Neutrophils and Monocytes Express High Levels of PU.1 (Spi-1) But Not Spi-B

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PU.1 (the Spi-1 oncogene) and Spi-B are closely related members of the ets transcription factor family, sharing similar DNA binding specificities mediated by similar DNA binding domains. PU.1 and Spi-B have been previously described as being predominantly expressed coordinately in macrophages and B cells, but their expression in early hematopoietic stages and during the course of myeloid differentiation to monocytes and macrophages or to neutrophils has not been extensively investigated. Here, we report that PU.1 mRNA is upregulated during myeloid differentiation of human purified CD34+ cells and murine multipotential FDCP-mix A4 cells, suggesting that PU.1 is upregulated as an early event during differentiation of multipotential progenitor cells. PU.1 expression is maintained at stable levels during differentiation of myeloid cell lines U937 and HL-60 to monocytic and neutrophilic cells. PU.1 is expressed at highest levels in mature human monocytes and human peripheral blood neutrophils. In contrast to PU.1, significant levels of Spi-B mRNA and protein are found only in some B-cell lines and spleen but are not found in myeloid cell lines, neutrophils, or macrophages. In vitro translated Spi-B protein can bind to PU.1 binding sites in myeloid promoters and transactivate these promoters in nonmyeloid cells. Therefore, although PU.1 and Spi-B may bind to similar DNA control elements and have redundancy of transactivation function in vitro, the lack of significant levels of Spi-B in myeloid cells makes it unlikely that Spi-B plays a significant role in myeloid lineage development and gene expression. In contrast, PU.1 is expressed at high levels not only in monocytes and macrophages but also in neutrophils, indicating that PU.1 can activate gene expression in both major myeloid lineages.

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THE RELATED TRANSCRIPTION factors PU.1 and Spi-B have been implicated in playing important roles in hematopoietic cells.1,3 PU.1 is the product of the Spi-1 oncogene whose expression is activated by the acute leukemogenic retrovirus spleen focus-forming virus in murine erythroleukemia.1,3 Overexpression of PU.1 in developing erythroblasts results in a block of erythroid differentiation.5 Spi-B was isolated from a B-cell library using the conserved 3’ region of the PU.1 cDNA as a probe.6 These two genes share similar DNA binding domains and binding specificities.

PU.1 and Spi-B belong to the Ets family and share a DNA binding motif that recognizes a purine-rich sequence along with the other members of this family, including Ets-1, Ets-2, Elk-1, Erg, Elf-1, and PEA3.3,19 PU.1 has been shown to bind to the promoters of multiple macrophage genes, including the macrophage colony-stimulating factor receptor (M-CSFR), the macrophage scavenger receptor, and CD11b, and is critical for their macrophage-specific expression.11-13 In B cells, PU.1 also regulates the J-chain promoter and the Ig A2-4 and k3’-enhancers.14,16 PU.1 is a cell type-specific activator and is expressed at high levels in macrophages and B cells, implying that it may play an important role in their differentiation and/or activation. Previous studies indicated that PU.1 was expressed in early granulocytic, erythroid, and megakaryocytic cells, but not in mature erythroid cells.11,14-16 and was downregulated in the course of differentiation of erythroid cell lines.7 Our own studies in human CD34+ cells indicated that PU.1 was upregulated during myeloid (granulocyte-macrophage CSF [GM-CSF]-induced) differentiation but not during erythroid differentiation of these cells.4 In contrast to its high levels of expression in macrophages, PU.1 protein was not detected in mature murine neutrophils, suggesting that its expression was upregulated during monocytoic and possibly downregulated during neutrophilic differentiation of myeloid progenitors.19 The pattern of PU.1 expression in mature monocytoic cells, combined with the role of PU.1 in the expression of the myeloid-specific integrin CD11b and the M-CSFR (a key growth factor receptor for the macrophage lineage), suggested that PU.1 might have a critical role in the differentiation of stem cells to the macrophage lineage, just as GATA-1 is essential for erythroid-lineage development.19 Recent studies using competitor oligonucleotides to block PU.1 function showed its critical role in myeloid and colony formation.4 Studies of the expression of Spi-B have been more limited but have suggested that its expression might be identical to that of PU.1.7

To study the possible role of PU.1 and Spi-B in commitment and differentiation of bone marrow (BM) stem cells to the myeloid lineage and, eventually, to mature cells, we analyzed their expression (1) during myeloid commitment of multipotential CD34+ cells and in murine FDCP cells, (2) during myeloid differentiation of myeloid cell lines, and (3) in primary human myeloid cells and murine tissues. In contrast to Spi-B, which is expressed at significant levels only in B cells, PU.1 is upregulated during myeloid differentiation of multipotential human and murine cells and is expressed at highest levels in myeloid cells, most prominently in human neutrophils. These findings suggest that PU.1 and Spi-B are likely to play very distinct roles in hematoopoiesis and that PU.1, not Spi-B, is likely to regulate neutrophil as well as macrophage gene expression.
**MATERIALS AND METHODS**

**Preparation, enrichment, and culture of CD34\(^+\) progenitor cells.** Human BM mononuclear cells were obtained from discarded filter sets from normal donors or from patients with solid tumors without BM involvement, undergoing BM harvest for transplantation. CD34\(^+\) progenitors were enriched by an immunomagnetic selection technique\(^{20}\), modified as follows. BM mononuclear cells, obtained by a Ficoll (Pharmacia, Uppsala, Sweden) density gradient were depleted of the adherent fraction, followed by fractionation on a 3-step Percoll (Pharmacia) gradient (densities: 1,054, 1,066, and 1,077 g/mL). Cells with the lowest density were incubated with a CD34 antibody (IMMU133; a generous gift from Immunotech, Marseille, France) and, subsequently, with goat antirabbit IgG1-coated immunomagnetic beads (DynaBeads; Dynal, Oslo, Norway). The CD34\(^+\) population was then isolated on a magnetic particle concentrator.

CD34-enriched cells were cultured in a liquid medium at a concentration of 10,000/mL in RPMI 1640, supplemented with 2 mmol/L glutamine, 100 \(\mu\)g/mL penicillin-streptomycin, 10% heat-inactivated fetal bovine serum (55°C for 30 minutes; Hyclone, Logan, Utah), human recombinant interleukin-3 (IL-3; 0.01 U/mL), and human GM-CSF (10 ng/mL). Cells were then harvested daily. To obtain a CD34\(^+/\)CD38\(^-\) population, the low-density cells from the Percoll gradient fractionation described above were subjected to cell-sorting on a Coulter EPICS 750 Flow Cytometer (Coulter, Hialeah, FL). The cells were first separated with anti-CD34-fluorescein isothiocyanate (FITC)-conjugated antibody (HPCA-2; Becton Dickinson, San Jose, CA). The CD34\(^+\) population was then further separated into CD34\(^+/\)CD38\(^-\) and CD34\(^+/\)CD38\(^+\) populations using a CD38-phyceroerythrin (PE)-conjugated antibody (Becton Dickinson). The CD34\(^+\)/CD38\(^-\) population represented 2% of the starting population of non-adherent Ficoll-compurified cells.

**Reeve transcription-polymerase chain reaction (RT-PCR) analysis of CD34\(^+\) cell cultures.** Total RNA was extracted from up to 100,000 cells according to the single-step isolation method\(^{21}\) using Tri-Reagent (Molecular Research Center, Inc, Cincinnati, OH). First-strand cDNA was synthesized by RT (Moloney murine leukemia virus RT) using random hexamers as primer (GIBCO-BRL, Gaithersburg, MD). Each cycle of PCR included 1 minute of denaturation at 95°C, 1 minute of primer annealing 3°C below the melting temperature (Tm) required by the set of oligo-nucleotides used, and 90 seconds of extension/synthesis at 72°C. The sequences of the PCR primer set for human PU.1 were 5'-TGGAAGGTTTCCTCCGGTCGTC-3' (bp 201 to 220 of the cDNA\(^{A}\)) and 5'-TGCTGTCTCTTTGATGTCGG-3' (bp 746 to 727); for CD11b, they were 5'-GCCATTGCTGCTTTCGGA-3' (bp 2304 to 2320 of the CD11b cDNA\(^{A}\)) and 5'-TCCAAGAAACGCTTGGC-3' (bp 2755 to 2739); for human Spi-B, they were 5'-ACCATGTCTGCCCTGGGA-3' (bp 4 to 20 of the cDNA\(^{A}\)) and 5'-GCCAGCGAGGTTCCMSCC-3' (bp 342 to 326); and for GAPDH, the primer set was 5'-CCATGGAGAAGCTGGG-3' (bp 388 to 405 of the GAPDH cDNA\(^{A}\)) and 5'-CAAGTGTCGATGACC-3' (bp 582 to 563). A negative control reaction, containing water instead of cDNA, was included in each PCR amplification to exclude the presence of possible DNA contamination.

cDNA from different time points was amplified using PCR conditions in the exponential range of amplification. The PCR amplification is linear so long as the reaction is performed in the exponential range of amplification, at a constant efficiency. For these reasons, we performed kinetic studies of RT-PCR amplification for the different genes studied (PU.1, CD11b, and GAPDH), using an equivalently low input of RNA (12 ng) from human myeloid lines that we estimated we had harvested from CD34\(^+\) cell cultures. The same amount of RNA was reverse-transcribed and amplified with specific primer sets for different genes, and one eighth of the reaction was harvested every 5 cycles. Once a linear range was established for each set of primers, increasing amounts of RNA (from 50 pg to 100 ng) were amplified in a linear fashion. Quantitation of RNA extracted from an estimated equal number of CD34\(^+\) cells and their progeny was performed according to the GAPDH-band intensity. The cDNA resulting from the same RT was then diluted according to the GAPDH signal intensity and amplified as described above. PCR products were separated in a 1.5% agarose gel, stained with ethidium bromide, and transferred to a positive-charged nylon membrane (Biotrans; ICN, Costa Mesa, CA). PU.1 and GAPDH messages were hybridized to the corresponding cDNA labeled with \(^{32}\)P-deoxyctydine triphosphate by the hexanucleotide random priming method\(^{24}\). Internal oligonucleotides, labeled as described,\(^ {25}\) were used for CD11b hybridization.

**Cell culture and induction of differentiation.** Human promyelocytic U937 cells (American Type Culture Collection [ATCC] no. CRL 1593; ATCC, Rockville, MD), human promyelocytic HL-60 cells (ATCC no. CCL 240), T-lymphoblastic Jurkat cells (ATCC no. TIB 152), CML cell line K-562 (ATCC no. 243), Burkitt’s lymphoma cell line RAJ1 (ATCC no. CCL86), B-lymphoblastic BJA-B cells,\(^ {26}\) murine monocye-macrophage cell line RAW 264.7 (ATCC no. TIB 71), and murine B-lymphoma cell line A20 (ATCC no. TIB 208) were maintained in RPMI-1640 medium (GIBCO-BRL) supplemented with 10% fetal calf serum (FCS; Hyclone) and 2 mmol/L L-glutamine. Human cervical carcinoma HeLa cells (ATCC no. CCL 2) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum and 2 mmol/L L-glutamine. The human myeloma line Sp2/0-Ag14 (ATCC no. CRL 1581) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 4.5 g/L glucose and 10% FCS. Human monocytic cell line Mono Mac 6 and murine pro-B-lymphocyte cell line LyD9 were maintained as described.\(^ {27,28}\)

**FDCP-mix A4** is a multipotent IL-3-dependent cell line derived from murine long-term BM culture and maintained in Fisher’s medium supplemented with 10% WEHI-3B-conditioned medium as a source of IL-3 and with 20% horse serum.\(^ {29}\)

U937 and HL-60 cells were induced for 6 days with 10\(^{-8}\) mol/L retinoic acid or with 3 \times 10\(^{-8}\) mol/L 12-O-tetradecanoylphorbol-13-acetate (TPA). FDCP-mix A4 cells were induced to differentiate in Iscove’s Medium (BRL) containing 10% FCS, 0.1% WEHI-3B-conditioned medium, and 2.5 ng/mL murine GM-CSF (kindly provided by Glaxo, Geneva, Switzerland).

**RNA preparations and Northern blot analysis.** Total cellular RNA was isolated by guanidium isothiocyanate extraction followed by cesium chloride gradient purification.\(^ {30}\) RNA (10 \(\mu\)g/lane) was denatured in formamide/formaldehyde followed by electrophoresis in 1% agarose/formaldehyde gels. RNA was then transferred to Bio-trans nylon membrane (ICN, Costa Mesa, CA), and was hybridized with species-specific PU.1 or Spi-B cDNA probes and 18S or 28S rRNA oligonucleotide probes. The probes for murine PU.1 mRNA were either the full-length mouse PU.1 cDNA\(^ {31}\) or the most 431 bp of the cDNA (the Xho I/Not I fragment). The probe for murine Spi-B mRNA was the 5’-496 bp Hpa I/Xho I fragment of the murine Spi-B cDNA (Gonzalez et al, manuscript in preparation). The probe for human PU.1 mRNA was the 5’-438 bp (the EcoRI/Pst I fragment) of the human PU.1 cDNA.\(^ {2}\) The probe for human Spi-B mRNA was the 5’-362 bp (the EcoRI/Nde I fragment) of the human Spi-B cDNA.\(^ {3}\) The oligonucleotide probe for 18S rRNA was 938 to 921 of the human 18S ribosomal RNA gene\(^ {31}\) (5’- TCCGGCCTGGTTGAACA-3’). The oligonucleotide for the 28S rRNA was bp 4036 to 4020 of the human 28S ribosomal RNA\(^ {27}\) (5’-AGTTAGGCCAAATGCTCC-3’). 18S and 28S probes were used as internal controls for RNA loading. The PU.1 and Spi-B cDNA probes were labeled by the random priming as previously described.\(^ {24}\) Hybridization was performed at 65°C in 0.5 mol/L sodium phosphate buffer (pH 7.2), 7% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin for 15 hours. Membranes were washed.
twice in 2X SSC and 0.2% SDS at room temperature for 5 minutes and once in 0.2X SSC and 0.2% SDS at 65°C for 20 minutes. Autoradiography was performed at −80°C with Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for times indicated in the figure legends. Subsequently, probes were removed by shaking in 10 mmol/L sodium phosphate buffer (pH 6.5) and 0.5% deionized formamide for 1 hour at 65°C, followed by washing in 2X SSC and 0.1% SDS for 15 minutes at room temperature. Blots were then hybridized to other cDNA probes or to the [α-32P]-deoxyctydine triphosphate 3′-end-labeled 18S or 28S oligonucleotide overnight in 5X SSC, 5X Denhardt’s solution, and 0.5% SDS at 42°C (for the 28S oligonucleotide) or 48°C (for the 18S oligonucleotide) and were washed twice in 2X SSC and 0.2% SDS at room temperature for 5 minutes and once in 0.2X SSC and 0.2% SDS at 42°C (for 28S oligonucleotide) or 48°C (for 18S oligonucleotide) for 30 minutes. Autoradiography was performed at room temperature for 2 to 20 minutes. Quantitation was performed on Northern blots exposed in the linear range of the film with an LKB (Uppsala, Sweden) ultrascan II Densitometer or using Imagequant Phosphorimagenger software (Molecular Dynamics).

**Neutrophil and monocyte purification.** Peripheral blood (PB) was obtained from normal donors. Dextran T70 (Pharmacia) was added, and the buffy coat was collected by gravity sedimentation. After a Ficoll-Hypaque density centrifugation, monocytes were collected at the interface and further purified by adherence. Neutrophils and red blood cells were pelleted, and red blood cells were lysed in 0.15 mol/L NH4Cl, 10 mmol/L KHCO3, and 0.1 mmol/L EDTA for 1 to 3 minutes. Neutrophils were washed twice in ice-cold solution of Hanks’ balanced salt solution (Sigma, St Louis, MO) and were resuspended in the same medium. Slides of cytocentrifuged cells were stained with Wright-Giemsa according to standard protocol, and neutrophils were counted by light microscopy. This purified neutrophil fraction contained over 94% mature neutrophils, 5% eosinophils, and not more than 0.5% monocytes. Further purification of neutrophils was performed with anti-CD16 antibody (MACS CD16 Protein Kit, Miltenyi Biotec, Auburn, CA) to a specific activity of 2 × 10^6 cpm/µg as previously described.11 A total of 0.5 ng of probe was incubated with 2 to 5 µg of nuclear extract in 20 µL containing a final concentration of 10 mmol/L HEPES (pH 7.5), 50 mmol/L KCl, 5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 0.1 mg/mL poly(dI: dC), 0.1 mg/mL bovine serum albumin, and 5% glycerol on ice for 15 minutes. As described in the figure legends, in some lanes, PU.1 and Spi-B were translated in vitro using a rabbit reticulocyte lysate system (Tnt system, Promega) as previously described,1 and 1 µL was added in a 20-µL reaction instead of adding nuclear extract. For competition experiments, unlabeled competitor oligonucleotides were added to the reaction at 100-fold molar excess immediately before the addition of the radioactive probe. For supershift experiments, 1 µL of antisera raised against a peptide representing amino acid 33-45 of murine PU.1,2 against PU.1 DNA binding domain (amino acids 157 to 272),11 or against a full-length PU.1 protein produced in bacteria1 was added to the reaction. Reactions were electrophoresed at 10 V/cm on a 6% polyacrylamide gel in 0.5× TBE (45 mmol/L Tris-borate, 45 mmol/L boric acid, 1 mmol/L EDTA) at 4°C.

**RESULTS**

**PU.1 is expressed in purified human CD34+ cells and upregulated during commitment to myeloid precursors.** The pattern of expression of PU.1 in the early stages of hematopoiesis has not been extensively investigated. Because of the relatively scarce number of CD34+ cells in the BM,28 we used RT-PCR for this analysis. We first established conditions in which we could measure changes in PU.1 expression by RT-PCR in human CD34+ cells. PU.1 mRNA is present in small amounts in untreated BM CD34+ progenitor cells and increases twofold by day 1 and 10-fold by day 7 after myeloid differentiation induced by GM-CSF and IL-3. The increase in PU.1 mRNA precedes the increase in mRNA expression of its target genes, such as CD11b (data not shown and Voso et al).29

In these studies, we detected PU.1 expression in day-0 CD34+ cells, which are approximately 70% CD34+ after a single immunomagnetic selection. Therefore, we wanted to ask whether this low level of PU.1 mRNA represented expression in the earliest progenitors or was a result of contaminating CD34- cells. To answer this question, we further fractionated BM using fluorescence-activated cell sorter and antibodies to CD34 and CD38. Previously, it has been shown that CD34+ /CD38- cells represent a more primitive population that differentiates into a CD34+/CD38− and, subsequently, to a CD34+ population, during which time GATA-1 mRNA, undetectable in CD34+ /CD38− cells, increases.30 When we fractionated BM in this manner, we could still detect PU.1 mRNA in the CD34− /CD38− fraction, although it was increased in the more mature CD34+ fraction (Fig 1). These results confirm the expression of PU.1 mRNA in primitive progenitor cell populations.

**PU.1 but not Spi-B is upregulated during myeloid commitment of murine multipotential cells.** To confirm our results showing that PU.1 is upregulated during multipotential progenitor commitment, we used a cell line model of early hematopoietic development. FDCP-mix A4 cells are a multipotential, diploid, nonleukemic, nontransformed murine BM cell line that can be induced into different lineages with specific CSFs.28 When we induced FDCP-mix A4 with GM-CSF in the presence of low doses of IL-3, the cells differentiated from blast-like morphology with less than 10% monocytes on day 1 to 90% macrophage-like cells on day 5. We
found, as reported previously,\textsuperscript{18} that PU.1 mRNA is expressed in FDCP-mix A4 cells. We detected a very low level of PU.1 mRNA in undifferentiated day-0 FDCP-mix cells, which increased in association with this monocytic differentiation of the cells (Fig 2). Using a 5'-specific probe for murine Spi-B, we detected no expression of Spi-B mRNA in these cells, even after monocytic differentiation (data not shown).

\textbf{PU.1 expression is maintained during differentiation of myeloid cell lines.} The demonstration of significant upregulation of PU.1 mRNA during the commitment of multipotential progenitors to the myeloid lineage led us to ask the question of what happens to PU.1 mRNA during the next stage of myeloid differentiation, from promyelocytic to neutrophilic cells. For these studies, we used RA treatment of HL-60 and U937 cells as a model.\textsuperscript{16,19} In the uninduced state, these cells represent promyelocytic/promonocytic cells. Addition of $10^{-16}$ mol/L RA induces neutrophilic differentiation, as assessed by morphology and upregulation of markers such as CD18.\textsuperscript{35,36} Northern blot analysis showed no significant change in PU.1 mRNA levels during RA induction (Fig 3). As a control to show that we, indeed, induced differentiation, analysis of the same RNA preparations showed that CD18 mRNA increased significantly during RA induction (Fig 3).

Although PU.1 mRNA does not change in a significant way during RA-induced neutrophilic differentiation of HL-60 or U937 cell lines, it is still possible that the amount of PU.1 protein changes through posttranscriptional regulation or alterations in DNA binding activity. To assess PU.1 DNA binding activity during myeloid differentiation, we performed DNase-I protection of a CD11b promoter fragment PU.1 binding site, comparing the difference between uninduced and RA- or TPA-induced U937 and HL-60 nuclear extracts. We could detect no significant difference between

\begin{align*}
\text{CD34} & \quad + \quad + \quad - \\
\text{CD38} & \quad - \quad + \\
\text{PU.1} & \quad \text{GAPDH} \\
\end{align*}

\textbf{PU.1/GAPDH Ratio} 1 1 4

Fig 1. PU.1 expression in human CD34$^+$/CD38$^-$, CD34$^+$/CD38$, and CD34$^-$ cells. Human BM nonadherent light density cells were separated by fluorescence-activated cell sorting into CD34$^+$/CD38$, CD34$^+$/CD38$, and CD34$^-$ populations, and amplification by RT-PCR and hybridization were performed as described in Materials and Methods. The third lane contains CD34$^-$ cells, including both CD38$^+$ and CD38$^-$ populations. The relative PU.1/GAPDH ratio, determined by scanning densitometry, is shown under the autoradiograph.

PU.1 DNA binding activity in uninduced or induced nuclear extracts in either cell line, with either monocytic (TPA) or neutrophilic (RA) differentiation of the cells (data not shown). In summary, these results, combined with our mRNA expression studies, suggest that PU.1 is upregulated during the process of stem cell/multipotential progenitor cell differentiation to myeloid precursors and is not downregulated during differentiation of myeloid precursors to neutrophilic and/or monocytic cells.

\textbf{PU.1 is expressed at high levels in mature human neutrophils.} As described above, PU.1 mRNA and protein was not downregulated during neutrophilic differentiation of human myeloid cell lines, suggesting that it might be expressed in human neutrophils at significant levels. Furthermore, PU.1 is an important regulator of the CD11b promoter, and neutrophils, like monocytes, express high levels of CD11b.\textsuperscript{31,32} Therefore, we asked whether neutrophils, like monocytes, expressed PU.1 mRNA. Northern blot analysis with a full-length mouse PU.1 cDNA probe showed that human PB neutrophils expressed significant levels of PU.1 mRNA, approximately twice that of human PB monocytes (Fig 4A).
HeLa cell RNA showed no expression of PU.1, a result similar to our inability to detect PU.1 DNA binding activity in these cells.\(^{13}\) Using a specific 5' human PU.1 cDNA probe on Northern blots confirmed that human neutrophils expressed high levels of PU.1 mRNA, higher than that observed in human myeloid lines (Fig 4B).

To show that human neutrophils also synthesized functional PU.1 protein, we performed EMSA using neutrophil nuclear extracts. We first determined the ability of two different PU.1 antisera to supershift specific PU.1 and Spi-B complexes. As shown in Fig 5A, antisera raised against a PU.1 amino-terminal peptide (Fig 5A, lanes 7 and 8) and polyclonal antiserum raised against full-length PU.1 protein (Fig 5A, lanes 3 and 6) specifically supershifted in vitro translated PU.1 but not Spi-B, although both proteins bound well to the CD11b PU.1 site probe (Fig 5A, lanes 1 and 4). The same amino-terminal antibody supershifted nearly all of the full-length PU.1 protein in U937 nuclear extracts (Fig 5A, lane 12) but did not supershift the faster migrating species (A\(^*\)) we previously described in U937 cells,\(^{11}\) which is a carboxyl-terminal cleavage product that retains the PU.1 DNA binding domain but has lost the amino-terminal peptide sequence that reacts with the amino-terminal antiserum (Fig 5A, lane 12). However, the antiserum against full-length PU.1 supershifted both complexes in U937 nuclear extract (Fig 5A, lane 16). These results indicate that both antisera specifically recognize PU.1 and not Spi-B and that U937 myeloid cell extracts contain little or no Spi-B DNA binding protein. A third antiserum, raised against the PU.1 DNA binding domain and reacting with PU.1 but not Spi-B,\(^{13}\) also abolishes all of the specific DNA binding activity in U937 nuclear extracts (Fig 5A, lanes 17 and 18).

When this same CD11b probe was used with neutrophil nuclear extracts (Fig 5B), we failed to observe a full-length PU.1 complex formed by either in vitro translated PU.1 (Fig 5A, lane 1; Fig 5B, lanes 1 and 20) or the slowest migrating species in U937 nuclear extracts (Fig 5A, lane 9 and 13; Fig 5B, lane 4; complex "S"). We did observe small amounts of specific complex A\(^*\) found in some (Fig 5A, lane 9 and Fig 5B, lane 4) but not all (Fig 5A, lane 17) preparations of U937 cells. Significantly, in neutrophils we also observed an even faster migrating specific complex ("N"; Fig 5B, lanes 7 and 13) that was competed specifically with oligonucleotides known to bind PU.1 (Fig 5B, lanes 8, 10 and 14) but not with mutant (Fig 5B, lanes 9 and 15) or irrelevant (Fig 5B, lane 11) oligonucleotide competitors. Complex N was not affected by addition of the amino-terminal PU.1 peptide antibody (Fig 5B, lane 12) or by an anti-Oct antiserum (Santa Cruz Biotechnologies, Santa Cruz, CA) used as a negative control (data not shown). However, addition of the full-length PU.1 antiserum abolished the complex (Fig 5B, lane 16) and increased the amount of radioactivity at the top of the gel, which was similar to that observed for in vitro translated PU.1 and U937 nuclear extract (Fig 5A, lanes 3 and 16). Antiserum raised against the PU.1 DNA binding domain, previously shown to be specific for PU.1 and not recognizing Spi-B,\(^{13}\) almost completely abolished complex N (Fig 5B, lane 18). Consistent with the notion that complex N in Fig 5B represents a cleaved form of PU.1 found in human neutrophils is the fact that we can generate a complex migrating with identical mobility from in vitro translated PU.1 protein after the addition of neutrophil nuclear extracts (Fig 5B, lanes 19 and 21).

**Spi-B is expressed in B cells but not at significant levels in myeloid cells.** Northern blots using a full-length human Spi-B cDNA probe had indicated that Spi-B was expressed at levels comparable with that of PU.1 in both myeloid and B cells.\(^{7}\) However, this finding was in conflict with our previous supershift studies in myeloid lines,\(^{14}\) in which the amino-terminal PU.1 peptide antibody, which recognizes PU.1 and not Spi-B (Fig 5A, lanes 7 and 8), was capable of supershifting almost all of the PU.1/Spi-B DNA binding activity in U937 cells (see also Fig 5A, lane 12, and Fig 5B, lane 6). We have observed similar findings in myeloid THP-1 cells.\(^{13}\) Because Spi-B binds to sites such as the CD11b PU.1 site (Fig 5A, lane 4), then, if Spi-B is expressed at significant levels in myeloid cells, we would expect a significant residual Spi-B complex in EMSA using U937 extracts, which is, however, not observed (Fig 5A, lanes 12, 16, and 18; Fig 5B, lane 6).
To more accurately assess the differential expression of Spi-B in comparison with PU.1 on Northern blots, we used 5' human and murine cDNA probes for Spi-B and PU.1. Figure 6A shows the DNA sequence comparisons between murine and human PU.1 and Spi-B cDNAs. The similarity of the 5' cDNA regions used as specific probes for murine PU.1 and Spi-B is 48%, whereas that of the 3' DNA binding regions is 70%. For human PU.1 and Spi-B, the similarity of the 5' probes is 52%, whereas that of the 3' DNA binding regions is 75%. Figure 6B shows the species similarity between human and murine PU.1 and Spi-B cDNAs. Again, the similarity is higher in the DNA binding domains. In contrast to full length probes which include the highly conserved carboxyl-terminal DNA binding domain, these 5' probes distinguish between Spi-B and PU.1 and also show species specificity in that murine probes do not cross-react with human mRNA and vice versa (see Fig 7 and discussed below).

When these gene- and species-specific probes were used, Spi-B and PU.1 showed a very different pattern of expression, both in human and murine cells. Human Spi-B was expressed only in a human pro-B–cell line, BJA-B, and was not expressed in three myeloid cell lines that are strongly positive for PU.1 mRNA (Fig 7A). In addition, we could detect no hybridization of the human Spi-B probe to human PB neutrophil mRNA using the blots shown in Fig 4, although a strong signal was detected in BJA-B cells in Fig 4B, lane 3 (data not shown). These findings are consistent with our EMSA data, in which little or no DNA binding Spi-B protein was observed in U937 cells (Fig 5A, lanes 12, 16, and 18; Fig 5B, lane 6). In the B-cell line, we did detect three human Spi-B mRNA species (Fig 7B, lane 5), including (1) a predominant 1.4-kb form, which comigrates with the single PU.1 mRNA species; (2) a minor 5-kb form, which migrates just below the 28S rRNA; and (3) a barely detectable 3-kb form running between the 28S and 18S rRNA bands.

These probes were also species-specific. For example, the human 5' Spi-B probe failed to detect murine Spi-B mRNA present in abundance in murine B-cell lines (compare Fig 7A, lanes 6 and 9 [upper panel], with Fig 7B, lanes 1 and 3 [upper panel]). The human PU.1 5' probe detected a single 1.4-kb species in human myeloid and B-cell lines and did not significantly cross-react with murine PU.1 mRNA under the conditions used in these experiments (Fig 7A, lower panel).

Northern blot analysis of murine lines and tissues with specific 5' murine probes for Spi-B and PU.1 showed a similar lack of significant levels of Spi-B expression in myeloid cells, consistent with our findings in human cells (Fig 7B). Murine Spi-B was detected only as a single 3-kb mRNA species. Significant levels of expression were found only in B-cell lines and spleen. Trace amounts of Spi-B mRNA were also found in peritoneal macrophages and lung. In contrast, murine PU.1 is expressed as a single 1.4-kb species at high levels not only in B cells but also in murine myeloid cell lines and is expressed at highest levels in peritoneal macrophages. Lower levels were also detected in lung. Because of the difficulty with isolation of intact mRNA from significant numbers of purified murine neutrophils, we have been unable to assess expression of Spi-B and PU.1 in these cells.
**Fig 5.** EMSA (gel shift) analysis of DNA binding of PU.1 and Spi-B. (A) Reactivity of in vitro translated PU.1 and Spi-B and U937 extracts to PU.1 antisera is shown. EMSA was performed using the CD11b promoter PU.1 site as a probe as described in Materials and Methods. The legend above the autoradiograph indicates the source of protein, competitor, and antibody used: PU.1 protein and Spi-B protein, in vitro translated protein; U937 NE, U937 nuclear extract. Competitors are the wild-type CD11b oligonucleotide (wt) and the CD11b M1 mutant (m), which does not compete PU.1 or Spi-B binding. Antibodies added are either antisera raised against full-length, bacterially synthesized, PU.1 protein (F), or against an amino terminal peptide (N); or against the DNA binding domain only (G). No change in the PU.1 (lane 1), Spi-B (lane 4), or U937 (lanes 9 and 17) complexes was observed with the addition of normal rabbit serum (data not shown). The relative amount of full-length PU.1 (lane 1) versus Spi-B (lane 4) complex correlates with the amount of protein observed on SDS analysis of the in vitro translated protein, not with a difference in relative affinity for the CD11b probe. W, location of the well; SS, supershifted PU.1 complex; S, complex formed by in vitro translated PU.1 or Spi-B; A*, cleaved form of PU.1 detected in U937 extracts; and F, free probe. Lanes 1 through 6, 7 and 8, 9 through 12, 13 through 16, and 17 through 18 represent different experiments performed under identical conditions. Lane 17 is identical to lanes 9 and 13, except a different U937 extract was used that does not have the cleaved (A*) PU.1 complex. (B) Binding of neutrophil extracts to the CD11b PU.1 site oligonucleotide. EMSA analysis was performed as described in Materials and Methods and in the legend to Fig 5A. Neutrophil NE; human PB neutrophil nuclear extract; PU, the wild-type CD11b PU.1 site oligonucleotide competitor; Sv, the SV40 PU.1 site (both of which bind PU.1); M1 and Sp, the CD11b M1 PU.1 site and -60 CD11b promoter Sp1 wild-type competitors (neither of which bind to PU.1); G, antibody, an antisera raised against the PU.1 DNA binding domain; and N (on the left side), the location of a complex found in human neutrophils. Lanes 1 through 12, 13 through 16, and 17 through 22 represent different experiments performed under identical conditions. In lanes 19 and 21, 2.8 μg of neutrophil nuclear extract was added to 4 μL of in vitro translated PU.1 protein (shown in lane 20) and incubated for 20 minutes at either 0°C (lane 19) or room temperature (lane 21) before performing the EMSA analysis.
**Journal Article**

**PU.1 AND SPI-B EXPRESSION IN MYELOID CELLS**

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### A

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<tr>
<th></th>
<th>Human PU.1</th>
<th>Human SPI-B</th>
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<td>75%</td>
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<table>
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<tr>
<th></th>
<th>Murine PU.1</th>
<th>Murine SPI-B</th>
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<td>70%</td>
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### B

<table>
<thead>
<tr>
<th></th>
<th>Human PU.1</th>
<th>Murine PU.1</th>
<th>Human SPI-B</th>
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<tbody>
<tr>
<td></td>
<td>80%</td>
<td>95%</td>
<td>78%</td>
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**100 bp**

- **5' probe region**
- **DNA binding domain**

![Fig 6](image)

**Fig 6. DNA similarity of human and murine PU.1 and SPI-B cDNAs.**

(A) Comparison between PU.1 and SPI-B. Sequence alignment of murine or human PU.1 and SPI-B (Gonzalez and Tenen, unpublished sequence, and Ray et al.) was performed with the align program (DNASTAR, Inc., Madison, WI). The 5' probe region portion of each cDNA was used in Northern blots in Figs 4B and 7. (B) Comparison between human and murine PU.1 and SPI-B is shown. Sequence alignment was performed as for (A).

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In summary, when gene- and species-specific 5' cDNA probes are used, Spi-B appears to be expressed at high levels only in certain B-cell lines and in spleen (an organ which contains large numbers of B cells), but not in myeloid cells. PU.1, in contrast, is expressed not only in all the B-cell lines we tested, but also in all myeloid cell lines and, specifically, in neutrophils at high levels.

**DISCUSSION**

In this report, we have studied the expression of the related transcription factors PU.1 and SPI-B during the course of myeloid development. PU.1 is expressed in early human CD34+ precursors and upregulated early during GM-CSF--induced differentiation of these cells, suggesting that it may play a role in the early stages of proliferation and/or differentiation of stem cells. In these cells, SPI-B mRNA was also initially upregulated and then downregulated by day 11 of GM-CSF culture (data not shown), whereas PU.1 mRNA remains stable. This upregulation of PU.1 mRNA is associated with differentiation of blasts to a population that is mostly promyelocytes and myelocytes (56% at day 11) and monocytic cells (24% at day 11). In this culture system, we can induce myeloid but not B-cell development. It is possible that, were we able to induce B-cell differentiation of the human CD34+ BM cultures, we might see sustained or increased levels of SPI-B at later times.

Because of the relatively small numbers of purified CD34+ cells in the BM, we analyzed PU.1 mRNA in these cultures by RT-PCR. In CD34+ cell cultures, PU.1 is expressed in the most immature CD34+/CD38- cells (Fig 1). When we investigated expression of PU.1 and SPI-B by Northern blot analysis in multipotential FDCP-mix cells, we observed significant PU.1 mRNA expression only after induction of myeloid differentiation (Fig 2); however, no SPI-B mRNA expression was observed. We also observe upregulation of PU.1 but not SPI-B mRNA by Northern blot analysis during G-CSF--induced neutrophilic differentiation of the murine progenitor 32D line (data not shown).

Expression of PU.1 was not significantly changed during differentiation of HL-60 or U937 cells and suggests that increases in PU.1 expression cannot account for the maturation of promyelocytic cells to neutrophilic or monocytic cells. These findings are consistent with studies of the CD11b promoter, in which mutations that block PU.1 DNA binding and function fail to block CD11b promoter upregulation during myeloid differentiation (Satterthwaite and Tenen, unpublished observations). Because it is expressed at highest levels in mature neutrophils, monocytes, and murine peritoneal macrophages, PU.1 may be strongly upregulated late during myeloid maturation (Figs 4 and 7). The levels observed in these mature primary cells are significantly higher than those observed in myeloid cell lines such as HL-60, which do not undergo terminal differentiation characteristic of actual mature neutrophils or monocytes.

In human cells, PU.1 mRNA was expressed at highest levels in mature PB neutrophils. This finding was consistent with its lack of significant downregulation during neutrophilic differentiation of myeloid cell lines, as well as its regulation of genes expressed in neutrophils as well as macrophages, such as CD11b. Previous investigators also identified a specific DNA binding activity in neutrophils binding to a site in the IL-1β promoter, which has recently been identified as a PU.1 site. We believe that our findings are not inconsistent with a previous report, in which immunohistochemical studies using an antisera directed against an amino-terminal peptide failed to detect significant PU.1 protein in murine neutrophils. In our gelshift studies (Fig 5B), we also failed to detect full-length PU.1 protein capable of reacting with this antibody. We instead detected specific faster migrating complexes that represent PU.1 carboxy-terminal peptides, in which the amino-terminal domain has been cleaved and the DNA binding domain retained. These complexes were detected because they react with specific antisera raised against either the entire PU.1 protein or the DNA binding domain.

It is possible that the cleaved form exists in intact neutrophils and monocytes and has biologic significance. However, we suspect that PU.1 more likely exists as an uncleaved protein in the body and is cleaved during the process of isolation of myeloid cells, because (1) U937 extracts rarely do not have the cleaved form (Fig 5A, lane 17), and (2) we observe the faster migrating complex in myeloid cells (U937,
Fig 7. Relative human and murine PU.1 and Spi-B mRNA expression using species- and gene-specific 5' cDNA probes. (A) Northern blot analysis using human probes is shown. Northern blot analysis was performed as described in Materials and Methods using 10 μg of total RNA isolated from the cell lines and tissues indicated. Under the conditions used, we do not detect cross-reaction between human and murine probes or between PU.1 and Spi-B (see Results). The arrows at the left of the top panel indicate the rare 5-kb (---), the rarer 3-kb (-----), and the abundant 1.4-kb (----) human Spi-B mRNA species. The arrows at the left of the middle panel indicate the 1.4-kb human PU.1 mRNA. Autoradiography was performed for 4 days for human Spi-B and for 22 hours for human PU.1. The panel at the bottom shows the 18S oligonucleotide hybridization as a control for loading. (B) Northern blot analysis using murine probes is shown. Northern blot analysis was performed as described in (A) using 10 μg of total RNA isolated from the cell lines and tissues indicated. The arrows at the left of the top panel indicate the 3-kb murine Spi-B mRNA species. The arrow at the left of the middle panel indicates the 1.4-kb murine PU.1 mRNA. Autoradiography was performed for 4 days for murine Spi-B and for 3 days for murine PU.1. The panel at the bottom shows the 18S oligonucleotide hybridization as a control for loading. A faint 3-kb mRNA signal is detected in the upper panel in lanes 5 and 9.

HL-60, neutrophils; which contain significant levels of proteases) and not, as a rule, in B-cell extracts, a finding which has been observed by other laboratories as well. Further support for the idea that PU.1 is cleaved by a protease during extraction comes from our experiment in which addition of neutrophil nuclear extracts to in vitro translated PU.1 protein induces the loss of full-length PU.1 complex S and the induction of a new faster migrating complex that migrates with a mobility identical to the complex N found in human neutrophils (Fig 5B, lanes 19 and 21). Addition of trypsin to U937 extracts also induces the loss of full-length PU.1 complex S and the induction of a complex with a mobility very similar to the complex N found in human neutrophils (data not shown). Of interest is that it appears that the DNA binding activity of the smaller complex in U937 extracts is significantly stronger than that of the intact protein. Other investigators have previously described an inhibitory domain in ets-1, in which deletion of the domain led to a similar increase in DNA binding activity of faster migrating complexes. In one of these studies, deletion of similar regions of the PU.1 protein did not augment DNA binding. Our studies, in contrast, suggest the presence of an inhibitory domain in PU.1 located in a distinct site not previously investigated by this group. Further mapping of the cleavage site will be necessary to show directly the presence of such an inhibitory region.

Our most significant finding is that the closely related transcription factor Spi-B is not expressed at significant lev-
els in myeloid cells. Using highly species- and gene-specific 5′ cDNA probes, we can distinguish between human and murine PU.1 and Spi-B on Northern blots and fail to detect significant amounts of Spi-B mRNA in murine or human myeloid lines or cells (Figs 4 and 7). Consistent with these distinct patterns of expression is the fact that, although the murine and human PU.1 promoters are highly conserved (with 90% similarity in the proximal promoter regions and 100% identity in a 100-bp region encompassing the transcriptional start site), we can detect no similarity between the murine PU.1 and Spi-B promoters (Gonzalez et al., manuscript in preparation). Interestingly, Spi-B shares DNA binding specificity similar to that of PU.1 for a number of myeloid promoters, including CD11b and the M-CSFR.12 At levels similar to the transactivation observed with PU.1,12 Spi-B can transactivate the M-CSFR promoter fivefold and the CD11b promoter twofold in cells that do not express PU.1 or Spi-B (Burn and Tenen, unpublished observations). These observations suggest that PU.1 and Spi-B may have at least some redundancy in function in vitro but clearly have very different patterns of expression in vivo and, therefore, are likely to play different roles in hematopoietic development and differentiation. In conclusion, we think it unlikely that Spi-B will play a significant role in myeloid gene expression, but its roles in early hematopoiesis and B-cell development are yet to be determined.

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