RAPID COMMUNICATION

Characterization of the Myeloid-Specific CD11b Promoter

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The CD11b/CD18 heterodimeric surface antigen is expressed exclusively on human monocytes, macrophages, granulocytes, and natural killer cells. During differentiation of myeloid cell lines, CD11b steady state messenger RNA levels increase significantly; we show here that CD11b transcription rates increase commensurately. A 1.7-kb fragment of CD11b 5' flanking sequence directs expression of a reporter gene specifically in myeloid cell lines. Deletion analysis localizes elements directing high levels of tissue-specific reporter gene expression to the 412 bp proximal to the transcriptional start site. This sequence contains two consensus binding sites for Sp1, a GATA motif, and a purine-rich sequence that presents potential binding sites for members of the ets family of genes. Analysis of this promoter should result in the isolation of myeloid-specific transcription factors and the development of methods to direct the myeloid-specific expression of heterologous genes.

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A CENTRAL OBJECTIVE in the study of hematopoiesis is the isolation of factors governing cell commitment to differentiation along a specific lineage. In several other systems, transcription factors have been shown to play a role in cellular differentiation. For example, a "master" transcription factor, GATA-1, appears to be essential for erythroid cell development.1 More recently, transcription factors have also been implicated in normal myeloid (monocytic, neutrophilic) differentiation and in the etiology of myeloid leukemia.2,3 Cloning of myeloid-specific genes, such as CD11b, and the isolation of myeloid transcription factors may enhance the understanding of molecular mechanisms governing myeloid differentiation and ultimately explain the aberrant development leading to acute myelogenous leukemia.

CD11b, the α subunit of the CD11b/CD18 heterodimer,5 is an integrin cell surface receptor whose expression is tightly regulated in both a developmental- and a tissue-specific manner.6 CD18, the β subunit, is expressed on all leukocytes in combination with one of the three CD11α subunits; however, the CD11b/CD18 heterodimer (previously referred to as Mac-1 or Mo-1) is expressed exclusively on the surface of mature monocytes, macrophages, neutrophils, and natural killer (NK) cells,7 reflecting the more restricted tissue distribution of CD11b. Immature precursors do not express the antigen on their surface, and undifferentiated myeloid leukemic lines such as HL-60 and U937 cells show little or no detectable CD11b messenger RNA (mRNA). Upon induction of differentiation with either 12-O-tetradecanoylphorbol-13-acetate (TPA) or retinoic acid,8 steady state levels of CD11b mRNA increase significantly. In an effort to determine regulatory regions, we have recently isolated and determined the structure of the CD11b gene, which is encoded by 30 exons spanning 55 kb on the short arm of chromosome 16, at the locus 16 p11-13.9

Because expression of CD11b is restricted to mature myeloid cells, we have characterized the CD11b promoter to study myeloid transcription factors that may be involved in normal and aberrant, i.e., leukemic, differentiation.

MATERIALS AND METHODS

RNA isolation and Northern blot analysis. Total cellular RNA was isolated and CD11b Northern blot analysis performed as described from the following cells: HeLa (ATCC no. CCL 2; ATCC, Rockville, MD), human primary foreskin fibroblasts, K562 (ATCC no. CCL 243), KG-1 (ATCC no. CCL 246), HL-60 (ATCC no. CCL 240), Laz509 (kindly provided by James D. Griffin, Dana-Farber Cancer Institute, Boston, MA), Jurkat, fetal human thymocytes, human peripheral blood monocytes. HL-60 cells were induced with TPA and dimethyl sulfoxide (DMSO) as described.9 Equivalent RNA loading (10 μg/lane) was confirmed by visual inspection of ethidium bromide staining of ribosomal bands and by hybridization with a 28S ribosomal RNA probe.10

Run-on assays. Nuclear run-on assays were performed as previously described.11 The following DNAs were used to prepare slot blots: the plasmid CD8p12; a 4.1-kb Xba 1 fragment of CD11b cDNA containing the complete coding region in the vector CD8p; a 3.1-kb Sph I/Xba I fragment containing the full-length CD18 cDNA in pUC1816; a 1.6-kb EcoRI/Cla I fragment of the human c-myc exon 3 in pSP6517; a 2-kb Psi I fragment of the chicken actin cDNA in pBR3225; a 1.5-kb Xba I fragment containing the cDNA encoding CD33 in the vector CD8p; and a 1.3-kb EcoRI fragment the 3' end of the myeloperoxidase cDNA in the vector bluescript.20 Autoradiograms were exposed at ~80°C with an intensifying screen and quantitated on a densitometer whereby background hybridization to the plasmid CD8p was subtracted from all other values.

Transfections. Transfections were performed as described18 with one alteration: 3 x 10^5 cells were used per electroporation. Results were normalized for transfection efficiency to the amount of growth hormone expressed from 2 μg of cotransfected plasmid (cytomegalovirus [CMV] early promoter driving growth hormone transcription). Growth hormone levels correlated with the amount of transfected plasmid as detected by quantitative slot blot hybridization of DNA isolated from transfected cells by Hirt extraction.22

Deletion and point mutants of the CD11b promoter. The −1704-bp CD11b promoter/luciferase construct was generated by cloning a HindIII/Sma I fragment from CD11b genomic λ phage 65-311 into the luciferase vector pXP2.21 This fragment extends from bp −1704 to +83 in the genomic sequence, whereby bp +1 denotes the...
transcriptional start site.\textsuperscript{11} Deletion mutants at bp $-1287$, bp $-654$, and bp $-412$ were generated using restriction endonucleases $Sst$ I, $Sat$ I, and $Ssp$ I, respectively. Deletions ending 3' of bp $-412$ were generated using Exonuclease III digestions.\textsuperscript{24} The GATA site at bp $-41$ to bp $-38$ was mutated by oligonucleotide directed polymerase chain reaction (PCR) mutagenesis\textsuperscript{25} to the sequence GTTA. For DNA binding studies, a 122-bp fragment, extending from bp $-90$ to bp $+32$, either containing the wild-type GATA or mutated was placed in the context of the 1.7-kb CD11b promoter fragment. All deletion end points and mutants were determined and confirmed by sequence analysis.\textsuperscript{26}

RESULTS

Tissue-specific expression of CD11b mRNA. Northern analysis of RNA from several cell lines as well as normal thymus and peripheral blood monocytes shows the presence of CD11b mRNA only in induced myeloid cell lines and peripheral blood monocytes (Fig 1). Very low levels of CD11b mRNA are detected in K562 cells, which are derived from a patient with chronic myeloid leukemia (CML) and show some characteristics of granulocytic cells.\textsuperscript{27} Therefore, CD11b mRNA expression parallels the presence of surface antigen,\textsuperscript{2,28} suggesting that tissue-specific expression of CD11b may be controlled at the mRNA level.

Transcriptional regulation of CD11b expression. To determine whether CD11b is transcriptionally regulated, we performed nuclear run on assays on uninduced HL-60 and U937 cells as well as cells induced for 24 hours with $10^{-7}$ mol/L TPA (HL-60 and U937) or 6 days with $10^{-8}$ mol/L retinoic acid (HL-60 only). Figure 2 shows that CD11b transcription rate increases approximately eightfold in U937 cells and 14-fold in HL-60 cells after differentiation with TPA; induction of HL-60 with retinoic acid results in a fivefold increase in transcription. These increases are similar in magnitude to the increases in the steady state levels of CD11b mRNA seen after induction,\textsuperscript{8} showing that CD11b mRNA expression is largely controlled at the level of transcription. Increased rates of CD18 transcription and decreased rates of myc and MPO transcription after differentiation have previously been reported and were used as controls.\textsuperscript{29,32}

Characterization of the CD11b promoter in transient transfections. A 1.7-kb fragment of CD11b 5' flanking DNA extending to bp $+83$ of the cDNA (just proximal to the translational start site ATG at bp $+84$) was cloned in front of a luciferase reporter gene. This construct directed reporter gene activity 600-fold above background (promoterless luciferase vector, Fig 3) when transfected into U937 cells that were induced to differentiate with $3 \times 10^{-8}$ mol/L TPA immediately after transfection; we observed activity 144-fold above background in uninduced U937 cells. The $-1704$-bp promoter also directed luciferase activity 25-fold above background in uninduced HL-60 cells (HL-60 cells are 100-fold less transfectable than U937 cells), which increased to 480-fold above background when the cells were induced with TPA.\textsuperscript{31} We confirmed by 5' rapid amplification of cDNA ends (RACE)\textsuperscript{33} that initiation of reporter gene transcription occurred at the predicted CD11b start site (data not shown). The promoter for the proto-oncogene c-myc, whose transcription rate is downregulated upon induction of myeloid cells with TPA\textsuperscript{34} (see also Fig 1), directed luciferase activity that decreased twofold upon induction with TPA, indicating that the TPA-induced upregulation of CD11b promoter activity represents a specific effect. A 3.2-kb fragment containing the transcriptional start site and 2 kb of 5' flanking DNA of the myeloperoxidase gene failed to direct any measurable reporter gene activity in either U937 or HL-60 cells (data not shown). This finding indicates that a functional promoter is needed to direct reporter gene activity in this assay.

Seven deletion mutants of the $-1704$-bp CD11b promoter were generated. The results shown in Fig 3 indicate that a construct extending from bp $-412$ to bp $+83$ is three times more active in U937 cells than a construct extending from bp $-1704$ to bp $+83$. However, deletions of the promoter ending 3' of bp $-412$ show diminished activity, suggesting the presence of positive regulatory elements in

![Fig 1. Northern blot analysis of CD11b mRNA expression. Ten micrograms of total cellular RNA from HeLa (cervical carcinoma; He), fibroblasts (Fi), K562 (erythroleukemia; K5), KG-1 (early myeloid; KG), uninduced HL-60 cells (Un), HL-60 cells induced with $5 \times 10^{-7}$ mol/L Vitamin D3 for 3 days (D3), HL-60 cells induced with 1.1% DMSO for 3 days (DM), LAZ 509 (B cells; La), Jurkat (T cells; Ju), Thymus (Th), or peripheral blood monocytes (Mo) was probed with a 1.9-kb Xba I fragment of the CD11b cDNA. The blot was exposed for 18 hours with an intensifying screen at $-80$°C.](image-url)
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Fig 2. Nuclear run on transcription assay. (A) U937 uninduced and induced for 24 hours with 10^{-7} mol/L TPA; (B) HL-60 uninduced and induced for 24 hours with 10^{-7} mol/L TPA; (C) HL-60 uninduced and induced for 6 days with 10^{-8} mol/L retinoic acid.

this region (Fig 3). The construct retaining only the first 92 bp of CD11b 5' flanking DNA, although 100-fold less active than the 412-bp construct, still directs high levels of reporter gene activity (30-fold above background), showing the presence of strong promoter elements immediately adjacent to the transcriptional start site. Transfection into uninduced U937 cells yields similar relative results; however, for all deletion mutants tested, the levels of reporter gene activity were increased fivefold with TPA induction.

Nucleotide sequence of the CD11b promoter. The first 412 bp of the CD11b promoter were sequenced on both strands. The result is shown in Fig 4. The CD11b promoter contains two consensus binding sites for Spl at bp -64 and -103 and a purine-rich potential PU.1 at bp -134. The CD11b promoter, like other myeloid promoters, such as CD13 and CD18, contains neither a TATAA box nor a CCATT box. In addition, the transcriptional start site that we have determined does not fit a consensus cap site sequence, nor is there an "initiator" sequence, as recently described in the TATA-less promoter for terminal transferase. The CD11b promoter is responsive to TPA, there are no consensus-binding sites for AP-1 or NF-kB, transcription factors known to mediate TPA inducibility in other promoters.

The GATA site is nonfunctional in U937 cells. The consensus sequence GATA is found at bp -41 to -38 of the CD11b promoter. A GATA site at bp -50 in the integrin platelet factor IIb promoter is necessary for efficient transcription of this gene. We therefore used a point mutant to test whether the GATA site in the CD11b promoter is functional. A point mutation in the 1.7-kb CD11b promoter fragment changing the sequence GATA to GTTA did not reduce the level of reporter gene activity obtained in transient transfection of U937 cells (data not shown). An electrophoretic mobility shift assay was used to determine that GATA-1 protein binds the wild-type CD11b promoter and that binding was obliterated by the point mutation (data not shown).

Tissue specificity of the CD11b promoter in vitro. To determine the tissue specificity of the CD11b -1.7-kb flanking region, we transfected several constructs into the HeLa cell line, which does not express CD11b. The -412-bp promoter directs 7.5 x 10^4 RLU in U937 cells and 7.7 x 10^3 RLU in HeLa cells, and the -92-bp promoter directs 9.2 x 10^4 RLU in U937 cell and 849 RLU in HeLa (values corrected for transfection efficiency). Therefore, both the -412-bp and the -92-bp construct yield 100-fold less activity in HeLa cells than in U937, and

Fig 3. Deletion analysis of the CD11b promoter. Twenty micrograms of each deletion mutant was transiently transfected into U937 cells that were then immediately induced with 3 x 10^{-8} mol/L TPA. Luciferase activity was determined 14 hours post-transfection and is reported in RLU. Values are corrected for transfection efficiency. Representative values of duplicate experiments are shown.
Fig 4. Sequence analysis of the CD11b promoter. The transcriptional start site is indicated as bp +1.1 The Sp1 sites at bp -64 and bp -103, the GATA site at bp -42, and the PU.1 site at bp -134 are underlined. The myeloid consensus sequence is overlined. The italicized single letter code, and the first intron, beginning at bp +112, is indicated in lower case letters. The sequence has been deposited in the Genbank data base (accession no. M80772).

DISCUSSION

Expression of the CD11b/CD18 surface antigen is limited to mature monocytes, granulocytes, and NK cells. We show here that CD11b mRNA is only present in cells expressing the antigen, indicating that the tissue specificity of CD11b expression is controlled at the mRNA level. Two different inducing agents, TPA and retinoic acid, upregulate the rate of CD11b transcription in U937 and HL-60 differentiating cells or that it functions redundantly in combination with another site that must also be destroyed in order to reduce promoter function. Furthermore, Shapiro et al.16 noted that the sequence CCCCTTCC is found in several myeloid-specific promoters, such as myeloperoxidase,43 cathepsin G,44 c-fes,47 and CD13.38 This sequence is also found in the CD11b promoter, on the antisense strand at bp -132 (Fig 4). Interestingly, on the sense strand, these nucleotides overlap with the potential PU.1 binding site. A second sequence noted by Hohn et al.46 and Shapiro et al.36 to be present in myeloid promoters (CCCCACCC or the related CCCCTTCCC) is not present in the CD11b promoter.

A fragment extending from bp -1704 to bp -76 does not enhance transcription from an enhancerless tk promoter, nor does a fragment extending from bp -1704 to bp -265 activate an enhancerless mmtv promoter.48 Thus, this DNA fragment does not contain a classical enhancer. Repressors and silencers may also play a role in the regulation of CD11b expression. Deletion analysis (Fig 3) indicates the presence of a negative element between bp -1287 and bp -654. However, this element does not behave as a classical silencer element in that it will not repress transcription from a heterologous (tk) promoter.51-53 In addition to allowing the isolation of myeloid transcription factors, the description of a myeloid promoter that directs tissue-specific transcription in vivo offers the possibility of targeting heterologous gene expression. Initial results using the 1.7-kb CD11b promoter to drive the expression of a Thy 1.1 reporter in transgenic mice show high level expression of the transgene in mouse peripheral blood monocytes.49 The effect of upregulated expression of proto-oncogenes, usually downregulated during myeloid differentiation (such as mmyc),50 or of expression of activated oncogenes found in acute myeloid leukemia (AML) cells (such as N-ras),51 or of expression of novel fusion proteins generated by translocations, such as PML/RAR,45 can now be tested in myeloid cells. Such experiments may provide new insights into the mechanism by which aberrant oncogene expression leads to neoplasia.

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