The myeloid integrin CD11b is expressed selectively on the surface of mature monocytes, macrophages, granulocytes, and natural killer cells. Tissue-specific and developmentally regulated expression of CD11b is controlled at the level of mRNA transcription, and recent characterization of the human CD11b promoter indicates that the first 92 bp of 5'-flanking DNA are sufficient to direct tissue-specific expression of a reporter gene. Here we show that the sequence AAAAG-GAGAAG at base pair -20 of the CD11b promoter binds the proto-oncogene PU.1 in vitro and that mutation of this site significantly reduces the ability of the CD11b promoter to direct expression of a reporter gene in myeloid cells but not in nonmyeloid cells. PU.1 may thus represent a major determinant of the myeloid expression of CD11b.

A major focus in the study of cellular differentiation is the isolation of factors governing cell commitment to lineage-specific differentiation. During hematopoiesis, pluripotential stem cells in the bone marrow give rise to committed progenitor cells, which in turn differentiate along lineage-specific pathways to form mature, circulating blood cells (Metcalf, 1989). Several transcription factors have been implicated in cell commitment and differentiation. For example, GATA-1, an erythroid transcription factor, is essential for cell differentiation along the erythroid lineage (Pevny et al., 1991).

Little, however, is known about factors governing myeloid (monocyte, neutrophilic) differentiation. For example, no myeloid-specific transcription factors regulating myeloid promoters have been described to date. However, commonly expressed transcription factors have been implicated in normal myeloid differentiation (Collins et al., 1990) and in the etiology of acute myelogenous leukemia (de Thé et al., 1990; Kastner et al., 1991; Kakizuka et al., 1991), which may arise when immature myeloid precursors fail to differentiate. Patients with acute promyelocytic leukemia (M3 subtype of acute myelogenous leukemia) carry a reciprocal translocation of chromosomes 15 and 17, fusing the α chain of the retinoic acid receptor, a member of the steroid receptor family of transcription factors, to the PML gene (de Thé et al., 1990; Kastner et al., 1992; Kakizuka et al., 1991). However, the mechanism by which this translocation may cause acute promyelocytic leukemia remains unclear. In addition, the etiology of other subtypes of acute myelogenous leukemia is not understood.

Recently, PU.1, a member of the ets family of transcription factors, was shown to be expressed principally in B-cells and macrophages (Klemsz et al., 1990). PU.1 is identical to the Spi-1 proto-oncogene, which was isolated as the site of Friend erythroleukemia virus integration in 95% of virally induced tumors. Viral insertion results in transcriptional activation and enhanced expression of PU.1/Spi-1 mRNA (Goebel et al., 1990; Moreau-Gachelin et al., 1988). DNA binding studies have identified the consensus sequence for PU.1 binding as purine-rich segments containing a 5'-GGAA-3' core sequence (Karim et al., 1990). Although PU.1 binding to B-cell and erythroid enhancers has been demonstrated, the effect of PU.1 binding site mutations on the function of complete promoters/enhancers has not been investigated (Galson et al., 1988; Pongubala et al., 1992). Moreover, no macrophage targets for this transcription factor are known.

Several approaches leading to the characterization of lineage-specific transcription factors can be taken. One possible approach is the identification of regulatory elements and factors responsible for the tissue-specific expression of a lineage-specific marker gene. Such a marker gene is CD11b, a myeloid-specific antigen (Pahl et al., 1991; Todd et al., 1981). An integrin cell surface receptor, CD11b is expressed selectively on the surface of mature monocytes, macrophages, granulocytes, and natural killer cells. CD11b surface expression increases concurrently with CD11b mRNA levels during myeloid differentiation, showing developmental regulation (Rosmarin et al., 1989). Using nuclear runoff transcription, we recently demonstrated that the rate of CD11b transcription increases during differentiation of the myeloid cell lines U937 and HL-60 (Pahl et al., 1992), indicating that CD11b expression is controlled at the level of mRNA transcription. Subsequently we isolated the CD11b promoter and demonstrated that it directs myeloid-specific expression of a reporter gene in transient transfection assays (Pahl et al., 1992). A 412-bp fragment of 5'-flanking DNA showed maximal activity in CD11b-expressing myeloid cells (U937); it directed 100-fold less activity in CD11b negative cervical carcinoma cells.

The abbreviations used are: bp, base pair(s); EMSA, electrophoretic mobility shift assay; RLU, relative light units; TPA, 12-O-tetradecanoylphorbol-13-acetate; kb, kilobase(s).
(HeLa). Deletion analysis shows that the first 92 bp of 5'-flanking DNA are sufficient to direct high levels of tissue-specific reporter gene activity (Pahl et al., 1992). In the present study, we investigated protein/DNA interaction in the CD11b promoter and subsequently tested whether mutation of protein binding sites could lower CD11b promoter activity. We show here that the transcription factor and proto-oncoprotein PU.1 binds at bp -20 of the CD11b promoter and that mutation of this site significantly reduces CD11b promoter activity.

**MATERIALS AND METHODS**

**Nuclear Extracts**—The human promonocytic cell line U937 (ATCC CRL 1593) was grown to 2 x 10^6 cells/ml and induced with 3 x 10^{-6} M TPA (Sigma) for 24 h prior to harvest for nuclear extraction. TPA induces U937 cells to differentiate into macrophage-like cells (Larrick et al., 1980). The human epithelial carcinoma cell line HeLa (ATCC CCL 2) was harvested in early logarithmic growth phase. Nuclear extracts were prepared as previously described (Dingham et al., 1983) with one modification: the following protease inhibitors were added at the final concentration indicated to every buffer immediately prior to use: 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin A, 0.5 mM chymostatin, 1 mg/ml antipain, 1 mM PMSF, 4 U/ml aprotinin (all from Sigma). Protein concentrations were determined using the Bradford assay (Bio-Rad) and bovine serum albumin standards (Sigma).

**Electrophoretic Mobility Shift Assay**—The probes for the EMSAs were 30-bp oligonucleotides, either wild type or carrying 3- or 4-bp mutations (see Fig. 1E for sequence), extending from bp -26 to bp +2 of the CD11b promoter, labeled with [γ-32P]ATP (Du Pont-New England Nuclear) to a specific activity of 2 x 10^6 cpm/μg as previously described (Maxam and Gilbert, 1980). An additional AG or GCT were included at the 5'- or 3'-end of the oligonucleotides, respectively, to create restriction endonuclease compatible overhangs. A single major translated protein was analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. In vitro translation of the 33-45 amino acid 33-45 of the mouse PU.1 protein (Klemsz et al., 1990), corresponding to amino acid 33-45 of the human PU.1 protein (Ray et al., 1990), was conserved across the two species.

**Transfections**—U937 and HeLa cells were transfected by electroporation in Lacoste's modified Eagle's medium at 960 μF and 250 or 150 V, respectively, as previously described (Pahl et al., 1991). Cells were induced with 3 x 10^{-6} M TPA (Sigma) immediately following transfection, and luciferase activity was determined 14 h post-transfection. Luciferase assays were performed precisely as previously described (Pahl et al., 1992). In the present study, we investigated protein/DNA interactions in the CD11b promoter and subsequently tested whether a known ets family binding site competed for binding to the CD11b promoter. The proto-oncoprotein PU.1, a member known PU.1 site raised the possibility that the protein binding to the CD11b promoter is the PU.1 transcription factor. Fig. 1B shows an EMSA using a probe containing the CD11b promoter oligonucleotide from bp -144 to -115 of the CD11b promoter, indicating that bp -20 to -15 on the noncoding strand to CCCG, while CD11b m2 and CD11b m3 changed the sequence GAGAA at bp -17 to -21 on the noncoding strand to TGGCA and CTGCA, respectively (Fig. 1E). Mutant CD11b promoter fragments were generated by polymerase chain reaction and cloned into the vector pXp2 (Nordeen, 1988). Sequence of the mutant constructs was confirmed by the dideoxy chain termination method (Sanger et al., 1977).

**In Vitro Footprint—**In vitro footprinting, a 370-bp piece extending from the EcoRI site at bp -264 of the CD11b promoter to the Hpal site at bp +106 (Pahl et al., 1992) was subcloned into pGem 3zf(+) (Promega). The plasmid was digested with EcoRI and HindIII to release the insert, which was radiolabeled at the HindIII site (coding strand) or the EcoRI site (noncoding strand) using [γ-32P]ATP as previously described (Maxam and Gilbert, 1980). 80 μg of HeLa or U937 cell nuclear extract were incubated with 10 ng of DNA probe, labeled to a specific activity of 1.5 x 10^6 cpm/μg, and 4 μg of poly(dI-dC) (Pharmacia LKB Biotechnology Inc.) in a total volume of 20 μl in 10 mM HEPES (pH 7.8), 30 mM KC1, 12% glycerol, 5 mM MgCl2, 0.5 mM dithiothreitol, 0.1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride on ice for 30 min. The DNA was cleaved with 180 ng of DNase I at room temperature for 1 min. Reactions were terminated by the addition of 100 μl of stop solution (20 mM EDTA, 0.5% SDS, 200 mM NaCl (pH 7.5), 75 μg/ml sheared salmon DNA, 100 μg/ml proteinase K) and subsequent incubation at 37 °C. Reactions were extracted with an equal volume of phenol/chloroform and ethanol-precipitated, and products were analyzed on a 6% denaturing polyacrylamide gel. Chemical sequencing reactions (Maxam and Gilbert, 1980) of the same DNA were used as size standards.

**RESULTS**

A Myeloid Factor Binds the CD11b Promoter—In order to characterize transcription factors that regulate CD11b promoter activity, we used EMSA to locate proteins interacting specifically with the CD11b promoter. Fig. 1A shows that a protein in the myeloid cell line U937, but not in cervical carcinoma HeLa cells, specifically bound the CD11b promoter between bp -26 and bp +2 (bands A and A', Fig. 1A, lanes 2 and 8). Binding was competed by the addition of excess unlabeled probe (Fig. 1A, lane 4), but not by a probe containing a 4-bp mutation at bp -12 to -15 (mutant m1, Fig. 1A, lane 3). In addition, the mutant probe did not bind the protein (Fig. 1A, lane 1), indicating that bp -12 to -15 of the CD11b promoter are critical for the interaction with this protein. An oligonucleotide from bp -144 to -115 of the CD11b promoter, containing a very similar sequence (Fig. 1E), likewise neither competed for nor bound this protein (Fig. 1A, lanes 5 and 9). Because of the resemblance of the sequence AAAAAA-GAGA to the consensus binding sequence for members of the ets family of transcription factors (Klemsz et al., 1990), we tested whether a known ets binding site competed for binding to the CD11b promoter. The proto-oncopogene PU.1, a member of the ets family, is specifically expressed in B-cells and macrophages (Klemsz et al., 1990). An oligonucleotide containing a characterized PU.1 binding site from the β-globin gene (Gaison and Houseman, 1985)3 (see Fig. 1E) competed binding to the CD11b promoter (Fig. 1A, lane 6); however, an oligonucleotide containing a 3-bp mutation in the globin PU.1 site, analogous to the mutation created in the CD11b promoter, did not compete (Fig. 1A, lane 7). Competition by a known PU.1 site raised the possibility that the protein binding the CD11b promoter is the PU.1 transcription factor.

**Tissue Distribution of the CD11b Promoter Binding Activity**—Since PU.1 is expressed in macrophages and B-cells, we investigated the tissue distribution of the CD11b promoter binding activity. Fig. 1B shows an EMSA using nuclear extracts from several different cell lines and the CD11b -26 to +2 bp probe. Specific binding to the CD11b promoter oligo-

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**PU.1 Regulates CDllb Expression**

A Probe: CDllb bp -26 to +2 

Extract: U937 

Competitor: m1 wild type

Lane: 1 2 3 4 5 6 7 8 9

B Probe: CDllb bp-144 to -115

Extract: U937 

Competitor: m1 wild type

Lane: 1 2 3 4 5 6 7 8 9 10 11 12

C Lane 1: U937 Extract Pu Competitor

Probe: CDllb bp -26 to +2

Extract: U937

Antibody: - PU pre

Lane: 1 2 3

D Probe: CDllb bp-26 to +2

Extract: U937

Antibody: - PU pre

Lane: 1 2 3

E Name | Abbreviation | Sequence

| CDllb wild type | -26 to +2 | (wt-26) |
| CDllb mutant 1 | -26 to +2 | (m1) |
| CDllb mutant 2 | -26 to +2 | (m2) |
| CDllb mutant 3 | -26 to +2 | (m3) |
| globin wild type | PU.1 site | (wt IVS) |
| globin mutant | PU.1 site | (m IVS) |

Fig. 1. A, a myeloid factor binds bp -26 to +2 of the CDllb promoter. 10 μg of nuclear extracts from TPA-induced U937 cells (lanes 1-7) or HeLa cells (lane 8) were used in an EMSA. Lanes 2-8 contain bp -26 to +2 of the wild type CDllb promoter as a probe (for sequence see Fig. 1E). In lane 1 the mutant m1 probe is used, in lane 9 a 30-bp oligonucleotide from bp -144 to -115 of the wild type CDllb promoter is used as a control. Unlabeled competitor oligos indicated were added at 50-fold molar excess over probe oligo. A and A* refer to complexes specifically bound to the CDllb wild type bp -26 to +2 probe (see “Results”). In order to achieve adequate separation of bands A and A*, the gel was electrophoresed sufficiently long so that unbound (free) probe was runoff the bottom; similar results were obtained with gel runs of shorter duration, in which the free probe remained on the gel (see C and D). B, tissue distribution of the CDllb promoter binding activity. 10 μg of nuclear extracts from TPA-induced U937 cells (lanes 1-3), Haftl B-cells (lanes 4-6), Jurkat T-cells (lanes 7-9), or glioma cells (lanes 10-12) were added to an EMSA using bp -26 to +2 of the wild type CDllb promoter as a probe. Unlabeled competitor oligos added at 50-fold molar excess over probe oligo were: none (lanes 1, 4, 7, 10); m1 (lanes 2, 5, 8, 11); and the CDllb wild type -26 to +2 oligo (lanes 3, 6, 9, 12). C, the CDllb promoter binding activity comigrates with in vitro-translated PU.1. The probe in all lanes was the CDllb wild type -26 to +2 oligo (see “Results”). In lane 1 the mutant m1 probe is used, in lane 9 a 30-bp oligonucleotide from bp -144 to -115 of the wild type CDllb promoter is used as a control. Unlabeled competitor oligos indicated were added at 50-fold molar excess over probe oligo. A and A* correspond to the two bands identified in A and B; FP indicates free (unbound) probe. In lanes 2 and 3, approximately 10 fmol of in vitro-translated PU.1 was incubated with the oligonucleotide. In lane 3, unlabeled CDllb wild type -26 to +2 competitor oligonucleotide was added at 50-fold molar excess over radiolabeled oligonucleotide. In lane 4, a volume of unprogrammed rabbit reticulocyte lysate was incubated with the probe. D, identification of the CDllb binding activity as PU.1. 10 μg of TPA-induced U937 extract were used in an EMSA containing bp -26 to +2 of the wild type CDllb promoter as a probe (lane 1). Rabbit polyclonal antibody to a peptide consisting of amino acids 33-45 of PU.1 (lane 2) or preimmune serum (lane 3) was added to the EMSA reaction prior to incubation on ice for 15 min. S refers to the supershifted band in lane 2. The unlabelled band near the bottom of the gel represents unbound (free) probe. E, names, abbreviations (used in Figs. 1 and 4), and sequences of oligos used in EMSAs. Mutated nucleotides in oligonucleotides m1, m2, and m3 are indicated with an asterisk (*) above the base, and the core binding region for ets family transcription factors is underlined. The CDllb sequences in the -26 to +2 region are presented as the antisense strand so as to facilitate visual comparison with the CDllb -130 site as well as the mouse β-globin IVS2 site.
nucleotide (bands A and A*) occurred only when extracts from TPA-induced U937 cells (macrophage) or Hafli (B-cell) were used, not in extracts from Jurkat (T-cell), U2-SIMG (glioma) (all Fig. 1B) or HeLa (Fig. 1A, lane 8). The faint band observed in Jurkat extracts between bands A and A* is not competed by an excess of cold competitor oligo, indicating that it represents nonspecific binding (Fig. 1B, lane 9). Thus, the tissue distribution of the CD11b promoter binding activity resembles that of PU.1.

The Proto-oncogene PU.1 Binds the CD11b Promoter—To provide further evidence that the CD11b promoter binding activity was indeed PU.1, we compared the electrophoretic mobility of complex A and A* with that formed by in vitro translated PU.1 protein. Fig. 1C demonstrates that complex A comigrates with a specific complex observed when in vitro-translated PU.1 is incubated with the CD11b wild type -26 to +2 probe. For final confirmation that PU.1 binds the CD11b promoter, we obtained an antibody which reacts specifically against the amino-terminal end of PU.1 and produces a "supershift" of bound protein when added to an EMSA. Fig. 1D shows that addition of anti-PU.1 peptide antibody (lane a) bound to the CD11b promoter, identifying it as PU.1. The second, lower band (A*) did not supershift; this lower band was only seen in extracts from U937 cells, not B-cells (compare lanes 1 and 4 of Fig. 1B), and may be the result of proteolysis, which we have previously observed during nuclear extract preparation from myeloid cells (Galsin and Housman, 1988). If the relevant epitope is lost, the anti-PU.1 antibody may not recognize a proteolytically cleaved PU.1. Alternatively, the lower band (A*) may represent a second, distinct protein binding the CD11b promoter.

The PU.1 Binding Site at bp -20 Is Protected from DNase I Digestion in Myeloid Cells in Vitro—In order to fully characterize the PU.1 binding site, we performed DNase I footprinting assays on the CD11b promoter with nuclear extracts. Fig. 2 shows that the pattern observed after DNase I digestion of plasmid DNA (Fig. 2, A and B, lane 2) remained unchanged after the addition of HeLa nuclear extract (Fig. 2, A and B, lane 5). However, after addition of U937 nuclear extract, bp -9 to bp -26 on the coding strand (Fig. 2A, lanes 3 and 4) and bp -10 to bp -29 on the noncoding strand (Fig. 2B, lanes 3 and 4) are protected from DNase I digestion. In addition, bp -8 and -43 became hypersensitive to DNase I digestion. This footprint was competed by the addition of 50-fold molar excess of the nonradioactive β-globin PU.1 binding site oligonucleotide (Fig. 2C). Therefore, PU.1 is the only DNA binding activity we can detect in this region. TPA induction of U937 cells does not alter PU.1 binding; the slight differences between lanes 3 and 4 in Fig. 2, A and B, were not consistently seen. Similarly, Northern blotting and EMSA revealed no change in either PU.1 mRNA or DNA binding activity following TPA induction (data not shown). Thus, DNase I footprinting places the PU.1 site between bp -9 and bp -29 of the CD11b promoter and is consistent with the results obtained from EMSA.

The PU.1 Binding Site Is Essential for High Level CD11b Promoter Function—Finally, we determined whether binding of PU.1 to the CD11b promoter is functionally important for the ability of this promoter to direct transcription. We constructed a mutation in the PU.1 site and compared the ability of the wild type and mutant CD11b promoter to direct reporter gene activity in transient transfection assays. A mutation of the PU.1 site, which abolished both competition for and binding to the PU.1 protein (see m1, Fig. 1A and 1E), was introduced both in the context of the 412 and the 92-bp promoter (Pahl et al., 1992) and resulted in a 3-4-fold decrease in reporter gene activity compared to the wild type promoter following transient transfections into the myeloid cell line U937 (Fig. 3). In contrast, transfections into HeLa cells, which do not express PU.1, showed no decrease following mutation of the PU.1 site (Fig. 3).

Characterization of the Nonconsensus PU.1 Core Binding Site—Because the PU.1 binding site in the CD11b promoter does not contain the core consensus sequence 5'-GGAA-3' (Karim et al., 1990), and because a sequence at bp -130 of the CD11b promoter, containing this consensus, does not bind PU.1 (Fig. 1A, lanes 5 and 9), we investigated the importance of the core region for PU.1 binding. We tested two point mutations (m2 and m3); both disrupt the sequence 5'-GGAA-3' between bp -16 and -21 of the CD11b promoter (Fig. 1E). Fig. 4 shows that both mutant m2 and m3 neither compete for (Fig. 4A, lane 2; Fig. 4B, lane 2) nor themselves bind PU.1 (Fig. 4A, lane 4; and 4B, lane 4). Fig. 5 summarizes the mutations demonstrated to interfere with DNA binding by PU.1 from EMSA (Figs. 1 and 4) and in vitro (Fig. 2) footprinting assays.

**DISCUSSION**

The PU.1 proto-oncogene was first identified distal to the site of Friend erythroleukemia virus integration in 95% of virally induced tumors (Moreau-Gachelin et al., 1988). Viral integration results in the abnormal expression of the PU.1 mRNA in erythroid cells (Moreau-Gachelin et al., 1988; Ray et al., 1990), presumably inhibiting the normal pathway of erythroid differentiation. A member of the ets family of transcription factors, PU.1 is expressed primarily in B-cells and macrophages (Klemz et al., 1990). Although PU.1 has been shown to bind the mouse β-globin intervening sequence 2
(IVS2) and immunoglobulin kappa 3' enhancer in vitro (Galson and Housman, 1988; Pongubala et al., 1992), the functional importance of this interaction remains to be elucidated. To date, no macrophage targets for this transcription factor are known. Moreover, no other myeloid factors activating expression from myeloid-specific promoters have been described.

We demonstrate here that a sequence around bp -20 of the myeloid-specific CD11b promoter binds PU.1, and that a mutation of this site, which no longer binds PU.1, reduces the ability of the CD11b promoter to function in myeloid cells (U937) but not in cervical carcinoma cells (HeLa). Thus, tissue-specific expression of the 92-bp CD11b promoter is regulated to a large extent by the PU.1 site: mutation of the PU.1 site reduces the ratio of reporter gene activity between myeloid cells and cervical carcinoma cells from 15:1 to 2.5:1. However, since PU.1 is expressed in both B-cells and myeloid cells, and CD11b expression is restricted to myeloid cells, PU.1 cannot represent the sole determinant of tissue specificity of the CD11b gene. Additional transcription factors may interact with PU.1 to modulate its activation of tissue-specific transcription. For example, Pongubala et al. (1992) have recently identified a transcription factor in B-cells (NF-5) which specifically associates with PU.1 via protein/protein interaction.

Mutation of the PU.1 site in the CD11b promoter significantly reduced promoter activity; however, it did not entirely abolish activity. Eukaryotic promoters are often regulated by multiple, interacting transcription factors (Sen and Baltimore, 1986). This most likely will also be true of the CD11b promoter. In an attempt to eliminate possible redundant transcription factor activity, we tested the m1 mutation in the context of only the first 92 bp of the CD11b promoter. If PU.1 interacts with transcription factors which bind the promoter between bp -92 and -412, deletion of these sequences may exacerbate the effect of PU.1 mutations on promoter activity. No such effect was seen; mutation m1 in both the context of the 92- and the 412-bp promoter reduced activity 3-4-fold. PU.1 may nonetheless act synergistically with proteins which bind within the 92-bp promoter. Sequence analysis shows several additional putative transcription factor binding sites within the first 92 bp: an SP1 box at bp -64 and a GATA motif at bp -42. We are currently investigating binding to these sites, as well as possible interactions between these and the PU.1 site. Mutations of the Sp1 site significantly reduce CD11b promoter activity in U937 cells and, in contrast to the m1 mutant, in HeLa cells as well.

EMSA using nuclear extracts from U937 cells (Figs. 1 and 4) consistently show two complexes (A and A*) binding the CD11b promoter. A* may represent a proteolytic cleavage product of band A (identified as PU.1 by supershift in Fig. 1D); alternatively, the two bands may represent distinct proteins. Although we cannot conclusively distinguish between the two possibilities, several observations suggest that A* represents a proteolytic cleavage product of A, and therefore is likely to be a cleaved PU.1 protein. Firstly, both complexes bind specifically to both the CD11b promoter at -20 and mouse β-globin IVS2 PU.1 site, and not to the CD11b upstream sequence GAGGAA at bp -144. Both complexes react identically with respect to mutations; i.e. mutations m1, m2, and m3 in the CD11b promoter and a mutation in the β-globin IVS2 do not compete for binding of either complex to the wild type promoter nor do they bind either complex A or A*. Secondly, myeloid cells such as U937 contain significant protease activity, and isolating nuclear extracts without any proteolytic cleavage is extremely difficult (Galson and Housman, 1988). Moreover, the PU.1 protein is exquisitely sensitive to proteolysis, as it contains a PEST region, an amino acid segment rich in proline (P), glutamic acid (E), serine (S), and threonine (T), implicated in targeting proteins for degradation (Klemsz et al., 1990). We have previously observed two complexes similar to A and A* in macrophage lines (HL-60 and WEHI-3) and not in B-cell lines, and demonstrated that the two complexes involved the same DNA binding site (Galson and Housman, 1988). Likewise, nuclear extracts from

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 binding by EMSA (Figs. 1 and 4). Brackets span sequences protected by an EMSA. This data demonstrate that complex A and A* have indis-tinguishable DNA binding activities. We have produced full-length PU.1 protein. Klemisz et al. (1990) demonstrated that the carboxyl-terminal portion of the PU.1 molecule contains the DNA binding activity. The antibody used in our supershift experiment is raised against the amino-terminal region (amino acids 33–45). Our data indicates that the larger complex (A) reacts with the amino-terminal antibody, while the smaller (A*) does not. If PU.1 is cleaved at the PEST site in macrophage cells, and complex A represents a complex formed by uncleaved PU.1, then one would predict that complex A* would not react with the antibody but would bind DNA in a manner indistinguishable from complex A, which is precisely what we observed.

Although unlikely, it is yet possible that complex A* represents a protein distinct from PU.1. Although the anti-PU.1 antibody used does not recognize any other known ets family protein (Pongubala et al., 1992), we cannot exclude the possibility that the protein binding the CD11b promoter is distinct from, but highly similar to PU.1. The ets family of transcription factors is divided into subfamilies; although members of distinct subfamilies are similar to each other, similarity between subfamilies is low, especially at the amino-terminal region (Xin et al., 1992). PU.1 is the only known member of its subfamily identified to date, but these experiments cannot distinguish PU.1 from a yet undiscovered subfamily member with whom antibodies raised against a peptide from the amino terminal region of PU.1 may cross-react.

Interestingly, the PU.1 binding site at bp −20 (AAAAG-GAGAA) does not conform to the previously described consensus binding site for PU.1 and other ets family proteins (GGAA) (Karim et al., 1990), whereas a purine-rich sequence at bp −140 of the CD11b promoter, which contains the consensus sequence (GGGGAGGAAGGG; see also Fig. 1E), is not bound by PU.1 nor competed for PU.1 bound at bp −20 (Fig. 1 A, lanes 5 and 9). The presence of the GGAA core is therefore not sufficient for PU.1 binding. Mutations of the nonconsensus core region of the CD11b PU.1 binding site (mutations m2 and m3) disrupt PU.1 binding (Fig. 4). In addition, the m1 mutation (Fig. 1E), which no longer binds PU.1 (Fig. 1A), does not disrupt the core, but the adjacent 5′ purine-rich stretch of DNA. We therefore submit that the bases outside the PU.1 core consensus region influence PU.1 binding, as previously described (Galsom and Housman, 1988; Wasylyk et al., 1992.). Our data also suggests that the core site itself contains more variation than the previously defined consensus. Furthermore, in in vitro footprinting assays, bp −8 and −43 become hypersensitive to DNase I digestion following PU.1 binding (Fig. 2). Interestingly, this hypersensitivity is not competed by the addition of unlabeled oligonucleotide which abolished PU.1 binding. This hypersensitivity may reflect binding of other factors on either side of the PU.1 site.

As other myeloid promoters, from genes such as CD13 (Shapiro et al., 1991), CSF-1 receptor (Roberts et al., 1992), and CD18 (Rosmarin et al., 1992), which contain putative PU.1 binding sites, are investigated, it will be interesting to see whether PU.1 also plays a role in the regulation of these promoters. If so, PU.1 may represent a putative myeloid "master transcription factor," similar to the role of GATA-1 in the erythroid lineage. Following this hypothesis, mutation or inhibition of PU.1 activity should interfere with myeloid differentiation, a possibility currently under investigation.

\[\text{\textsuperscript{4}} R. J. Scheibe and D. G. Tenen, manuscript in preparation.\]
PU.1 Regulates CD11b Expression

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