Characterization of a Specific Erythromegakaryocytic Enhancer within the Glycoprotein IIb Promoter

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The gene coding for glycoprotein IIb (GPIIb), the α subunit of platelet integrin GPIIb/IIIa, is an early and specific marker of the megakaryocytic lineage. Thus, studies on the regulation of this gene may provide helpful information on the mechanisms controlling cell specificity and differentiation in this lineage. The promoter region of this gene was isolated and analyzed to understand its tissue-specific transcriptional activity. A region extending from nucleotides -414 to -554 was found to be extremely important for the promoter function. Deletion of this region results in a 70% decrease of the promoter activity, as measured in CAT assays. This region has the properties of an enhancer. It is able to activate a heterologous promoter, in a distance- and orientation-independent manner, in both megakaryocytic and erythroid cells. This enhancer contains binding sites for nuclear factors and mutation of these sites, individually or together, abolishes the enhancer activity. These nuclear factors are present in megakaryocytic and erythroid cell lineages, but they are absent in the other tested cells. One of the sites, named domain D, contains a TTATC motif that may interact with the transcription factor GATA1, active in erythroid and megakaryocytic cells. These results indicate that the promoter of a megakaryocytic gene contains a tissue specific enhancer, active in both the erythroid and the megakaryocytic lineages, and may implicate the erythroid factor GATA1.

The commitment of a totipotent hematopoietic cell and its terminal development into the different hematopoietic lineages are certainly controlled by switching on and off a number of genes and by controlling the extent of transcription of these genes. Modulation of gene expression in response to intra- or extracellular cues can be influenced by different combinations of nuclear transcription factors. Despite the bulk of information concerning the effect of growth factors and cytokines on hematopoiesis (1), the role of transcription factors in the developmental program of hematopoietic cells is less documented than in the hepatocyte or the muscle cell system. One of the few transcription factors that have been identified and most extensively characterized is GATA1 (2-6). This factor is implicated in the transcription of erythroid genes and was shown to play a key role in the development of the erythroid lineage (7).

Identification of lineage restricted factors can be achieved through their binding capacity to cis-acting elements of promoter regions of cell-specific genes. To characterize factors that may be implicated in the establishment of the megakaryocyte phenotype, we have used the gene encoding the platelet glycoprotein IIb (GPIIb). This gene encodes the α subunit of the platelet adhesion receptor GPIIb/IIIa and belongs to the family of RGD-sensitive integrins (8-12). While GPIIIa, or integrin β3 subunit, is expressed in a variety of cells, including fibroblasts (13), endothelial cells (14), macrophages (15, 16), and megakaryocytes (17), expression of GPIIb is restricted to megakaryocytes and is detected at an early stage of the development of this lineage (18-21). Therefore, GPIIb is a good candidate as a marker for megakaryocyte development. For this reason, the GPIIIa gene was isolated (22) and its 5'-flanking region was examined (23). We found that a genomic fragment extending 1080 bp upstream from the transcription initiation start site is able to drive the expression of a reporter gene in a tissue-specific manner. Different binding sites for nuclear proteins were identified within this domain, including consensus sequences for the binding of the erythroid factor GATA1 and sites for proteins present in megakaryocytic cells. This region has the properties of an enhancer. It is able to activate a heterologous promoter, in a distance- and orientation-independent manner, in both megakaryocytic and erythroid cells. This enhancer contains binding sites for nuclear factors and mutation of these sites, individually or together, abolishes the enhancer activity. These nuclear factors are present in megakaryocytic and erythroid cell lineages, but they are absent in the other tested cells. One of the sites, named domain D, contains a TTATC motif that may interact with the transcription factor GATA1, active in erythroid and megakaryocytic cells. These results indicate that the promoter of a megakaryocytic gene contains a tissue specific enhancer, active in both the erythroid and the megakaryocytic lineages, and may implicate the erythroid factor GATA1.

Materials and Methods

Plasmid Construction—The basic CAT plasmids used were pBLCAT3 (24), pRSVCAT (25), and pCAT promotor plasmid (Promega Biotech). The pBLCAT3 series of constructs was obtained after unidirectional deletions using the exonuclease III/mung bean system (Stratagene), as described elsewhere (23). The pRSV-Luciferase plasmid was used as internal standard expressing firefly luciferase under the control of the Rous sarcoma virus promoter (26). All plasmids used for transfection were purified by double banding on CsCl gradients.

Cell Culture and DNA Transfection—HEL, K562, and HeLa cells were grown in RPMI 1640 medium (GIBCO) and 10% fetal calf serum (Boehringer Mannheim). HEL and K562 were transfected by electroporation, using a gene pulsar (Bio-Rad) set at 960 microfarads, 400 V. Each assay was done with 10 μg of one of the CAT constructs, 10 μg of pRSV-Luciferase, and 50 μg of salmon sperm DNA/10⁶ cells in a total volume of 800 μl. HeLa cells were transfected at 960 microfarads and 200 V, in a total volume of 180 μl.

Luciferase and CAT Assays—Cells were harvested 48 h after transfection, and cell extracts were obtained by three cycles of freeze and thaw lysis. Luciferase activity of the extracts was measured using the luciferase assay system (Promega Biotech). CAT assays were performed essentially as described by Gorman et al. (25). The amount of protein in the extract tested was normalized in function of luciferase activity.

The abbreviations used are: GPIIb, glycoprotein IIb; bp, base pair(s); CAT, chloramphenicol acetyltransferase.

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Platelet GPIIb Promoter

**Gel Retardation Assays**—The gel retardation assays were performed as already described (23) by a combination of the procedures of Halligan and Desiderio (27) and Singh et al. (28). The oligonucleotides D and E corresponding to the footprinted areas D and E have already been described (23). Their sequences were 5'-CTTACAGGGTTT-TATCCGGGGAAGCAGCT for oligo D and 5'-TCTTACAGGGGAAGAATGTTTTAAATG for oligo E, respectively. These oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer, 5' end-labeled and incubated with HEL, K562, and HeLa nuclear protein extracts, as previously described (23).

**Methylation Interference Assay**—The oligonucleotide probes, 32P-labeled at one 5' end, were partially methylated with dimethyl sulfate (Maxam and Gilbert sequencing kit, Du Pont-New England Nuclear). The methylated probes were incubated with nuclear extracts of HEL cells, and the DNA-protein complexes were used in gel retardation assays, under conditions previously described (23). After autoradiography, the protein-bound and free oligonucleotide probes were cut out and recovered from the gel. DNA was then purified by electrophoresis, cleaved in piperidine at 90 °C for 30 min, electrophoresed on 15% acrylamide, 8 M urea gels, and autoradiographed.

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed with the "oligonucleotide-directed in vitro mutagenesis system, version 2" (Amersham Corp.) using the instructions of the manufacturer. The sequence of the oligonucleotides used were CTTACAGGTTT-TATCCGGGGAAGCAGCT for the mutant oligo D and TCTTACAGGGAAGAATGTTTTAAATG for the mutant oligo E.

**RESULTS**

**Analysis of 5' Deletion Mutants of the GPIIb Promoter**—To delineate the functional DNA sequences within the 5' region of the GPIIb gene, a genomic fragment containing the domain of the gene extending from +33 to -1050 and including the initiation start site was inserted into the XbaI site of the pBLCAT3 vector containing the CAT gene. We have already reported that the fusion mRNA produced by this construct is initiated at the correct transcription start site (23).

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**Analysis of the Enhancer Region**—In our previous studies (23), we have shown that the regulatory enhancer domain for platelet GPIIb expression consists of 192 bp of DNA containing the sequence 5'-CCTCAGGTTT-TATCCGGGGAAGCAGCT for the target oligo D and TCTTACAGGGAAGAATGTTTTAAATG for the mutant oligo E. These data suggested that the region between -554 and -414 contains a positive acting DNA element necessary for platelet GPIIb expression. To verify this conclusion, the CAT activity was measured in extracts of transfected HEL cells. As shown in Fig. 4, both constructs, either without or containing the previous studies of the GPIIb promoter, contained the nucleotide sequence corresponding to the region -554/-414 is foot-printed by nuclear proteins of HEL cells but not by nuclear proteins from HeLa cells (23). These protected areas, corresponding to cell-specific interactions were designated domains D and E and found to form a DNA cluster essential for the activity of the GPIIb promoter.

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To verify if this conclusion was still valid when the enhancer was located within the sequence of the GPIIb promoter, we examined its potential enhancer properties.

**Characterization of the Enhancer Domain**—A 192-bp DNA fragment, corresponding to the sequence between -598 and -406, including the -554/-414 region, was produced by digestion of the 1050 plasmid with HindIII and PvuII, purified, and fused to the CAT gene, driven by the SV40 promoter in the pCAT promoter plasmid. Three different constructs were made. In two of them, the 192-bp fragment was inserted in the direct or opposite direction upstream of the SV40 promoter, whereas the third construct contained the fragment in direct orientation downstream from the CAT gene (Fig. 2). These plasmids were introduced into HEL, K562, and HeLa cells to evaluate the enhancer activity and the tissue specificity of the IIb promoter DNA fragment. The CAT activity obtained with the enhancerless vector containing the CAT gene and the SV40 promoter alone was arbitrary given the value 1. Addition of the 192-bp fragment in either position produced a 4.4-5.9-fold increase of this basal activity when the plasmids were introduced into HEL or K562 cells (Fig. 2). In contrast, the CAT activity measured in extracts of transfected HeLa cells remained close to the basal level (0.7-1.4).

These results indicated that the 192-bp DNA fragment, containing the GPIIb nucleotide sequence between -406 and -598, is able to increase the activity of an heterologous promoter in megakaryocytic or erythroid cells but not in HeLa cells, in a position- and orientation-independent manner.

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Platelet GPIIb Promoter

Deletion analysis of the human GPIIb gene promoter region. Construct -1050, containing 1050 bp upstream and 33 nucleotides downstream from the transcription start site, was inserted at the XbaI site of the pBLCAT3 vector in the direct or the reverse orientation. 5' deletion mutants were generated by digestion of this fragment with exonuclease III and blunted with mung bean nuclease as previously described (23). Each plasmid was transfected into HEL cells, and the respective CAT activities were measured 48 h after transfection. In each assay, the pRSVL plasmid was cotransfected, and the CAT assays were normalized according to the luciferase activity. The CAT values obtained with the different plasmids were expressed relatively to the -813 plasmid, which was taken as the 100% value. Each value is an average of at least three independent experiments. The pRSVCAT plasmid was used as a positive control, and the promoterless plasmid pBLCAT3 was used to measure the background level.

Enhancer activity of the -598/-406 promoter fragment. This fragment was produced by digestion with HindIII and PstI of the 1050-bp GPIIb genomic fragment. It was inserted in direct or reverse orientation, upstream or downstream from the CAT gene driven by the SV40 promoter in the pCAT promoter plasmid. The different constructs were introduced into HEL, K562, and HeLa cells for transient transfection assays. The volumes of protein extract for each CAT assay were normalized according to the luciferase activity. CAT activity was expressed relative to the basal activity obtained with the enhancerless pCAT promoter plasmid. The values reported represent the mean value of at least three different experiments.

Active in K562 cells, the CAT activity was measured after transfection of K562 cells, with the different constructs containing mutations at the contact sites. As shown in Fig. 4, the mutations produced an inhibition of the enhancer activity similar to that observed in HEL cells.

Taken together, these results indicate that the enhancer domain interacts with similar positive transcription factors present in erythrocytic and megakaryocytic cells and that the enhancer exhibits an identical activity in both cell lines.

DISCUSSION

The production of circulating platelets is the ultimate reaction of a complex multistep differentiation process. It involves the commitment of an hematopoietic early pluripotent stem cell to the megakaryocytic lineage, the mitotic development of the megakaryoblast, and the polyploidization reaction prior to the destruction of the mature megakaryocyte to generate a large number of platelets. This unique phenomenon occurs in the bone marrow and is certainly mediated by positive or negative regulatory elements controlling the establishment or the maintenance of the megakaryocytic phenotype. Identification of these regulatory elements would help the understanding of thrombopoiesis and the etiology of
In the present study, we describe the presence of a tissue-specific regulatory element within the promoter region of the platelet-specific GPIIb gene. The presence of a positive regulatory element within this promoter, centered at position -484, was suggested by the observation that deletion of the region between -554 and -414 produces a 70% decrease of the CAT activity in HEL cells but not in HeLa cells. A DNA segment corresponding to this sequence was able to activate the enhancerless ubiquitous SV40 promoter in a position- and orientation-independent manner in HEL but not in HeLa cells. Additional 5' deletions down to the nucleotide -113 had a minimal effect on the residual activity of the promoter. When the sequence between -113 and -29 was deleted, however, an additional 50% inhibition of the residual GPIIb promoter activity was observed. This loss of activity may be partly due to the deletion of a consensus binding site for the erythroid factor GATA1 centered at position -54. We have already shown that this binding site interacts with a nuclear protein similar to this erythroid factor and is functional when fused to the CAT gene and transfected into HEL cells (29). Interestingly enough, the construct that contains the sequence between +33 and -13 exhibited a significant residual activity, as compared with the promoterless CAT construct. This suggests that the GPIIb promoter that does not have an obvious TATA motif may contain sequences that are critical for the positioning of the transcriptional protein complex close to the transcription start site.

These experiments indicate that the GPIIb promoter contains at least four distinct functional domains. One of these domains, centered at -484, exhibits an enhancing activity. This DNA region is composed of multiple binding sites for nuclear proteins that are present in megakaryocytic cells but not in HeLa cells. One of these sites, designated domain D, interacts with only one factor, whereas the other site, called domain E, can form two DNA-protein complexes, as detected by mobility shift assays. When these sites were mutated either in the isolated fragment or within the complete promoter sequence, the activity of the enhancer was lost, establishing a functional implication of these DNA-protein complexes. Alteration of these sites, either alone or in combination, produced the same effect, indicating that these DNA-protein complexes do not function separately, and are cooperative rather than synergistic.

To verify whether this enhancer domain was megakaryocyte-specific or not, the K562 cell line was used as a model for the erythroid lineage. This cell line does not express the platelet GPIIb protein, although the enhancer domain was active at a level similar to that observed with HEL cells. Furthermore, the nuclear proteins that interact with this region are also present in nuclear extracts of K562 cells, but not in myelomonocytic or fibroblastic cells (23). Two series of independent results suggest that the K562 nuclear proteins are similar to the factors present in megakaryocytic cells. 1) The electrophoretic pattern of the DNA complexes were similar. 2) Mutagenesis of the contact sites produced a comparable inhibition of the enhancer activity in both HEL and K562 cells. One of the binding sites, domain D, contains a TTATC motif, which is equivalent to the binding site of the erythroid factor GATA1. This factor has been shown to be implicated in both promoter and enhancer activity of erythroid gene. The present study suggests that it may also be implicated in the enhancer of a megakaryocytic gene. Further studies, however, are needed to determine if the observed positive effect of this IIb enhancer is mediated by GATA1 in association with an as yet unidentified factor.
A number of independent observations indicate that megakaryocytic and erythroid lineages share a number of phenotypic features. Both cells express the receptor for erythropoietin, a major regulator of the production of erythrocytes, and chromosomal markers have been used to demonstrate the existence of a bipotent progenitor cell (30, 31). Thus, understanding the switch between the two lineages is an interesting challenge. The complete GPIIb promoter is inactive in K562 cells, but our results indicate that the GPIIb enhancer domain is tissue-specific but is not lineage-specific. Therefore, this enhancing activity is not solely responsible for the megakaryocyte-specific expression of the gene. Then, why is the GPIIb gene not expressed in erythroid cells? Selectivity of expression can be achieved by negative, as well as positive regulatory elements. Negative regulation has been shown, for instance, to repress the a-globin gene in nonerythroid cells (32). A similar mechanism can control gene expression in the megakaryocyte. Though from the results presented in this study we can conclude that the GPIIb gene contains a classical enhancing activity, this element must function in synergy with another positive or negative element. In support of this hypothesis, a recent observation that the promoter region of another megakaryocyte marker, the PF4 gene, also contains positive and negative regulatory domains (33). More recently, we were able to identify a silencing domain within the GPIIb enhancer element, this element must function in synergy with another megakaryocyte marker, the PF4 gene, also contains positive and negative regulatory domains (33). Thus, it is likely that the establishment of the megakaryocyte lineage is mediated by a specific mechanism that controls the transcription of megakaryocytic genes.

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