Identification of Essential GATA and Ets Binding Motifs within the Promoter of the Platelet Glycoprotein Ibα Gene*

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Platelet glycoprotein (GP) Ib-IX-V is a multisubunit adhesion receptor that supports platelet attachment to thrombogenic surfaces at sites of vascular injury. The congenital absence of the receptor results in a bleeding disorder associated with “giant” platelets, a condition linking the expression of the complex to platelet morphogenesis. To understand better the expression of the GP Ib-IX-V complex, studies were undertaken to define the essential genetic elements supporting the expression of the α-subunit of the complex (GP Ibα). GP Ibα promoter activity was evaluated by transfection of human erythroleukemia cells with reporter plasmids coding for the enzyme, luciferase. Studies were initiated with a fragment extending 2,738 nucleotides 5′ to the transcription start site and lead to the identification of 253 nucleotides retaining full promoter activity in human erythroleukemia cells. In cells of nonhematopoietic lineage, human endothelial and HeLa cells, the GP Ibα promoter activity was no greater than background levels obtained with promoterless constructs. Gel shift assays and site-directed mutagenesis studies defined essential GATA and Ets binding motifs 93 and 150 nucleotides upstream of the transcription start site, a finding which further substantiates these elements as important determinants of megakaryocytic gene expression. The results define essential cis-acting elements responsible for the expression of GP Ibα and provide insights into molecular events coinciding with the release of normal platelets into the bloodstream.

Basic information on the differentiation of megakaryocytes and the mechanisms responsible for the release of platelets into the bloodstream has significantly lagged behind basic studies of other cellular components of myeloid tissue. Major reasons for the lag have been the lack of suitable cell lines mimicking the unique properties of megakaryocytes in vitro. Certainly, the recently described c-Mpl ligand is essential for the differentiation of the pluripotent stem cell to a megakaryocyte (1–3), but the late events of the process, specifically those that result in the release of platelets from the mature megakaryocyte, are still poorly understood. To this end, the expression of megakaryocytic or platelet-specific antigens is one unique aspect of megakaryocytosis and platelet production that can be examined and the importance of such studies has been validated by the recent work identifying the transcription factor, NF-E2, as an essential genetic factor for platelet production (4).

A role for the platelet membrane receptor, glycoprotein (GP)1 Ib-IX-V, in platelet morphogenesis is suggested by the congenital absence of GP Ib-IX-V, a condition associated with the release of abnormal or “giant” platelets and referred to as the Bernard-Soulier syndrome (5, 6). The platelet GP Ib-IX-V complex is assembled from four distinct gene products, the α- and β-subunits of GP Ib (GP Ibα and GP Ibβ), GP IX, and GP V (7–11). Considerable progress has been made defining the essential role of this receptor in hemostasis (12, 13), but little is known about the molecular events and/or factors associated with the transcription and expression of the individual genes of the complex. Moreover, nothing is known about the mechanisms which link the expression of the complex and platelet morphogenesis. Recent studies have supported the hypothesis that expression of the complex on the megakaryocyte, and ultimately on the platelet surface, is dependent upon the coordinated assembly of at least three individual gene products (14). This implies that (i) similar genetic regulatory elements exist within each of the three components, or (ii) the megakaryocytic-specific expression of one, or perhaps two, units, controls the surface expression of the complete complex.

We previously reported the generation of a transgenic mouse colony expressing the human gene for GP Ibα as part of a human-murine chimeric GP Ib-IX-V receptor complex (15). Our results documented the expression of the human transgene and established among bone marrow cells that in vivo gene expression is dependent upon a 6-kilobase DNA sequence containing the entire human GP Ibα gene. Thus, within this fragment are the promoter and enhancer sequences necessary for the in vivo expression of GP Ibα protein. In the present study we have extended the characterization of the GP Ibα gene identifying the transcription start site and the promoter elements responsible for megakaryocytic gene expression. Using the megakaryocytic-like cell line, HEL (human erythroleukemia cells), essential GATA and Ets consensus sequences were identified, a finding which further documents these elements as important determinants of megakaryocytic gene expression (16). The results provide insights into the regulatory elements involved in megakaryocytic expression of the GP Ibα gene and provide a better understanding of the events leading to the commitment of cells to the megakaryocytic lineage and the release of platelets.

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1The abbreviations used are: GP, glycoprotein; HEL, human erythroleukemia; HUVECs, human umbilical vein endothelial cells; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus.
EXPERIMENTAL PROCEDURES

Prime Extension Reaction—Human erythroleukemia cells (HEL 92.1.7, American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum under conditions containing 5% CO2 (17). Total RNA was prepared from HEL cells lysates by ultracentrifugation through cesium chloride cushions (18). Subsequently, poly(A)+ RNA was isolated by affinity chromatography on an oligo(dT) cellulose column (Life Technologies Inc., Gaithersburg, MD). Dideoxynucleotides were synthesized for use in primer extension analysis. The first, RPEX1 (37-mer), is within the first exon of GP Ibα and corresponds to the antisense strand of nucleotides 2788–2820 (numbering according to the genomic sequence reported in Ref. 19 and in Genbank accession No. M22403). The second oligonucleotide, RPEX2 (37-mer), spans the GP Ibα intron and corresponds to nucleotides 2809–2829 and nucleotides 3063–3078. Each oligonucleotide was purified by polyacrylamide gel electrophoresis and resuspended in 1 mM dNTP, 0.5 mM spermidine in a final volume of 50 μL.

RNA was mixed with labeled oligonucleotide (2 × 10^6 cpm) in a buffer composed of 50 mM Tris (pH 8.3), 50 mM KCl, 10 mM MgCl2, 10 mM dithiothreitol, 1 mM dNTP, and 0.5 mM spermidine in a final volume of 20 μL. The reaction mixtures were incubated at 80 °C for 10 min and 40 °C for 2 h to allow annealing. Two-hundred units of reverse transcriptase (Moloney murine leukemia virus, Life Technologies Inc.) were added and the mixture was incubated at 42 °C for another 30 min. The reaction products were directly analyzed by electrophoresis through a denaturing 6% polyacrylamide-urea sequencing gel.

RNAse Protection Assay—A recombinant plasmid containing approximately 1.3 kilobases of the GP Ibα gene was constructed in the vector, pBS/ExI (Stratagene). La Jolla, CA. Designated pBS/I, this plasmid contains 1142 base pairs 5’ to the transcription start site (as defined by primer extension analysis) and includes exon I of the GP Ibα gene (Fig. 1). pBS/ExI was linearized by digestion with StuI and used to synthesize an RNA probe in an in vitro transcription reaction. The RNA transcript was synthesized in a 30-min reaction (37 °C) using 10 units of T7 RNA polymerase in a buffer composed of 40 mM Tris (pH 8.8), 8 mM MgCl2, 2 mM spermidine, 50 mM NaCl, 0.4 mM rATP, 0.4 mM rCTP, 0.4 mM rGTP, 30 mM dithiothreitol, 40 units of RNase inhibitor (Stratagene), and 50 μCi of [α-32P]UTP. The resultant radioactive transcript (approximately 200 nucleotides in length) was purified using a NucTrap purification column (Stratagene). The RNase protection assay was performed using a modified protocol provided by U. S. Biochemical Corp. (Cleveland, OH) accompanying their Lysate Ribonuclease Protection Kit. The modification was the use of purified poly(A)+ RNA instead of a cellular lysate suspension. Briefly, 2.5 × 10^6 cpm of RNA probe was incubated overnight with HEL poly(A)+ RNA (1.5 μg) in 4 μL guanidine thiocyanate, 0.5% Sarkosyl (45 °C) and subsequently digested with 10 units of RNase A and 0.5 units of RNase T1 (37 °C, 30 min). The digested RNA was resolved and analyzed after denaturation by electrophoresis through a 6% polyacrylamide-urea sequencing gel.

Generation of Promoter Constructs of the Human GP Ibα Gene—The isolation of a 6.6 kilobase EcoRI fragment containing the entire GP Ibα gene and its subcloning into pBS/KS is described (15). This fragment contains the entire coding sequence for GP Ibα protein along with approximately 3 kilobases of DNA 5’ to the initiating methionine codon (Fig. 1A). Previously reported experiments established the ability of the EcoRI fragment to support the megakaryocytic expression of the human GP Ibα subunit on the surface of mouse platelets (15). Thus, this fragment served as a starting point to identify the critical components of the GP Ibα promoter. First, the methionine codon of GP Ibα was converted to a HindII restriction site via site-directed mutagenesis (20). This manipulation allowed the isolation of a HindII fragment containing approximately 3.1 kilobases of DNA 5’ to the initiating methionine codon. The HindII fragment was subcloned into a p19LC plasmid containing a promoterless luciferase gene. This parent plasmid was designated p-2788/LUC since it contained 2,738 base pairs of the GP Ibα gene and was digested with HindIII to support the megakaryocytic expression of the human GP Ibα 3’ flanking sequences. Each HindIII fragment containing a critical 5’ element was inserted into a plasmid containing the entire EcoRI fragment and the HindIII site at the 3’ end of the fragment held various points through the promoter. In all cases, the nomenclature for each new recombinant plasmid is based on the number of nucleotides 5’ to the transcription start site. For example, p-253-LUC contains 253 nucleotides 5’ to the transcription start site. All plasmids used in transfection analysis were purified by equilibrium banding in cesium chloride gradients prior to their use in cell transfections (18). Cell Lysates—Cell lysates were established from a pool of human umbilical vein cells (HUVECs) that were derived from a human umbilical vein as described (21) and cultured in EGM medium (Clonetics, San Diego, CA) supplemented with 10% fetal bovine serum. HUVECs were transfected using Lipofectin (Life Technologies, Inc.) according to the manufacturer’s recommended conditions. Briefly, cells were seeded in 60-mm dishes to obtain 50% confluency at the time of transfection. Six μg of each plasmid was diluted in OPTI-MEM (Life Technologies, Inc.) and mixed with 36 μg of Lipofectin reagent. After a 30-min incubation, the mixture was added to the cells for a 3-h (37 °C) incubation in a 5% CO2 incubator. Following the 3-h incubation, the medium was replaced with complete medium and left until time to harvest the cell lysate for analysis.

HEL cells (CCL 2, American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transfection of HEL cells was performed using a standard calcium phosphate procedure (22).

Luciferase and CAT Assays—The plasmid, pRSV/LUC, served as positive control plasmid to establish 100% promoter activity. pRSV/LUC contains the Rous sarcoma virus (RSV) promoter driving the expression of the coding sequence for luciferase. pRSV/LUC was transfected into each cell line as an internal control to normalize transfection efficiency. pRSV/CAT contains the RSV promoter driving the expression of chloramphenicol acetyltransferase (CAT). Cells were harvested 72 h after transfection and lysed in 100 μL of 50 mM K-HPO4, 50 mM KH2PO4, and 0.1 mM dithiothreitol. After removing cell debris by centrifugation the extract was assayed for luciferase and CAT activity.

Luciferase activity produced by 20 μL of cell lysate was determined by mixing with 100 μL of assay buffer (50 mM K-HPO4, 50 mM KH2PO4, 15 mM MgSO4, 5 mM ATP, 1 mM dithiothreitol) and analyzing with a Monolight 2010 luminometer in which α-luciferin was automatically injected (Analytical Luminescence Laboratory, San Diego, CA). A similar amount of cell extract was assayed for CAT activity in an assay buffer composed of 132 mM Tris (pH 7.8), 0.64 mM acetyl-CoA, and 0.1 mM of [14C]chloramphenicol. The conversion of chloramphenicol to acetylated chloramphenicol was allowed to proceed for 45 min (37 °C) and then separated by thin layer chromatography. CAT activity was determined by scintillation counting and by radioimaging using a PhosphorImaging screen (Molecular Dynamics, Sunnyvale, CA).

Gel Shift Assay—Nine synthetic DNA fragments were generated as described in Fig. 4. These fragments were synthesized as individual single-stranded oligonucleotides mixed and dialyzed to generate each double-stranded fragment. The mutants oligonucleotides used in gel shift assays were constructed in a similar manner after synthesis of the corresponding oligonucleotides with the base changes. Prior to their use in gel shift assays the oligonucleotides were labeled with [γ-32P]ATP and purified through microspin columns from Pharmacia Biotech Inc. (Piscataway, New Jersey).

HEL and HEL cell nuclear extracts were prepared by washing cells with phosphate-buffered saline followed by a buffer composed of 10 mM Tris (pH 7.5), 130 mM NaCl, 5 mM KCl, 8 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonfyl fluoride. The washed cell pellet was resuspended in a hypotonic buffer (20 mM HEPES (pH 7.9), 5 mM KCl, 0.5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonfyl fluoride for 10 min. Cells were collected on a Dounce homogenizer and the nuclei were collected by centrifugation at 1500 × g (10 min). The nuclei were resuspended in an extraction buffer (20 mM HEPES (pH 7.9), 0.5 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonfyl fluoride, and 5 μM spermidine) and incubated at 4 °C for 1 h. The extracts were again centrifuged (10,000 × g, 10 min) to pellet the cellular debris followed by resuspending the binding buffer composed of 25 mM HEPES (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 10% (v/v) glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonfyl fluoride. Approximately 1.5 ml of nuclear extract was dialyzed against 1.5 liters of binding buffer for 16 h followed by a change of buffer and an additional dialysis for 8 h.

Each labeled double-stranded fragment (2 × 10^6 cpm) was mixed with 5 μg of nuclear extract, 2 μg of poly(dI-dC)poly(dI-dC) (Pharmacia Biotech Inc.), and 0.2 μg of sonicated human umbilical vein cell DNA as a competitor. The resultant mixture was incubated at room temperature for 30 min. Samples were analyzed by electrophoresis in 6% polyacrylamide gels prepared in a running buffer.
composed of 50 mM Tris (pH 8.5), 0.38 M glycine, 5 mM EDTA. After electrophoresis, gels were dried and exposed to x-ray film.

RESULTS

Mapping the Site of Transcription Initiation—The nucleotide sequence of a 6062-base pair EcoRI fragment encompassing the GP Ibα gene has been previously reported (19, 23). This analysis revealed at least two exons, one exon encoding the 626 amino acids of the GP Ibα precursor protein, preceded by a relatively short intron (233 base pairs) and a 5'-untranslated exon (Fig. 1A). Our previous results established that this 6-kilobase fragment contains the necessary promoter elements to express human GP Ibα on the surface of transgenic mouse platelets. To determine the exact 5' boundary of the GP Ibα mRNA, we purified poly(A)⁺ RNA from the human erythroleukemic cell line, HEL, and performed primer extension analysis. HEL cells have properties similar to cells of erythroid and megakaryocytic lineages and have served as RNA sources for cloning cDNAs for a variety of platelet-specific antigens, including the components of the GP Ib-IX complex (7–9). Antisense oligonucleotides were synthesized corresponding to GP Ibα mRNA near the 5' end of the published cDNA sequence, designated RPEX1, and to sequence spanning the two exons, designated RPEX2 (Fig. 1A). The results of primer extension experiments using HEL poly(A)⁺ RNA revealed major extension products of 82 and 107 nucleotides using RPEX1 and RPEX2, respectively (Fig. 1B). These fragment lengths map the

Fig. 1. Identification of the transcription start site of the human GP Ibα gene. A, the GP Ibα gene and its two known exons are schematically shown (19). The coding sequence for the GP Ibα precursor is contained within exon II and is represented by the filled black box. Primer extension analysis (panel B) used two oligonucleotides corresponding to the antisense sequence within exon I (RPEX1) and antisense sequence spanning exons I and II (RPEX2). An RNA probe of approximately 200 nucleotides in length was synthesized in an in vitro transcription reaction for the RNase protection assay shown in panel C. B, each oligonucleotide was end labeled with [γ-32P]ATP and annealed to 3 μg of poly(A)⁺ RNA purified from HEL cells. After incubation with reverse transcriptase, the primer extension products were analyzed on a 6% denaturing polyacrylamide gel. The results are shown in the absence (-) or presence (+) of HEL poly(A)⁺ RNA (Template). A sequencing reaction using the M13-40 universal primer labeled with [γ-32P]ATP and M13 mp19 was included as a size marker and is shown on the left. Major extension products of 82 nucleotides and 107 nucleotides in length were observed using RPEX1 and RPEX2, respectively. C, an RNase protection assay was performed using a radioactive RNA probe generated from an in vitro transcription reaction and subsequently purified as described under “Experimental Procedures.” The RNA probe was annealed overnight to poly(A)⁺ RNA from HEL cells and subsequently digested with RNase A and RNase T1. The digested products were denatured and electrophoresed through a 6% polyacrylamide-urea sequencing gel. The marker (left) is a dideoxy sequencing reaction using M13 mp19 and predicts a protected RNA species of 92 nucleotides in length.
5' boundary of the first GP Ibα exon as nucleotide 2739 in accordance with the numbering scheme in Genbank accession No. M22403.

Additionally, we performed an RNase protection assay to confirm the primer extension results. A protected RNA fragment of 92 nucleotides in length was identified as the dominant protected fragment (Fig. 1C). This result mapped the 5' boundary of the first GP Ibα exon to be one nucleotide further 5' as compared to the results from primer extension analysis. However, the molecular weight determination of RNA by denaturating gel electrophoresis may vary up to 10% given the anomaly of the first GP Ibα exon to be one nucleotide further 5'.

Promoter Activity of the GP Ibα Gene—As described under "Experimental Procedures" a GP Ibα promoter cassette fragment was generated via site-directed mutagenesis to contain 2,738 nucleotides 5' to the transcription initiation site. Additionally, this fragment contains the nontranslated exon I, the single intron, and the first 6 base pairs of exon II which immediately precede the initiating methionine codon (Fig. 2). Luciferase constructs were generated to evaluate the promoter strength of this fragment. The parent plasmid, p-2738/LUC, and 5' unidirectional deletions of p-2738/LUC were generated and characterized for their ability to drive the expression of luciferase activity in HEL cells. The results are shown in Fig. 2 and suggest the core promoter elements to be within the sequence spanning −253 and −63 upstream of the transcription initiation site. Recombinant constructs containing 140 (p-140/LUC), 63 (p-63/LUC), and 6 (p-6/LUC) base pairs preceding the transcription initiation site displayed a progressive loss of promoter activity (Fig. 2). The polarity of the promoter was established with a construct, designated p-1142inv/LUC, in which the promoter cassette containing 1,142 base pairs 5' to the transcription start site was inverted. As shown, this construct produces a basal luciferase activity comparable to the background obtained with p-6/LUC (Fig. 2).

Cell Specificity of the GP Ibα Promoter—To evaluate promoter strength in cell types of nonhematopoietic lineage, luciferase assays were performed with three different promoter constructs in HEL, HeLa, and endothelial cells. As presented in Fig. 3, the GP Ibα constructs had dramatically higher promoter activity in HEL cells as compared to HeLa or endothelial cells, the later displaying an activity which was indistinguishable from background. To achieve the highest level of transfection efficiency in these experiments it was necessary to transfect the cells via different procedures. The HEL cells were transfected by electroporation, HeLa cells via a calcium-phosphate procedure, and the endothelial cells using Lipofectin. The results are presented as a percent of the 100% activity obtained when the same cell type was transfected with a luciferase gene under the control of a Rous sarcoma virus promoter (pRSV/LUC).

Consensus Binding Motifs within the 253 Nucleotides Preceding the Transcription Initiation Site—A number of previously described promoter binding motifs are present in the 253 nucleotides preceding the GP Ibα transcription initiation site (Fig. 4). To determine if any of these putative core elements contain sequences capable of specifically interacting with transcription factors, we performed gel shift analyses. For this purpose we synthesized 9 overlapping double-stranded fragments, as represented in Fig. 4 and labeled 1 through 9. Individual digonucleotides were labeled with [γ-32P]ATP, mixed with nuclear extracts from HEL or HeLa cells, and electrophoresed through nondenaturing polyacrylamide gels. Specific
ity for the protein-DNA complexes was established by the ability of the cold oligonucleotide (approximately 10- and 100-fold molar excess) to be a competitive inhibitor of the radiolabeled fragment.

Fig. 5 is a representative gel shift analysis using oligonucleotides 1 through 3 which correspond to nucleotides 212 to 264 of the GP Ibα promoter (Fig. 4). Four major bands, their relative mobility positions referred to as A through D, are considered specific DNA-protein complexes since they disappear in the presence of cold competitor (Fig. 5, panels A and B). The bands at position A were generated with oligonucleotide 1 and are present in both HEL and HeLa nuclear extracts. However, HeLa nuclear extracts are slightly different exhibiting a doublet band as compared to a single band observed with HEL nuclear extracts. Bands at positions C and D are observed using oligonucleotide 2 and are specific for HEL nuclear extracts (a comparison of panels A and B, Fig. 5). In similar experiments using oligonucleotides 4–9, several bands were observed that would disappear upon competition with cold oligonucleotides (Fig. 6, A and B). However, we observed no HEL-specific complexes using oligonucleotides 4–9 (comparing panels A and B, Fig. 6).

The observation that oligonucleotide 2 produced protein-

Fig. 3. Cell specificity of the GP Ibα promoter. As described in Fig. 2, the promoter activities of three different constructs were evaluated by transient transfection analysis of HEL, HeLa, and human umbilical vein endothelial cells (HUVECs). As before, the results are presented as a percentage of the activity obtained with parallel transfections using pRSV/LUC. Raw data from individual experiments using pRSV/LUC ranged from 50,000 to 300,000 light units from HEL cells; 35,000–100,000 light units from HeLa cells; 30,000–150,000 light units from HUVECs; and less than 300 light units from nontransfected cells.

DNA complexes using nuclear extracts from HEL cells, but not HeLa cells, prompted further investigation into the binding sequence(s) within oligonucleotide 2. In particular, we tested a potential GATA recognition sequence by generating a mutant oligonucleotide 2 containing the consensus 5'-GATAA-3' sequence changed to 5'-GAGCA-3' (Fig. 8A). As shown in Fig. 7, the mutant oligonucleotide 2 was unable to compete with the labeled normal oligonucleotide 2 for the DNA-protein complexes at positions C and D produced using HEL cell nuclear extracts. Thus, these bands most likely represent a specific GATA binding protein interacting with oligonucleotide 2.

Based on studies using other platelet-expressed genes which suggest that GATA proteins interact with other regulatory complexes, such as Ets motifs (16, 25, 26), we also examined the role of two potential Ets motifs (5'-GGAA-3') within the sequence of oligonucleotide 5 by generating a mutant oligonucleotide 2 containing the consensus 5'-GATAA-3' sequence changed to 5'-GAGCA-3' (Fig. 8A). As shown in Fig. 7, the mutant oligonucleotide 2 was unable to compete with the labeled normal oligonucleotide 2 for the DNA-protein complexes at positions C and D produced using HEL cell nuclear extracts. Thus, these bands most likely represent a specific GATA binding protein interacting with oligonucleotide 2.

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Promoter Strength of GATA and Ets Mutant Constructs—To evaluate directly the relevance of the GATA motif within the GP Ibα promoter we performed site-directed mutagenesis...
within the promoter construct containing 253 nucleotides preceding the transcription initiation site (p-253/LUC). The disruption of the potential GATA binding site reduced the promoter strength from 21 to 8% (Fig. 8). This experiment provided direct evidence that the GATA motif is required for full promoter activity, but also suggested that additional motifs can still support promoter activity even in the absence of a fully functional GATA.

To test the role of the potential Ets motif, we prepared a mutant construct and performed transfection assays using the same parent plasmid, p-253/LUC (Fig. 8). The disruption of the potential Ets motifs reduced the promoter strength from 21 to 6%, similar to that observed with the GATA mutations alone (Fig. 8). However, the coordinated effects of the GATA and Ets motifs were evident when the double mutation, containing both GATA and Ets mutant sequences, was evaluated in a similar experiment. This construct resulted in luciferase activity of less than 0.5%, a level indistinguishable from background (Fig. 8). Thus, the luciferase activity driven by the plasmid containing both GATA and Ets mutations was completely diminished identifying these motifs as essential elements of the GP Ibα core promoter.

**DISCUSSION**

Our parent plasmid, p-2738/LUC, contained 2,738 nucleotides 5' to the transcription start site (Fig. 2). Additionally, this plasmid contained all the DNA sequence preceding the initiating methionine codon of the precursor protein, which includes the sequence of exon I, sequence of the only intron, and the first 6 nucleotides of exon II (Fig. 2). Thus, p-2738/LUC contained...
sequences downstream of the transcription start site in the event that these sequences contributed to the activity of the promoter. Unidirectional deletion analysis of p-2738/LUC identified the shortest construct containing full promoter activity (p-253/LUC) as one containing 253 nucleotides 5' to the transcription start site. The progressive deletion from -2738 to -253 did result in a 2-fold, yet reproducible, difference in promoter activity (Fig. 2). Thus, the possibility exists for positive and negative enhancer sequences outside the 253 nucleotides containing the core elements of the GP Ib promoter. Our studies suggest for endothelial and HeLa cells that the GP Ib promoter is only capable of a basal level of transcription that is indistinguishable from background levels. Indeed, the in vivo expression of GP Ibα has not been systematically evaluated in nonhematopoietic cells, but a number of in vitro observations suggest that GP Ibα mRNA and/or protein expression may be induced by cytokines to detectable levels in endothelial cells and smooth muscle cells (27, 28). Additionally, some indirect evidence for GP Ibα antigen on the surface of activated endothelial cells has been shown by the ability of these cells to bind von Willebrand factor in a ristocetin-dependent manner (29), a characteristic property of platelet GP Ibα-V and its soluble ligand, von Willebrand factor (30, 31). The only in vivo corollary for these observations is an immunological identification of GP Ibα protein in inflamed tonsillar endothelium (27). Indeed, we have observed that unstimulated endothelial cells contain a very low, yet detectable, level of GP Ibα mRNA as seen by Northern blot analysis (data not shown), a finding originally reported by Rajagopalan et al. (28). With respect to our presented results (Fig. 3), it must be recognized that each of the three cell types (HEL, HUVECs, and HeLa) were transfected via different procedures in order to obtain the highest transfection efficiency for each cell type. Additionally, the determination of GP Ibα promoter activity within each cell type is based on the activity, assumed to be maximal, generated from a transfection with the viral promoter in pRSV/LUC. It is possible that each cell type responds to this viral promoter in a slightly different manner which would invalidate comparative conclusions among the three cell types (Fig. 3). If fact, a more likely explanation is that the GP Ibα promoter is somewhat "leaky" and capable of a low level of transcription in cells of nonhematopoietic lineage. However, in light of the subunit requirements for efficient surface expression of a GP Ib-IX complex (14), the in vivo relevance of such low levels of GP Ibα expression is questionable.

The choice of HEL cells as a model for evaluating gene expression for components of the GP Ib-IX-V complex is based on the fact that cDNAs for 3 subunits of the complex were synthesized from HEL mRNA, the exception being GP V. It can argued that HEL cells are not a perfect "model" megakaryocytic cell. In fact, studies evaluating megakaryocytic-specific promoters have lagged behind similar studies for other hematopoietic cells simply because the "perfect" cell line has not been identified. It is well known that HEL cells express genes of both the erythroid and megakaryocytic lineages, and the evidence is quite strong that erythrocytic and megakaryocytic lineages derive from a common progenitor cell (17, 32). However, studies of other platelet-specific promoters, specifically gene promoters for rat platelet factor 4 and the human platelet GP IIb receptor (α1b, integrin), have utilized HEL cells and have been merito-
rious (16, 26, 33, 34). Interestingly, Block et al. (26) have determined promoter strength for the rat GP I bb promoter by transfecting rat megakaryocytes. Comparing their results to that obtained by others studying the human GP I bb gene trans- fected into HEL cells corroborated the same promoter elements originally identified using HEL cells, but the actual promoter strength or quantitative conclusions were significantly differ- ent for the two cell types. One explanation for the conflicting quantitative results is that HEL cells express the same tran- scription factors as megakaryocytes, but the levels of individual transcription factors differ resulting in different quantitative conclusions.

The identification of essential GATA and Ets binding ele- ments within the GP I bb promoter is consistent with previous reports identifying these elements as major regulatory regions in megakaryocytic-specific genes (16). The two previously studied platelet-specific genes, platelet factor 4 and the platelet integrin subunit, GP II bb (cII bb), have confirmed the important role of GATA-binding proteins for megakaryocytic-specific gene regulation (16, 26, 33, 34). The GATA family of proteins are related by a high degree of sequence similarity throughout their zinc finger-binding domains (35). GATA-1 is primarily expressed in endodermally-derived tissues and heart (35–37). GATA-3 is most abundant in T lymphocytes; and GATA-4 is expressed in platelet factor 4 that some GATA proteins, such as GATA-2, may act-

Our results also identified an essential binding site for an Ets-related protein. The Ets family of transcription factors recognize a 5'-GGA(A/T)-3' motif (25). The GP I bb promoter contains two potential Ets motifs between −138 and −153 (Fig. 4). Our mutagenesis targeted both potential binding sites by substituting the consensus 5'-GGA(A/T)-3' (5'-GAT(A/T)-3'). However, the expression of an individual GATA binding factor cannot be the sole factor for megakaryo-

cytic-specific expression; in fact, the situation may be much more complex (39). Aird et al. (39) have proposed for platelet factor 4 that some GATA proteins, such as GATA-2, may actu-

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