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GATA and Ets *cis*-Acting Sequences Mediate Megakaryocyte-Specific Expression

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The human glycoprotein IIB (GPIIB) gene is expressed only in megakaryocytes, and its promoter displays cell type specificity. We show that this specificity involved two *cis*-acting sequences. The first one, located at −55, contains a GATA binding site. Point mutations that abolish protein binding on this site decrease the activity of the GPIIB promoter but do not affect its tissue specificity. The second one, located at −40, contains an Ets consensus sequence, and we show that Ets-1 or Ets-2 protein can interact with this −40 GPIIB sequence. Point mutations that impair Ets binding decrease the activity of the GPIIB promoter to the same extent as do mutations that abolish GATA binding. A GPIIB 40-bp DNA fragment containing the GATA and Ets binding sites can confer activity to a heterologous promoter in megakaryocytic cells. This activity is independent of the GPIIB DNA fragment orientation, and mutations on each binding site result in decreased activity. Using cotransfection assays, we show that c-Ets-1 and human GATA1 can transactivate the GPIIB promoter in HeLa cells and can act additively. Northern (RNA) blot analysis indicates that the *ets*-1 mRNA level is increased during megakaryocyte-induced differentiation of erythrocytic/megakaryocytic cell lines. Gel retardation assays show that the same GATA-Ets association is found in the human GPIIB enhancer and the rat platelet factor 4 promoter, the other two characterized regulatory regions of megakaryocyte-specific genes. These results indicate that GATA and Ets *cis*-acting sequences are an important determinant of megakaryocytic specific gene expression.

Cellular differentiation is determined in part by cell-type-specific gene activation achieved through the complex interactions of *cis*- and *trans*-acting regulatory elements (for a review, see reference 28). These interactions involve DNA-binding proteins that may be expressed ubiquitously or may be restricted to a particular cell or tissue type. Transcription of a particular gene is regulated by the binding of such *trans*-acting proteins to its regulatory sequences. Hematopoiesis is one of the model systems used to examine cell-type-specific gene activation and regulation as a pluripotent stem cell differentiates through a series of committed progenitors toward terminally differentiated cells of each blood cell lineage. This process involves specific activation of particular sets of target genes during cellular commitment and terminal differentiation and therefore expression of transcription factors specific for the different hematopoietic lineages.

Studies on the regulatory elements found in the promoters and enhancers of erythroid cell-specific genes have revealed two *cis*-acting sequences which are necessary for regulated expression of cloned genes introduced into erythroid cells (12, 19, 26). The first motif, GGTGG, can bind either SP1 or the CCACC-binding protein (44) and is found in close association with a second motif (for a review, see reference 26). This second motif, the WGATAR (W = A or T; R = A or G) or GATA motif, has been found in the promoters or enhancers of all characterized erythroid genes. This WGA TAR sequence can bind a family of *trans*-acting factors now called the GATA family. Within this family, GATA1 is expressed in the erythrocytic lineage. This factor seems to be indispensable for erythropoiesis, as site-specific disruption of the X-linked GATA1 gene in mouse embryo-derived stem cells and subsequent formation of chimeric mice results in the absence of contribution of the embryo-derived stem cells to erythropoiesis (25).

GATA1, which was previously thought to be erythroid cell specific (40), is in fact also expressed in megakaryocytes (36) and bone marrow-derived mast cells (17). These data support previous data indicating that the erythrocytic and megakaryocytic pathways of hematopoietic differentiation are closely related and might arise from a common cellular progenitor. However, despite the presence of GATA1 in erythroid cells, these cells do not express megakaryocyte-specific genes. This result rules out the hypothesis that GATA1 is sufficient for specific gene expression but suggests that restricted expression of a gene in the megakaryocytic or erythrocytic lineage could be mediated by interactions between GATA1 and other transcription factors. Such factors could be expressed ubiquitously or in only one of the two lineages.

Only two genes specific for the megakaryocytic lineage have been studied in detail. The rat platelet factor 4 (PF4) gene has been cloned (8), and a 1.1-kb DNA fragment located upstream from the cap site seems sufficient to confer megakaryocyte-specific expression to a reporter gene in transgenic mice (32). Molecular analysis of the PF4 promoter has shown a complex interaction between a minimal promoter (−97 to +1), which is sufficient for efficient megakaryocyte-specific expression, and upstream sequences (33). Sequence analysis of the minimal PF4 promoter reveals a GATA motif around −30 which seems to be involved in the tissue specificity of the promoter (33). The position of this GATA sequence is not conserved in the human PF4 promoter (10), indicating either that GATA1 may bind elsewhere in the human PF4 promoter or that the GATA binding site may be replaced by another control region. A similar analysis has been done on the human gene

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encoding glycoprotein IIB (GPIIB), a megakaryocyte-specific membrane protein. Two positive responsive elements involved in megakaryocytic expression have been defined (41). These two elements contain GATA consensus sequences and a possible binding site for a megakaryocyte-specific protein which has not been further characterized.

We have previously shown that the −55 GATA binding site of the GPIIB promoter is involved in the activity of this promoter (36). However, GATA1 is not necessary for tissue specificity, as a GPIIB promoter mutated in the GATA site was still active in megakaryocytic cells. This result differed from those for mutations on proximal GATA binding sites of erythroid cell-specific promoters, which always resulted in complete extinction of the activity of these promoters (19, 12), and indicated a megakaryocyte-specific combination of *cis*-acting sequences absent from erythroid regulatory sequences. In this report, we present a detailed analysis of the minimal *cis*-acting sequences that allow megakaryocytic activity and describe an association of sequences that is found in regulatory regions of megakaryocyte-specific genes.

MATERIALS AND METHODS

Cell culture, transfection, and CAT assays. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. HEL, KU812, MEG01, and K562 cells were grown in the same conditions except that RPMI medium was used. HEL cells were transfected by electroporation with 10 μg of reporter gene plasmid and 2 μg of the Rous sarcoma virus-luciferase plasmid. Cotransfection of reporter genes was performed by electroporation of HeLa cells with the indicated human GATA1 (hGATA1) and/or *ets* construct, the reporter plasmid, the Rous sarcoma virus-luciferase plasmid. Twenty-four hours after transfection, the cells were harvested and chloramphenicol acetyltransferase (CAT) activity was assayed as described previously (14), using amounts of extract containing the same level of luciferase activity. Stimulation was measured by comparison with results for the pECE or ΔEB vector as a mock transactivator for hGATA1 or *ets*-1 and *ets*-2. The *Spodoptera frugiperda* SF9 cells were grown in TC100 medium (GIBCO) supplemented with 10% fetal calf serum. For infection with the *Autographa californica* nuclear polyhedrosis virus c-*ets*-1 recombinant and h-*ets*-2 recombinant, cells were seeded at 20×10^6 cells per 150-mm petri dish and infected at a multiplicity of infection of 20. Whole cell extracts were prepared as described previously (3).

Construction of reporter plasmids. The GPIIB-CAT parental vector was constructed by fusion of a GPIIB promoter sequence (−109 to +26) to the bacterial CAT coding sequence into the ptkAGPT-CAT vector (18a). For deletion analysis, oligonucleotides that defined the 5′ end of the deletion and a +7 to +26 (+7/+26) oligonucleotide were used as primers for polymerase chain reaction to generate the GPIIB DNA fragments used. Those fragments were cloned into the ptkAGPT-CAT vector and sequenced before use. For site-directed mutagenesis, the −75 to +26 region was subcloned into phage M13mp18 and used as a the template for mutagenesis using the Amersham oligonucleotide-directed in vitro mutagenesis system. All GPIIB mutants were then fused to the CAT coding sequence, and the resulting constructs were sequenced before use.

The hybrid GPIIB-glycophorin B (GPB) promoters were constructed by inserting the wild-type and indicated mutated GPIIB DNA fragments spanning nucleotides −63 to −29 upstream from the −37/+43 GPB promoter. The GPIIB DNA fragments were blunt ended and inserted at the unique *Sph*I site of the vector containing the −37/+43 GPB promoter upstream from the CAT coding sequence. All of the constructs were sequenced before used.

Gel shift assays. HeLa, HEL, and KU812 nuclear extracts were prepared by the method of Dignam et al. (7) from exponentially growing cells. Gel shift assays were performed with the end-labelled oligonucleotides indicated. Sequences of the oligonucleotides used are as follows (coding strand): U1, 5′-TCTGAAGGGAAGGAGGAGGAGCTGG-3′; Mut U1, 5′-TCTGAAGGTACGGTGGAGTAGCTGG-3′; SP1, 5′-CGCAGAGGGGCGGGCCTGG-3′; M1, 5′-AAAGACTTC CTGTGGAGGAATCTGA-3′; Mut M1, 5′-AAAGACTTAC TGTGGAGTAATCTGA-3′; Wt Ets, 5′-ATAAACAGGAA GTGGT-3′; Mut Ets 5′-ATAAACACCAAGTGGT-3′; PF4, 5′-GGATCACTTCCTCATCCC-3′; and GPIIB enhancer, 5′-AGAAGGAGGAAGTGGGTA-3′.

Construction of expression vectors. A plasmid that constitutively expresses hGATA1 was constructed by using the pECE vector (11), in which the inserted cDNA is transcriptionally directed by the simian virus 40 promoter and enhancer. An *Eco*RI DNA fragment containing 120 bp of the 5′ untranslated region, the complete coding sequence, and 60 bp of the 3′ untranslated region of hGATA1 was inserted at the unique *Eco*RI site of the pECE vector, and its orientation was checked by *Bam*HI digestion.

Plasmids that express *ets*-1 and *ets*-2 were constructed as previously described (3).

Preparation and blot analysis of RNA. Total RNA was isolated from the various cell lines as described previously (6). RNA denaturation, fractionation in formaldehyde-agarose gels, transfer to nitrocellulose, hybridization, and washing were performed as described by Thomas (39).

RESULTS

A −47/+26 DNA fragment of the GPIIB promoter drives activity in megakaryocytic cells but not in HeLa cells. To delimit the sequences involved in regulation of the GPIIB promoter, we first performed sequential 5′ deletions of this promoter and linked the remaining sequences to the bacterial CAT coding sequence. We used constructs −109/+26 CAT, −75/+26 CAT, −47/+26 CAT, and −27/+26 CAT (coordinates are relative to the transcription initiation site of the human GPIIB gene). These constructs were introduced for transient transfection in megakaryocytic (HEL) or nonhematopoietic (HeLa) cells lines together with a plasmid expressing firefly luciferase as an internal control for transfection efficiency. HEL was chosen, as this cell line expresses many megakaryocyte-specific genes, including the GPIIB gene (18, 38). As expression of −109/+26 CAT was identical to that of −75/+26 CAT (i.e., five- to sevenfold above the CAT activity obtained with the vector alone), we considered this latter construct to represent 100% CAT activity. By primer extension analysis, we showed that the GPIIB-CAT fusion mRNA is correctly initiated in HEL cells (not shown).

The results of this deletion analysis are shown in Fig. 1. In HeLa cells, none of the constructs were active (data not shown). In HEL cells, sequential removal of sequences resulted in a loss of activity of the GPIIB promoter. The −47 deletion mutant, which did not contain any GATA binding site, expressed 65% CAT activity compared with the −75 construct, whereas the −27 deletion mutant expressed only 30% CAT activity. These results indicated that the −47 GPIIB construct, which contains no GATA binding site, was

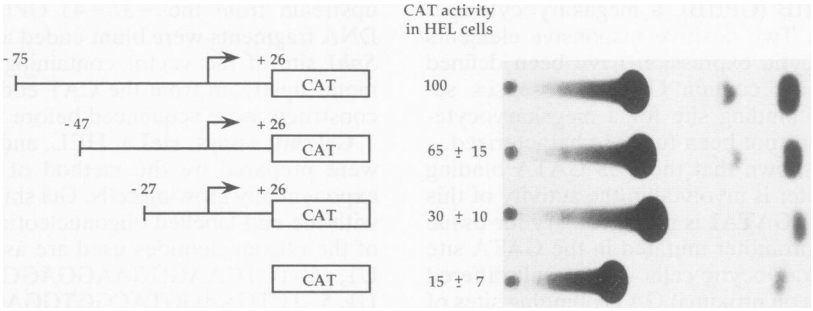

FIG. 1. Effects of 5′ deletions on expression of the GPIIB promoter transfected in HEL cells. HEL cells were transfected with the constructs indicated. Twenty-four hours after electroporation, the transfections were normalized and the level of CAT activity was measured. CAT activities are averages of four experiments with different preparations of plasmids. The boxed CAT region in the constructs represents the vector without promoter.

still active in HEL cells, suggesting that sequences located between −47 and −27 were important for driving megakaryocytic expression of the GPIIB promoter. We therefore investigated the ability of the −47/+26 GPIIB construct to bind nuclear factors.

Transcription factors binding to the −47 DNA fragment. We have previously investigated the ability of a −305/+26 GPIIB promoter fragment to bind nuclear factors by DNase I footprinting (36). Since this study showed that the −55 region could bind hGATA1, we focused on the other two footprints. Between −47 and +26, two regions were protected (Fig. 2A). The first one (U1), around −15, was obtained when the DNA fragment was incubated with nuclear extracts from nonhematopoietic cells (HeLa) or hematopoietic cells (HEL or KU812). The second one (M1), centered around −40, was detectable only with nuclear extracts from megakaryocytic or erythrocytic cell lines.

To determine which factors interact with the −15 and −40 regions, we performed gel shift assays. We obtained identical gel retardation patterns when HeLa, KU812F, or HEL nuclear extracts were incubated with the U1 probe (Fig. 2B, lanes a to c). This finding indicates that a general transcription factor bound to this −15 region. As this region contains a GGGAAGG sequence reminiscent of an SP1 binding site, we analyzed whether these complexes could correspond to SP1. When a known SP1-binding oligonucleotide was used as a competitor, complex formation over the −15 probe was reduced (lanes g and h). Similarly, SP1 binding to a consensus GGGCGGG oligonucleotide could be abolished by an excess of the cold −15 oligonucleotide and was not affected when we used a −15 oligonucleotide mutated in the SP1 motif (lanes i to l). Finally, we found that oligonucleotide U1 could not compete for the specific CCACC-binding protein (not shown). We conclude from these experiments that SP1 or a related protein but not the CCACC protein can interact with the −15 region of the GPIIB promoter.

Incubation of an oligonucleotide corresponding to footprint M1 with HeLa, KU812, or HEL nuclear extracts results in the formation of two complexes (Fig. 2C, lanes, a, c, and e). Close inspection of the sequence covered by footprint M1 revealed that this sequence contained a 5′-CAGGAAGT-3′ motif known as an Ets binding site, and thus the −40 sequence was a putative DNA binding site of Ets family proteins. The lower-mobility complex was indeed competed for when we used a high-affinity Ets-binding oligonucleotide as a competitor (lane j). Furthermore, a GG→CC conversion in this competitor oligonucleotide, a mutation that is known to suppress Ets binding, did not compete for this lower-mobility complex (lane k). This result indicated that the −40 region could interact with members of the Ets family. Although the higher-mobility complex was also competed for by oligonucleotide M1 itself (lane h) and by a high-affinity Ets-binding oligonucleotide (lane j), it was also competed for by mutated oligonucleotides (lanes i and k), indicating that this complex is nonspecific.

Recombinant Ets-1 and Ets-2 proteins bind to the −40 region of the GPIIB promoter. To investigate whether Ets family members indeed bind to the −40 region, we analyzed the binding of probe M1 by recombinant Ets-1 and Ets-2 proteins obtained from baculovirus-infected cell extracts. Both Ets-1 and Ets-2 were able to bind the −40 region (Fig. 3, lanes c and d), whereas no binding was obtained when we used extracts from noninfected cells (lane b). The binding observed with extracts from infected cells is specific since it was competed for by an excess of the high-affinity Ets binding site but not by the mutated nonbinding version of this oligonucleotide (lanes e and f).

Effects of point mutations on the activity of the GPIIB promoter. Although GPIIB promoter regions further upstream have been shown to contribute to the activity of the GPIIB promoter (41), we used the −75 GPIIB construct as the minimal DNA fragment that retains megakaryocytic activity. To characterize the respective contribution of the GATA, Ets, and SP1 binding sites identified in this region, we performed site-directed mutagenesis on each site. In accordance with our previous results (36), we found that point mutations on the GATA binding site resulted in a 60% decrease in activity of the −75 construct (Fig. 4, mutant 1). Mutations that impaired Ets-binding resulted in similar decrease in activity (mutant 2), while mutations that destroyed −15 binding site resulted in an increased activity of the −75 GPIIB promoter (mutant 3). As none of these mutants were active in HeLa cells (not shown), these results indicated that the GATA and Ets binding sites are equally important for the activity of the GPIIB promoter in megakaryocytic cell lines.

A 40-bp GPIIB DNA fragment containing the GATA and Ets binding sites confers activity to a heterologous promoter. Since GATA and Ets binding sites are also found in regulatory regions of other megakaryocyte-specific genes (see below), we tested the effect of a 40-bp GPIIB DNA fragment containing only the GATA and Ets binding sites on the activity of a heterologous promoter. We used the minimal (−37 to +43) human GPB promoter, which is completely inactive when transfected in HEL cells (our unpublished data). Upstream from this heterologous promoter, we cloned, in both orientations, the 40-bp GPIIB DNA frag-

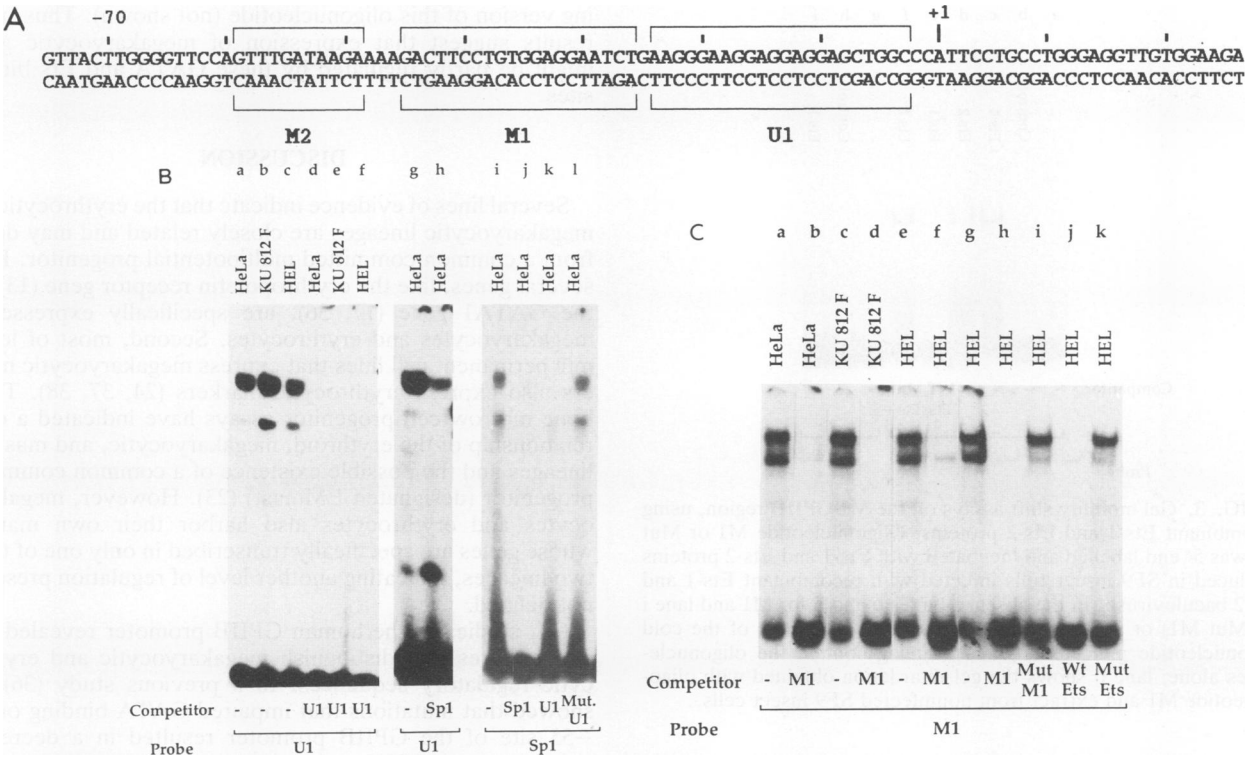

FIG. 2. Gel mobility shift assays using oligonucleotides spanning the GPIIB proximal promoter and nuclear extracts from HeLa, KU812F, and HEL cells. (A) Footprints previously observed (36) are summarized on the sequence of the GPIIB promoter. M indicates sequences protected with megakaryocytic and erythrocytic nuclear extracts, and U indicates sequences protected with nuclear extracts from hematopoietic and nonhematopoietic cell lines. +1 indicates the cap site. (B) An oligonucleotide spanning the footprint U1 (nucleotides −28 to −4) was 5′ end labelled and incubated with HeLa, KU812, or HEL nuclear extracts in the absence (lanes a to c and g) or presence of a 200-fold excess of the same cold oligonucleotide (lanes d to f) or a cold oligonucleotide that was known to bind only SP1 (lane h). This SP1-binding oligonucleotide was 5′ end labelled and incubated with HeLa nuclear extract in the absence (lane i) or presence (lanes j to l) of a 200-fold excess of the cold oligonucleotide indicated. Mut U1 indicates the mutant oligonucleotide used for site-directed mutagenesis of the GPIIB promoter (see Materials and Methods). The U1 probe appears to have a higher affinity for the upper of the two complexes (lane a), while the SP1 probe has similar affinities for the two complexes (lane i). These data could reflect two different proteins or two forms of the same protein. (C) An oligonucleotide spanning footprint M1 (nucleotides −48 to −24) was 5′ end labelled and incubated with HeLa, KU812F, and HEL nuclear extracts in the absence (lanes a, c, e, and g) or presence (lanes b, d, f, h, and j) of a 200-fold excess of the cold oligonucleotide indicated. Mut M1 is the mutated oligonucleotide used for site-directed mutagenesis of the GPIIB promoter. Wt Ets is a known Ets-binding oligonucleotide (see Materials and Methods), and Mut Ets is a mutated Wt Ets oligonucleotide that cannot bind Ets proteins.

ment. We also analyzed the properties of two 40-bp GPIIB DNA fragments mutated in either the Ets or GATA binding site. The results of the transfections performed with those constructs are shown in Fig. 5. The 40-bp GPIIB DNA fragment was sufficient to confer activity to the heterologous promoter. Mutation of either the GATA or Ets binding site decreased but did not abolish this activity, indicating that each of these sites could contribute to this activity.

The GPIIB promoter is efficiently transactivated by hGATA1 and Ets-1 but not Ets-2. We have shown that the −75 GPIIB construct was inactive in HeLa cells, and we investigated the ability of hGATA1, Ets-1, and Ets-2 to restore GPIIB promoter activity in these cells. The −75 GPIIB construct was transfected into HeLa cells together with a plasmid containing the hGATA1, c-Ets-1, or h-Ets-2 cDNA expressed from the SV40 enhancer/promoter. In control experiments, expression vectors without inserts were used. As shown in Fig. 6, the −75 GPIIB promoter was stimulated threefold when cotransfected with hGATA1 or c-Ets-1 compared with the vector alone and was inefficiently transactivated by Ets-2. Finally, we found that the activation effects of hGATA1 and c-Ets-1 were additive, as cotransfection of the −75 GPIIB CAT reporter with hGATA1 and c-Ets-1 resulted in a fivefold activation. Indeed, no transactivation was obtained with −75 GPIIB promoters mutated in either the GATA or Ets binding site (not shown).

***ets*-1 mRNA is present in megakaryocytic cell lines.** We performed Northern blot analysis on RNA isolated from human cell lines known to express (HEL and MEG01) and not express (K562, KU812, and HeLa) megakaryocyte-specific markers. Since the addition of tetradecanoyl phorbol acetate (TPA) to HEL or MEG01 cells induces megakaryocytic differentiation, we analyzed the expression of the *ets*-1 and *ets*-2 genes before and after TPA treatment of these cell lines. We found that *ets*-2 mRNA was present in all cell lines tested irrespective of whether they expressed megakaryocyte-specific markers (Fig. 7). Furthermore, *ets*-2 mRNA levels were not increased after TPA addition. In contrast, *ets*-1 mRNA was detected primarily in cell lines with megakaryocytic features (HEL and MEG01), and its level was increased after TPA treatment (Fig. 7). These results

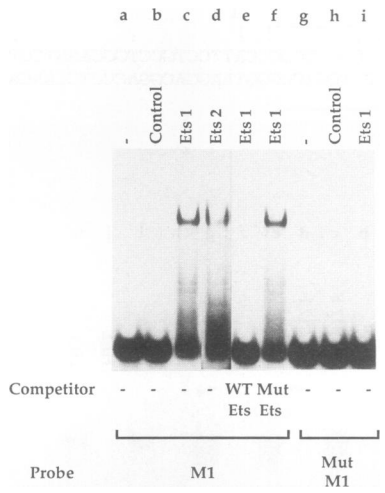

FIG. 3. Gel mobility shift assays on the M1 GPIIB region, using recombinant Ets-1 and Ets-2 proteins. Oligonucleotide M1 or Mut M1 was 5′ end labelled and incubated with Ets-1 and Ets-2 proteins produced in SF9 insect cells infected with recombinant Ets-1 and Ets-2 baculoviruses in the absence (lanes c and d for M1 and lane i for Mut M1) or presence (lanes e and f) of an excess of the cold oligonucleotide indicated. Lanes a and g contain the oligonucleotides alone; lane b shows the gel retardation obtained with oligonucleotide M1 and extract from noninfected SF9 insect cells.

indicate a potential function of Ets-1 in megakaryocytic gene expression.

Functional GATA and Ets motifs are present in regulatory regions of other megakaryocyte-specific genes. Two other regions of megakaryocyte-specific genes have been shown to confer cell type specificity. The first one lies within the promoter of the rat PF4 gene (−97 to +1), and the second was a region located between −501 and −526 in the human GPIIB promoter (33, 41). Analysis of these two sequences revealed that in addition to harboring a GATA sequence, they both harbored a potential Ets binding site (Fig. 8A). Using oligonucleotides that covered these two regions, we found that Ets-1 and Ets-2 could bind those regulatory sequences with different apparent affinities (Fig. 8B). Using competition experiments, we found that Ets binding was specific, as it was competed for by an excess of cold Ets-binding oligonucleotide but not by the mutated nonbinding version of this oligonucleotide (not shown). Thus, these results suggest that expression of megakaryocytic genes might be partly regulated by these GATA and Ets binding sites.

DISCUSSION

Several lines of evidence indicate that the erythrocytic and megakaryocytic lineages are closely related and may derive from a common committed multipotential progenitor. First, several genes, like the erythropoietin receptor gene (13) and the GATA1 gene (17, 36), are specifically expressed in megakaryocytes and erythrocytes. Second, most of leukemic permanent cell lines that express megakaryocytic markers also express erythrocytic markers (24, 37, 38). Third, bone marrow cell progenitor assays have indicated a close relationship of the erythroid, megakaryocytic, and mast cell lineages and the possible existence of a common committed progenitor (designated EMmast) (23). However, megakaryocytes and erythrocytes also harbor their own markers whose genes are specifically transcribed in only one of those two lineages, indicating another level of regulation presently not defined.

Our studies of the human GPIIB promoter revealed several features that distinguish megakaryocytic and erythrocytic regulatory sequences. In a previous study (36), we showed that mutations that impaired GATA binding on the −55 site of the GPIIB promoter resulted in a decreased activity of the GPIIB promoter. A similar result has been obtained for another megakaryocyte-specific promoter, the rat PF4 promoter. This promoter contains a GATA site around −30, and mutations that convert this GATA site to a TATA box resulted in a decreased tissue specificity and moderately affected expression in megakaryocytes (33). However, the effect of a mutation that impaired protein binding to this −30 GATA site has not been reported, and the precise function of this proximal GATA site is still unclear. Furthermore, the putative core promoter of the major human PF4 gene possesses a GATA sequence in place of the GAATA sequence in the rat promoter. As hGATA1 cannot interact with this GAATA sequence (our unpublished result), the precise implication of GATA1 in the activation of the PF4 promoter, although probable, is not clear.

To clarify the function of GATA1 in the megakaryocytic expression of the GPIIB promoter, we have carried out a detailed analysis of this promoter. We have defined a mini-

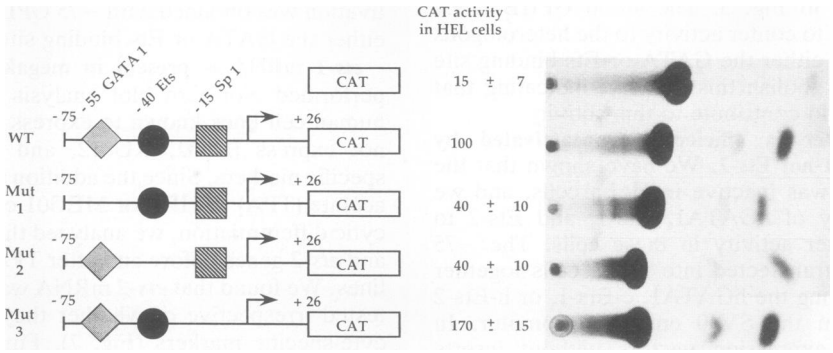

FIG. 4. Effects of point mutations on the activity of the GPIIB promoter transfected into HEL cells. Point mutations on the three protein binding sites indicated were performed, and the resulting promoters were transfected into HEL cells. After normalization of the transfections, CAT activities were measured; the values shown are averages of three different experiments. ◇, ●, and ▭ indicate GATA, Ets, and SP1 binding sites, respectively. WT, wild type; Mut, mutant.

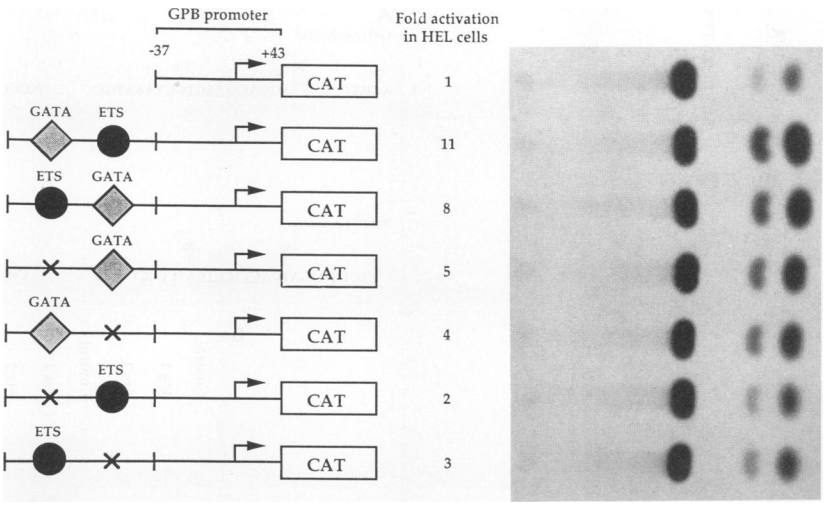

FIG. 5. Effects of GATA and Ets binding sites on the activity of a heterologous promoter transfected into HEL cells. The indicated constructs were cotransfected with a plasmid expressing the luciferase coding sequence into HEL cells. Cells were incubated for 24 h, and lysates were assayed for luciferase activity. After normalization, CAT activities were measured. The results are expressed as fold stimulation of CAT activity compared with the value for the −37/+43 GPB promoter. This minimal promoter gave CAT activity similar to that obtained with the vector (pBL CAT3) alone. CAT activities are averages of three experiments with different preparations of plasmids and do not deviate by more than 10%. ◇ and ● indicate GATA and Ets binding sites, respectively.

mal promoter (−75 to +26) that displayed megakaryocytic expression, and we studied the relative contribution of each *cis*-acting sequence previously shown to bind nuclear factors (36). We found that point mutations that impaired GATA1 binding in the −75 GPIIB construct resulted in a 60% decrease of activity in megakaryocytic cell lines and did not affect the tissue specificity of this promoter. Compared with the almost complete extinction of expression of erythroid cell-specific promoters mutated on their proximal GATA binding sites (12, 19), this result indicated that at least one other *cis*-acting sequence present in the GPIIB promoter could also promote efficient transcription in megakaryocytic cells.

Two sequences were studied for such a function, first by gel retardation assays and then by functional analysis in vivo. The first sequence, located at −15, bound SP1 or a related factor, and mutations that impaired this binding resulted in increased transcriptional activity of the mutated promoter. Again, this result differed from these obtained with erythroid cell-specific promoters. In these studies, mutation of the CCACC or SP1 binding site strongly affected promoter activity, and the existence of a synergy between GATA1 and SP1/CCACC has been proposed (12; our unpublished data). Interestingly, the activity of the murine erythropoietin receptor gene, which is expressed in both the megakaryocytic and erythrocytic lineages, is also strongly affected by mutation of its SP1 (−20) or GATA (−48) sequence when transfected in erythrocytic cell lines (46). These results indicate that megakaryocyte- and erythrocyte-specific promoters may share only the GATA *cis*-acting sequence as a major specific regulatory element.

The second sequence that we studied is located at −40 and

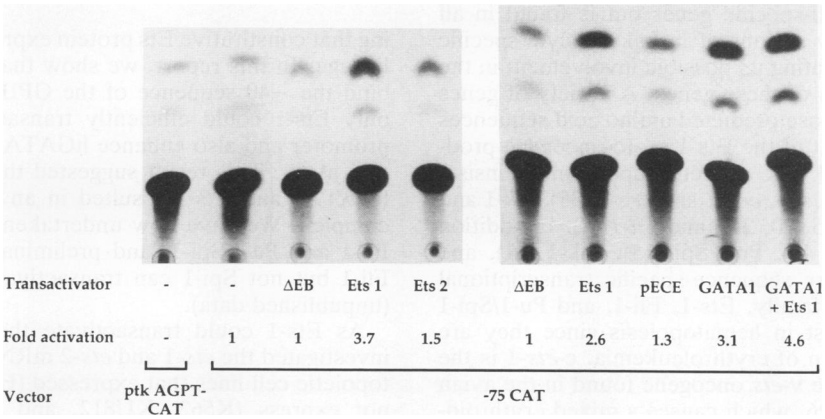

FIG. 6. Transcriptional activation of the GPIIB promoter by hGATA1, Ets-1, and Ets-2. Transactivation analysis of the −75 GPIIB promoter was performed by transfecting into HeLa cells the −75 GPIIB construct with plasmids expressing Ets-1, Ets-2, or hGATA1. As a control, the −75 GPIIB construct was cotransfected with ΔEB or pECE, the expression vectors used to express Ets-1, Ets-2, or hGATA1. Transactivation was calculated as the ratio of CAT activity in the presence of GATA1, Ets-1, or Ets-2 to CAT activity in the presence of the expression vector alone. These experiments were done three times, and the fold activation values obtained do not deviate by more than 15%.

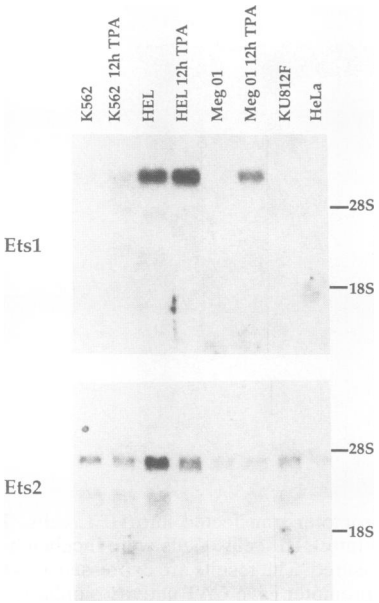

FIG. 7. Northern (RNA) analysis of *ets*-1 and *ets*-2 mRNAS. A Northern blot containing 20 μg of total RNA from the indicated cell lines was hybridized with ^{32}P-labeled *ets*-1 and *ets*-2 probes. K562 and KU812F are erythrocytic cell lines; HEL and MEG01 are megakaryocytic cell lines; HeLa is a nonhematopoietic cell line. The amount of RNA loading was checked by ethidium bromide staining of the Northern blot (not shown). TPA treatment was performed by adding TPA (30 nM, final concentration) to the cells and extracting total RNA 12 h after treatment.

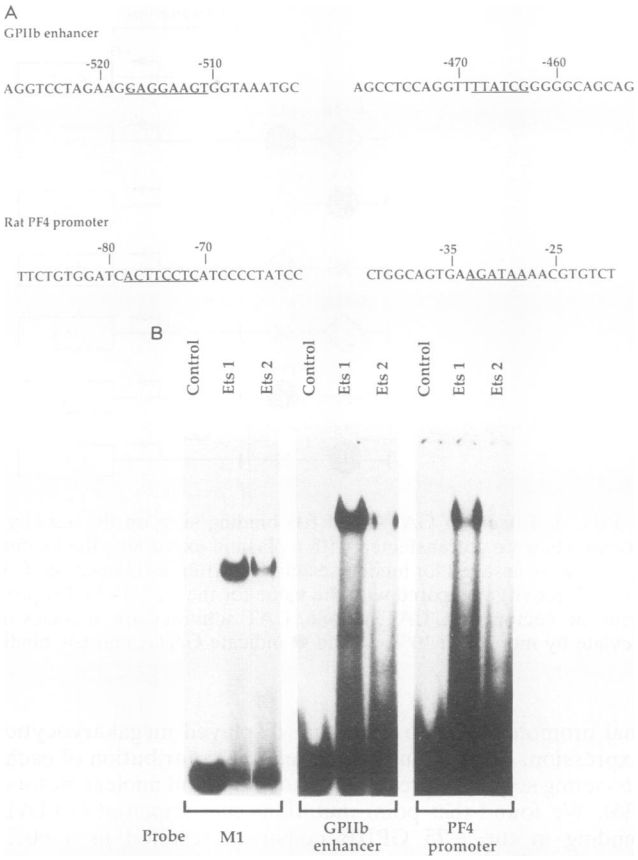

FIG. 8. Ets-1 and Ets-2 binding to the rat PF4 promoter and human GPIIB enhancer region. (A) Sequences of the GPIIB enhancer and of the PF4 promoter. The GATA binding sites located in these regions and the putative Ets binding sites are underlined. The −465 GATA site of the GPIIB promoter/enhancer does not conform to the WGATAR consensus sequence but was shown to bind GATA1 (27, 40a). (B) Oligonucleotides spanning the GPIIB footprint M1 (nucleotides −48 to +24), the GPIIB enhancer Ets binding site, and the PF4 promoter Ets binding site were 5′ end labelled and incubated with Ets-1 and Ets-2 proteins produced in SF9 insect cells. Controls are gel shifts obtained with the same oligonucleotides and extracts from noninfected SF9 insect cells.

consists of an inverted repeat of a purine-rich region. Analysis of this sequence revealed a 5′-ACTTCCTG-3′ motif which perfectly matched an Ets consensus sequence. We showed that recombinant Ets-1 and Ets-2 proteins could both bind this −40 sequence. Mutation that impaired Ets binding to the −40 sequence had an effect of the same magnitude as that produced by mutation of the GATA binding site, i.e., a 60% decrease in activity of the GPIIB promoter in megakaryocytic cells, indicating that the −40 Ets binding site was as important as the −55 GATA binding site for full activity of the GPIIB promoter. This Ets consensus sequence has never been described in regulatory regions of erythroid cell-specific genes but is found in all characterized regulatory regions of megakaryocyte-specific genes (see below), indicating its possible involvement in the specificity of expression of these genes. A variety of genes that encode proteins whose predicted amino acid sequences show similarity with that of the Ets-1 proto-oncogene product have been described. The *ets* gene superfamily consists of c-*ets*-1 (5, 9), *ets*-2 (4, 43), *erg*-1 and *erg*-2 (35), *elk*-1 and *elk*-2 (30), *Pu-1/Spi-1* (15, 20, 21), and *Fli-1* (1). In addition c-Ets-1 and h-Ets-2 (3, 42), Pu-1/Spi (15), Elk-1 (31), and Erg-1 and Erg-2 (34) are sequence-specific transcriptional activators. Among this family, Ets-1, Fli-1, and Pu-1/Spi-1 are of particular interest in hematopoiesis since they are involved in the induction of erythroleukemia. c-*ets*-1 is the cellular progenitor of the v-*ets* oncogene found in the avian acute leukemia virus E26, which causes a mixed erythroid-myeloid leukemia in chicken and transforms erythroblasts and myeloblasts in culture (22, 29). *Fli-1* and *Pu-1/Spi-1* are involved in erythroleukemia induction by various strains of Friend leukemia virus (1, 20). In both cases of leukemias, no alteration of megakaryopoiesis has been described, suggesting that constitutive Ets protein expression did not affect this lineage. In this report, we show that Ets-1 and Ets-2 could bind the −40 sequence of the GPIIB promoter. However, only Ets-1 could efficiently transactivate the −75 GPIIB promoter and also enhance hGATA1 effect in a cotransfection assay. This result suggested that interactions between hGATA1 and Ets-1 resulted in an efficient transcriptional complex. We have now undertaken a similar analysis with Fli-1 and Pu-1/Spi-1, and preliminary results indicate that Fli-1 but not Spi-1 can transactivate the GPIIB promoter (unpublished data).

As Ets-1 could transactivate the GPIIB promoter, we investigated the *ets*-1 and *ets*-2 mRNA distribution in hematopoietic cell lines that expressed (HEL and MEG01) or did not express (K562, KU812, and HeLa) megakaryocytic markers. *ets*-2 mRNA was found in all hematopoietic cell lines, but *ets*-1 mRNA was found only in cell lines that expressed megakaryocytic markers. In addition, the amount of *ets*-1 mRNA was increased when the megakaryocytic cell lines were treated with TPA, an agent known to induce

megakaryocytic differentiation (38). This result indicated that Ets-1, which has already been shown to transactivate T-cell-specific genes (2), might also be involved in the expression of terminal megakaryocytic markers. However, the precise determination of the *ets* member(s) expressed during megakaryopoiesis will require purification of megakaryocytic progenitors and analysis of their gene expression.

Finally, we investigated the presence of Ets binding sites in the regulatory regions of megakaryocyte-specific genes and found Ets consensus sequences in all regions that have been described as important for megakaryocyte-specific expression (33, 41). In addition, sequence analyses of the human β-thromboglobulin promoter (16) and the human PF4 promoter (10) have also revealed two *ets* consensus sequences located at −70 and −60 close to two GATA binding sites. These observations further strengthen the hypothesis that the GATA-Ets association is a major determinant of megakaryocytic gene expression.

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