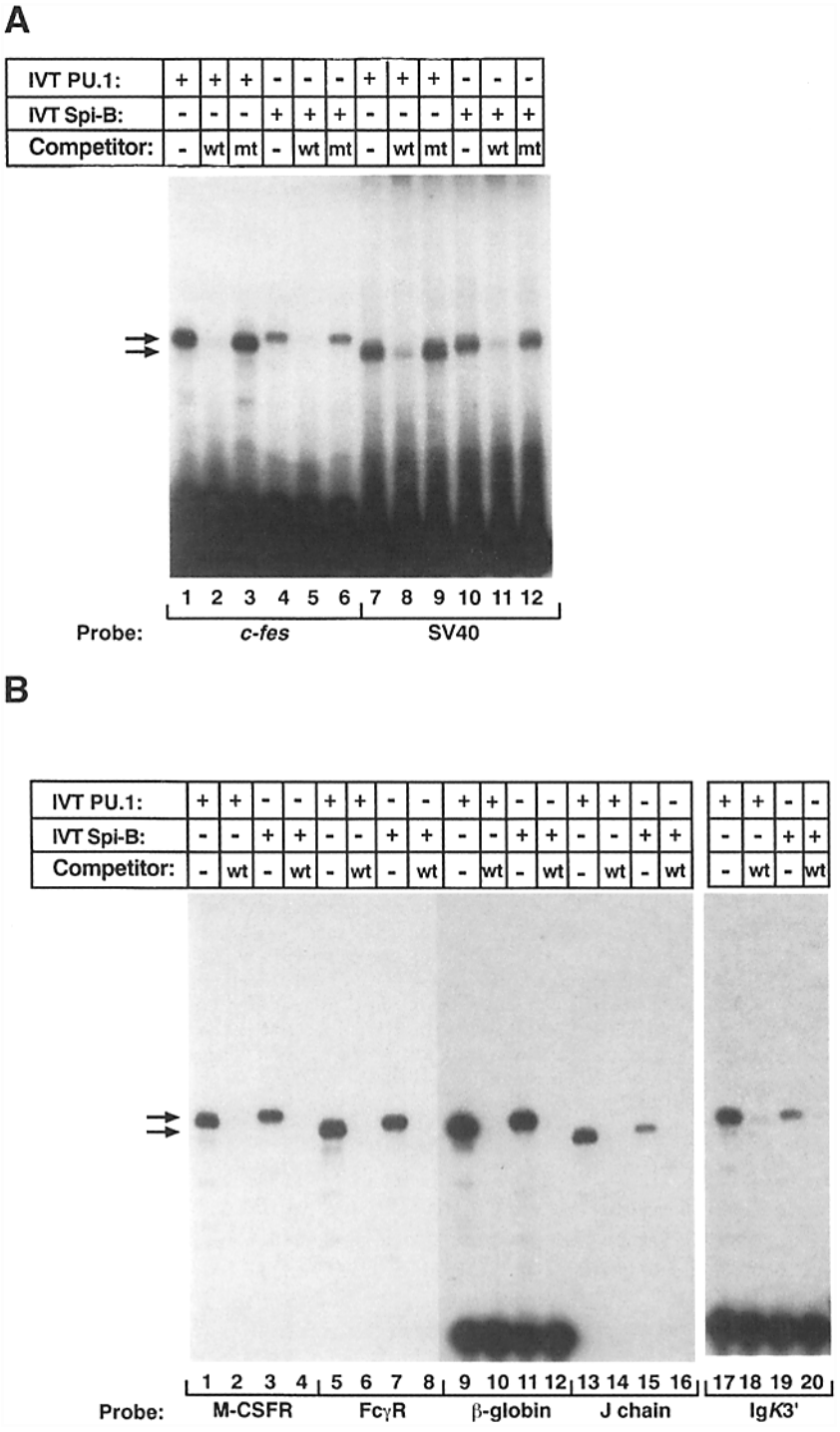

Figure 8. EMSAs of Spi-B binding to published PU.1 binding sites in macrophage and B cell target genes. (*A*) Radiolabeled *c-fes* and SV40 enhancer oligonucleotides were used in gel retardation assays with IVT PU.1 and Spi-B. Where indicated, a 200–300 ng wild type (*wt*) or PU.1 site mutant (*mt*) oligonucleotides were added to the DNA-binding assays. The arrows indicate the rapidly migrating PU.1/DNA complexes and slowly migrating Spi-B/DNA complexes. (*B*) Radiolabeled M-CSFR promoter, FcγRI promoter, β-globin intron, J chain promoter, and Igκ 3′ enhancer oligonucleotides were used in gel retardation assays. The arrows indicate the various PU.1/DNA and Spi-B/DNA complexes.

lower affinity than PU.1 (lanes *13–20*). Interestingly, both PU.1 and Spi-B bind strongly to the β-globin intervening sequence 2, (lanes *9–12*). Table 1 summarizes the results of the binding assays performed and displays the oligonucleotide sequences used. We observed strong Spi-B-binding to the PU.1 sites in β-globin, M-CSFR, FCγRI and weaker binding to the PU.1 sites in SV40, *c-fes*, Igκ 3′, and J chain. We did not find any PU.1-binding sites that failed to bind IVT Spi-B.

To determine if Spi-B in B cell nuclear extracts can also bind to a PU.1-binding site in the Igκ 3′ enhancer, a series of binding experiments were performed on extracts obtained from clone 13 human B cells. First, antibodies specific for both PU.1 and Spi-B were tested for cross reactivity on purified proteins. As shown in Fig. 9 *A*, the anti-PU.1 antibody inhibited PU.1 binding to a *c-fes* promoter oligonucleotide and did not interfere with the binding of IVT Spi-B (lanes *3* and *4*). Furthermore, the anti-Spi-B anti-

Table 1. *Compilation of the PU.1 and Spi-B Binding Sites*

β-globin	GATCACCTTCCTATCAGAAAAAAAGG**GGAA**GCGATTAT	Galson et al. 1993
M-CSF receptor	TCGACCTAGCTAAAAGGGGA**AGAA**GAGGATCAGC	Zhang et al. 1994
Fcγ receptor	CT**AGGCAA**TTTCCCTTCCTCTTTTCTAA	Perez et al. 1994
SV40 enhancer	GATCTCGGGCTCGAGTCTGAAA**GAGGAA**CTTGGTTA	Ray et al. 1992
C-fes promoter	GATCAAACCGCGGGAG**GAGGAA**GCGCGGAATCAGGA	Heydeman et al. 1996
Ig κ-3′ enhancer	GATCCCTTT**GAGGAA**CTGAAAACAGAACCTAGATC	Pongubala et al. 1992
Ig J chain	CTAGATTTTAAGAAAGC**AGAA**GCAGCAT	Shin et al. 1993

body ablated Spi-B binding but did not affect PU.1 binding (lanes *5* and *6*). As expected, pre-immune sera did not affect the binding of either protein (lanes 7 and *8*).

Having demonstrated that these antibodies are specific for either PU.1 or Spi-B, binding assays were performed on human B cell nuclear extracts. As shown in Fig. 9 *B*, two specific complexes were detected using the Igκ 3′ oligonucleotide (bands 2 and 3 in lane *1*). Both of these complexes were competed by 200 ng of cold Igκ 3′ oligonucleotide while band 1 was not (lane *2*). Pre-immune sera did not affect either the specific or non-specific DNA-protein complexes (lane *3*). However, the antibody specific for Spi-B allowed the detection of a DNA–protein complex with a rapid mobility and the antibody specific for PU.1 allowed the detection of a complex with a slower mobility (lanes *4* and *5*). We conclude that the addition of anti-Spi-B antibody allowed the detection of the rapidly migrating PU.1-DNA complex and the more slowly migrating Spi-B-DNA complex was revealed by the addition of the anti-PU.1 antibody. When both Spi-B and PU.1 antibodies were added to these reactions, both PU.1- and Spi-B-binding were eliminated (lane *6*). The migration of PU.1 and Spi-B was confirmed by their co-migration with IVT PU.1 and Spi-B (lanes 7 and *8*). We conclude that both PU.1 and Spi-B in clone 13 B cell nuclei are capable of binding the PU.1-binding site in the Igκ 3′ enhancer in an in vitro assay.

Spi-B Functions as a Transcriptional Activator of a B Cell-specific Gene. PU.1 and Pip (PU.1 interaction partner) form a B cell–specific heterodimeric protein complex that regulates the expression of the Igλ enhancer $E_{\lambda 2\text{-}4}$ (21). PU.1 and Pip bind to a composite element in this enhancer that is essential for enhancer activity (20). Furthermore, PU.1 and Pip function as mutually dependent transcriptional activators of this element when coexpressed in NIH-3T3 cells (21). Having shown that Spi-B can bind to multiple PU.1-binding sites in B cell–specific genes, we wanted to learn if Spi-B can also function as a transactivator of the lambda $E_{\lambda 2\text{-}4}$ element. The λB element of $E_{\lambda 2\text{-}4}$ when multimerized and placed upstream of the thymidine kinase promoter, is transactivated by PU.1 and Pip when coexpressed in NIH-3T3 cells (21). As shown in Fig. 10, neither PU.1 nor Pip transactivated the λB reporter construct (B_4TKCAT) when expressed alone in NIH-3T3 cells. However, if both PU.1 and Pip were cotransfected with the B_4TKCAT reporter DNA, chloramphenical acetyl transferase (CAT) activity was elevated 20–30-fold. If Spi-B and Pip were cotransfected with B_4TKCAT, CAT expression was also elevated 15-fold. Spi-B expression alone failed to elevate CAT activity (see Fig. 10). These results show that Spi-B acts as a transcriptional activator in conjunction with Pip. Eisenbeis et al have shown that PU.1 is required for Pip to bind to the $E_{\lambda 2\text{-}4}$ element (21). We conclude that Spi-B can also allow Pip binding and activate transcription.

Discussion

The Ets family of DNA-binding proteins have become the subject of extensive investigation as key regulators of immune response genes and cell division (24). The Ets family now includes ~20 members and their cellular functions and target genes are currently being analyzed. Spi-B and PU.1 share a 67% amino acid Ets-domain identity and 43% overall amino acid identity (12). It is essential to learn if Spi-B and PU.1 have similar patterns of expression to begin to understand their respective functions in development and immune cell function.

In contrast to previous reports that Spi-B and PU.1 are coexpressed in a variety of hematopoietic cells (12, 25), our data clearly showed that Spi-B expression is restricted to T and B lymphocytes. We detected no human Spi-B RNA or protein by poly-A^+ Northern and Western blot analysis in monocytic or erythroid cells. We attribute the divergent results to the different probes used. The 3′-UTR of Spi-B was used in all of our Northern blot assays as opposed to the full-length cDNA used in the study by Ray et al. (12). Because the nucleotide sequence similarity between Spi-B and PU.1 human cDNAs in the DNA-binding domain is 75% (12), we conclude that the myeloid cell expression observed in the previous report represents cross reaction with PU.1. Contrary to previous reports (12), we also detected Spi-B expression in some human T cell lines at the level of both RNA and protein. The polyclonal antiserum raised against a human Spi-B-GST fusion protein was able to Western blot, immunoprecipitate, and ablate DNA binding activity of human Spi-B specifically without any cross-reactivity with PU.1. In Western blot analysis, Spi-B protein was detected in all B cell lines and one T cell line, but not

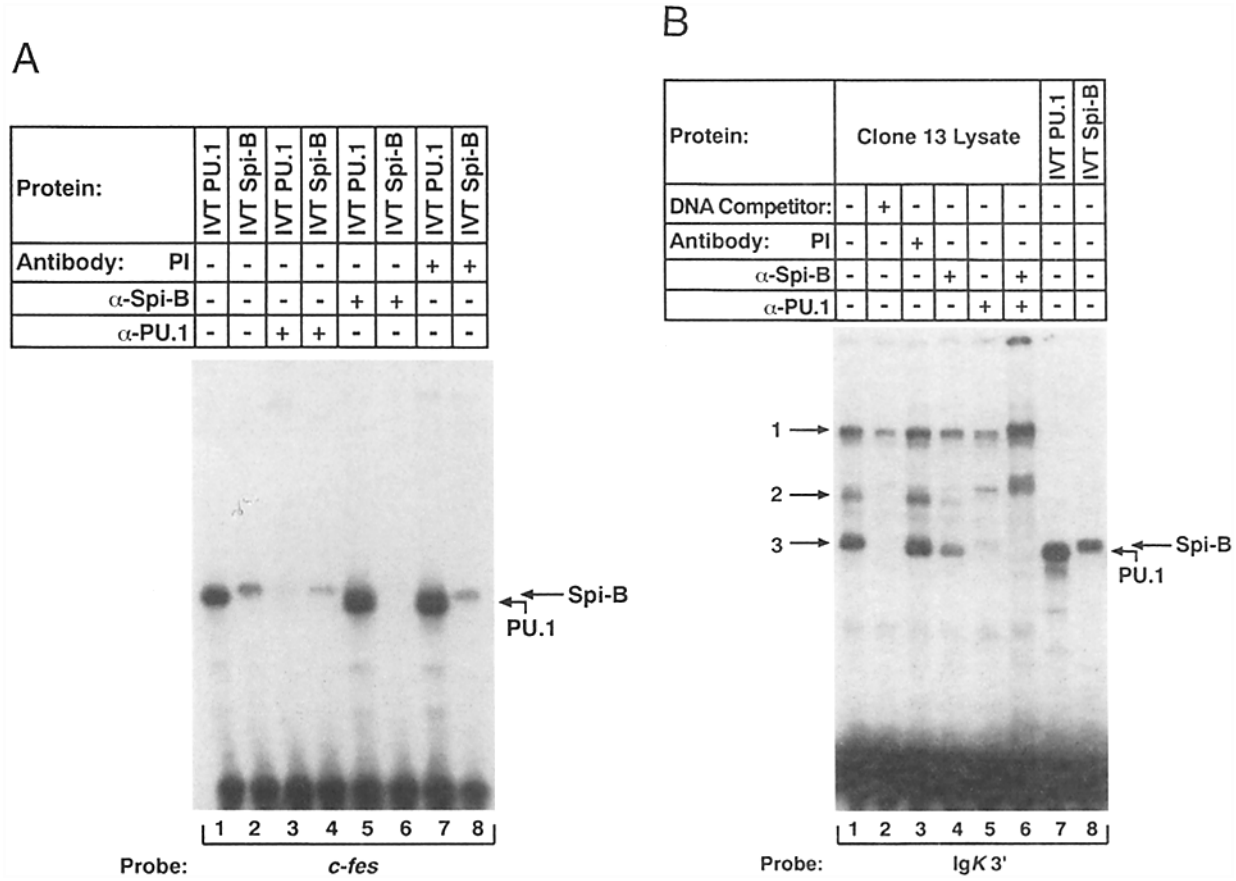

Figure 9. Binding of PU.1 and Spi-B from clone 13 B cell nuclear extracts to the 3′ enhancer of Igκ. (*A*) A radiolabeled *c-fes* promoter oligonucleotide was used in EMSAs with IVT PU.1 and Spi-B. Where indicated, pre-immune serum (*PI*) or antisera specific for PU.1 or Spi-B were added to DNA-binding reactions. (*B*) A radiolabeled oligonucleotide from the Igκ 3′ enhancer was used in EMSAs with nuclear extracts obtained from clone 13 B cells. Three DNA/protein complexes were detected and are numbered *1*, *2*, and *3*. Where indicated pre-immune (*PI*) or anti-PU.1 and anti-Spi-B antisera were added to DNA-binding assays. 200 ng of unlabeled competitor oligonucleotide was added to the binding reaction shown in lane *2*. The mobility of PU.1/DNA and Spi-B/DNA complexes are indicated at the left.

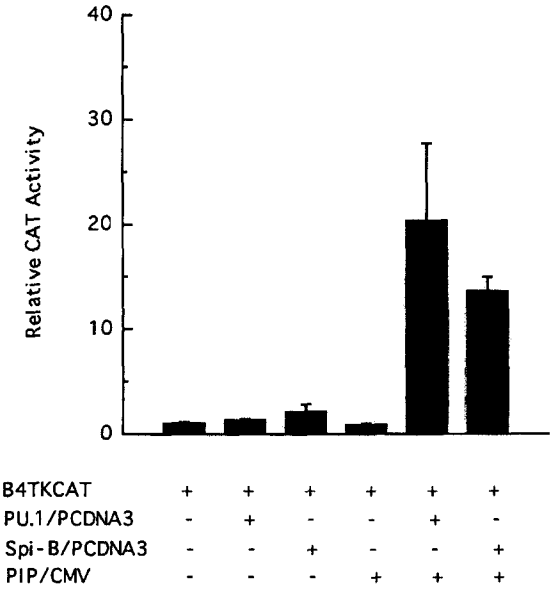

Figure 10. Functional analysis of the Spi-B protein. PU.1, Spi-B, and Pip were cloned into a eukaryotic expression vector containing the CMV promoter and enhancer. The reporter is the B_4TKCAT construct described in (20) containing four κB sites upstream of the thymidine kinase promoter. The indicated DNAs were transiently cotransfected into NIH-3T3 cells and extracts were analyzed for CAT activity 48 h later. The activity of B_4TKCAT alone has been set at 1.0. The results reported are an average of four independent transfections.

in any other lineages. Spi-B protein levels in B and T cells corresponded to RNA levels; only those cells with high Spi-B mRNA expression had detectable Spi-B protein.

Spi-B expression in both B cells and T cells appears to be tightly regulated during development. Pro-B cells had the lowest levels of Spi-B mRNA and expression increased in pre-B and mature B cells. The notion that mature B cells display high levels of Spi-B expression was also evident in tissue studies. Spleen, lymph nodes, and purified splenic B cells exhibited high levels of Spi-B expression. Spi-B expression was highest in immature $CD4^-$ $CD8^-$ T cells and decreased during maturation to the $CD4^+$ $CD8^+$ stage of development. These findings were consistent with human T cells and murine and human T cell lines. The medullary

staining observed for Spi-B in the murine thymus would appear to be inconsistent with high levels of Spi-B RNA in $CD4^-$ $CD8^-$ T cells. However, it is not clear that the positive selection of developing thymocytes is restricted to the thymic cortex (26). It remains a formal possibility that $CD4^-$ $CD8^-$ T cells reside in the medulla. Alternatively, the medullary staining observed may represent expression in antigen presenting cells (B cells and dendritic cells). Other than the thymus, spleen, and lymph nodes, Spi-B was virtually undetectable in any other organ of either embryonic or adult mice by both poly-A^+ Northern blot analysis and in situ hybridization.

The expression of Spi-B is clearly distinct from that of PU.1. In situ hybridizations of fetal and adult mouse tissues with the 5′ untranslated region of PU.1 mRNA previously detected PU.1 expression in bone marrow, liver, and spleen of adults and the fetal liver of day 18.5 embryos (11). Further analysis showed that this was due to high levels of PU.1 in macrophages, neutrophils, mast cells, B cells, and immature erythroid cells (9, 11, 12). In contrast, Spi-B expression in murine tissues is limited to the lymphoid organs (thymus, spleen, and lymph nodes) and is not detected in the bone marrow or liver. The lack of Spi-B expression in the fetal liver suggests that Spi-B is not present in immature proerythroblasts that are very abundant in the liver during days 12.5 to 16.5 of gestation. Therefore, macrophages, neutrophils, and red blood cells which express high levels of PU.1 completely lack Spi-B. Furthermore, Spi-B was detected in T cells where no PU.1 expression was observed. Therefore, only B cells and $CD4^-$ $CD8^-$ T cells express high levels of both PU.1 and Spi-B.

When the binding affinities of IVT PU.1 and Spi-B were compared, Spi-B was able to bind to all published PU.1-binding sites albeit with less affinity in some cases. Since both PU.1 and Spi-B are present in B cells, it is interesting that both were capable of binding to the 3′ enhancer of Igκ. Although, Spi-B bound efficiently to the PU.1 binding sites of M-CSFR, FcγRI, and the scavenger receptor promoters (27), it is unlikely to regulate these genes as it is not present in monocytes or macrophages. It is intriguing that Spi-B can also transactivate a reporter construct containing the PU.1 binding site in the lambda chain $E_{\lambda 2\text{-}4}$ enhancer. It will be of interest to learn what Spi-B target genes exist in B cells and early T cells. We have previously shown that PU.1 and Spi-B play nonredundant roles in B cells and T cells: gene targeting of PU.1 leads to mice lacking B cells, T cells, monocytes, and neutrophils (13). It will be of interest to learn how lymphocytic development and function are affected in Spi-B-deficient mice.

We thank Harinder Singh and Abe Brass for the B_4TCAT and Pip/CMV plasmids and Mike Parmacek for helpful discussions. We also thank Cheryl Small for her careful preparation of this manuscript.

This research was supported by the National Institutes of Health (HL52094-02) and the Howard Hughes Medical Institute. G.H. Su is supported by a Genetics training grant to the University of Chicago.

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Received for publication 24 January 1996 and in revised form 2 May 1996.

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