The CD34 surface antigen is a highly glycosylated transmembrane protein expressed on hematopoietic stem cells and lineage-specific progenitor cells, on a subset of bone marrow stromal cells and on small vessel endothelium of a variety of tissues (1-4). About 1-4% of normal bone marrow cells and ~30% of blasts from acute leukemia patients express CD34 (1, 2, 5). To date, CD34 remains the only well-defined human stem cell marker. CD34-positive cells have self-renewal capacity and ability to reconstitute hematopoiesis in sublethally irradiated primates and marrow-ablated humans (6, 7). The function of CD34 is not yet known, although this protein has been proposed to play a role in hematopoietic cell adhesion to stromal cells, perhaps facilitating the interaction with locally released growth factors, and to directly function as a signal transducer (3, 4, 8).

The mechanisms regulating CD34 expression in hematopoietic cells are not well understood, although there is evidence of transcriptional and posttranscriptional regulation of CD34 expression (9, 10). In light of putative Myb binding sites present in the 5′ flanking region of the CD34 gene (9, 11), and the fundamental role of c-myb in hematopoietic cell proliferation and/or differentiation (12-15), most likely through the transcriptional regulation of Myb proteins (16-19), we investigated the possibility that c-myb is directly involved in transcriptional regulation of CD34 gene, possibly by inducing expression of its mRNA and synthesis of the surface membrane protein in nonexpressing cells.

Materials and Methods

Gel Retardation Assay. HB101 cells containing the parental pFlag (IBI, New Haven, CT) expression vector only or HB101 cells containing the pc-Myb Flag vector (20) were incubated for 4 h in the presence of 1.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) to an OD 600 of 0.500. Myb expression was determined in bacterial lysates by Western blot using an anti-Myb-specific antibody (Upstate Biotechnology Incorporated [UBI], Lake Placid, NY). 32p-labeled double-stranded probes were synthesized by PCR and corresponded to three different segments of the human CD34 5′ flanking region from -294 to -115; -132 to 54, and 67 to 234, based on the published sequence (9, 11). Gel retardation assays were performed as described (20).

Transient Chloramphenicol Acetyl-Transferase (CAT) Analysis. Constructs in which different CD34 promoter regions drive the bacterial CAT gene were prepared by PCR amplification of human placental genomic DNA and by cloning the different fragments of the CD34 5′ flanking region first into the pCRII vector (Invitrogen Corp., San Diego, CA) or directly into the pUCCAT vector (Promega Corp., Madison, WI). CD34 SM-CAT and CD34 LM-CAT contain identical nucleotide substitutions in the Myb consensus sequence, from nucleotide 75 to 78 and 92 to 95 (CAAC to TGGC and GTTA to GCCC, respectively), of the published sequence of the human CD34 gene (9, 11). TK-ts13 hamster fibroblasts were transfected, using the calcium-phosphate precipitation method (21), with 1 μg of CAT reporter plasmid with or without 5 μg of effector plasmid (pMB1-dhfr, named pSV myb, containing the human c-myb cDNA driven by SV40 early promoter) plus 1 μg of a plasmid containing the bacterial β-galactosidase gene driven
by the DNA polymerase-α promoter, as an internal control of transfection efficiency. Cells were harvested 48 h after transfection. Proteins were extracted by freeze-thawing and normalized for transfection efficiency using the β-galactosidase assay, as suggested by the manufacturer (Promega). Cellular lysates were incubated with [14C]chloramphenicol and acetyl-CoA for 1 h at 37°C. Transactivation of the reporter constructs was assayed by measuring the amount of acetylated [14C]chloramphenicol by thin-layer chromatography followed by autoradiography and scintillation counting.

**Detection of c-myb Protein in Transfected Cells.** Cells of c-myb protein were determined in total cell extracts from 6 × 10⁶ transfected TK-ts13 hamster fibroblasts and human glioblastoma T98G cells by Western blot analysis with a monoclonal anti-mouse c-myb antibody (UBI) and then with a peroxidase labeled sheep anti-mouse Ig antibody (Amersham Corp., Arlington Heights, IL). Bound antibodies were revealed with the Enhance Chemiluminescence Detection System (ECL; Amersham Corp.).

**Expression of CD34 mRNA in Transfected Cells.** TK-ts13 cells were transfected with no plasmid, 5 μg of pSV β-gal containing bacterial β-galactosidase DNA under the control of the SV40 early promoter or 5 μg of pSVmyb. RNA was extracted as described (22) 24, 36, or 48 h after transfection. Using 0.7 μg of total RNA and 40 cycles CD34 mRNA was amplified by reverse transcriptase (RT)-PCR, as described (23), with a pair of synthetic primers corresponding to nucleotides from 461 to 482 (5' primer) and from 246 to 267 (3' primer) of the published murine CD34 cDNA sequence (24). Amplified DNA was subjected to electrophoresis, transferred to Zetabind nylon filters (Cuno, Inc., Meriden, CT) and detected by Southern hybridization with a γ-[32P]ATP end-labeled oligoprobe corresponding to nucleotides 366 to 395 (24). In TK-ts13 cells constitutively expressing c-myb, CD34 mRNA levels were also measured by RT-PCR using 0.7 μg of total RNA and 40 cycles. In the different T98G cell lines CD34 mRNA levels were also determined by RT-PCR. Primers used correspond to nucleotides 957 to 982 (5' primer) and 1165 to 1190 (3' primer); the oligoprobe includes nucleotides 1109 to 1134, of the published human cDNA sequence (25). Amplification products were run on 1% agarose gel, blotted and probed with the 32P-end-labeled oligoprobe.

**Cell Surface CD34 Expression.** Exponentially growing cells were harvested and incubated (30 min on ice in PBS containing 0.1% gelatin, 0.01% sodium azide, 5% fetal calf serum) with antibodies to CD34 (mouse IgG1 anti-HPCA1; Becton Dickinson & Co., Mountain View, CA), β2-microglobulin (BBM1) as positive controls, or CD16 (3G8, irrelevant IgG1), as negative controls. Cells were washed and incubated (30 min on ice) with FITC-conjugated goat anti-mouse Ig F(ab')₂. Cells were washed and analyzed by flow-cytometry on an EPICS Profile Analyzer (Coulter Corp., Hialeah, FL).

**Results**

c-myb Protein Interacts with the 5' Flanking Region of the CD34 Gene. To determine whether Myb interacts with putative Myb binding sites in the 5' flanking region of the CD34 gene, gel retardation assays were performed with bacterial lysates containing or not containing Myb protein, and probed with different 32P-labeled DNA fragments of the CD34 5' flanking region (Fig. 1). One retarded complex was detected in the lysate containing Myb protein (Fig. 1, lanes 2, 5, and 8), but none in the lysate that lacked Myb protein (Fig. 1, lanes 3, 6, 9, and 12). No binding was detected when a probe with a 3-base substitution in each of the two potential Myb consensus sequences was used (Fig. 1, lane 11), demonstrating the specificity of the interactions.

**CD34 Promoter Activity Is Transactivated by c-myb**

To determine whether c-myb transactivates the CD34 5' flanking region, constructs in which different fragments of the CD34 promoter drive bacterial CAT gene expression were prepared by cloning the segments corresponding to nucleotides -666 to 234 (CD34 L-CAT), -132 to 234 (CD34 M-CAT), and 31 to 187 (CD34 S-CAT) (9, 11) into the pUC19 vector (Fig. 2 A), and transfected into wild-type TK-ts13 Syrian hamster fibroblasts or in cells constitutively expressing c-myb (SV-mybTK-ts13). TK-ts13 cells were transfected at a 5:1 effector-to-reporter ratio with plasmid pMBm-dhfr. This plasmid, which contains the human c-myb cDNA under the control of the SV40 early promoter and enhancer linked to the dehydrofolate reductase coding sequence for methotrexate selection (11) and the described above reporter plasmids, were used in the experiments. Levels of CAT activity were assayed 48 h later (Fig. 2 B). c-myb induced 8-, 10-, and 14-fold increases, respectively, in CAT expression driven by the CD34 L-CAT (Fig. 2 B, lane 3), CD34 M-CAT (Fig. 2 B, lane 5), and CD34 S-CAT (Fig. 2 B, lane 7) CD34 5' flanking region segments. Transactivation was abolished when mutations were introduced in the Myb binding sites of the CD34 S-CAT reporter vector (CD34 SM-CAT; Fig. 2 B, lane 9).

To determine whether the Myb binding sites not included in the segment corresponding to CD34 S-CAT (nucleotides 31 to 187) are also involved in CD34 5' flanking region transactivation, TK-ts13 and SVmybTK-ts13 cells were transfected with CD34 LM-CAT containing the 900-bp fragment of the CD34 5' flanking region with mutations in the two most proximal Myb binding sites (9, 11). Transactivation was unaffected (Fig. 2 B, lane 4), compared with the wild-type L-CAT construct (Fig. 2 C, bar 2), indicating that the more distal Myb consensus sequences are also functional binding sites.

**Figure 1.** Myb protein binding to CD34 5' flanking region. Lanes 1, 4, 7, and 10, free probe only; lanes 3, 6, 9, and 12, probe plus 1 μg of parental bacterial lysate; lanes 2, 5, 8, and 11, probe plus 1 μg of bacterial lysate containing the human c-myb protein. The probe used in lanes 10-12 contains three nucleotide substitutions in both Myb binding sites. The different regions of the CD34 promoter used as probe are indicated on the top.

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of CAT activity were identical in TK-ts13 cells transfected with pSVCAT in the presence or absence of an excess (100:1 molar ratio) of competitor (not shown).

**CD34 Expression Is Regulated by c-myb** To test the prediction, from the above results, that c-myb may upregulate CD34 expression, RT-PCR analysis of CD34 mRNA expression was performed in TK-ts13 cells 24, 36, and 48 h after transfection with pSVmyb. CD34 mRNA was detectable only in c-myb-expressing cells (Fig. 3 A, lanes 3–5), but not in mock-transfected TK-ts13 cells (Fig. 3 A, lane 1), or in TK-ts13 cells transfected with the pSVβ-gal plasmid (Fig. 3 A, lane 2) used as controls. In addition, CD34 mRNA expression was detected in SVmyb TK-ts13 cells constitutively expressing c-myb protein (Fig. 3 B, right, lane 2).

Antibodies are available for detection of human, but not rodent CD34 proteins; therefore, to prove that induced CD34 mRNA expression results in synthesis and expression of the encoded protein, human glioblastoma T98G cells (nonexpressing c-myb) were transfected with a c-myb cDNA and assayed for Myb protein and CD34 expression. Western blot analysis revealed the presence of a 75-kD protein corresponding to c-myb, in SVmyb T98G cells (Fig. 4 A, lane 2), in SVmybM T98G cells selected with increasing concentrations of methotrexate (final concentration 22 μM) to induce amplification

Transactivation of CD34 L-CAT and CD34 LM-CAT was abolished by the wild-type competitor (Fig. 2 C, bars 6 and 8) but was unaffected by the mutated oligomer (Fig. 2 C, bars 10 and 12), further demonstrating that the transactivation of the CD34 promoter depends directly on c-myb expression and interaction with Myb binding sites. The 36-bp competitor containing two Myb binding sites was not toxic, as levels of CAT activity were identical in TK-ts13 cells transfected
Figure 4. CD34 mRNA levels in T98G human glioblastoma cells constitutively expressing c-myc. (A) Levels of c-myc protein were determined by Western blot analysis in untransfected T98G cells (lane 1), in SV-myc-transfected T98G cells (lane 2), in SV-myc T98G cells after methotrexate selection (SV-myc M T98G) (lane 3), and in the control MEL cells (lane 4). (B) CD34 mRNA levels were determined by RT-PCR in the presence (+) or absence (−) of reverse transcriptase. Each lane is representative of three independent experiments with similar results. (Lane 1) negative control, HL-60 cells; (lane 2) T98G cells; (lane 3) SV-myc T98G cells; (lane 4) SV-myc T98G cells after methotrexate selection; (lane 5) positive control, KG-1a cells (only one-fifth of the reaction was used); (lane 6) RT-PCR reaction in the absence of RNA in the reaction mixture.

Discussion

The role of c-myc in hematopoiesis likely derives from its transactivating function, but the search for the relevant target(s) remains elusive, since two hematopoietic-associated targets, the MIM-1 and the lysozyme genes identified in avian cells, are expressed at a relatively late stage of myeloid differentiation. In contrast, expression of the CD34 stem cell marker declines during late stages of myeloid differentiation and maturation. Our data suggest that CD34 represents a primary target of c-myc regulation during the earliest recognizable stages of hematopoietic progenitor cell commitment and differentiation. A temporal relationship in which c-myc expression precedes that of CD34 in human stem and progenitor cells is difficult to establish because primitive CD34-positive cells express c-myc, and CD34-negative stem cells, if they exist, cannot be identified yet. A functional and temporal relationship between c-myc and CD34 expression might be explored during the differentiation of embryonic stem cells. In these cells a stage can be identified at which c-myc, but not CD34, is expressed (26); this observation may support the involvement of c-myc in regulating CD34 expression during embryonal hematopoietic development.

Our data clearly indicate that c-myc is a transcriptional factor involved in regulating CD34 expression, but do not exclude that others are, too. Binding sites for ets proteins are present in the CD34 5' flanking region, some in close proximity with Myb binding sites, and preliminary findings suggest that c-myc and ets-2 act synergistically to transactivate the CD34 promoter. In addition, it is likely that c-myc expression is the only prerequisite for CD34 expression, as implied by the lack of CD34 expression in the HL-60 cells promyelocytic leukemia cells that express c-myc at high levels.
KG-1a and HL-60 represent relatively close stages of myeloid differentiation and express c-myb at high levels, and yet only KG-1a cells express CD34. This suggests either positive regulation of CD34 expression by a c-myb partner in KG-1a cells, or negative regulation in HL-60 cells by a suppressor of CD34 expression. In this regard, a functional binding site for the myeloid-specific transactivator NF-M contiguous to a c-myb binding site appears to be necessary for c-myb regulation of the MIM-1 promoter (27); a similar mechanism may operate to control CD34 expression in early progenitor cells. Finally, the increased fraction of CD34-positive cells in acute leukemia patients correlates with the enhanced clonogenic potential of these cells and their high levels of c-myb expression. In conjunction with such observations, our data on c-myb regulation of CD34 expression might provide insights into the coupling of differentiation arrest and growth advantage in leukemic cells.

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