

Regulation of the Expression of the Hematopoietic Stem Cell Antigen CD34: Role of *c-myb*

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Summary

The CD34 antigen defines a subset of hematopoietic progenitor cells with self-renewal capacity and the ability to reconstitute hematopoiesis in irradiated primates and marrow-ablated humans, but its function remains unknown. The *c-myb* protooncogene plays a fundamental role in hematopoiesis, most likely via its transcriptional regulator function. We report that *c-myb* protein transactivates the CD34 promoter via specific interaction with multiple Myb binding sites in the 5' flanking region of the gene and induces expression of the endogenous CD34 mRNA in rodent fibroblasts. Also, constitutive expression of *c-myb* in CD34-negative human glioblastoma cells induces expression of CD34 mRNA and synthesis of the surface membrane antigen. These data directly demonstrate that *c-myb* regulates the expression of the hematopoietic stem cell antigen CD34 and raise the possibility that *c-myb* regulates hematopoiesis inducing a cascade of differentiation-related events.

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 transcription regulator function 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). 32 P-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 μ g of CAT reporter plasmid with or without 5 μ g of effector plasmid (pMb1-dhfr, named pSV myb, containing the human *c-myb* cDNA driven by SV40 early promoter) plus 1 μ g 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 [14 C]chloramphenicol and acetyl-CoA for 1 h at 37°C. Transactivation of the reporter constructs was assayed by measuring the amount of acetylated [14 C]chloramphenicol by thin-layer chromatography followed by autoradiography and scintillation counting.

Detection of *c-myb* Protein in Transfected Cells. Levels of *c-myb* protein were determined in total cell extracts from 6×10^6 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 μ g of pSV β -gal containing bacterial β -galactosidase DNA under the control of the SV40 early promoter or 5 μ g of pSVmyb. RNA was extracted as described (22) 24, 36, or 48 h after transfection. Using 0.7 μ g 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 -[32 P]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 μ g 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 32 P 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), β_2 -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 a 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 32 P-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

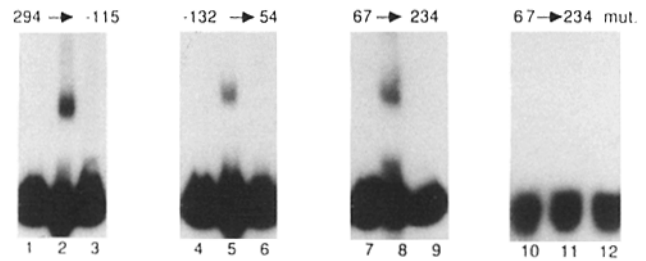


Figure 1. Myb protein binding to CD34 5' flanking region. Lanes 1, 4, 7, and 10, free probe only; lanes 3, 6, 9, and 12, probe plus 1 μ g of parental bacterial lysate; lanes 2, 5, 8, and 11, probe plus 1 μ g of bacterial lysate containing the human *c-myb* protein. The probe used in lanes 10–12 contains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

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 pUCCAT 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. CAT assays were also performed using TK-ts13 or SVmybTK-ts13 cells transfected with CD34 L-CAT and CD34 LM-CAT in the presence of an excess (100:1, molar ratio) of a 36-bp fragment of the CD34 5' flanking region (nucleotides 31 to 67) containing two wild-type or two mutated Myb binding sites.

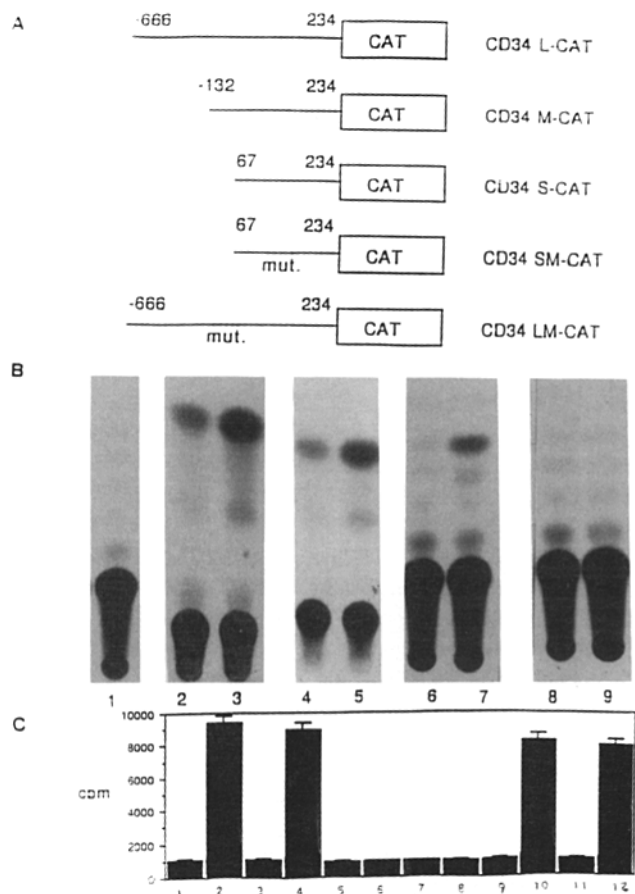


Figure 2. *c-myb* transactivation of CD34 5' flanking region in Tk-ts13 hamster fibroblasts. (A) Constructs in which different CD34 promoter regions drive the bacterial CAT gene. (B) Autoradiograms of CAT activity in lysates of TK-ts13 transfected with the negative control puCCAT (lane 1), CD34 L-CAT (lane 2), CD34 M-CAT (lane 4), CD34 S-CAT (lane 6), CD34 SM-CAT (lane 8), or cotransfected with pSVmyb (lanes 3, 5, 7, and 9, respectively). (C) Scintillation counts of acetylated [¹⁴C]chloramphenicol in lysates of: TK-ts13 (lane 1), and SVmyb-TK-ts13 cells (lane 2) transfected with CD34 L-CAT; TK-ts13 (lane 3) and SV-Myb TK-ts13 cells (lane 4) transfected with CD34 LM-CAT, TK-ts13 (lane 5), and SVmyb TK-ts13 cells (lane 6) transfected with CD34 L-CAT plus a synthetic competitor containing two wild-type Myb binding sites; TK-ts13 (lane 7) and SVmyb TK-ts13 cells (lane 8) transfected with CD34 LM-CAT plus a synthetic competitor containing two wild-type Myb binding sites; TK-ts13 (lane 9) and SVmyb TK-ts13 cells (lane 10) transfected with CD34 L-CAT plus a synthetic competitor containing two mutated Myb binding sites; and TK-ts13 (lane 11) and SVmyb TK-ts13 cells (lane 12) transfected with CD34 LM-CAT plus a synthetic competitor containing two mutated Myb binding sites.

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

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 pSV β -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 μ M) to induce amplification

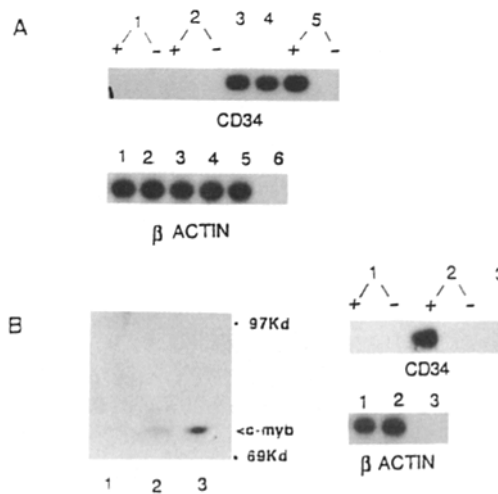


Figure 3. CD34 mRNA levels in TK-ts13 cells transfected with pSVmyb. (A) Expression of CD34 mRNA in transiently transfected TK-ts13 cells. (Lane 1) cells transfected with no plasmid; (lane 2) cells transfected with 5 μ g of pSV β -gal; (lanes 3–5) cells transfected with 5 μ g of pSVmyb. RNA was extracted as described (22) 48 h after transfection except in lanes 3 and 4 (RNA extracted 24 and 36 h after transfection, respectively). To exclude amplification from genomic DNA, RT-PCR reactions were done in the absence of reverse transcriptase (–). In controls, reactions were also performed in the absence of RNA. Endogenous β -actin mRNA levels were also measured to ensure that a similar amount of RNA was analyzed for CD34 mRNA expression. (B) Expression of CD34 mRNA in TK-ts13 cells constitutively expressing *c-myb*. (Left) levels of *c-myb* protein determined by Western blot analysis. (Lane 1) untransfected TK-ts13 cells; (lane 2) SVmybTK-ts13 cells; (lane 3) control HL-60 cells. (Right) CD34 mRNA levels determined by RT-PCR in untransfected TK-ts13 (lane 1) and in SVmyb TK-ts13 cells (lane 2) in the presence (+) or absence (–) of reverse transcriptase; (lane 3) RT-PCR reaction in the absence of RNA in the reaction mixture.

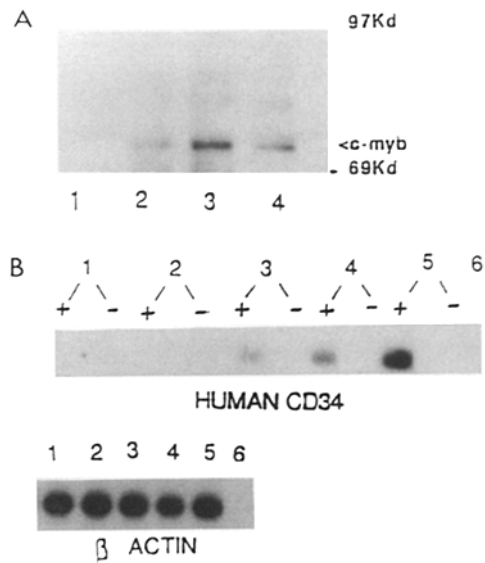


Figure 4. CD34 mRNA levels in T98G human glioblastoma cells constitutively expressing *c-myb*. (A) Levels of *c-myb* protein were determined by Western blot analysis in untransfected T98G cells (lane 1), in SV-myb-transfected T98G cells (lane 2), in SV-myb T98G cells after methotrexate selection (SVmyb 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-myb T98G cells; (lane 4) SV-myb 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.

of the *c-myb* transcription unit (Fig. 4 A, lane 3), but not in parental T98G cells (Fig. 4 A, lane 1). In SVmybM T98G cells, levels of *c-myb* protein expression were at least sevenfold greater than in SVmyb T98G cells, as revealed by densitometric scanning of the film (Fig. 4 A, compare lanes 3 and 4).

Expression of CD34 mRNA was not detected in control HL-60 cells (Fig. 4 B, lane 1) or in nontransfected T98G cells (Fig. 4 B, lane 2), but was found in SVmyb T98G (Fig. 4 B, lane 3), and, at higher levels, in SVmybMT98G cells (Fig. 4 B, lane 4). Phenotypic analysis performed to monitor surface expression of the human CD34 antigen on T98G, SVmyb T98G and SVmybMT98G cells (Fig. 5 B) indicated expression of CD34 antigen on a small proportion ($4.3 \pm 1.2\%$) of T98G cells. Low density CD34 expression was detected on SVmyb T98G cells, and most SVmybMT98G expressed CD34 at a variable level, with average density approximately half of that detected in KG-1a myeloblastic leukemia cells used as positive control (Fig. 5 A).

Discussion

The role of *c-myb* 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 differentia-

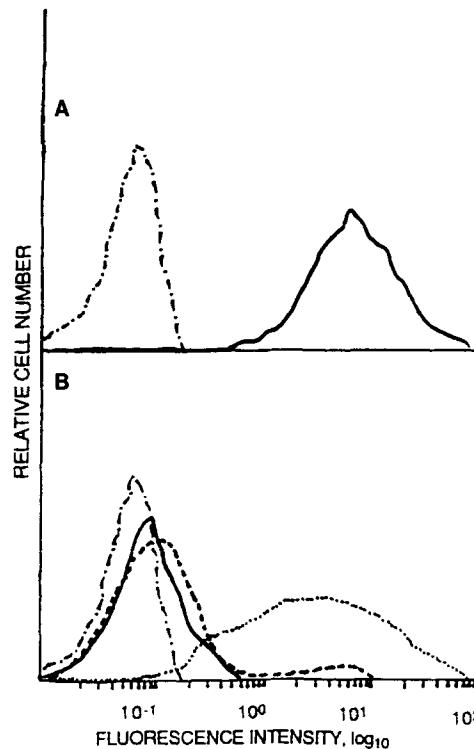


Figure 5. Cell surface expression of CD34 in T98G constitutively expressing *c-myb*. (A) KG-1a cells: ---, negative control; —, anti-CD34. (B) —, T98G; anti-CD34; ---, SVmyb T98G; anti-CD34, ····, SVmybMT98G; anti-CD34, -·-·, negative control analysis, attached for the three cell lines, is reported. x-axis, number of cells; y-axis, fluorescence intensity, log scale. The analysis was repeated twice with identical results.

tion. 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-myb* regulation during the earliest recognizable stages of hematopoietic progenitor cell commitment and differentiation. A temporal relationship in which *c-myb* expression precedes that of CD34 in human stem and progenitor cells is difficult to establish because primitive CD34-positive cells express *c-myb*, and CD34-negative stem cells, if they exist, cannot be identified yet. A functional and temporal relationship between *c-myb* and CD34 expression might be explored during the differentiation of embryonic stem cells. In these cells a stage can be identified at which *c-myb*, but not CD34, is expressed (26); this observation may support the involvement of *c-myb* in regulating CD34 expression during embryonal hematopoietic development.

Our data clearly indicate that *c-myb* 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-myb* and *ets-2* act synergistically to transactivate the CD34 promoter. In addition, it is likely that *c-myb* 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-myb* 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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