CD18 (β2 leukocyte integrin) is a leukocyte-specific adhesion molecule that plays a crucial role in immune and inflammatory responses. A 79-nucleotide fragment of the CD18 promoter is sufficient to direct myeloid transcription. The CD18 promoter is bound by the B lymphocyte- and myeloid-restricted ets factor, PU.1, and disruption of the PU.1-binding sites significantly reduces promoter activity. However, PU.1 alone cannot fully account for the leukocyte-specific and myeloid-inducible transcription of CD18. We identified a ubiquitously expressed nuclear protein complex of extremely low electrophoretic mobility that also binds to this region of the CD18 promoter. This binding complex is a heterotetramer composed of GABPα, an ets factor, and GABPβ, a subunit with homology to Drosophila Notch. GABPα competes with the lineage restricted factor, PU.1, for the same critical CD18 ets sites. The CD18 promoter is activated in myeloid cells by transfection with both GABPα and GABPβ together, but not by either factor alone. Transfection of non-hematopoietic cells with the two GABP subunits together with PU.1 is sufficient to activate CD18 transcription in otherwise non-permissive cells. Thus, GABP and PU.1 compete for the same binding sites but cooperate to activate CD18 transcription.

Cellular differentiation is linked to tightly regulated gene expression, and most developmentally regulated genes are controlled at the transcriptional level (1). In some tissues, e.g., erythroid cells (2), cellular differentiation and gene expression are governed by lineage restricted transcription factors. However, many lineage-specific or developmentally controlled genes are regulated by transcription factors that are expressed in a broader range of cell types. In such cases, specific combinations of these more widely expressed factors, or their lineage restricted modification, may contribute to tissue-specific gene expression.

CD18 (β2 leukocyte integrin) is a cell surface molecule that plays a crucial role in cell-cell and cell-matrix interactions. It is expressed exclusively by lymphocytes and myeloid cells (monocytes and granulocytes). During myeloid differentiation, CD18 expression increases due to increased transcription (3, 4). We and others have cloned the gene encoding CD18 and isolated its promoter (4–7). The CD18 promoter directs expression of a linked reporter gene when transfected into myeloid cells (4, 6). A 79-nucleotide (nt)1 region of the CD18 promoter that is crucial for its myeloid expression contains three potential binding sites for ets transcription factors. We have shown that this region of the promoter is bound by PU.1, an ets-related transcription factor that is expressed by B lymphocytes and myeloid cells. Mutagenesis of these PU.1-binding sites in the CD18 promoter decreases promoter activity and commensurately decreases PU.1 binding (8).

CD18 and PU.1 are co-expressed in some cell types, e.g., myeloid cells and B lymphocytes. However, other transcription factors must contribute to the regulated transcription of CD18. T lymphocytes express CD18 in the absence of PU.1 expression (9–11), and transfection of PU.1 is not sufficient to activate the CD18 promoter in non-hematopoietic cells. CD18 expression increases 4-fold during myeloid differentiation while PU.1 binding activity is not appreciably altered (8, 12). Thus, PU.1 alone does not fully account for the leukocyte-specific and myeloid-inducible expression of CD18.

We have found, using the electrophoretic mobility shift assay (EMSA), that multiple nuclear proteins bind to the crucial regulatory region of the CD18 promoter. We characterized these factors in order to better define the regulated transcription of CD18. The sequence GGAA, which is the core binding site for several members of the ets transcription factor family, is present at three locations in the CD18 79-nt promoter. We identified a protein complex of extremely low electrophoretic mobility that binds to these crucial regulatory elements. This complex contains both GABPα, an ets-related factor, and GABPβ, a subunit with homology to Drosophila Notch (13, 14). GABPβ alone does not bind to DNA, but together these proteins form a heterotetrameric complex on the CD18 promoter. GABPα competes with PU.1 for binding to regulatory elements in the CD18 promoter. Transfection into myeloid cells of GABPα and GABPβ together, but of neither factor alone, activates the CD18 promoter. Transfection of both GABP subunits along with PU.1 into non-hematopoietic cells is sufficient to activate a CD18 reporter construct. Thus, two different ets-related transcription factors, the lineage restricted factor PU.1 and the ubiquitously expressed factor GABPα, compete for binding to crucial regulatory sites in the CD18 promoter, yet they functionally cooperate to activate CD18 transcription.

1 The abbreviations used are: nt, nucleotides; EMSA, electrophoretic mobility shift assay; CMV/hGH, cytomegalovirus promoter/human growth hormone construct; GST, glutathione S-transferase; HSV IE, herpes simplex virus immediate early.
GABP Activates CD18 Promoter

MATERIALS AND METHODS

Transfection—U937 (American Type Culture Collection (ATCC) catalog no. CRL 1593), Raji (ATCC no. CCL 86), and Jurkat (ATCC no. CRL 8163) cells were passaged twice weekly in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum (ICN, Costa Mesa, CA) in an atmosphere of 5% CO2. HeLa cells were passaged twice weekly in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) 10% fetal calf serum. About 5 x 10^5 cells were transfected in a Bio-Rad Gene Pulser at 900 microfarads, 300 V with 20 μg of CD18/luc constructs and 1 μg of cytosomalgivirus promoter/human growth hormone construct (CMV/HGH). Where indicated, 5 μg of the human homologues of GABPα and GABPβ1–3 (E4TFTF-60 and E4TFTF-53, respectively) (14) in the expression vector pCAGGS (15) were co-transfected with 20 μg of CD18/luc constructs and pEGM3Z' was added to bring the total amount of transfected DNA to 30 μg. Fourteen h after transfection, luciferase activity was assayed, and growth hormone was measured from 100 μl of supernatant. Promoter activity is expressed as normalized relative light units, by dividing luciferase activity by growth hormone content (CMV/HGH). Where indicated, 5 μg of the human homologues of GABPα and GABPβ1–3 (E4TFTF-60 and E4TFTF-53, respectively) (14) in the expression vector pCAGGS (15) were co-transfected with 20 μg of CD18/luc constructs and pEGM3Z' was added to bring the total amount of transfected DNA to 30 μg. Fourteen h after transfection, luciferase activity was assayed, and growth hormone was measured from 100 μl of supernatant. Promoter activity is expressed as normalized relative light units, by dividing luciferase activity by growth hormone, to account for transfection efficiency (8). Transfection results represent the mean and S.E. from three separate experiments. Preparation of Nuclear Extracts and Proteins—Nuclear extracts were prepared as described (8). In vitro translated proteins were prepared with 1 μg of the following supercoiled DNAs in the TNT Coupled Reticulocyte Lysate System (Promega Corporation, Madison, WI): PU.1 (a gift of Richard Mak), La jolla Cancer Research Institute, La jolla, CA); Elf-1 and Erts-1 (gifts of eff Leiden, University of Chicago); Fil-1 (a gift of Michael Klemsz, University of Indiana). GABPα and GABPβ1–3 (16), purified by nickel chelate chromatography, were gifts of Tom Brown (Pfizer, Inc., Groton, CT) and GST-PU.1 (a gift of Tony Kouzarides, WellcomeCRC Institute, Cambridge, United Kingdom) and GST-GABPα (as E4TFTF-60, the human homologue of GABPα) protein extracts were expressed in Escherichia coli, isolated with glutathione-Sepharose, and quantitated by staining with known protein standards in SDS-polyacrylamide gel electrophoresis. EMSA—EMSA probes are schematized in Fig. 2A. The GGA1 sites in the EMSA probes presented below are underlined (note that the GGA1 sequence at −72/−75 is encodce on the bottom probe). Probes that correspond to −85/−37 of the CD18 promoter (ACACCACACCTCCTGAGAGGAGCTGCAGGGAAGTTGTCAG and its complement) are annealed and 5' end-labeled with γ-32PATP (ICN) by Polyuridylate kinase (New England Biolabs, Beverly MA). Probes that correspond to −89/−58 (GGCTGAGAGGAACTGGAAGCAAGGAAGCTTGTTGCTCA and its complement) and −59/−32 (CGTGGAGGAACTGGGAAGCTTGTTGCTCA and its complement) and corresponding mutant versions that contain GGA → CC mutations in the underlined sites, include SalI and BamHI compatible sites at either end and were labeled with [γ-32P]ATP by the Klenow fragment of DNA Polymerase I. Radio-labeled probe (0.1 ng) was incubated for 10 min on ice with 1 μl of unpurified reticulocyte lysate, 1 μl of in vitro translated or purified protein, or 10 μg of nuclear extract in a 15-μl reaction containing 10 μl Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM mercaptoethanol, 1% Ficoll and poly(dI-dC) (Pharmacia Biotech Inc.; 2.0 μg for nuclear extracts, 0.5 μg for proteins). Where indicated, a 100-fold molar excess of homologous DNA or irrelevant probe that lacks an ets-binding site (corresponding to CD18 −903/−883, CGCAAGTTGCAGTAGCTGGATCACGAGATGCGTTGCTC and its complement) were used as unlabeled competitors. Where indicated, up to 10-fold greater amounts of competing GST fusion proteins were added to binding reactions. Products were electrophoresed at 180 V in a 5% acrylamide/12% gel in 0.5 × TBE (1 × TBE is 90 mM Tris-base, 90 mM boric acid, 2 mM EDTA) prior to autoradiography. Because binding by GABP protein is sensitive to the ionic content of the electrophoretic gel (12), EMSA with purified GABP was performed in 0.25 × TBE, where indicated. For supershift experiments, reactions were preincubated for 10 min on ice with either 1 μl of preimmune rabbit serum or 1 μl of polyclonal rabbit antiserum raised against either GABPα or GABPβ (gifts of Tom Brown).

DNase I Footprinting—A probe corresponding to −148/−28 relative to the dominant start site of CD18 transcription, radio-labeled on the non-coding strand, was prepared by a modification of the polymerase chain reaction, as described (8). One μl of purified GABPα protein was equilibrated in 10 μl Tris, pH 7.4, 40 mM KCl, 1 mM EDTA, 1% Ficoll, 1 mM β-mercaptoethanol for 10 min at room temperature, incubated with 5,000 counts/min of probe for 30 min at room temperature, and digested with 1 μl of DNase I (Sigma from temperature for 1 min, phenol, extracted, and ethanol-precipitated. Identical samples, which lacked added protein, were treated similarly, except they were digested with 0.5 μg/ml DNase I at room temperature for 20 s. Half of the resultant products were electrophoresed in a 6% polyacrylamide, 7 M urea gel alongside the same probe subjected to chemical footprinting with 0.5 μg/ml DNase I (Fig. 2B). The DNase I digestion tracks were scanned, and the positions of the protected sites were identified and plotted.

RESULTS

CD18 Minimal Promotor Directs Lineage Restricted Expression—A 79-nt fragment of the CD18 promoter is active after transient transfection into U937 myeloid cells. The B lympho-

cyte and myeloid-restricted ets transcription factor PU.1 binds to this region of the promoter, and mutagenesis of each PU.1 site reduces promoter activity and commensurately decreases PU.1 binding (8). Because PU.1 alone cannot fully account for its lineage restricted expression, we sought to identify other factors that may control CD18 transcription. In order to localize its leukocyte-specific activity, we transfected a variety of cell types with constructs that contain either 918 or 79 nt of the CD18 promoter; cells were co-transfected with CMV/HGH as an internal control for transfection efficiency. Figure 1 indicates that both the 918- and 79-nt CD18 promoter fragments are active in myeloid cells (U937), B lymphocytes (Raji), and T lymphocytes (Jurkat). In Raji cells, the 79-nt construct is significantly less active than the full-length promoter, indicating the presence of an element between −918 and −79 that enhances CD18 activity in B lymphoid cells. Neither CD18 promoter construct is active following transfection into non-hematopoietic HeLa cells (cervical carcinoma). These studies indicate that the 79-nt CD18 minimal promoter encodes the cell type-specific expression of the endogenous gene.

Lineage specific and Ubiquitously Expressed Factors Bind to the CD18 Promoter—We performed EMSA with a radiolabeled, double-stranded probe corresponding to −85/−37 of the CD18 promoter, which contains three potential ets-binding sites (see Fig. 2A; GGA1 at −72/−75, −53/−50, and −47/−44). Using nuclear extracts from U937, Raji, HeLa, and Jurkat cells, we found that several different protein species bind to this region of the CD18 promoter. Consistent with its known pattern of expression, PU.1 binding activity was present only in extracts of myeloid cells and B lymphocytes (Fig. 2B, lanes 9 and 10, right arrow) (8). An as yet unidentified cell-specific band (lane 12, curved arrow) is observed with the Jurkat nuclear extract. All other binding species are present in all of the nuclear extracts tested (lanes 9–12).

Multiple ets-related Transcription Factors Bind to the CD18 Promoter—ets transcription factors bind to related sequences, typically containing a core of GGAA. Therefore, we sought to determine if any of the ets family of transcription factors bound to the CD18 promoter. Nuclear extracts were prepared from U937, Raji, HeLa, and Jurkat cells. ets family proteins were immunoprecipitated from nuclear extracts using antibody to Ets-1, GABP, PU.1, and GABPβ1–3. Protein was electrophoresosed on a 10% SDS-polyacrylamide gel alongside the same probe subjected to chemical foot printing. The positions of the protected sites were identified and plotted. We found that several different protein species bind to this region of the CD18 promoter.
ATP. EMSA was performed with 10,000 counts/min probe in 0.5
mL of TBE. Proteins used in the binding assay include unprogrammed in vitro
translated PU.1, Ets-1, Fli-1, and Elf-1. In addition to
species from Jurkat nuclear extracts. None of these factors co-migrated with the unidenti
fied binding species (data not shown). GABP Activates CD18 Promoter

FIG. 2. *ets* factors bind to CD18 promoter. *A,* the sequence of the
CD18 promoter between −89 and −30 is displayed. The three potential
*ets*-binding sites and the orientation of the sequence GGAA are indi
cated by arrows. Note that the GGAA sequence at −72/−75 is encoded
on the bottom strand. Double-stranded probes used for EMSA are indi
cated by brackets. *B,* a double-stranded oligonucleotide probe cor
responding to −85/−37 of the CD18 promoter was radiolabeled with
[^32P]ATP. EMSA was performed with 10,000 counts/min probe in 0.5 ×
TBE. Proteins used in the binding assay include unprogrammed reticu
locyte lysate (RL), in vitro translated PU.1, Ets-1, Fli-1, Elf-1, purified,
bacterially expressed GABPα and GABPβ, or nuclear extracts from
U937 (myeloid), Raji (B lymphocyte), HeLa (cervical carcinoma), and
J urkat (T lymphocyte) cell lines. Right arrow indicates the location of
binding by PU.1 (lanes 3, 9, and 10), and the curved arrow indicates a
binding species unique to J urkat cells.

determine if other *ets* factors besides PU.1 bind to the CD18
promoter. We performed EMSA with the −85/−37 probe and in
vitro translated PU.1, Ets-1, Fli-1, and Elf-1. In addition to
PU.1 (Fig. 2B, lane 3), Elf-1 (lane 6), and Spi-B (not shown)
bound avidly to −85/−37. However, neither Elf-1 nor Spi-B
formed a complex that co-migrates with binding species from
nuclear extracts (lanes 9–12). Neither Ets-1 (lane 4), Fli-1 (lane
5), nor Ets-2 (not shown) bound to the CD18 −85/−37 probe,
although each of these in vitro translated transcription factors
bind to appropriate positive control probes (data not shown).
None of these factors co-migrated with the unidentified binding
species from J urkat nuclear extracts.

GABP Complexes Bind to the CD18 Promoter—We performed
EMSA with the CD18 −85/−37 probe and GABPα, a ubiqui
tously expressed member of the GABP family. Recombinant
GABPα, purified from bacteria, forms two binding complexes
with the CD18 promoter (Fig. 2B, lane 7). GABPβ, which ex
hibits homology to Drosophila Notch and is not related to the
*ets* transcription factors, interacts with GABPα via its ankyrin
repeats (13, 17). When purified GABPβ is added to GABPα
three distinct complexes are generated. Each complex co-
migrates with a binding species present in the myeloid, lymphoid,
and non-hematopoietic nuclear extracts (compare lane 8 to
lanes 9–12), suggesting that the ubiquitous CD18 binding spe
cies (lanes 9–12) is GABP.

FIG. 3. GABPα and GABPβ are present in the co-migrating nuclear binding species. EMSA was performed with radiolabeled
CD18 −85/−37 probe and GABPα alone (lane 2), GABPβ alone (lane 3),
GABPα and GABPβ together (lane 4), and U937 nuclear extract (lanes
5–8). Binding reactions were preincubated with antibody against
GABPα (lane 6), antibody against GABPβ (lane 7), or preimmune serum
(lane 8). The locations of monomeric, dimeric, and tetrameric binding
species are indicated by M, D, and T, respectively, and the open arrow
indicates the location of the supershifted proteins.

The multimeric pattern of binding by GABPα and GABPβ to
the CD18 promoter resembles GABP binding to the HSV IE
promoter and other genes that contain multiple *ets* sites. Gel
filtration, sedimentation, and cross-linking studies were used to
characterize the nature of each of these binding species on the
HSV IE promoter (13). We used UV cross-linking and
mutagenesis of the GABP subunits to confirm the multimeric
nature of the binding complexes on the adenovirus E4 promoter
(17). The two complexes formed on the CD18 promoter by
GABPα correspond to monomeric and homodimeric forms of the
protein (Fig. 3, lane 2, M and D). GABPβ alone does not
bind to CD18 (lane 3). However, GABPα and GABPβ together
can generate three distinct binding species. These correspond
to the monomeric GABPα (lane 4, M), heterodimeric GABPα/
GABPβ (D, which co-migrates with homodimeric GABPα2), and
heterotetrameric GABPα2/GABPβ2 (T) species demonstrated in
previous studies (13, 17).

GABPα and GABPβ Are Present in Myeloid Nuclear Ex
trates—We used antibodies to confirm the identity of the nu-
clear proteins that co-migrate with purified GABPα and
GABPβ. Preimmune serum does not appreciably alter binding
by myeloid nuclear proteins to CD18 −85/−37 (Fig. 3, lane 8).
However, an antibody against GABPα abrogates the mono-
meric and dimeric species and sharply reduces the intensity of
the tetrameric species (lane 6, M, D, and T, respectively).
Binding by this antibody results in a supershift of these pro-
teins, as indicated by the open arrow. Note that a binding
species of slightly slower electrophoretic mobility than the di-
meric species is unaffected by the GABPα antibody. Antibody
against GABPβ abrogates the dimeric and tetrameric species
and results in a supershift of the binding complexes (lane 7,
open arrow). These results demonstrate that myeloid nucl
GABP Activates CD18 Promoter

Fig. 4. Localization of GABP-binding sites and sensitivity of binding to mutations. The following EMSA studies were performed in 0.25 × TBE. A, EMSA was performed with purified GABPα and GABPβ and the following radiolabeled probes: −85/−37 (lanes 1–3), −89/−58 (lanes 4–6), and −59/−31 (lanes 7–9). The locations of monomeric, dimeric, and tetramer binding species are indicated by M, D, and T, respectively. B, EMSA was performed with purified GABPα and GABPβ and the following radiolabeled probes: wild type (wt) −89/−58 probe (lanes 1–3), −89/−58 probe containing GGAA CCMA mutation (mut) in the ets site (lanes 4–6), wild type −59/−31 probe (lanes 7–9), and −59/−31 probe with GGAA CCMA mutations in both of the ets sites (lanes 10–12).

contain CD18-binding proteins that are immunologically related to GABP.

Localization of GABP-binding Sites—We prepared additional EMSA probes to further localize the GABP-binding sites within the crucial region of the CD18 promoter. Because binding by GABP proteins is dependent on the ionic strength of the electrophoretic gel (13), the following experiments with purified proteins were performed in 0.25 × TBE. The region from −89/−58 contains one potential ets-binding site (GGAA −72/−75, labeled A in Fig. 2A), GABPα alone can form monomeric, but not dimeric binding species on this DNA fragment (Fig. 4A, lane 5, M). GABPα and GABPβ, together, can form the monomeric and dimeric (lane 6, M and D), but not the tetramer binding species.

The probe corresponding to −59/−31 of the CD18 promoter contains two potential ets sites (at −53/−50 and −47/−44, labeled B and C in Fig. 2A). GABPα alone forms predominantly a monomeric species (lane 8, M), with a small amount of homodimer (more apparent in Fig. 4B, lane 8). However, GABPα and GABPβ together generate all three species that form on the larger −85/−37 probe (compare lane 9 to lane 3). Thus, each of the three potential ets sites supports GABPα binding and can contribute to formation of tetrameric GABP binding on the CD18 promoter.

GABP Binding Is Sequence-dependent—We prepared EMSA probes with mutations in each of the ets-binding sites to determine if GABP binding is dependent on the integrity of the conserved ets sites. Mutagenesis of the ets site in the −89/−58 probe (GGAA CCMA at −73/−72 in site A) abrogates all GABP binding (Fig. 4B, compare lanes 5 and 6 to lanes 2 and 3). Similarly, mutagenesis of the two ets sites in the −59/−31 probe (GGAA CCMA at −53/−52 in site B and −47/−46 in site C) abrogates GABP binding (compare lanes 11 and 12 to lanes 8 and 9). Thus, GABP binding depends on integrity of the ets sites.

DNase I Footprinting—We performed DNase I footprinting in order to further define the binding sites of GABP. A probe which corresponds to −148/−28 relative to the start site of CD18 transcription was prepared by polymerase chain reaction and labeled on the non-coding strand. Purified GABPα protects three regions of the CD18 promoter from DNase I digestion (Fig. 5, vertical bars; compare lanes N (naked DNA) to lane α (GABPα)). The protected regions encompass the three ets-binding sites, labeled A–C in Fig. 2A, and they correspond to the footprints that we have previously demonstrated with U937 nuclear extract. The asterisk indicates a DNase I-hypersensitive site that is also seen with myeloid nuclear extracts (8). The addition of GABPβ to GABPα has little effect on the footprints (data not shown).

GABP and PU.1 Directly Compete for Binding to the CD18 Promoter—We sought to determine if GABPα and PU.1 compete for binding to the same ets sites in the CD18 promoter. These factors were expressed in E. coli as fusion proteins with GST and isolated with glutathione-Sepharose beads. No binding to the CD18 promoter, −85/−37 probe is seen with GST alone (not shown). EMSA with GST-GABPα generates two distinct bands, corresponding to monomeric and homodimeric binding species (Fig. 6A, lane 2, M and D, respectively). Their binding specificity is indicated by abrogation with 100-fold excess of unlabeled homologous probe (lane 3), but not by 100-fold excess of nonspecific competitor oligonucleotide (lane 4). Addition of increasing amounts of GST-PU.1 to a fixed amount of GABPα in the binding reaction recruits bound probe from GABPα to PU.1 (lanes 5–8). As PU.1 increases, there is preferential loss of binding to the monomeric form, relative to the dimeric form of GABPα, indicating the higher binding affinity of the dimeric GABPα species.

Similarly, EMSA with GST-PU.1 (Fig. 6B, lane 2, labeled PU.1) forms a distinct binding species with the CD18 −85/−37 probe that is abrogated by a 100-fold molar excess of homologous unlabeled probe but not by nonspecific probe (lanes 3 and 4, respectively). A species of high electrophoretic mobility (indicated by the asterisks in Fig. 6, A and B) is caused by binding to the probe by a proteolytic degradation product of PU.1 (8).
We co-transfected CD18 with expression vectors containing the human homologues of GABPα and GABPβ alone, which had no effect on CD18 promoter activity, and transfection complexes. Addition of increasing amounts of GABPα resulted in increased CD18 promoter activity compared to the vector control. GABPα binding (lane 2) was abrogated by a 100-fold excess of unlabeled specific competitor (lanes 5–8). GST-PU.1 binding (lane 2) is abrogated by a 100-fold excess of unlabeled specific competitor (lane 3), but not by nonspecific probe (lane 4). Addition of increasing amounts of GST-PU.1 (lanes 5–8) causes loss of GABPα binding, in exchange for increased binding by PU.1. GABPα monomeric (M) and dimeric (D) binding species and PU.1 (PU.1) are indicated. A binding complex of high electrophoretic mobility (indicated by the asterisk) represents a proteolytic degradation product of GST-PU.1, as described previously (8). B. GST-PU.1 binding (lane 2) is abrogated by a 100-fold excess of unlabeled specific competitor (lane 3), but not by nonspecific probe (lane 4). Addition of increasing amounts of GABPα (GST-E4TF1–60) recruits probe from PU.1 to GABPα complexes (lanes 5–9).

Addition of increasing amounts of GABPα to a fixed amount of PU.1 in the binding reaction recruits probe to the GABPα complexes.

Co-transfection of both GABPα and GABPβ into myeloid cells activates the CD18 promoter. We sought to determine if GABPα and GABPβ could activate the CD18 promoter in vivo. We co-transfected CD18 (–918)luc with mammalian expression vectors containing the human homologues of GABPα and GABPβ (E4TF1–60 and E4TF1–53, respectively). Neither GABPα nor GABPβ alone, activated the CD18 promoter construct following transfection into U937 myeloid cells (Fig. 7A). However, co-transfection of both GABPα and GABPβ with the CD18 promoter increased luciferase activity more than 10-fold. Interestingly, transfection into U937 cells of a PU.1 expression vector fails to substantially activate the CD18 promoter (not shown). Thus, the GABP complex activates myeloid expression from the CD18 promoter in vivo.

GABPα and GABPβ cooperate with PU.1 to activate the CD18 promoter in non-hematopoietic cells. The CD18 promoter encodes the lineage-specific activity of the endogenous CD18 gene, for it directs reporter expression in myeloid and lymphoid cells but not in non-hematopoietic cells (Fig. 1). We sought to determine if GABPα co-activates the CD18 promoter following transfection into otherwise non-permissive HeLa cells. Transfection into HeLa cells of either GABPα or GABPβ alone had no effect on CD18 promoter activity, and transfection.

**Fig. 6. GABPα and PU.1 directly compete for binding to CD18 ets sites.** A. EMSA was performed with radiolabeled CD18 (–85–37) probe and purified bacterially expressed GABPα (GST-E4TF1–60). GABPα binding (lane 2) is abrogated by a 100-fold excess of unlabeled specific competitor probe (lane 3), but not by nonspecific probe (lane 4). Addition of increasing amounts of GST-PU.1 (lanes 5–8) causes loss of GABPα binding, in exchange for increased binding by PU.1. GABPα monomeric (M) and dimeric (D) binding species and PU.1 (PU.1) are indicated. A binding complex of high electrophoretic mobility (indicated by the asterisk) represents a proteolytic degradation product of GST-PU.1, as described previously (8). B. GST-PU.1 binding (lane 2) is abrogated by a 100-fold excess of unlabeled specific competitor (lane 3), but not by nonspecific probe (lane 4). Addition of increasing amounts of GABPα (GST-E4TF1–60) recruits probe from PU.1 to GABPα complexes (lanes 5–9).

**Fig. 7. GABPα and GABPβ together with PU.1 activate CD18 transcription.** A. U937 cells were transfected with 20 μg of CD18 (–918)luc and no added effector, 5 μg of GABPα (pCAGGS-E4TF1–60) alone, 5 μg of GABPβ (pCAGGS-E4TF1–53) alone, or 5 μg each of both GABPα and GABPβ. Each sample was transfected with the internal control CMV/hGH and additional pGEM3zf to bring the amount of transfected DNA in each sample to 30 μg. Luciferase activity was measured 14 h later and was normalized to growth hormone expression. Data represent the mean ± S.E. from three separate experiments. B. HeLa cells were transfected with 20 μg CD18 (–918)luc and no added effector, 5 μg of GABPα (pCAGGS-E4TF1–60) alone, 5 μg of GABPβ (pCAGGS-E4TF1–53) alone, 5 μg of PU.1 (in the mammalian expression vector pCE) alone, or combinations of 5 μg each, as indicated. Each sample was transfected with the internal control CMV/hGH and additional pGEM3zf to bring the amount of transfected DNA in each sample to 40 μg. Luciferase activity was measured 14 h later and was normalized to growth hormone expression. Data represent the mean ± S.E. from three separate experiments.

**DISCUSSION**

In order to better define the molecular basis of myeloid differentiation, we are studying the factors that regulate transcription of the leukocyte β2 integrin, CD18. A 79-nt fragment of the CD18 promoter is required for transcription in myeloid cells (6, 8), and we have previously shown that the ets-related transcription factor PU.1 binds to this region (8). We now demonstrate that GABPα, a second ets factor, competes with PU.1 for binding to these sites in the CD18 promoter. GABPα forms multimeric complexes with its Notch-related partner GABPβ, and together these factors activate CD18 transcription in myeloid cells. Furthermore, we show that GABP and PU.1
are sufficient to activate the CD18 promoter in non-hematopoietic cells. Thus, although these ets factors compete for promoter binding, they functionally cooperate to activate CD18 transcription.

The region of CD18 that is required for promoter activity contains three potential ets sites. Because ets transcription factors bind to similar core DNA sequences, we sought to determine if other ets factors, besides PU.1, bind to CD18. We detected a widely expressed nuclear factor of extremely low electrophoretic mobility that binds to this region of the CD18 promoter. Böttinger et al. (6) suggested that GABP might bind to the CD18 promoter. We now demonstrate that purified GABPα binds to all three CD18 ets regulatory elements. Puriﬁed GABPα alone forms monomers and homodimers on these sites and its binding is sensitive to mutation (GGAA → CCAA) of the consensus ets sequence. GABPβ alone does not bind to the CD18 promoter, but in the presence of GABPα it forms a complex that co-migrates with the low mobility nuclear binding complex. We used antibodies to conﬁrm that this myeloid-binding complex contains proteins that are immunologically related to both GABPα and GABPβ.

GABP was originally identiﬁed as a factor from rat nuclei that binds to critical regulatory elements in HSV IE genes (13, 16). GABP is composed of two distinct polypeptides, which together are required for high afﬁnity DNA binding and transcriptional activation. The human equivalents of GABPα and GABPβ were independently cloned as regulators of adenovirus E4 gene transcription (E4TF1–60 and E4TF1–53/47, respectively) (14) and as nuclear respiratory factor 2 (NRF-2α and NRF-2β/γ, respectively) (18) GABPα is a DNA-binding protein with homology to the ets family of transcription factors. GABPβ is unrelated to the ets family, but it has homology to Drosophila Notch and Caenorhabditis elegans Glp-1 and Lin-1. Four ankyrin repeats in its amino terminus are required for its interaction with GABPα, and a coiled-coil motif in its carboxyl terminus permits GABPβ homotypic dimerization (13, 17). Whereas DNA binding is mediated by the GABPα subunit, transcriptional activation is controlled by GABPβ (17–19).

GABP generates a complex of extremely low electrophoretic mobility when it binds to CD18 promoter probes that contain at least two of its three ets-binding sites. Gel ﬁltration, gradient sedimentation, and cross-linking studies were used to conclusively demonstrate the heterotetrameric nature of GABP complexes on a similar arrangement of three ets sites on HSV IE genes (13). We used UV cross-linking and mutagenesis of the GABP subunits to conﬁrm the multimeric nature of the binding complexes to the adenovirus E4 transcriptional promoter (17). In the present study, we used mutagenesis of CD18 ets sites to demonstrate an identical pattern of binding by GABPα and GABPβ. Other genes with multiple ets-binding sites, including folate-binding protein (20), aldose reductase (21), and cytochrome c oxidase subunits IV and 5b (22, 23), are bound by GABP in a similar manner. As with each of these genes, the low mobility CD18 promoter binding complex represents GABPαβ/GABPβα heterotetramers.

The two ets factors that bind to the CD18 promoter, PU.1 and GABPα, are expressed in very different lineage-speciﬁc patterns. PU.1 is expressed by B lymphocytes and myeloid cells (9–11); in accordance with its pattern of expression, we detect PU.1 binding activity only in these cellular compartments. PU.1 appears to control the transcription of several leukocyte-speciﬁc genes including CD11b (24), M-CSF receptor (25), FcγR1 (26, 27), and macrophage scavenger receptor (28), as well as CD18 (8). PU.1 is required for myeloid colony formation (29), and its targeted disruption by homologous recombination abrogates lymphopoiesis and myelopoiesis (30). Thus, PU.1 is essential for normal hematopoiesis and may control transcription of some myeloid genes.

In contrast to PU.1, GABPα and GABPβ are widely expressed (16). We detected binding to CD18 by the heterotetrameric GABP species in all cell types examined. Thus, GABP binding activity is present both in cells that express CD18 (myeloid cells and B and T lymphocytes) and in cells that do not express CD18 (HELa). Although most genes whose activity is controlled by GABP are widely expressed, GABP also controls genes that are expressed in a lineage-restricted manner. For example, the gene encoding α4 integrin, which is expressed predominantly by leukocytes (31), and the F promoter of 6-phosphofructo-2 kinase (32) are transcriptionally regulated by GABP.

How can the widely expressed GABP transcription complex control the lineage-restricted expression of genes such as CD18 and α4 integrin? There are multiple isoforms of the GABPβ (14, 16, 22), the subunit that controls transcriptional activation by GABP (17, 18). These isoforms, which may be derived from alternative splicing of the mRNA transcript differ primarily in the region of the protein that is responsible for GABPβ homodimerization. Alternate forms of GABPβ may affect formation of GABP complexes and their interactions with other components of the transcriptional apparatus. Recently, GABPβ2–1, a distinct but related gene that is encoded on a different chromosome, was described (33). The two distinct GABPβ genes differ in the region of the protein that is responsible for transcriptional activation. GABPβ1 and GABPβ2 can form heteromorphic complexes with one another, thereby adding another level of complexity to the regulation to GABP function (33). The cell type-specific and differentiation-associated expression of the GABPβ genes and their various isoforms have not been well deﬁned. Thus, subtle lineage-speciﬁc alterations in GABPβ expression may control GABP complex formation and transcriptional activation and thereby contribute to the regulated expression of CD18. Cell type-speciﬁc transcriptional activation by the ubiquitously expressed GABP may also be inﬂuenced by lineage-restricted factors such as PU.1.

Other genes besides CD18 are also controlled by more than one ets factor. Immunoglobulin heavy chain enhancer function is regulated by binding of different ets factors including PU.1, Ets-1, and Erg-3 (34, 35). The α4 integrin promoter may be bound by a second ets factor besides GABPα (31). Although we cannot exclude the possibility that GABP and PU.1 physically interact on the CD18 promoter in vivo, we identiﬁed no novel binding complexes that are formed by these factors in the electrophoretic mobility shift assay. Rather, titration of increasing amounts of PU.1 competes for GABPα binding to the CD18 promoter and vice versa. Furthermore, antibodies to GABP do not supershift the EMSA probes that are bound by PU.1 and vice versa (8). Thus, PU.1 and GABPα appear to compete for the same CD18 promoter sites and their binding appears to be mutually exclusive.

J urkat (T lymphoid) cells express CD18, yet they lack PU.1 expression. These cells possess a unique nuclear binding species which does not co-migrate with other ets factors that are expressed by T lymphocytes (Fig. 2). Additional mutagenesis studies and supershift assays (not shown) suggest that this binding species likely represents an as yet unidentified ets factor. We propose that this T cell factor acts in lieu of PU.1 and contributes to T lymphoid expression of CD18. These ﬁndings suggest that at least one other ets factor may functionally cooperate with GABP to effect CD18 transcription.

Transfection of both GABPα and GABPβ into myeloid cells activates CD18 reporter activity more than 10-fold; we have found no such activation of CD18 myeloid transcription by
PU.1. Furthermore, co-transfection of both GABP and PU.1 activates CD18 transcription in non-hematopoietic cells. This confirms our previous proposal that although PU.1 is necessary for myeloid expression of CD18, it alone is not sufficient to control CD18 transcription. Although these two ets factors compete for the same CD18 promoter-binding sites, they functionally cooperate to activate its transcription. GABP and PU.1 may have different roles in the process of transcriptional activation. For example, because PU.1 directly interacts with TATA-binding protein (36), it may recruit the basal transcriptional apparatus to the TATA-less CD18 promoter, while GABP may have different roles in the process of transcriptional activation. Further analysis of these factors should demonstrate the means by which these two ets factors cooperate to control CD18 transcription.

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