Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 2105–2109, March 1992
Immunology

Identification of the promoter of the myelomonocytic leukocyte integrin CD11b

(adherence/receptor/complement)

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Communicated by Paul B. Beeson, December 16, 1991

ABSTRACT **The CD11b (or macrophage-1 antigen; MAC-1) subunit of the leukocyte integrin family forms a noncovalently associated heterodimeric structure with the CD18 (β) subunit on the surface of human granulocytes and monocyte/macrophages, where it enables these myeloid cells to participate in a variety of adherence-related activities. Expression of the CD11b subunit is restricted to cells of the myelomonocytic lineage and depends upon the stage of differentiation with the most mature myeloid cells expressing the highest levels of CD11b. To study the regulation of CD11b expression, a genomic clone corresponding to the 5′ region of the CD11b gene was isolated from a human chromosome 16 library. Primer extension and RNase protection assays identified two major transcriptional start sites, located 90 base pairs and 54 base pairs upstream from the initiation methionine. DNA sequence analysis of 1.7 kilobases of the 5′ flanking sequence of the CD11b gene indicated the absence of a "CAAT" or "TATA" box; however, potential binding sites for the transcription activators Sp1, PU.1, ets, and AP-2 are present, as well as retinoic acid response elements. The 1.7-kilobase CD11b promoter sequence displayed functional activity in transient transfection assays in the monocytic cell line THP-1 and the myeloid cell line HL-60. In contrast, this 1.7-kilobase promoter sequence did not display functional activity in the Jurkat T-lymphoid cell line. Detailed characterization of the CD11b promoter sequence should provide insight into the molecular events regulating the tissue-specific and developmental stage-specific expression of the CD11b molecule in myelomonocytic cells.**

The CD11b (or macrophage-1 antigen; MAC-1) leukocyte integrin subunit exists on the surface of human granulocytes (polymorphonuclear leukocytes or PMNs) and monocyte/macrophages coupled with the CD18 (β) subunit in an α1/β1 heterodimer; this heterodimer mediates multifaceted adherence reactivity of these myeloid cells, including the ability to adhere to endothelial cells (for review, see refs. 1 and 2). The expression of the CD11b subunit is restricted to myeloid cells, and this expression depends upon the stage of differentiation with mature granulocytes (PMNs) and macrophages expressing the highest levels of CD11b surface antigen and mRNA (3–5). Expression of the CD11b subunit is inducible in *in vitro* models of myeloid differentiation. For example, undifferentiated HL-60 promyelocytic leukemia cells do not express CD11b surface antigen or mRNA; however, exposure of these cells to retinoic acid or phorbol 12-myristate 13-acetate results in the appearance of CD11b mRNA and surface CD11b/CD18 (6, 7). Several monocytic leukemia cell lines, such as THP-1, express the CD11b/CD18 receptor on the cell surface.

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To investigate the basis of regulation of CD11b gene expression and to provide a foundation upon which to examine the relationship between myeloid differentiation and CD11b expression, we identified and characterized the promoter region of CD11b. The region upstream from the two major CD11b start sites lacks the "TATA" box frequently present in class II eukaryotic promoters; however, there are several potential binding sites for transcriptional activators, including Sp1 (8), PU.1 (9), ets (10), and AP-2 (11). Several retinoic acid response elements (12, 13) are also present. In transient transfection assays, a 1.7-kilobase (kb) fragment of the 5′ flanking sequence of CD11b demonstrated functional activity when transfected into the monocytic cell line THP-1 and the myeloid cell line HL-60 but did not demonstrate functional activity when transfected into the Jurkat T-lymphoid cell line (14).

MATERIALS AND METHODS

Isolation and Characterization of a CD11b Genomic Clone. We screened a human chromosome 16 library (American Type Culture Collection no. 57758) by plaque hybridization using a ^{32}P-labeled 39-mer oligonucleotide corresponding to a region of the CD11b 5′ untranslated region (5′-TCC AGG TTC TGG CTC CTT CCA GCC ATG GCT CTC-3′). Positive clones were plaque-purified and appropriate fragments were subcloned into Bluescript II (Stratagene) plasmids for detailed restriction mapping and into M13 vectors for DNA sequencing. The DNA sequence of the M13 subclones was determined using the dideoxynucleotide chain-termination method with Sequenase (United States Biochemical) (15).

Primer-Extension Analysis. Primer-extension studies were performed according to published methods (16). For primer-extension studies, two 39-mer oligonucleotide primers complementary to the CD11b mRNA were synthesized. These oligonucleotides were designated P (5′-GTT CTC TTG GAA GGT CAT TGC GTT TTC AGT GTC CAA GTT-3′), located 90 base pairs (bp) 3′ of the ATG of the signal peptide, and S (5′-CTG CCC GAA GCC CCT TGC GTT CTC TTG GAA GGT CAT TGC), located 109 bp 3′ of the ATG of the signal peptide (17, 18). Each oligonucleotide was ^{32}P-labeled using T4 polynucleotide kinase and 5 ng of labeled primer was hybridized to 14 μg, 28 μg, and 42 μg of total RNA extracted from the peripheral blood leukocytes of an individual with chronic myelogenous leukemia (CML) in chronic phase, as described (4). The hybridization reaction was carried out at 30°C for 15 h. The annealed primers were extended with 20 units of SuperScript reverse transcriptase (BRL) for 120 min at 42°C. The reaction mixture was digested with RNase A,

Abbreviations: PMN, polymorphonuclear leukocyte; CML, chronic myelogenous leukemia.

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phenol/chloroform-extracted, and ethanol-precipitated. The reaction products were separated by 8 M urea/6% polyacrylamide gel electrophoresis followed by autoradiography. The sizes of the primer-extended DNA products were determined by comparison to a sequencing reaction of M13 template primed with the −40 primer.

RNase Protection Assay. For this study a 444-bp *Eco*RI–*Apa* I restriction fragment from the CD11b genomic clone was subcloned into Bluescript II (Stratagene). Approximately 14 μg of plasmid DNA was digested with *Eco*RI, and a labeled RNA probe was synthesized using [^{32}P]UTP according to the manufacturer's instructions. The RNA probe was hybridized to 12 μg of total RNA from CML PMNs or human fibroblasts at 45°C for 15 h. RNA digestions were performed using DNase-free RNase A (Boehringer Mannheim) and RNase T1 (Sigma). RNase digestion products were analyzed on a denaturing polyacrylamide gel followed by autoradiography (16). The digestion products were electrophoresed alongside a dideoxynucleotide sequencing ladder of an M13 template primed with −40 sequencing primer.

CD11b Promoter Constructs. The CD11b promoter sequence was amplified from the template plasmid using the polymerase chain reaction (PCR). This template plasmid consisted of a 5.2-kb *Hin*dIII fragment of CD11b subcloned into Bluescript II. The 5′ primer (5′-GGT TCA AGT GAT TCT GCT GC-3′) hybridized ≈1.7 kb 5′ to the transcription start sites. The 3′ primer (5′-AGA ACC TGG AAG AGT GAA CC-3′) hybridized 15 bases 5′ of the initiation ATG. A *Hin*dIII site was added to the 5′ primer during synthesis and a *Bam*HI site was added to the 3′ primer to facilitate forced orientation cloning into the pϕGH (growth hormone) vector to produce pCD11b-GH. The pϕGH plasmid was obtained commercially (Nichols Institute, Los Angeles). The CMV-GH construct was obtained by ligating the cytomegalovirus immediate/early gene promoter into pϕGH as described (19, 20).

DNA Transfection and Transient Expression. Jurkat, THP-1, and HL-60 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM)/10% (vol/vol) fetal calf serum. All cell lines were transfected by electroporation using a Bio-Rad Electroporation apparatus (Bio-Rad). Approximately 1 × 10^7 cells were suspended in 0.5 ml of Hepes buffer (pH 7.5), placed in a 0.4-ml cuvette, and 100 μg of circular plasmid DNA was added. No carrier DNA was used. Plasmid DNA was prepared using equilibrium centrifugation in cesium chloride/ethidium bromide gradients (16). Jurkat and THP-1 cells were electroporated at 250 V and 960 μF. HL-60 cells were electroporated at 300 V and 960 μF. The cells were then transferred to 5 ml of DMEM and incubated at 37°C in 5% CO_2/95% air. After 3 days, 100 μl of supernatant was collected and growth hormone levels were assayed using a ^{125}I-labeled anti-growth hormone antibody (Allegro Human Growth Hormone Assay Kit, Nichols Institute).

RESULTS

Isolation of a CD11b Genomic Clone. Using oligonucleotides that hybridized to the 5′ untranslated region of the CD11b cDNA, we screened a human chromosome 16 library. Approximately 1 × 10^6 plaques were screened, and a single positive clone was isolated. This clone contained a 5.2-kb *Hin*dIII fragment. DNA sequencing of an *Eco*RI–*Apa* I restriction fragment of the *Hin*dIII insert indicated identity between a region of this restriction fragment and the 5′ end of the CD11b cDNA. A map of the genomic clone and its relationship to the cDNA are shown (Fig. 1). The first exon of CD11b contains the 5′ untranslated region and 28 bp of the signal sequence.

Identification of the Transcription Start Sites for CD11b. A combination of primer-extension and RNase protection assays was used to define the 5′ end of the CD11b mRNA. For the primer-extension assay, two staggered single-stranded 39-mers complementary to the mRNA were used as primers in the DNA elongation reaction. The extended products were 180 bp and 144 bp using oligonucleotide P and 198 bp and 162 bp using oligonucleotide S, indicating the presence of two major transcriptional start sites (Fig. 2). The most 5′ transcription start site is located 90 bp upstream from the initiation ATG, and the second start site is located 54 bp upstream from the initiation ATG.

An RNase protection assay was also used to identify the start site of transcription (Fig. 3). Two major fragments were identified in the RNase protection assay, indicating the presence of two major transcriptional start sites. The lengths of the fragments indicate two transcription start sites located ≈120 bp and 84 bp 5′ to the exon 1–intron 1 boundary (or ≈90 bp and 54 bp upstream from the initiation ATG). These distances are approximate because of the slight differences in migration of DNA and RNA fragments. The transcription start sites identified by the RNase protection assay are consistent with those identified by the primer extension.

Identification of Potential Transcriptional Activator Binding Sites. As a preliminary step in the functional analysis of the CD11b gene, we searched the 1.7 kb of the DNA sequence 5′ to the transcription start site for consensus binding sites for transcription factors. The CD11b gene lacks a TATA or "CAAT" box typical of eukaryotic class II promoters but contains several other putative binding sites for transcriptional activators. These putative binding sites are identified in

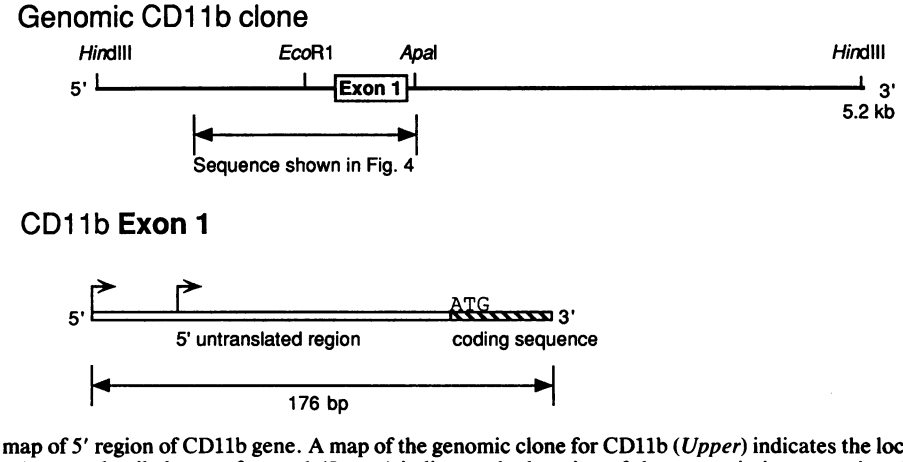

FIG. 1. Restriction map of 5′ region of CD11b gene. A map of the genomic clone for CD11b (*Upper*) indicates the location of restriction sites and exon 1 boundaries. A more detailed map of exon 1 (*Lower*) indicates the location of the transcription start sites (right-angle arrows) and the initiation ATG. The portion of exon 1 that codes for 28 bp of the signal sequence is hatched.

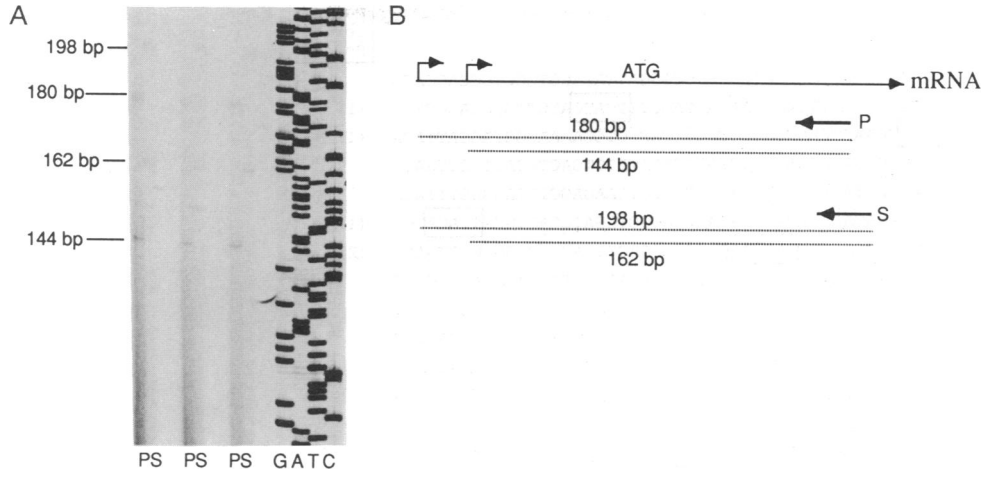

FIG. 2. Primer-extension assay of CD11b. For the primer-extension assay, the 39-mer oligonucleotides P and S were ^{32}P-labeled and hybridized to CML PMN RNA. (*A*) Locations of the primer-extension products on a 6% polyacrylamide sequencing gel. Oligonucleotide P hybridized 90 bp 3′ of the initiation ATG, and oligonucleotide S hybridized 109 bp 3′ of the initiation ATG. On the gel, the distances of 180 bp and 144 bp represent the length of the extended products from oligonucleotide P, and the bands at 198 bp and 162 bp represent the length of the extended products from oligonucleotide S. The three sets of reactions represent increasing concentrations of CML PMN RNA (14 μg, 28 μg, and 42 μg). The DNA sequence on the right side in *A* was derived from a single-stranded M13 template using the −40 primer. This DNA sequence was used to calculate the length in nucleotides of the primer extension products. (*B*) Results in the primer-extension assay are shown. The location of the two major transcription sites identified by primer extension appear as right-angle arrows on the mRNA (top line). The lengths of the extended products from oligonucleotides P and S are shown as dotted lines with the distances listed.

Fig. 4. This 1.7-kb 5′ flanking region of CD11b contains one potential binding site for the transcription factor Sp1 (8), two potential binding sites for PU.1 (9), a potential binding site for ets (10), and a potential binding site for AP-2 (11). Numerous retinoic acid response elements are also present (12, 13).

Functional Activity of the CD11b Promoter. To determine whether the 1.7-kb 5′ flanking sequence of CD11b displayed promoter activity, transfection studies were performed. A CD11b–growth hormone fusion gene containing ≈1.7 kb of CD11b 5′ flanking sequence was transfected into the Jurkat T-lymphoid cell line (14), the THP-1 monocytic cell line (21), and the HL-60 myeloid cell line (7). For a positive control, the growth hormone expression plasmid containing the cytomegalovirus immediate early gene promoter was transfected into all cell lines, where it uniformly produced high levels of immunoreactive growth hormone (Fig. 5). For a negative control, the pφGH construct, which contains no promoter sequence, was transfected into all cell lines and uniformly produced very low levels of growth hormone (Fig. 5). The CD11b–GH construct produced low levels of growth hormone in the Jurkat T-lymphoid cell line. However, the CD11b–growth hormone construct produced high levels of growth hormone in the monocytoid cell line THP-1 and the myeloid cell line HL-60, indicating that the CD11b promoter is active in these myelomonocytic cells.

DISCUSSION

This report describes the identification of the promoter for the leukocyte integrin CD11b. For these studies, a genomic clone encompassing the 5′ flanking region of the CD11b gene was isolated from a human chromosome 16 library. We identified two transcription start sites using primer-extension and RNase protection assays. Approximately 1.7 kb of this

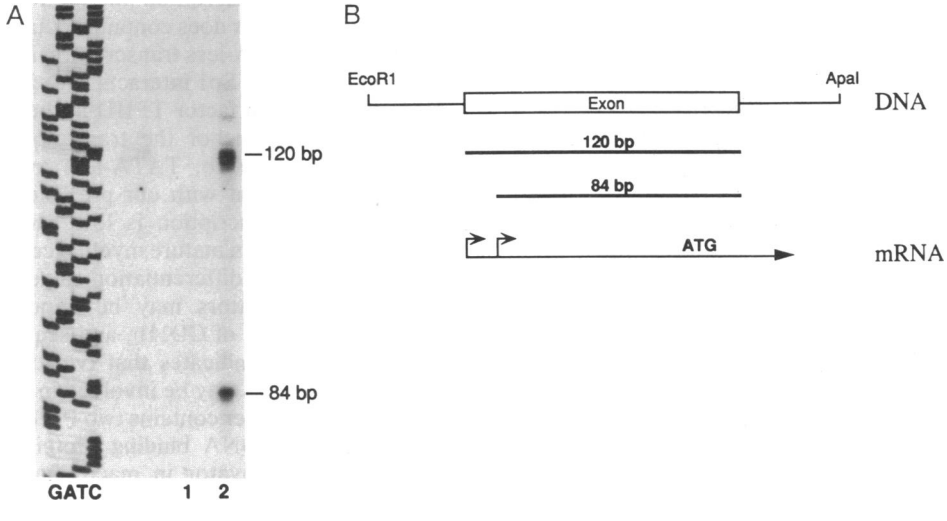

FIG. 3. RNase protection assay of CD11b. (*A*) A 444-bp *Apa* I–*Eco*RI RNA probe was synthesized, annealed to fibroblast RNA (lane 1) or human CML PMN RNA (lane 2), and digested with RNases A and T1. The DNA sequencing reaction using M13 template DNA and the −40 primer was used to calculate distance in nucleotides (*Left*). The length of the two bands corresponding to the protected fragments is shown (*Right*). (*B*) The RNase protection assay is shown schematically with the length of the protected fragments and the location of the two transcription start sites. The intron–exon boundary closest to the *Apa* I site is located 28 bp 3′ to the adenine of the initiation methionine ATG.

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                10        20        30        40        50        60  Notes for Boxes
                |         |         |         |         |         |
   1 GGTTCAAGTGATTCTGCTGCCTCAGCCTCCCAGGCGGGATTACAGGTGCCTGCCACCACG
  61 CCTGGCTAATTTTTTTGTCTTTTTAGTAAAGATGAGGTTTCACCATGTTGGGCAGGCTGG  (1)
 121 TTTCAATTGCTGACCTCAAGTGAGCCACCCCGCCTCAGCCTCCAAAATGCTAGGATTACA  (1)
 181 GGCATGAGCCACCGCACCCAGCCAAGTTTGTACATATATTTTTGACTACACTTCTTAACT
 241 ATTCTTAGGATAAATTACTAGAAGTGAAAATTCTTGGGTGAAGAGCTTGAGGCCTTTACA
 301 CACACACACACACACACACACACACACACACACAAATAGGCTGGATCGAGTGGCTCACACCT  (1)
 361 GTAATCTCAGCAGTTTGGGAGGCTCAGGAAGGAGGATCACTTGAGTCCAGGAGGTTGAGA  (2)
 421 ATAGCCTGAACAACATAGCAAGATCTTGTCTCTACAAAAAAGTTTAAAAAAAATTAGCTG
 481 GCCATGGCAGCATGTGCCTGTAGTACCAGCTACTCGGAAGGCTGAGGTAGGAGGATCGCT
 541 TGAGCCCAGGAGGTGATTGAAGCTGCAGTGAGCTGTGATTACACCACTGCACTCCAGCCT
 601 GGGCAACAGAGCTAGACTCTGTCTCTAAAAAAAGGCACAAAATAATATTTAAAAAGCACC
 661 AGGTATGCCTGTACTTGAGTTGTCTTTGTTGATGGCTACAAATGAGACAGCTCTGGCTGA
 721 AGGGCGGCTTCCATTTCCATGGGCTGGAGGAGGACATTTTGCAAAGTGTGTTTTCAGGAA
 781 GACACAGAGTTTTACCTCCTACACTTGTTTGATCTGTATTAATGTTTGCTTATTTATTTA
 841 TTTAATTTTTTTTTTGAGACAGAGTCTCACTCTGTCACCTGGGCTGGAGTGCAGTGGCAT  (1)
 901 TATTGAGGCTCATTGCAGTCTCAGACTCCTGAGCTCAAACAATCCTCCTGCCTCAGCCTC
 961 TGGAGTAGCTAGGACTACAGGCATGTGCCACCATGCCTGGCTAATTTTTTAAATGTATTT
1021 TTTTGTAGAGTCGGGGTCTCCCTATGTTGCCCAGGCTGGAGTGCAGTGGTGTGATCCTAG
1081 CTCACTGCAGCCTGGACCTCGGGCTCAAGWAATTCTCACACCTCAGCCTGTCCAGTAGCA  (1)
1141 GGGGCTACAGGCGCGCACCACCATCCCAGCTAATTAAAAATATTTTTTTGTAGAGACAGG
1201 GTCTCTCTATGTTGCCCAGGCTGGTTTCAAACTCCCAGGCTCAAGCAATCCTCCTGCCTT
1261 GCCTCCCAAATGACATCGGATTACAGGCGTGAGCCACTGAGCCTGGCCCGTATTAATGTT  (1),(3)
1321 TAGAACACGAATTCCAGGAGGCAGGCTAAGTCTATTCAGCTTGTTCATATGCTTGGGCCA
1381 ACCCAAGAAACAAGTGGGTGACAAATGGCACCTTTTGGATAGTGGTATTGACTTTGAAAG  (1)
1441 TTTGGGTCAGGAGCTGGGGAGGAAGGGTGGGCAGGCTGTGGGCAGTCCTGGGCGGAAGAC  (2),(4)
1501 CAGGCAGGGCTATGTGCTCACTGAGCCTCCGCCCTCTTCCTTTGAATCTCTGATAGACTT  (5)
1561 CTGCCTCCTACTTCTCCTTTTCTGCCCTTCTTTGCTTTGGTGGCTTCCTTGTGGTTCCTC
1621 AGTGGTGCCTGCAACCCTGGTTCACTCTTCCAGGTTCTGGCTCCTTCCAGCCATGGCTCT
                                                        MetAlaLe
1681 CAGAGTCCTTCTGTTAACAGgtgcatgggggtggggtgggggactctgggtggggaggag
     uArgValLeuLeuLeuThr
1741 ggtaacttttgggtctgtcataaatagagggccc
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FIG. 4. Sequence of the CD11b promoter. The sequence of the 1.7-kb fragment is displayed. The two transcription start sites identified by the primer-extension and RNase protection assays are denoted by arrows. Coding sequence amino acids are displayed underneath the corresponding nucleotide sequence. The first intron is denoted by lowercase letters. Putative binding sites for regulatory elements are boxed, and notes for the boxes are as follows: 1, retinoic acid response element—T(G/C)AC(C/A); 2, PU—GAGGAA; 3, AP-2—G(CG)(CG)-(TA)G(GC)CC; 4, Ets-1—GCGGAAG; 5, Sp1—CCGCCC.

sequence immediately upstream from the most 5′ transcription start site conferred tissue-specific promoter activity when transfected into myelomonocytic cells (THP-1 and HL-60). Very little promoter activity was detectable when the 1.7-kb CD11b promoter sequence was transfected into a hematopoietic cell line of lymphoid lineage (Jurkat). These

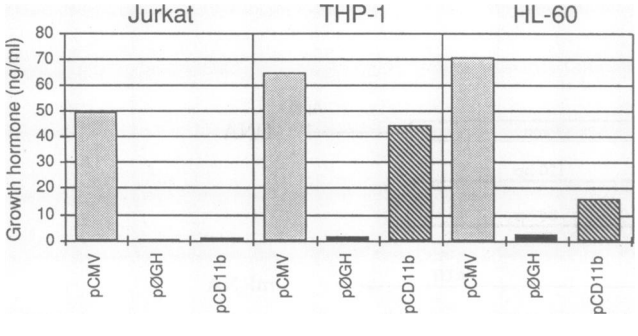

FIG. 5. Transient transfection assay of the CD11b promoter. Transient expression of a human growth hormone reporter gene was used to assess functional activity of the CD11b promoter in Jurkat and uninduced THP-1 and HL-60 cell lines. For these studies, 1×10^7 target cells were electroporated with 100 μg of a reporter construct containing the cytomegalovirus immediate early gene promoter (pCMV), the 1.7-kb fragment of the 5′ region of CD11b (pCD11b), or no promoter (pϕGH). After electroporation, cells were incubated in DMEM at 37°C, 5% CO_2/95% air for 96 h; culture supernatants were assayed for human growth hormone protein by a radioimmunoassay. At least three transfections into each cell type have been performed. A representative experiment is shown, each value was determined in duplicate, and the average value is displayed on the graph. Duplicate assays varied by <10%.

experiments demonstrate that promoter elements important for tissue-specific CD11b transcription are located within 1.7 kb of the 5′ end of the CD11b coding sequence.

DNA sequence analysis of the 1.7-kb CD11b promoter reveals several potential binding sites for transcriptional activators that may be involved in CD11b transcription. Although the CD11b promoter lacks a TATA box, which is responsible for transcription initiation in many other genes, the CD11b promoter does contain a putative Sp1 binding site. In a model of TATA-less transcription initiation proposed by Pugh and Tjian (8), Sp1 interacts with a tethering factor that binds transcription factor TFIID in the absence of a TATA box, serving to anchor the transcription complex to the promoter. For CD11b, TATA-less transcription initiation would be consistent with our previous work showing that basal CD11b transcription is low and that the enhanced CD11b expression in mature myeloid cells must require other tissue-specific and differentiation stage-specific factors (6).

Upstream activators may be important in the tissue-specific expression of CD11b, and sequence analysis of the CD11b promoter indicates that two transcriptional activators, PU.1 and ets, may be involved in CD11b transcription. The CD11b promoter contains two PU boxes. PU boxes bind a tissue-specific DNA binding protein, PU.1, which is a transcriptional activator in macrophages and B cells (9). Macrophages display high levels of CD11b, and although normal B cells do not express CD11b, PU.1 could be one of a group of transcriptional activators involved in the myeloid-specific expression of CD11b. The CD11b promoter also contains a putative ets binding site. Ets is a sequence-specific transcriptional activator that is known to bind to the T-cell

receptor α gene enhancer and may be involved in the leukocyte-specific expression of CD11b (10).

Transcriptional activators may also be involved in the stage-specific expression of CD11b. The CD11b promoter contains numerous retinoic acid response elements that could be involved in regulating CD11b transcription. In HL-60 cells induced with retinoic acid to differentiate into mature granulocytes, CD11b steady-state mRNA increases markedly. In previous studies, we have shown (6) that this increase in CD11b expression is primarily posttranscriptional; however, basal transcription of CD11b does occur in HL-60 cells and some modulation of transcription may be involved. The CD11b promoter also contains a putative AP-2 binding site. AP-2 is a site-specific DNA binding protein that can activate transcription (22); binding sites for AP-2 have been demonstrated in cis-regulatory regions of several viral and cellular genes. The aspect of AP-2 most relevant to CD11b expression is that AP-2 mRNA expression is stimulated by retinoic acid-induced differentiation of human teratocarcinoma cells (23). Thus, AP-2 could be involved in stage-specific regulation of CD11b transcription. Further analysis of the CD11b promoter is required to elucidate which, if any, of the aforementioned transcription factors are truly involved in CD11b transcription.

A notable aspect of the transfection studies in this report concerns our results with HL-60 cells. Although undifferentiated HL-60 cells express very low levels of CD11b mRNA or surface expression, these same undifferentiated HL-60 cells can be transfected with the CD11b–growth hormone construct to demonstrate promoter activity. At least two possible explanations for this apparent inconsistency exist. The first possible reason is that undifferentiated HL-60 cells, although possessing the necessary cellular machinery for transcribing CD11b, have another level of regulation—such as selective methylation—that suppresses CD11b transcription *in vivo*. The second possible reason is that CD11b mRNA levels in undifferentiated cells are primarily controlled by posttranscriptional mechanisms.

Although these experiments do not precisely identify the mechanisms underlying the tissue-specific activity of the CD11b promoter, it is interesting to compare the CD11b promoter sequence to the CD18 promoter sequence reported by Agura *et al.* (20). *In vivo*, CD11b and CD18 are expressed on myeloid cell surfaces only as noncovalently bound heterodimers; this observation suggests the possibility that the subunits of the leukocyte integrins are regulated coordinately. Like the CD11b promoter, the CD18 promoter region lacks a TATA or CAAT box but has two potential Sp1 binding sites and numerous retinoic acid response elements. Further work will be required to ascertain whether these transcriptional activators, or perhaps others, participate in coordinate regulation of the leukocyte integrin subunits.

In conclusion, these studies indicate that the 1.7-kb CD11b promoter sequence described in this report contains potential regulatory elements appropriate to its function as a promoter that is tissue-specific for myeloid cells. Future studies should be designed to identify specific regions of the CD11b promoter/enhancer that are required for the tissue-specific and developmental stage-specific expression of this molecule.

This work was supported by the National Institutes of Health Grant DK43530 (D.D.H.), the March of Dimes Birth Defects Foundation (D.D.H.), and the Veterans Affairs Career Development and Merit Review Program (D.D.H.). A.L.B. is supported by a Clinical Investigator Award from the National Heart, Lung, and Blood Institute (HL02637) and by a fellowship from the American Society of Hematology.

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