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Ets-2 and c-Myb Act Independently in Regulating Expression of the Hematopoietic Stem Cell Antigen CD34*

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Paola Melotti‡ and Bruno Calabretta§

From the Department of Microbiology and Immunology, Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107

CD34 is currently the only well defined human hematopoietic stem cell marker and is expressed on 1–4% of normal bone marrow cells. Putative binding sites for Ets proteins, a family of transcription factors involved in the regulation of cell differentiation and proliferation in many cell systems, are present in the 5′-flanking region of the CD34 gene. Some of these sites are in close proximity to binding sequences of the encoded product of the proto-oncogene c-*myb*, which regulates CD34 expression by interacting with the Myb binding sites. Here we demonstrate that Ets-2 (i) transactivates the CD34 promoter in rodent fibroblasts upon interaction with Ets binding sites and (ii) induces expression of CD34 mRNA and protein in the CD34⁻ human glioblastoma T98G cells. Ets-2 and c-Myb transactivate the CD34 promoter independently because specific transactivation is abrogated by site-specific mutations of the binding sites or by competition with oligomers that include wild type but not mutated Myb or Ets binding sites. Ets-2 and c-Myb appear to have addictive effects on transactivation of the CD34 promoter and on induction of CD34 mRNA. Instead, CD34 surface protein levels might be induced synergistically, raising the possibility of a posttranslational mechanism of CD34 expression in cells constitutively expressing c-Myb and Ets-2.

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 (1–4). Besides stem cells and lineage-specific progenitors, a subset of bone marrow stromal cells and small vessel endothelium also expresses the CD34 surface antigen (5, 6). This highly glycosylated transmembrane protein might function as a ligand for L-selectin, which is involved in the interaction of leukocytes with endothelial cells (7); similarly, in hematopoietic cells, it may serve as ligand for stromal lectins, perhaps promoting the interaction with locally released growth factors (5, 6). CD34 has been also proposed to act as a signal transducer (8). The regulation of CD34 expression in hematopoietic cells involves both transcriptional and posttranscriptional mechanisms (9, 10). We showed that the proto-oncogene c-*myb*, whose function is required for hematopoietic cell growth, differentiation, and development (11–13), regulates CD34 expression upon binding to Myb binding sites in the 5′-flanking region of the CD34 gene (14). Because several putative Ets binding sites are also scattered in this region, some of which in close proximity to functional Myb binding sites (9, 15), we asked whether genes of the ets family, which act as transcriptional regulator of gene expression during differentiation, proliferation, and development (16), might also modulate CD34 expression. Moreover, in light of (i) the concurrence of v-*myb* and v-*ets* in the E26 virus (17, 18), (ii) the contribution of both v-*myb* and v-*ets* components to the transforming ability of E26 (19), and (iii) the recent demonstration that Myb and Ets-2 proteins synergize in transcriptional regulation of the Myb-regulated *mim-1* promoter (20), we also investigated whether Ets-2 and c-Myb cooperate to regulate CD34 expression.

MATERIALS AND METHODS

Gel Retardation Assay—HB101 cells containing the parental pFlag (IBI, New Haven, CT) expression vector only, the pc-Myb Flag vector, or the pc-ets-2 Flag vector were incubated for 4 h with 1.5 mM isopropyl-1-thio-β-D-galactopyranoside to an A_{600} of 0.500. Bacterial lysates were quantitated by the Bradford method (21). c-*myb* and *ets*-2 expression was determined in bacterial lysates by Western blot using anti-c-Myb- and anti-Ets-specific antibodies (Upstate Biotechnology Incorporated, Lake Placid, NY and Santa Cruz Biotechnology Inc., Santa Cruz, CA, respectively). [γ-^{32}P]ATP end-labeled probes corresponding to nucleotides −490 to −458 (containing c-Myb and Ets-2 binding sites), −293 to −114 (containing four c-Myb and two Ets-2 putative binding sites), −113 to +55 (containing three c-Myb and one Ets-2 putative binding sites), 67 to 102 (containing two closely spaced c-Myb binding sites), and 148 to 187 (containing one Ets-2 binding site) based on the published sequence (9) were used in gel retardation assays, performed as described (14).

Chloramphenicol Acetyltransferase (CAT)[1] *Analysis*—Constructs in which different CD34 promoter regions drive the bacterial CAT gene were synthesized by PCR amplification of human placental genomic DNA and by cloning the different fragements of the CD34 5′-flanking region first into the pCRII vector (Invitrogen Corp., San Diego, CA) or directly into the pUCCAT vector (Promega, Madison, WI). CD34 Sme-CAT and CD34 LM-CAT contain identical nucleotide substitutions in the c-Myb consensus sequences, from nucleotide 75 to 78 and 92 to 95 (CAAC to TGGC and GTTA to GCCC, respectively), and in the Ets-2 binding site, from 152 to 156 (AGGA to ATTA) of the published sequence of the human CD34 gene (9, 15). CD34 Sm-CAT and CD34 Se-CAT contain these mutations only in the c-Myb binding sites (75 to 78 and 92 to 95) or only in the Ets-2 consensus sequence (152 to 156), respectively. CD34 LDM-CAT contains mutations in two overlapping c-Myb binding sites, corresponding to nucleotides −472 to −467 (GTTAAC to GTCTTC), and in the Ets-2 consensus sequence, −479 to −475 (AGGAA to ATTAA).

TK-ts13 hamster fibroblasts (22) were transfected, using the calcium phosphate precipitation method (23), with 1 μg of CAT reporter plasmid with or without 5 μg of effector plasmid (pMb1-dhfr, named pSVmyb, containing the human c-*myb* cDNA driven by the SV40 early promoter, or pSVets, containing the human *ets*-2 cDNA driven by the SV40 early promoter, or a 1:1 mixture of both) plus 1 μg of a plasmid containing the bacterial β-galactosidase (*β-gal*) 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-

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§ Scholar of the Leukemia Society of America.

[1] The abbreviations used are: CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR.

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.

Cell Culture and Transfections—T98G human glioblastoma cells (kind gift of Dr. Mercer) were maintained in culture as described (24). The cells were transfected with pSVmyb or pSVets2 using the calcium-phosphate precipitation method (23). Briefly, 10^6 cells were cotransfected with 10 μg of plasmid pSVmyb or pSVets2 and 1 μg of plasmid pLHL4, which contains the gene encoding hygromycin resistance (25). After a 12-day selection in culture medium containing 0.5 mg/ml of hygromycin B (Calbiochem), colonies were pooled and cultured in medium containing 0.3 mg/ml of hygromycin B. To obtain pSVmyb+pSVets2 transfectants, pSVmyb-transfected T98G were cotransfected with 10 μg of pSVets2 and 1 μg of plasmid pRSVNEO. After a 14-day selection in culture medium containing 0.8 mg/ml of G418 (Life Technologies, Inc.), colonies were pooled and cultured in medium containing 0.4 mg/ml of G418 and 0.3 mg/ml of hygromycin B.

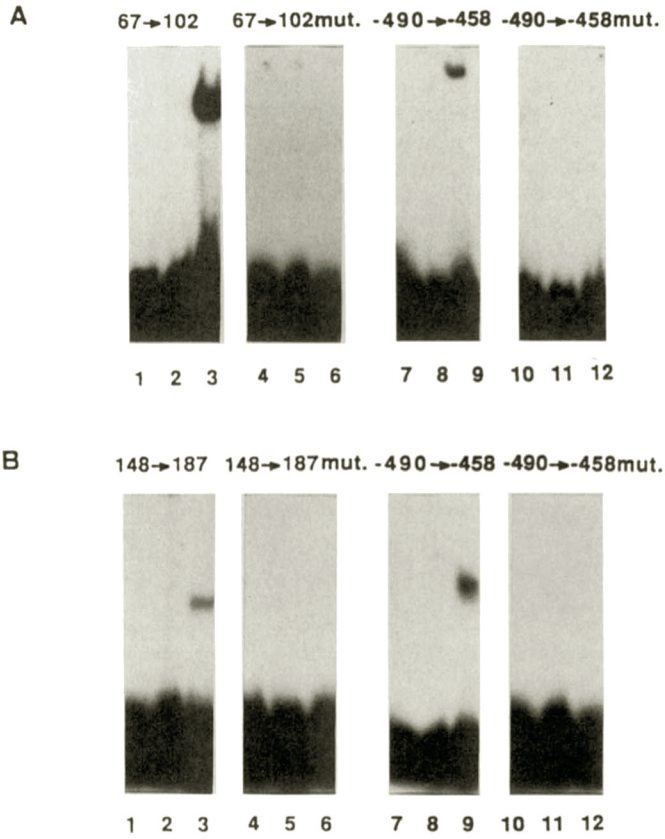

FIG. 1. **c-Myb and Ets-2 binding to CD34 5′-flanking region.** *Lanes 1*, *4*, *7*, and *10*, free probe only; *lanes 2*, *5*, *8*, and *11*, probe plus 1 μg of parental bacterial lysate; *lanes 3*, *6*, *9*, and *12*, probe plus 1 μg of bacterial lysate containing the human c-Myb protein (*A*) or the human Ets-2 protein (*B*). Probes used in *lanes 4–6* and *10–12* contain the same mutations in c-Myb binding sites (*A*) or in Ets-2 binding sites (*B*) as those present in CD34 LM-CAT and CD34 LDM-CAT (see below). The different regions of the CD34 promoter used as probe are indicated on the *top* of each *panel*.

Detection of c-Myb and Ets-2 Proteins in Transfected Cells—Levels of c-Myb and Ets-2 proteins were determined in total cell extracts from 6 × 10^6 human glioblastoma T98G cells by Western blot analysis with a monoclonal anti-mouse c-Myb antibody (Upstate Biotechnology Incorporated) and a peroxidase-labeled sheep anti-mouse Ig antibody (Amersham Corp.) or with a polyclonal anti-human Ets-2 antibody (Santa Cruz Biotechnology Inc.) and then with a peroxidase-labeled donkey anti-rabbit Ig antibody (Amersham Corp.). Bound antibodies were revealed with the Enhanced 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), 5 μg of pSVmyb or pSVets2, or a 1:1 mixture of both. RNA was extracted as described (22) at 24, 36, or 48 h after transfection. CD34 mRNA was detected by reverse transcriptase (RT)-PCR using a pair of synthetic primers corresponding to nucleotides 246 to 267 (5′ primer) and 461 to 482 (3′ primer) of the published murine CD34 cDNA sequence (25). 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 (26). CD34 mRNA levels were determined in the different transfected T98G cells by Northern blot analysis.

For Northern blot analysis, 20 μg of total RNA extracted as described (22) was subjected to electrophoresis, transferred to Zetabind nylon filters, and then detected by hybridization using the 1.5-kilobase *Xba*I CD34 cDNA fragment or the 1.5 *Bam*HI β-actin cDNA fragment, ^{32}P-labeled by the random priming technique (27).

Cell Surface CD34 Expression—Exponentially growing cells were harvested and incubated (30 min on ice in phosphate-buffered saline containing 0.1% gelatin, 0.01% sodium azide, 5% fetal calf serum) with antibodies to CD34 (mouse IgG1 anti-HPCA1; Becton Dickinson, Mountain View, CA), β2-microglobulin (BBM1, as positive control), or CD16 (3G8, irrelevant IgG1 as negative control). Cells were washed and incubated (30 min on ice) with fluorescein isothiocyanate-conjugated goat anti-mouse Ig F(ab)$_2$. Cells were washed and analyzed by flow-cytometry on a EPICS profile Analyzer (Coulter, Hialeah, FL).

RESULTS

Interaction of Myb and Ets-2 Proteins with the 5′-flanking Region of the CD34 Gene—Interactions of c-Myb and Ets-2 proteins to putative binding sites in the CD34 promoter were determined by gel retardation assays (Fig. 1). ^{32}P-labeled DNA fragments of CD34 5′-flanking region, corresponding to nucleotides −490 to −458 and 67 to 102 and containing putative c-Myb binding sites, were reacted with a bacterial lysate, containing or not containing Myb protein. One retarded complex was revealed in the lysates containing Myb protein (Fig. 1*A*, *lanes 3* and *9*), whereas no complex was seen with the lysate that lacked Myb protein (Fig. 1*A*, *lanes 2*, *5*, *8*, and *11*). The binding was abolished by excess of unlabeled wild type oli-

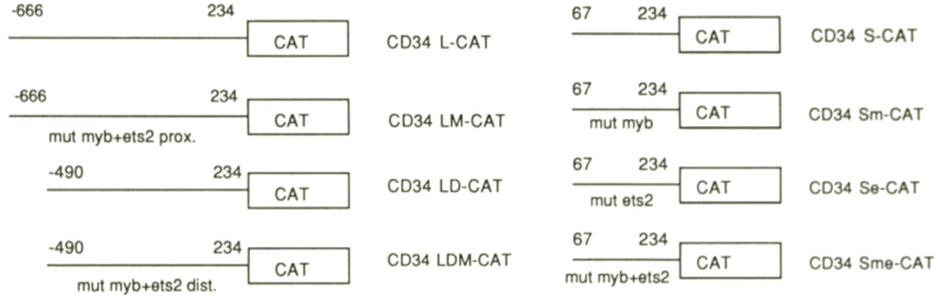

FIG. 2. **Constructs in which different CD34 promoter regions drive the bacterial CAT gene.** These segments correspond to nucleotides indicated in each construct according to the published sequence (17). In CD34 LM-CAT, two "proximal" c-Myb binding sites (75 to 78 and 92 to 95), and one proximal Ets-2 binding site (152 to 156) contain mutations. In CD34 LDM-CAT, two "distal" c-Myb binding sites (−472 to −467) and one distal Ets-2 binding site (−479 to −475) are mutated. Two c-Myb consensus sequences, one Ets-2 binding site, and c-Myb and Ets-2 consensus sequences contain mutations in CD34 Sm-CAT, CD34 Se-CAT, and CD34 Sme-CAT, respectively.

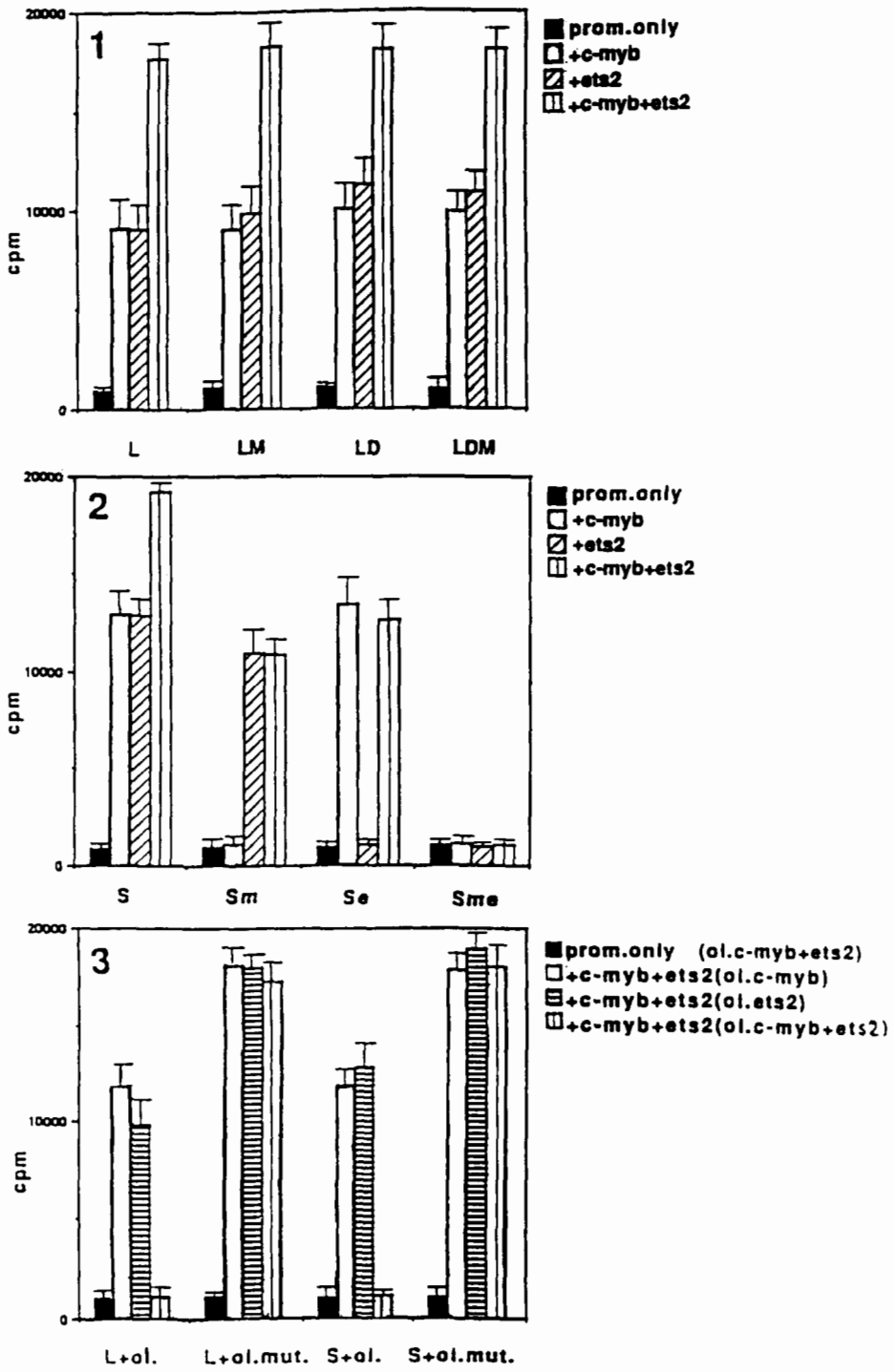

FIG. 3. **Transactivation of CD34 5′-flanking region by c-Myb and Ets-2 in Tk-ts13 hamster fibroblasts.** Scintillation countings of acetylated [^{14}C]chloramphenicol in lysates of Tk-ts13 cells transfected with the following: *panels A* and *B*: CD34 L-, LM-, LD-, LDM-, S-, Sm-, or Se- or Sme-CAT only (■) or cotransfected with pSVmyb (□), pSVets2 (▨), or pSVmyb plus pSVets2 (▥); *panel C*: with CD34 L- or S-CAT only in the presence of c-Myb and Ets-2 oligonucleotide competitors (■), or cotransfected with pSVmyb plus pSVets2 in the presence of c-Myb (□), Ets-2 (▤), or both (▥) oligonucleotide competitors. Bars designated *L+ol. mut.*, and *S+mut.* indicate the same experiments only using oligonucleotide competitors with mutations in binding sites for c-Myb, Ets-2, or both. *Error bars* indicate mean + S.D. of three independent experiments. The oligonucleotides from +67 to +102 (5′TTTGGGACCAACCAGGGGAGCTCAAGTTAGTAGCAG3′) and from 148 to 187 (15′AGAGAGGAAAAAAGCAAGAATCCCCCACCCCTCTCCCGGG3′) of the published sequence (9) were utilized as competitors for c-Myb and Ets-2 binding, respectively. The *underlined* consensus sequences were mutated, CAAC to TGGC and GTTA to GCCC in the c-Myb binding sites, and AGGAA to ATTAA in the Ets-2 binding sites. The mutated oligomers were used as irrelevant competitors.

gomers used as competitors, but not by excess oligomers containing mutations of the Myb binding site (not shown). No binding was observed when probes containing mutated Myb consensus sequences were used, further demonstrating the specificity of the interactions (Fig. 1*A*, *lanes 6* and *12*). Analogously, gel retardation assays using bacterial lysates containing or not containing Ets-2 protein and ^{32}P-labeled DNA oligomers containing putative Ets-2 binding sites (−490 to −458 and 148 to 187 of the CD34 5′-flanking region) revealed one retarded complex (Fig. 1*B*, *lanes 3* and *9*), but no complex was observed with bacterial lysates lacking Ets-2 protein (Fig. 1*B*, *lanes 2*, *5*, *8*, and *11*). The specificity of Ets-2 protein binding to these fragments was demonstrated by the absence of complex formation when radiolabeled probes with nucleotides substitutions in the Ets-2 binding sites were used as probes (Fig. 1*B*, *lanes 6* and *12*). Also, excess unlabeled oligomers containing the wild type, but not the mutated, Ets binding site were able to compete out formation of the DNA-protein complex (not shown).

Transactivation of CAT Gene Expression Driven by the Human CD34 Promoter by c-Myb and Ets-2—To examine the ability of c-Myb and Ets-2 to transactivate CAT reporter constructs containing different segments of the CD34 promoter able to interact with these proto-oncogene encoded proteins, transient expression assays were performed in which TK$^-$ts13 fibroblasts were transfected at a 5:1 effector-to-reporter ratio with the reporter plasmids (Fig. 2) or cotransfected with effector plasmid pSVmyb, pSVets2, or both and assayed for CAT activity 48

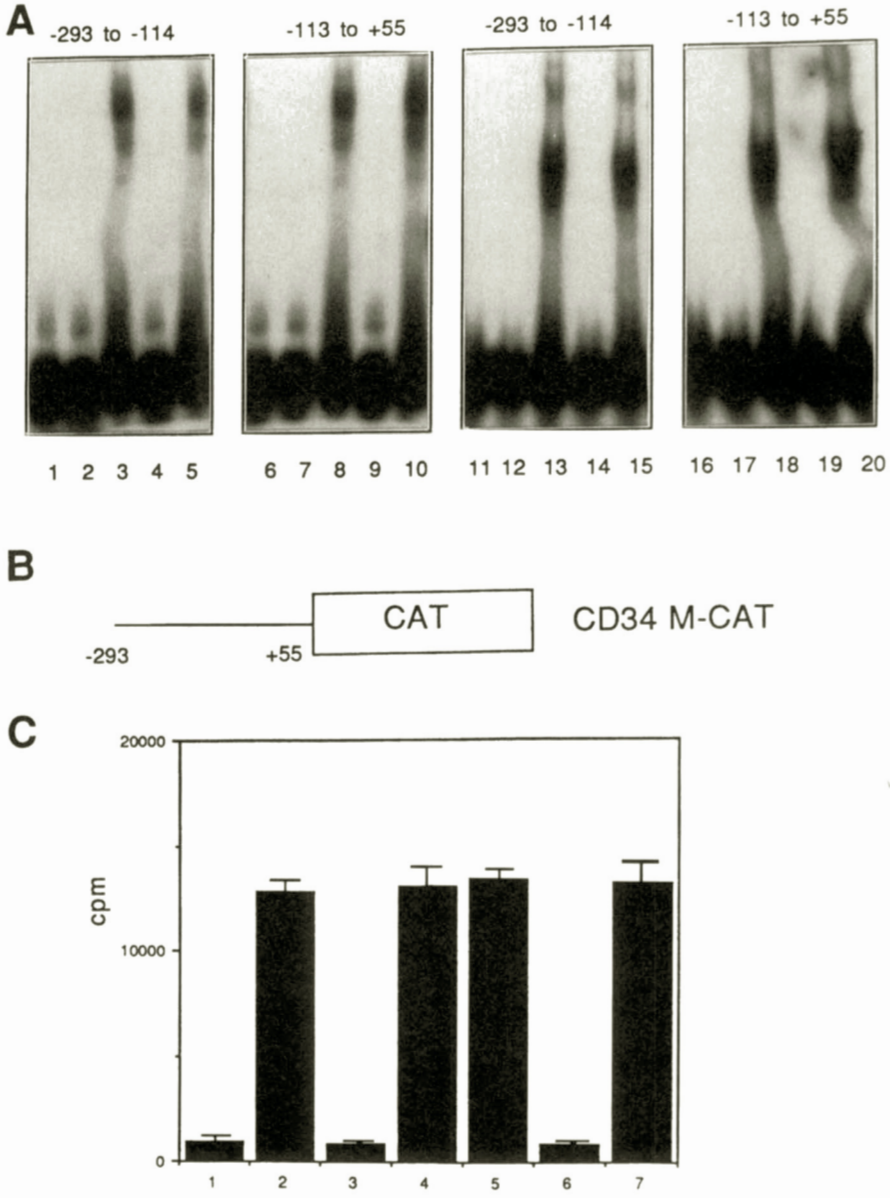

FIG. 4. **Transactivation of the CD34 promoter region from nucleotide −293 to +55 upon binding of Myb or Ets-2 proteins.** *A*, gel shift experiments were performed using bacterial extracts containing the Myb or the Ets-2 protein and ^{32}P-labeled probes corresponding to nucleotides −293 to −114 and −113 to +55, respectively. Free probe only (*lanes 1*, *6*, *11*, and *16*); probe plus 1 μg of parental bacterial lysate (*lanes 2*, *7*, *12*, and *17*); probe plus 1 μg of bacterial lysate containing the human c-Myb or Ets-2 proteins (*lanes 3* and *8* and lanes *13* and *18*, respectively); probe plus 1 μg of bacterial lysate containing the human c-Myb or Ets-2 proteins in presence of specific unlabeled competitors (*lanes 4*, *9*, *14*, and *19*, respectively); probe plus 1 μg of bacterial lysate containing the human c-Myb or Ets-2 proteins in presence of irrelevant unlabeled competitors described in Fig. 3 legend (*lanes 5* and *10* and *lanes 15* and *20*, respectively). A mixture of synthetic oligomers including putative Myb binding sites at position −266 to −263, −249 to −246, (oligomer I), −190 to −187, −178 to −175 (oligomer II), −88 to −85, −81 to −78 (oligomer III), and +39 to +42 (oligomer IV) was used as specific competitor. Similarly, a mixture of synthetic oligomers including putative Ets binding sites at positions −206 to −202 (oligomer V), −152 to −148 (oligomer VI), and −125 to −121 (oligomer VII), was used as specific competitors. The different regions of the CD34 promoter used as probe are indicated on the *top* of each *panel*. *B*, construct in which the CD34 5′-flanking region from −293 to +55 drives the bacterial CAT gene (CD34 M-CAT). *C*, scintillation countings of acetylated [^{14}C]chloramphenicol in lysates of Tk-ts13 cells transfected with CD34 M-CAT only (*lane 1*), or cotransfected with CD34 M-CAT plus pSVmyb or pSVets2 (*lanes 2* and *5*, respectively), or with CD34 M-CAT plus SVmyb in the presence of specific or nonspecific competitors (*lanes 3* and *4*, respectively), or with CD34 M-CAT plus SVets-2 in the presence of specific or nonspecific competitors (*lanes 6* and *7*, respectively). *Error bars* indicate mean + S.D. of three independent experiments.

h later (Fig. 3). c-Myb and Ets-2 induced an 8- and 9-fold increase, respectively, in CAT expression driven by CD34 L-CAT or CD34 LD-CAT CD34 5′-flanking region segments; together, c-Myb and Ets-2 induced a 17-fold increase in CAT expression by these segments (Fig. 3, *panel A*). Similar increases in CAT expression were induced by c-Myb, Ets-2, or both when CD34 LM-CAT and CD34 LDM-CAT, containing mutations in the more proximal or more distal c-Myb and Ets-2 binding sites, respectively, were transfected as reporter plasmid (Fig. 3, *panel A*). These results suggest that distal c-Myb and Ets-2 binding sites are involved in transactivation of the CD34 promoter. c-Myb and Ets-2 induced a 14- and 15-fold increase in CAT expression, respectively, driven by the CD34 S-CAT CD34 5′-flanking region; coexpression of c-*myb* and *ets*-2 induced a 23-fold increase in CAT expression driven by this segment (Fig. 3, *panel B*). These increases were abolished by introducing nucleotide substitutions in c-Myb (CD34 Sm-CAT); Ets-2 (CD34 Se-CAT) or in c-Myb and Ets-2 binding sites (CD34 Sme-CAT) (Fig. 3, panel 2) included in the CD34 S-CAT CD34 5′-flanking region.

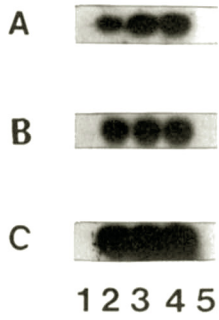

FIG. 5. **CD34 mRNA levels in TK-ts13 cells transiently transfected with pSVmyb and/or pSVets2.** Expression of CD34 mRNA determined by RT-PCR in TK-ts13 cells transiently transfected with pSVβgal (*lanes 1*), with pSVmyb (*A*, *lanes 2–5*), with pSVets2 (*B*, *lanes 25*), and with pSVmyb+pSVets2 (*C*, lanes *2–5*). RNA was extracted 48 h after transfection except in *lanes 2* and *3* (RNA extracted 24 and 36 h after transfection, respectively). To exclude amplification from genomic DNA, RT-PCR reactions were also performed in the absence of reverse transcriptase (*lane 5*).

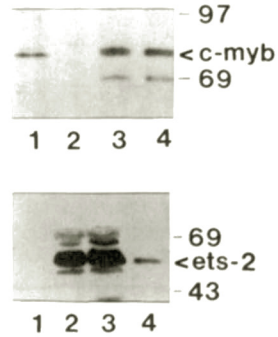

FIG. 6. **Levels of c-Myb and Ets-2 protein in T98G cells stably transfected with pSVmyb and/or pSVets2.** *Top*, levels of c-Myb protein were detected by Western blot analysis in HL60 cells as positive control (*lane 1*), in untransfected T98G cells (*lane 2*), in pSV-myb transfected T98G cells (*lane 3*), and in pSVmyb+pSVets2 cotransfected T98G cells (*lane 4*). *Bottom*, levels of Ets-2 protein were detected by Western blot analysis in untransfected T98G cells (*lane 1*), in pSVets2 transfected T98G cells (*lane 2*), in pSVmyb+pSVets2 cotransfected T98G cells (*lane 3*), and in 2 μg of bacterial lysate containing Ets-2 protein (*lane 4*).

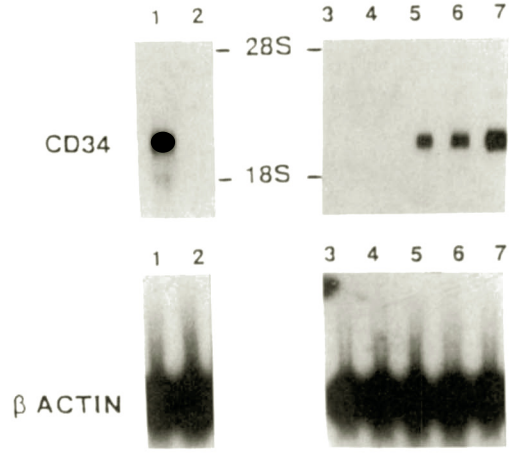

FIG. 7. **CD34 mRNA levels in T98G human glioblastoma cells constitutively expressing c-*myb* and/or Ets-2.** CD34 mRNA levels were detected by Northern blot. *Lane 1*, KG1-a cells; *lane 2*, HL60 cells; *lane 3*, T98G parental cells; *lane 4*, insertless transfected T98G cells; *lane 5*, SVmyb T98G cells; *lane 6*, SVets T98G cells; *lane 7*, SVmyb+ets T98G cells. β-actin mRNA levels are shown (*bottom*).

CAT assays were also performed using TK$^-$ts13 transfected with CD34 L-CAT or CD34 S-CAT transiently coexpressing c-*myb* and *ets*-2 in the presence of a 100-fold molar excess of fragments of CD34 5′-flanking region containing two wild type or mutated c-Myb binding sites, one wild type or mutated Ets-2 binding site, and both wild type or mutated c-Myb and Ets-2 binding sites (Fig. 3, *panel C*). Transactivation of CD34 L-CAT and CD34 S-CAT was abolished by the wild type competitor for both proto-oncogenes and reduced by competitors with mutations in only c-Myb or Ets-2 binding sites. Under these conditions, levels of transactivation were similar to those induced by individual proto-oncogenes on wild type CAT constructs (Fig. 3, compare *panel C* with *panels A* and *B*). Transactivation levels were unaffected by the mutated competitors, further suggesting that the transactivation of the CD34 promoter directly depends on c-Myb or Ets-2 interaction with their binding sites. Since the CD34 promoter region between "distal" and "proximal" Myb and Ets-2 binding sites contains other putative Ets and Myb binding sites, we assessed the ability of Myb and Ets-2 proteins to interact with and to transactivate a CAT construct, including the region from nucleotides −293 to +55 of the CD34 promoter. Gel shift assays using bacterially synthesized Myb or Ets-2 protein and two ^{32}P-labeled fragments (from −293 to −114 and from −113 to +55) revealed specific complexes (Fig. 4*A*, *lanes 3*, *8*, *13* and *18*), as demonstrated by competition with a 100-fold excess of unlabeled oligomers containing Myb (Fig. 4*A*, *lanes 4* and *9*) or Ets (Fig. 4*A*, *lanes 14* and *19*) binding sites, but not with a 100-fold excess of irrelevant unlabeled oligomers (Fig. 4*A*, *lanes 5*, *10*, *15*, and *20*). The potential for such DNA-protein interactions correlated with the ability of c-Myb or Ets-2 to transactivate a CAT construct including the −293 to +55 promoter region (Fig. 4*B*). A 12-fold increase in CAT expression was induced by c-Myb or Ets-2 when CD34 M-CAT (Fig. 4*B*) was used as reporter plasmid in cotransfection experiments (Fig. 4*C*, *lanes 2* and *5*). These increases were abolished by a 100-fold molar excess of the same specific competitors of c-Myb and Ets-2 binding utilized in gel retardation assays (Fig. 4*C*, *lanes 3* and *6*, respectively), but unaffected by an 100-fold excess of irrelevant oligomers (Fig. 4*C*, *lanes 4* and *7*).

Regulation of CD34 Expression by c-Myb and Ets-2—The regulation of CD34 expression by c-Myb and Ets-2 suggested by the effects observed on promoter activity was confirmed by RT-PCR in TK$^-$ts13 fibroblasts 24, 36, and 48 h after transfection with pSVmyb or pSVets2, with pSV-βgal-transfected TK$^-$ts13 cells used as controls (Fig. 5). CD34 mRNA expression was detected only in c-*myb*- or *ets*-2-expressing cells, and the levels were similar at 24, 36, and 48 h after transfection (Fig. 5). Similar studies were performed to assess CD34 expression in CD34-negative T98G cells transfected with pSVmyb, pSVets2, or both. In transfected cells, Western blot analysis revealed the presence of a 75-kDa protein corresponding to c-Myb or of a 56-kDa protein corresponding to Ets-2, or of both proteins (Fig. 6). Expression of CD34 mRNA in parental T98G cells, and in T98G cells transfected with pSVmyb, pSVets2, or both was compared by Northern blot analysis. CD34 mRNA expression was not detectable in parental and insertless transfected T98G cells (Fig. 7, *lanes 3* and *4*); in marked contrast, CD34 mRNA levels were readily detected in SVmybT98G and SVetsT98G cells (Fig. 7, *lanes 5* and *6*, respectively). Higher levels of CD34 mRNA expression, similar to those in positive control KG-1a cells (Fig. 7, *lane 1*), were detected in SVmyb+etsT98G cells (Fig. 7, *lane 7*).

Indirect immunofluorescence was performed to monitor surface expression of human CD34 antigen on T98G, SVmybT98G, SVetsT98G, and SVmyb+etsT98G cells (Fig. 8). Such analysis indicated no expression of CD34 antigen on T98G cells, whereas most SVmybT98G and SVets-T98G cells expressed the antigen (Fig. 8*B*) at density levels one-fourth to one-tenth of those detected on positive control KG-1a cells (Fig. 8*A*). Most SVmyb+etsT98G cells expressed CD34 antigen at density levels similar to those on KG-1a cells (Fig. 8, *A* and *B*).

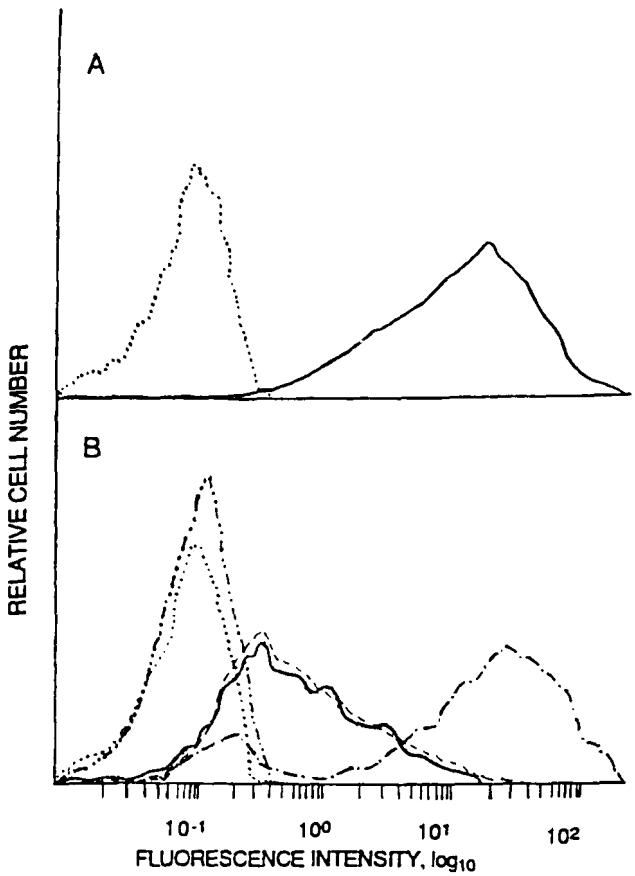

FIG. 8. **Cell surface expression of CD34 in T98G cells constitutively expressing c-*myb* and/or *ets*-2.** *A*, KG-1a cells (····, negative control; ——, anti-CD34); *B*, –·–·–, T98G, anti-CD34; ——, SVmyb T98G, anti-CD34; – – – SVetsT98G, anti CD34; –·–·–, SVmyb+etsT98G; ····, negative control. *x* axis, number of cells; *y* axis, fluorescence intensity, log scale. The analysis was repeated twice with identical results.

DISCUSSION

Our recent demonstration that c-Myb is a transcriptional regulator of CD34 antigen expression (14) suggests that this gene exerts an important transactivating function during the earliest stages of hematopoietic progenitor cell commitment and differentiation. Preliminary findings in our laboratory suggest Myb regulation of CD34 expression also in embryonic stem cells, a system that is probably more relevant for assessing Myb function of rodent fibroblasts or human glioblastoma cells that lack detectable levels of endogenous c-Myb protein. In this report, we searched for additional regulators of CD34 expression, because not all cells expressing high levels of c-*myb* express CD34 and because transcription regulation during differentiation, proliferation, or development is a finely tuned process that usually involves multiple transcription factors interacting, individually or in combination, with cis-regulatory elements of the genes of interest (28, 29). Our data show that CD34 is an Ets-2 target, an observation that extends the range of hematopoietic-specific genes regulated by members of the *ets* family (30–32). However, the most notable finding is the correlation between transactivation of promoter activity by transiently expressed Myb and Ets-2 proteins in TK-ts13 fibroblasts with levels of induced CD34 expression in human glioblastoma T98G cells stably transfected with c-*myb* and *ets*-2. Based on the observations that (i) the transactivating effect of either Ets-2 or c-Myb appears to be additive in TK-ts13 cells, (ii) mutations in either the Myb or the Ets binding sites affect only the ability of c-Myb or Ets-2 to transactivate the specifically mutated promoter, and (iii) competition with wild type, but not mutated Myb or Ets binding sites only abrogates specific transactivation, we conclude that Myb and Ets-2 proteins transactivate the CD34 promotor independently. In addition, the interaction Ets-2-Myb protein does not seem to be required for optimal transactivation via either Myb or Ets binding sites. This conclusion is consistent with the reported simultaneous interaction of Myb and Ets-2 proteins with a segment of the *mim-1* promoter cooperatively activated by Ets-2 and c-Myb, and the inability to detect protein-protein interaction involving Myb and Ets-2 (20).

There is no precise information on c-Myb and Ets-2 levels in hematopoietic progenitors and, most important, on the requirements of CD34 levels for hematopoietic progenitor cell function. Overexpressed c-*myb* and *ets*-2 can, on their own, induce CD34 expression; however, based on the much higher CD34 protein levels found in T98G cells coexpressing *ets*-2 and c-*myb*, it is tempting to suggest that the c-Myb-Ets-2 cooperation is essential for the regulation of CD34 levels in hematopoietic progenitor cells. The apparent synergistic effect of c-Myb and Ets-2 in inducing expression of the surface protein suggests that cells expressing constitutive levels of c-*myb* and *ets*-2 are endowed with posttranslational mechanisms of regulation of CD34 expression. The significance of this finding remains, however, uncertain, because T98G cells do not normally express the CD34 antigen.

Our findings of independent regulation of CD34 expression by Myb and Ets-2 upon specific interaction with closely spaced binding sites in the 5′-flanking region of the gene are reminiscent of a similar mechanism of transcriptional regulation in the expression of the megakaryocyte-specific glycoprotein IIb gene, where mutations in the erythromegakaryocyte GATA motif and in the Ets binding site impair promoter activity (31). Myb and GATA genes may have overlapping functions in the regulation of erythroid development as reflected by the phenotype of Myb- or GATA-1-deficient mice (13, 33). Myb mutations probably affect the pluripotent hematopoietic stem cell compartment, whereas GATA-1 mutations interfere with later stages of progenitor development leading to the erythroid lineage. This pattern of gene function is compatible with the ability of a *myb-ets*-containing retrovirus to transactivate the GATA-1 promoter (34).

The presence of closely spaced Myb and Ets binding sites might be a feature of hematopoietic promoters that are regulated during the earliest stages of hematopoietic differentiation. As differentiation proceeds, transactivators such as the GATA-1 gene may activate a lineage-restricted pattern of gene expression. It is also possible that different members of the Ets family regulate gene expression at different stages of hematopoietic differentiation, which would be reflected in a distinct pattern of *ets* expression in multilineage and lineage-restricted progenitors. Alternatively, Ets binding sites in the promoter region of genes with lineage- and stage-specific expression are bound by distinct members of the Ets family. Answers to these questions await additional studies of CD34 transcriptional regulation and of other genes regulated during hematopoietic differentiation.

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