Characterization of Regulatory Elements in the 5′-Flanking Region of the Rat GPIIb Gene by Studies in a Primary Rat Marrow Culture System

By Karen L. Block, Katya Ravid, Quy H. Phung, and Mortimer Poncz

Glycoprotein (GPIIb/IIa, an integrin complex found on the surface of platelets, is a receptor for fibrinogen and other ligands, and is involved in platelet aggregation. Because GPIIb is specifically expressed in megakaryocytes, we have studied the 5′-flanking region of the rat (r) GPIIb gene as a model of a megakaryocyte-specific gene. The studies presented here used a rat marrow expression system, which allows the study of primary cells undergoing terminal differentiation into megakaryocytes. The determination of megakaryocyte-specific expression of DNA constructs was possible by immunomagnetically separating megakaryocytes from total bone marrow cells. Transient expression constructs, containing varying lengths of the 5′-flanking region from -39 to -912 bp, localized a regulatory element between -460 and -439 bp upstream of the transcriptional start site. This region contains a GATA consensus binding element between -457 and -454 (GATAcore). Further constructs demonstrated that this GATA binding element was indeed essential for expression. A 25-bp substitution, covering the region -450 to -426 immediately downstream of the GATAcore, demonstrated that this region was essential for full expression, which suggests that this region may interact with the GATAcore site in promoting high-level lineage-specific expression. To define regulatory elements between the GATAcore and the transcriptional start site further, we tested additional constructs derived from the original -912 construct; each of which contained the GATAcore but had different 50-bp deletions from -450 to the start site. Virtually all of these constructs continued to show high-level tissue-specific expression. The deleted -150 to -101 construct had twice the level of expression of the full-length wild-type construct; therefore, this region may contain a negative regulatory element. Comparison of our data with expression studies performed with the 5′-region of the human GPIIb gene using HEL cells, a cell line with some megakaryocytic properties, demonstrates significant differences, which may reflect our use of primary rat bone marrow cells. In particular, our study points to the importance of the GATAcore for high levels of GPIIb expression in developing megakaryocytes.

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This system was used because it allowed studies of the tissue-specific expression of the \(5'\)-flanking region in megakaryocyte precursors undergoing normal differentiation into megakaryocytes. It was anticipated that regulatory regions would be conserved across species and that domains of biologic importance in the regulation of gene expression would be preserved.

We have previously published a partial sequence of the \(5'\)-flanking and untranslated regions of the \(r\)GPIIb gene and showed that the sequence is well preserved near the transcriptional start site when compared with its human counterpart. However, we show that domain A has a more potent effect (\(~15\)-fold) and that the GATA sequence in domain D is a very potent regulator of GPIIb expression (\(~50\)-fold). Furthermore, we demonstrate that a region immediately downstream from this domain also acts as a strong positive regulatory element. A potential suppressor site between -150 and -101 is also defined. These studies provide further insights into the regulatory elements involved in megakaryocyte-specific expression of the GPIIb gene.

**MATERIALS AND METHODS**

**Subcloning and characterization of the \(5'\)-flanking region of the \(r\)GPIIb gene.** \(5'\)-GPIIb genomic clones were isolated from a partial \(Sax3\)A rat genomic library in EMBL3A as described previously.
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A 2.2-kb SstI restriction fragment was then subcloned into linearized M13mp18. The 5'-flanking region of rGPIIb (~1.1 kb) was sequenced using the dideoxy chain termination technique.25 The rat amplification and subcloning, as indicated in the Materials and Methods.

**Plasmid construction.** The full-length 912-bp construct was made by overlap amplification,26 a polymerase chain reaction (PCR) technique, which fused 912 bp of the 5'-flanking region plus the 5'-untranslated region of the rGPIIb gene directly in front of the initiating methionine of the human growth hormone (hGH) gene in pOGH (Nichols Institute Diagnostics, San Juan Capistrano, CA) (Fig 2). The necessary portion of the hGH gene was amplified from the pOGH vector using a 5' primer containing a portion of the 5'-untranslated (5'UT) region of the rGPIIb gene fused to the beginning of the coding region of the hGH gene. The 3' primer was located downstream of an SstI site in pOGH. Nine hundred twelve base pairs of the rat 5'-flanking region was amplified from the 2.2-kb SstI fragment of the rGPIIb gene in single-stranded M13mp18, described above, using a 5' primer containing an artificial BamHI site near its 5' end. The 3' primer is the complementary sequence of the 5'UT region of the rGPIIb gene. These two PCR products were then unannealed and reamplified with the two outermost primers: the 5' 912 primer with the BamHI site and the 3' primer located downstream of the SstI site in the pOGH vector. The product was then cut with BamHI and SstI and ligated into a similarly cut pOGH vector. First PCR reactions

**Second PCR overlap reaction**

**Digest with Bam HI/Sst I**

**Subclone into Bam HI/Sst I cut pOGH**

**5'-GPIIb/pOGH**

Fig 2. Design of rGPIIb expression constructs. The strategy for expression vector construct design uses the 5'-flanking region of the rGPIIb gene in M13mp18 phage, the pOGH vector, and overlap amplification and subcloning, as indicated in the Materials and Methods.

**Cell culture and electroporation.** Rat bone marrow cells were isolated essentially as previously described.27 Femurs and tibias isolated from CO2-narcotized rats (~400 g) were placed into cold CATCH medium (Hank’s balanced salt solution [no. Ca2+ or Mg2+], 0.38% Na citrate, 1 mmol/L adenine, 2 mmol/L theophylline, 5% fetal bovine serum, pH 7.4). Marrow cells were flushed out of the bones using a 18-gauge needle filled with CATCH medium (5 mL per femur and tibia). Marrow cells were dispersed in CATCH medium with a syringe and filtered through a 100- to 200-μm mesh nylon filter (Tetko, New York, NY). The cells were pelleted at 400 × g for 5 minutes, resuspended in an equal volume of lysis buffer (Tris Base 2.06 g/L, NaCl 1.75 g/L), and incubated at 37°C for 10 minutes to lyse red blood cells. Cells were then pelleted as above, washed twice with CATCH medium, and the total yield of nucleated cells determined. The remaining cells were resuspended in electroporation buffer (30.8 mmol/L NaCl, 120.7 mmol/L KCl, 8.1 mmol/L Na2HPO4, 1.46 mmol/L KH2PO4, 5 mmol/L MgCl2). One hundred micrograms of circular plasmid DNA was added to 25 × 106 rat bone marrow cells in 0.4-cm cuvettes. This amount of DNA was comparable with that used in previous studies.28 The cells were incubated on ice for 15 minutes and then electroporated (Cell-Porator; Life Technologies, Gaithersburg, MD) at 230 V and 800 μF, surrounded by ice. After electroporation, cells were allowed to recover for 10 minutes on ice and then for 15 minutes at room temperature. One milliliter growth medium (Iscove’s Modified Dulbecco’s Medium [IMDM]; Life Technologies) supplemented with penicillin/streptomycin, L-glutamine, and 20% horse serum (JRH Biosciences, Lenexa, KS) was then added to each sample. The samples were spun at 380 × g for 5 minutes, and the pellet was resuspended in 3 mL of growth medium. Bone marrow cells after electroporation were grown in six-well, 35-mm plates for 3 days at 37°C in 5% CO2. These were then either assayed directly for hGH expression or immunomagnetically depleted of megakaryocytes and then assayed for hGH. The amount of DNA used for transfection, electroporation conditions, and culturing time posttransfection were based on preliminary titration experiments.

**Radioimmunoassay.** Nonadherent rat bone marrow cells were collected from the six-well plates 3 days posttransfection, washed with phosphate-buffered saline (138 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na2HPO4, 1.2 mmol/L KH2PO4, pH 7.1), resuspended in 0.3 mL of D2O, and lysed by freezing and thawing twice. Preliminary studies showed that only 1% of the cells were adherent to the plates, and these cells do not express hGH above background levels. One hundred microliters of lysed cells was then assayed for hGH by radioimmunoassay according to the protocol provided by the manufacturer (Nichols Institute Diagnostics).

**Hirt assay.** The Hirt assay29 was used to determine the relative transfection efficiencies of the different plasmids tested, as described previously.22 To this end, electroporated, cultured marrow cells (0.7 mL) were lysed by incubation with 0.7 mL of Hirt solution (0.6% sodium dodecyl sulfate, 0.01 mol/L EDTA, and 7 μg/mL calf thymus DNA, pH 7.5) at 4°C. Control rGPIIb/pOGH plasmid DNA (175 ng) was added. This control contained a GPIIb/hGH BamHI/SstI fragment of a different size compared with those constructs under study. After a 2-hour incubation, NaCl was then added to 1 mol/L and the
sample was incubated overnight at 4°C. The lysates were centrifuged at 7,000 X g for 20 minutes at 4°C. 0.5 mL of the supernatants were treated with DNase-free RNase (50 μg/mL), and the DNA was extracted with phenol-chloroform and chloroform, precipitated with 100% ethanol, and washed with 70% ethanol. After extraction and purification, the DNA was digested with SstI and BamHI and quantitated by Southern blot analysis using the 500-bp BamHI/SstI fragment from hGH as a probe. Because this region is common to all constructs, we were able to examine simultaneously both the sample DNA fragment and the different-sized control DNA fragment on the same gel. An Ambis β-Analyzer (Ambis Systems, San Diego, CA) was used to quantitate the bands. The result of each hGH assay was then normalized to the quantitated sample DNA to account for transfection efficiency.

Demonstration of megakaryocyte-specific expression. The primary method used for the determination of megakaryocyte specificity of expression involved immunomagnetic separation of the electroporated marrow followed by reporter gene assay. Forty microliters of primary antibody (0.898 mg/mL), a mouse antirat GPIIbIIIa monoclonal antibody (a generous gift from Drs Takamoto Suzuki and Hiroshi Miyazaki29), was added to 3-day posttransfection, electroporated, cultured marrow cells (4 X 10⁶ cells), then resuspended in 0.5 mL of modified growth medium (MGM) (IMDM; troporated, cultured marrow cells (4 X 10⁶ cells), then resuspended in 0.5 mL of modified growth medium (MGM) (IMDM; 5% horse serum, 1 mmol/L adenosine [Sigma, St Louis, MO], 2 mmol/L theophylline [Sigma]) and incubated for 45 minutes at room temperature on a gyrorotary platform. After incubation, the samples were washed twice with MGM. Iron beads (Dynal, Oslo, Norway) coated with bead-negative, megakaryocyte-depleted samples (supernatant and constructs, we were able to examine simultaneously both the sample and the different-sized control DNA fragment on the same gel. An Ambis β-Analyzer (Ambis Systems, San Diego, CA) was used to quantitate the bands. The result of each hGH assay was then normalized to the quantitated sample DNA to account for transfection efficiency.

RESULTS

Identification of regulatory domains within the 5'-flanking region of the rGPIIb gene. Nine hundred sixty-seven base pairs of the 5'-flanking region of the rGPIIb gene were determined. A dot matrix comparison of the rat 5'-flanking region and its human homologue was performed to determine overall homology. As shown in Fig 1B, homology was well conserved for the first 600 bp upstream of the transcriptional start site. There also appears to be additional homology between the rat sequences at ~750 to ~950 bp and the human sequences of ~950 and ~1150.

We used transient expression experiments to identify functionally significant regulatory regions within these conserved domains of the rGPIIb gene 5'-flanking region. Nine hundred twelve base pairs of the 5'-flanking region upstream from the transcriptional start site and the 32-bp 5'UT region were linked to the hGH gene, as described in the Materials and Methods, and served as the full-length, wild-type expression vector in the studies described below.

Transient expression studies in primary rat bone marrow demonstrated that the 912 construct had approximately similar levels of hGH expression as the previously described PF4 5'-flanking region construct (2) (data not shown). To define cis-acting regulatory elements in this 5'-flanking region, truncated constructs containing shorter lengths of the 5'-flanking region were generated using 5' primers for PCR amplification that were downstream of the -912 primer, following the same strategy as discussed above for the full-length construct (Fig 2). Rat bone marrow cells were transfected with these constructs. As shown in Fig 3A, there was a slight decrease in activity with the different constructs, gradually truncated to contain 460 bp upstream of the transcriptional start. However, a precipitous decrease in expression to the level of the promotorless vector (~1% of wild-type, full-length) occurred with constructs containing 439 bp or less of the 5'-flanking region. This suggested that the 21-bp region between -460 and -439 played an important role in the regulation of expression of the GPIIb gene.

Experiments were then performed to characterize further the domain between -460 and -439. A 912Δ construct, in which the -460 to -439 region of the rGPIIb gene was deleted, had background levels of expression (Fig 3B). Similarly, an additional truncated construct, beginning at base -453 immediately downstream of a GATA-binding consensus sequence, also had background levels of expression (Fig 3B). These constructs further localized this important regulatory element to a 7-bp region, containing a GATA-binding consensus sequence, which we have termed GATA₄₃₉. Elimination of this region has a marked effect on expression.

Regulatory elements between -453 and the transcription start site. Because all of the 5'-truncated constructs missing the GATA₄₃₉ element (containing <454 bp of the 5'-flanking region) had background levels of expression, we were not able to determine whether other regions closer to the start site would affect expression. To test for additional elements, 50-bp deletions were made 3' to the GATA₄₃₉ element in the full-length 912 construct, leaving the GATA₄₃₉ intact, as depicted in Fig 4A. Most of the constructs expressed at greater than 30% of the wild-type construct. However, deletion constructs, with the bases between -450 and -401 and between -400 and -351 removed, had background levels of expression comparable to the promotorless vector.

We then constructed four substitutions in the full-length.
wild-type expression construct, each containing a noncomplementary 25-bp inversion. These sequence substitutions covered the following regions: -450 to -426, -425 to -401, -400 to -376, and -375 to -351. The -450 to -426 substitution caused a decrease in expression to 17% of the full-length, wild-type, while the other three had approximately 68% activity (Fig 4B). These data suggest that the region immediately downstream of the GATA54 element, between -450 and -426, is important in the regulation of expression of the GPIIb gene.

As shown in Fig 4A, a deletion construct (100/51Δ), with the bases between -100 and -51 removed, had approximately 35% of wild-type expression levels. In addition, a deletion construct (50/-1Δ), with the bases from -50 to -1 removed, also had a low level of expression (7%). Of interest was the increased level of expression with 150/101Δ construct, which expressed at twice the level of the wild-type vector and over three times higher than any of the other deletional constructs. Such an increase in expression suggests that there may be a negative regulatory element in this region. This increase over the wild-type was statistically significant within 99% confidence limits.

Because elimination of the rat homologue of domain A, a region that contains a GATA-binding consensus sequence near -54, still had 35% of the wild-type expression, we wondered whether this GATA54 element had promoter activity in our rat expression system. Elimination of the GATA354 element in our truncation studies had a much more marked effect on expression, decreasing expression by 2 orders of magnitude, while elimination of the GATA54 element in our 50-bp deletion constructs decreased expression by only threefold. Therefore, two point-mutation constructs were made: the GATA54 construct contains a G to C mutation at -54 in the full-length 912 construct, and the GATA254 construct contains a similar change involving a C to G mutation at -454. These point mutations destroy the GATA binding sequence, because the G-A-T portion of the consensus sequence is important for GATA nuclear protein binding. As expected, the mutated GATA54 construct expressed at background levels (Fig 4B). However, the mutated GATA354 construct also expressed at low levels, approximately 11% of the full-length, wild-type construct (Fig 4B). This suggested that this GATA-binding consensus sequence may also be an important element in promoting GPIIb expression.

We also tested, by point mutation, whether either the Ets-consensus binding sequence at -512 and the complement Ets-consensus binding sequence at -35 affected expression. We mutated the consensus core GGAA sequence in these Ets-consensus binding sequences to GGTA in the full-length, wild-type construct. We found that the Ets512 mutation decreased expression to 32% of the full-length, wild-type (Fig 4B). This level of decreased expression was consistent with our truncated constructs in which the 460 construct expressed at 55% of the 540 construct, which contained the Ets512 element, suggesting that elimination of the Ets512 element was responsible for the decreased expression. The Ets512 mutation construct ex-
pressed at 5% of the full-length, wild-type construct (Fig 4B), which was consistent with our deletional construct in which removal of the region between -50 and -1 decreased expression to 7%. Again, these data suggest that the decreased expression observed with the deletional construct can be accounted for by the elimination of an Ets-consensus binding sequence. PU.1, a member of the Ets family of transcription factors, has been shown to interact with TFIID. It has been suggested that in TATA-less promoters, Ets proteins binding in regions normally containing TATA boxes
may be involved in tethering the transcriptional initiation complex.18

线表性表达不同rGPllb基因构。这些转基因表达研究在多重线性原血细胞中进行。因此有必要确定hGH表达在嵌合血细胞中的特异性。早期研究通过botrocetin移除嵌合血细胞，发现大于93%的hGH测量值表现在嵌合血细胞中，与PF4/hGH表达载体在类似条件下表达所见一致。这表明GPllb 5'-反向区域是嵌合血细胞特异性表达，类似于PF4。然而，由于botrocetin的有限可用性，我们开发了替代方法用于嵌合血细胞分离。为此，我们设计了一种免疫磁性分离方案，用于从3天后细胞培养中去除所有GPllb/IIIa阳性细胞。少于1%的总细胞附着在细胞上。形态学检查这些细胞显示它们是大型嵌合血细胞。当抗rat GPllb/IIIa抗体未包含时，没有这样的大型细胞存在。线表特异性被确定为所有大于10%表达的全长，野生型912构。用作金标准的抗GPIIb/IIIa抗体被不包括。线表特异性被确定为所有大于10%表达的全长，野生型912构。线表特异性被确定为所有大于10%表达的全长，野生型912构。

DISCUSSION

The data derived from our analyses of the 5'-flanking region of the rGPIIb gene in a transient assay primary cell system are summarized in Fig 1A, which compares our results with those derived for the human GPIIb gene based primarily on transient expression studies in HEL cells. The change in hGH expression resulting from elimination, deletion, or point mutations in defined regions is indicated. In general, our results corroborate some of the findings previously reported.9,10 Domains A, D, and E involve regions important for lineage-specific expression of the GPIIb gene. However, a number of important quantitative differences were noted. In studies with HEL cells, the various promoter elements all had approximately equal effects on expression, decreasing expression threefold. In our studies, elimination of domain E, which contains the Ets-1 site, decreased expression twofold (Fig 1A). In contrast, expression obtained with a mutation of the Ets-consensus site at -35 resulted in a 15-fold decrease in expression, as compared with a threefold decrease observed in studies performed with the human homologue in HEL cells. Moreover, deletion, truncation, or mutation of the GATA site in the rat 5'-flanking region in our primary rat marrow system decreased expression by approximately 50-fold (Fig 1A). Thus, our studies clearly demonstrate, for the first time, the crucial role of this GATA-binding element at -454 in GPIIb expression. Mobility-shift studies using HEL cell nuclear extracts and a polyclonal anti-GATA-1 antibody suggest that GATA-1 binds to the GATA site.37 Of interest, it appears that while GATA-1 is expressed during early megakaryocytopoiesis, this expression decreases with terminal differentiation.38,39 In contrast, GATA-2 is expressed in both megakaryocyte precursors and mature megakaryocytes.40 How these nuclear regulatory factors interact to result in high-level terminal expression of GPIIb remains to be elucidated. Also of interest is the finding that a GATA-binding consensus sequence at -31 in the PF4 gene promotes high-level tissue-specific expression of the PF4 gene in megakaryocytes and inhibits PF4 expression in other lineages.22,41 Thus, primary hematopoietic expression studies of two different platelet-specific genes both imply an important role for GATA-binding proteins in megakaryocyte-specific gene regulation.

The GATA(54) binding element also affected expression. Its
deletion in the 100/51Δ construct resulted in a threefold decrease in expression, while a point mutation in this GATA site within the full-length 912 construct decreased expression ninefold. In contrast, mutation of the homologous GATA-binding element in the human studies had no effect within the full-length, wild-type construct, and only decreased expression sixfold when combined with a mutation in the domain D GATA-binding element.37

The differences between the presented data and previous data on hGPIIb may be due to several reasons. Part of the difference may be caused by species differences, even though there is a high degree of sequence homology between rat and human GPIIb in these specific 5′-flanking regions, as illustrated in Fig 1B. The differences may also reflect the differences in the expression level of the full-length construct to which expression by the mutant constructs were compared. Previous publications8,9 used approximately 800 bp of the 5′-flanking region in their full-length expression construct. This is comparable to our 798 construct, which had only two thirds of the expression of our 912 construct (Fig 3A). However, this difference in full-length construct expression levels cannot account for the marked differences observed with the removal of important cis elements.

It is therefore likely that a significant portion of the quantitative differences in the importance of different cis elements demonstrated in the presented studies is due to the use of primary marrow cells undergoing normal differentiation into mature megakaryocytes, rather than cell lines such as HEL cells. HEL cells are aneuploid and while they exhibit some megakaryocytic features such as expressing GPIIIb and platelet factor 4 (PF4),41 the level of GPIIb/IIIa complex and PF4 in HEL cells is lower compared with those in platelets. Further, HEL cells do not express other megakaryocyte-specific genes such as Gα42 or βTG (unpublished observations), nor do they develop α-granules or a membrane demarcation system. They also express a number of erythroid43 and macrophage-like44 features, which may perturb expression levels of megakaryocytic genes.

In addition, our studies appear to have defined two new regulatory regions in the 5′-flanking region of the rGPIIb gene. One of these areas involves the region immediately downstream of the GATAα4-binding element. Deletions in the −450 to −401 and the −400 to −351 regions greatly decreased expression. Noncomplementary 25-bp inversions in these regions suggest that the −450 to −426 region may contain a regulatory element. Further studies are needed to confirm these findings in that the noncomplementary inversions would preserve any simple repeated sequences and palindromic structures. Confirmation of the above findings would be of interest in that this region is immediately downstream of the GATAα4 element, which suggests that there may be interactions between these two sites. It has been shown that bound trans-acting GATA proteins may interact with other nearby regulatory complexes such as Sp1, Ets, and transcription initiation complexes.5,46

Based on our deletional constructs, we note that the sequence between −150 and −101 bp upstream of the transcriptional start site may contain a negative regulatory element. Elimination of this sequence increases expression by threefold over other deletional constructs and twofold over the wild-type expression construct. A potential suppressor element containing a 26-bp stretch of pyrimidine residues has been described in the 5′-flanking region of the rPF4 gene.39 The −150 to −101 region contains a 13-bp pyrimidine stretch. Further studies are underway to define the minimal suppressor element and its role in the megakaryocyte-specific nature of GPIIb expression.

In summary, our transient expression studies of the rGPIIb 5′-flanking region in primary marrow cells demonstrate important features of the regulation of this gene (Fig 1A). We show that the GATAα4 element is an extremely important regulatory region in the 5′-flanking region, capable of affecting GPIIb expression in megakaryocytes by 50-fold. In addition, the Etsβ5 has a significant effect on expression, as does the GATAα4. The Etsβ5 has only a minimal effect on expression. In addition, we show that a region from −450 to −426 may contain a positive regulatory element. If confirmed, it is possible that this region may interact with the GATAα4 regulatory domain. Another regulatory region demonstrated by deletional constructs involves a potential silencer element from −150 to −101. Further characterization of these cis-acting elements and the trans-acting factors that bind to these regions are underway and should elucidate the mechanisms by which megakaryocyte-specific expression is achieved for GPIIb. Such studies may be helpful to determine the mechanisms by which other lineage-specific genes are expressed during megakaryocytopoiesis.

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