An Sp1-binding Silencer Element Is a Critical Negative Regulator of the Megakaryocyte-specific αIIb Gene*

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The Sp1 family of transcription factors are often involved in the regulated expression of TATA-less genes, frequently enhancing gene transcription. In this paper, we demonstrate that an Sp1-binding element inhibits the expression of the megakaryocyte-specific αIIb gene in all cell lines tested and that this inhibition is actively overcome only in megakaryocyte-like cell lines. We had noted previously in primary megakaryocytes that a 50-base pair (bp) deletion from −150 to −101 bp in the rat αIIb promoter region resulted in increased expression. We now show that deletion of this region markedly increased expression in both megakaryocytic and non-megakaryocytic cell lines, eliminating the tissue specificity of the αIIb promoter. Electrophoretic mobility shift assays (EMSA) defined a single complex, which bound to a −145 to −125 bp subregion. Point mutations within this region, localized the critical point of binding around bases −130/−135, and expression studies showed that introduction of the −130/−135 mutation into the rat αIIb promoter had a comparable result to that seen with the 50-bp deletion. EMSA studies with the homologous human αIIb promoter region gave an identical migrating band. Southwestern blots of HeLa nuclear proteins with a 101-kDa protein, the known molecular weight of Sp1. Confirmation that this region of the αIIb gene promoter bound Sp1 was accomplished using EMSA studies with an Sp1 consensus probe, anti-Sp1 and -Sp3 antibodies, and recombinant Sp1 protein. Further support for the role of Sp1 in the silencing of the αIIb promoter was obtained using a Gal4 binding site substitution for the silencer region of αIIb and co-expression vectors. Ectopic reinsertion of the −150 to −101 bp region, back into the −150 to −101 bp deleted promoter, enhanced rather than decreased expression, suggesting that Sp1’s inhibitory role at −130/−135 depends on its local interactions. In summary, we believe that we have identified a cross-species, non-consensus Sp1-binding site that binds Sp1 and that acts as a silencer of αIIb expression in many cell lines. A model is presented as to how this Sp1-binding silencer element contributes to the megakaryocytic-specific expression of αIIb gene.

Platelets have a central role in thrombus formation. These anuclear cytoplasmic fragments are derived from bone marrow megakaryocytes and are highly differentiated (1). One of the specialized features found on the platelet surface is the αIIbβ3 (also known as glycoprotein IIb/IIIa or CD41b) integrin receptor (2). This receptor is densely packed on the platelet surface. Following platelet activation, this receptor binds fibrinogen and plays an important role in platelet aggregation (2). Normally, αIIb/β3 is only found on developing megakaryocytes and circulating platelets. This is due to the tissue-specific nature of the αIIb subunit. While β3 is expressed in several different cell types (3), the αIIb gene is normally limited in its expression to megakaryocytes (4). We have shown previously that the αIIb gene is a TATA-less gene comprising 30 exons, extending over an 18-kilobase pair region of the long arm of chromosome 17 (5, 6).

The αIIb gene is presently the best studied megakaryocyte-specific gene. Better understanding of the regulation of αIIb transcription in a lineage-restricted fashion allows us to learn more about the molecular mechanisms controlling hematopoietic differentiation. It may also allows us to develop new approaches for regulating gene expression in developing megakaryocytes and modulating platelet thrombogenic tendency. Other investigators defined four important elements in the 5′-flanking region of the human αIIb gene that promote tissue-specific expression: two pairs of GATA- and Ets-binding sites, one proximal to the transcriptional start site and one more distal (7–10). Deletion or mutation of any of these GATA- or Ets-binding sites had a 2–3-fold effect on the level of reporter gene expression in transient expression studies in megakaryocytic cell lines. Studying the rat αIIb promoter in a primary rat marrow system, we found the same four regulatory regions, but demonstrated a significant quantitative difference in promoter activity; the distal GATA-binding site at −454 bp (GATAATG) had a 50-fold effect on expression and was essential for observing any promoter activity (11). We have since shown that these quantitative differences in promoter strength were not species-specific differences in the promoter region, but due to differences in the cell systems studied. When the same rat αIIb promoter constructs were restudied in a megakaryocytic cell line (HEL), the results seen were indistinguishable from the human data (12).

In addition, we used the same rat primary marrow transient expression system to define a GA-rich Sp1-binding site at −14 bp (Sp14) (13). We showed that the complex bound to this site interacted with the complex bound at the proximal Ets-binding site at −35 bp (Ets35). The Ets35 site appears to tether Sp1 to its binding site, and we proposed that this tethered Sp1 is

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1 The abbreviations used are: bp, base pair(s); EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction; CMV, cytomegalovirus.
imporant in mooring the transcriptional initiation complex to the TATA-less αIIb gene.

While carrying out these studies, we noted that, when a series of 50-bp deletions were made in a 912-bp αIIb 5'-flanking region reporter construct, leaving the GATAαIIb intact and beginning the deletions at −450 bp, all of the constructs had decreased activity compared with the wild-type construct, except for one construct that expressed twice as well as the wild type. We proposed that either this region between −150 bp and −101 bp contained a silencer element or the increase in expression was due to architectural disruption of the promoter region by the deletion (11). Since then, two other groups have defined silencer elements near this region in the human αIIb promoter region (14, 15). The common element between these two studies suggests that there is a region at −120 to −116 bp in the human αIIb 5'-flanking region 5'-ATGAG-3' (corresponding to the rat −113 to −109 bp region) that binds a silencer complex. In this paper, we demonstrate a different site upstream as being important in silencing αIIb expression. We show that an Sp1-binding site that is conserved across species is the center of this silencer element and that this site appears to bind Sp3 (16). Mutation of this site leads to high levels of expression in both megakaryocytic and non-megakaryocytic cell lines, and therefore eliminates the tissue specificity of the αIIb promoter. This site does not appear to bind to Sp3, a known negative regulator in the Sp1 family (17–19). Thus, it appears that the silencer domain of the αIIb gene involves an increasingly recognized role of Sp1 as a negative regulator. We show that ectopic reintroduction of the silencer domain into the silencer-deleted αIIb promoter enhanced rather than decreased expression, suggesting that Sp1 silencing effect may depend on local interactions with other bound nuclear proteins. A model is presented as to how the silencer element may function in αIIb expression.

MATERIALS AND METHODS

Cell Culture—CHRF-288–11 (called CHRF) (20), a megakaryoblastic cell line generously provided by Dr. Michael Lieberman (University of Cincinnati, Cincinnati, OH), was maintained in RPMI medium 1640 supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Sigma). All other cell lines used in this study were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). K562 (chronic myelogenous leukemia, ATCC CCL-2), HEL (erythroleukemia, ATCC TIB-180), and HL-60 (promyelocytic leukemia, ATCC CCL-240) cells were grown in RPMI medium 1640 containing 10% FBS. HeLa (cervical epitheloid carcinoma, ATCC CCL-2) and NIH 3T3 cells (murine embryonic fibroblast, ATCC CRL-1658) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. All media contain 100 units/ml penicillin, 100 units/ml streptomycin, and 200 μg/ml of t-glutamine. K562 cells were incubated with 40 μM phorbol myristate acetate (PMA) for 5 days to induce megakaryocytic differentiation (14).

Plasmid Construction—Both 912 and 453 base pairs of rat αIIb 5'-flanking region were PCR-amplified from a 2.2-kilobase pair BSfI fragment of the rat 5'-αIIb gene that was isolated from a partial Sau3A rat genomic library (5) and subcloned into single-stranded M13mp18 (21). The sense primers for PCR were designed according to published sequence (5) with a BamHI site flanking their 5' ends. The antisense primer 5'-AAGCTTTCTCCTCTCTCCCAATGTTG-3' includes the untranslated region of rat αIIb gene and a HindIII site (underlined). PCR products were cut with both BamHI and HindIII, and subcloned into BglII/HindIII-digested promoter-less luciferase reporter vector pGL3-basic (Promega Corp., Madison, WI). −150 to −101 bp deletion, and CC → AA substitutions at −140/−141 bp, −135/−136 bp, and −130/−131 bp, were then created by overlapping PCR (11) using 912bp-pGL3 or 453bp-pGL3 construct as templates. All the PCR-based constructs were sequenced to verify PCR-induced mutations.

A single Gal4 binding site 5'-GGGAGTACTGCCTCCCAGGGA-3' (21) was substituted for the rat αIIb promoter sequence between −145 bp and −125 bp in the 453bp-pGL3 construct using a similar overlapping PCR strategy. The Gal4/Sp1 expression vectors pSG424 (no Sp1 fusion), pSG-Sp1NT (containing Sp1 amino acid residues 89-778, which include domains A–D of Sp1), pSG-Sp1A&B (containing Sp1 domain A and B), and pSG-Sp1A (containing Sp1 domain A) were generously provided by Robert Tjian (University of California, Berkeley, CA). All these vectors contain the Gal4 binding domain, amino acids 1–147 of the Gal4 protein, in addition to the various domains of the Sp1 transcription factor (22) and are driven by an SV40 promoter.

We have ectopically reinserted the −150 to −101 bp region back into the 453bp-pGL3 vector (Δ) refers to −150 to −101 bp deletion) after digestion of the vector with SacI, whose restriction site is immediately upstream of the rat αIIb promoter region. The complementary sense and antisense oligonucleotides covering the sequence of −150 to −101 bp region were synthesized by Integrated DNA Technologies (Corvallel, CA) with over-hanging SacI ends. Annealed double-stranded DNA was T4 ligated into the cut 453bp-pGL3 vector. The orientation of the inserted 50-bp region and the copy number of this insert were determined by sequence analysis.

pGL3-basic vector was used as a negative control in transfection assays. The SV40-pGL3 vector (Promega Corp.) containing both SV40 promoter and enhancer sequence, was included as a positive control, since it results in strong expression of luciferase gene in many types of mammalian cells. The pCMVβ vector (CLONTECH), which contains the β-galactosidase gene driven by the CMV promoter, was used as an internal control for transfection efficiency.

Transfection and Reporter Gene Assays—For CHRF cell transfection, cells were seeded at 0.3×10⁶ cells/ml. After growing for 2 days, cells were collected and resuspended in electrophoresis buffer (30.8 mmol/liter NaCl, 120.7 mmol/liter KCl, 8.1 mmol/liter Na2HPO4, 1.46 mmol/liter KH2PO4, 5 mmol/liter MgCl2) at a concentration of 30 × 10⁶ cells/ml. Thirty micrograms of assay plasmid DNA and 20 μg of pCMVβ were added to 0.5 ml of cells in 0.4-cm electroporation cuvettes. After a 15-min incubation on ice, these cells were electroporated by Cell-Porator (Life Technologies, Inc.) at 220 V and 800 millifarads. Cells were then allowed to recover on ice for 10 min and at room temperature for 15 min. After washing with the complete growth medium, cells were resuspended in 3 ml of growth medium and grown in six-well tissue culture plates for 24 h before being assayed for luciferase activity. HEL, HeLa, NIH 3T3, and HL-60 cells were transfected using LipofectAMINE™ reagent from Life Technologies, Inc. Briefly, 2.5 μg of assay plasmid DNA and 0.5 μg of internal control DNA pCMVβ were incubated with 10 μl of LipofectAMINE™ reagent in Opti-MEM I reduced serum medium (Life Technologies, Inc.) for 30 min. The formed DNA-liposome complexes were added to either exponentially growing HEL and HL-60 cells with 1.5 × 10⁶ cells/sample or 60–70% confluent HeLa and NIH 3T3 cells grown in six-well tissue culture plates. All cells were washed with Opti-MEM medium before the addition of DNA-liposome mixture. A 5-h incubation at 37 °C in a CO2 incubator followed. After being washed with the appropriate complete growth medium, cells were resuspended in 3 ml (HEL and HL-60) or 2 ml (HeLa and NIH 3T3) of the same medium in six-well tissue culture plates for 48 h before the reporter gene assays. For Gal4/Sp1 cotransfection assays in HeLa cells, 2 μg of the 453Gal4-pGL3 vector (having the Gal4 binding site inserted into the −145 to −125 region of the 453bp rat αIIb promoter), 1 μg of the various Gal4/Sp1 expression vectors, and 0.5 μg of pCMVβ, were cotransfected to each well of cells using 12.5 μl of LipofectAMINE™ reagent.

Cells in each sample were collected, washed twice with phosphate-buffered saline, and lysed in 100 μl of lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.6, 1 mM diithiothreitol). For the luciferase assay, 20 μl of cell lysate were mixed with 350 μl of Reaction Mix (100 mM Tris, pH 7.8, 15 mM MgSO4) and 10 μl of 100 mM ATP in a luminometer cuvette. The relative light units for each sample were immediately measured in a luminometer that automatically pumps into each cuvette 100 μl of 1 mM β-luciferin (Analytical Luminescence Labs) solution. Ten seconds measuring time was used.

To account for variable transfection efficiency among different samples, the luciferase activity for each sample was normalized by its β-galactosidase counts. For β-galactosidase activity, the Galacto-Light Plus™ chemiluminescent reporter assay kit (TROPIX, Inc., Bedford, MA) was used. Briefly, 20 μl of cell lysate, diluted in 200 μl of Reaction Buffer Diluent, were incubated at 48 °C for 1 h to inactivate endogenous β-galactosidase activity in cell extracts. The Galacto-Light Plus™ substrate was incubated 100-fold with Reaction Buffer Dilluent to make Reaction Buffer. 200 μl of Reaction Buffer were added into 20 μl of the diluted and heat-inactivated cell lysate in a luminometer cuvette. After incubation at room temperature for 1 h, 300 μl of Accelerator was manually injected into each cuvette, and the light output was immediately counted by luminometer using a 5-s averaging time.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts...
were prepared as described previously (11) from HeLa, CHRF, and K562 cells with or without PMA induction. The single-stranded oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA), and the complementary sense and antisense strands were then annealed into double-stranded DNAs used for EMSA. The Sp1 consensus binding sequence was purchased from Promega Corp. These double-stranded DNAs were end-labeled by T<sub>4</sub> polynucleotide kinase and [γ-<sup>32</sup>P]ATP. Then, 0.1–0.2 ng of probes (2 × 10<sup>5</sup> cpm) were incubated with 5 μg of nuclear extract on ice for 20 min in a 20 μl binding reaction that contained 18 mM HEPES, pH 7.8, 40 mM KCl, 4 mM MgCl<sub>2</sub>, 0.5 mM dithiorthreitol, 3 μg of poly(dI-dC), 2 μg of bovine serum albumin, and 10% (v/v) glycerol. For competition studies, prior to the addition of radioactive probes, unlabeled competitors were added to the binding reaction and incubated with nuclear extract for 10 min on ice. Samples were then electrophoresed at 4°C on a 4% (v/v) polyacylamide, non-denaturing gel in 0.5× Tris-boric acid-electrophoresis (TBE) buffer. Rabbit anti-human Sp1, Sp3, and YY1 polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For gel supershift assays, 0.5–2 μl (1 μg/μl) of antibody were added to the binding reaction and incubated on ice for additional 15 min before loading. A total of 0.5 footnote units of recombinant human Sp1 protein (Promega Corp.) was used instead of nuclear extracts in EMSA.

**Southwestern Blotting Analysis**—Thirty micrograms of HeLa nuclear extract was electrophoresed on a 6% sodium dodecyl sulfate (SDS)-polyacylamide gel with prestained protein molecular weight standards (14,300–200,000 molecular range) from Life Technologies, Inc. The proteins were then blotted electotroblotted to nitrocellulose filters (Schleicher & Schuell BA85, 0.45 mm), and renatured on the filters by serial dilution from 6 μl guanidine hydrochloride to Z’ buffer (25 mM Hepes-KOH, pH 7.6, 12.5 mM MgCl<sub>2</sub>, 20% glycerol, 0.1% Nonidet P-40, 0.1 mM KCl, 10 μM ZnSO<sub>4</sub>, 1 mM dithiothreitol). The membrane was then incubated for 30 min in blocking solution, containing 5% nonfat dried milk in Z’ buffer, and probed with <sup>32</sup>P-end-labeled double-stranded rat α<sub>IIb</sub>-flanking region sense, its human homologue, and M2 mutant in binding buffer (Z’ buffer containing 0.25% nonfat dried milk) for 1 h at room temperature. The filter was then washed three times with Z’ buffer for a total time of 15 min and exposed to autoradiographic film. A parallel gel containing the same amount of HeLa nuclear extract was also stained with Coomassie Brilliant Blue.

**RESULTS**

**Functional Studies of the Potential Silencer Element in Both Megakaryocytic and Non-megakaryocytic Cell Lines**—Previous studies have shown that −150 to −101 bp region upstream of the transcriptional start site of rat α<sub>IIb</sub> gene, when deleted, caused a 2-fold increase in the reporter gene, human growth hormone expression in a rat primary marrow transient expression system (23). To confirm this finding in other expression systems and to examine the tissue-specific nature of this deletion on expression, we shatted the 453-bp and 912-bp 5′-flanking region of the rat α<sub>IIb</sub> gene into the pGL3-basic vector, which contains a luciferase reporter gene. The −150 to −101 bp region was then deleted from the wild-type constructs. The important positive regulator GATA<sub>545</sub> element is present in 912-bp constructs but not in 453-bp constructs. Transient transfection studies of all these constructs were carried out both in the α<sub>IIb</sub>-expressing CHRF and HEL cells, and in the α<sub>IIb</sub>-non-expressing cell lines, epitheloid HeLa, fibroblastic NIH 3T3, and myeloid HL60 cells.

In the megakaryocyte-like cell lines, CHRF and HEL cells (Fig. 1, top), we found that the 912-bp construct expressed well (1.8- and 4.5-fold, respectively, higher than the SV40-driven pGL3 control), while the 453-bp construct, lacking the GATA<sub>545</sub> element, expressed poorly (0.3- and 0.2-fold of the same control). The −150 to −101 bp deletion (Δ) in the 912-bp promoter construct led to a 2.0- and 1.7-fold further increase in expression, respectively, much as in the published rat primary marrow transient expression studies (11). Surprisingly, in the 453-bp construct, which lacks the GATA<sub>545</sub> site, the same deletion had a 5.1- and a 25.9-fold increase in expression, respectively, resulting in levels comparable to the 912-bp wild-type construct (1.4- and 4.9-fold of the positive control, respectively).
to the −150 to −101 bp region, EMSA was carried out using nuclear extracts prepared from CHRF, K562 (another megakaryocyte-like cell line that may represent a less differentiated megakaryocyte-stage; Ref. 14) with or without PMA induction, and HeLa cells. The results were very similar with all three cell lines. Because the silencer effect was seen with non-megakaryocytic as well as megakaryocytic cell lines, the data presented below focus on the HeLa mobility shift studies.

Fig. 2. Initial EMSA of HeLa nuclear extracts with two overlapping probes for the −150 to −101 bp region. A, a comparative analysis of the αIIb promoter sequences in this region between the available rat, human, and mouse sequences (5, 67) is shown at the top with numbers based on the rat sequence. The human silencer core element (15) is shown as a dark gray area between rat −113 and −109 bp. The three introduced CC → AA mutations in the rat sequence are also shown at the top as gray ovals. The subregions used as probes in this and other EMSA figures are indicated at the bottom. B, EMSA studies with the −150 to −116 probe. The arrow indicates the band of interest. Cold competition was done at both a 100-fold and a 500-fold molar excess for the −150 to −116 DNA and a 500-fold for the other cold competitors. C, EMSA studies of the −135 to −101 region with varying magnesium concentrations indicated.

Only the −150 to −116 bp DNA formed a complex (Fig. 2B), and this complex could be cold competed away by non-radio-labeled −150 to −101 DNA (lanes 3 and 4). The complex could not be competed away by the −135 to −101 bp region (lane 5) or by an unrelated Ets sequence (lane 6), indicating specific binding to the −150 to −116 region. The −135 to −101 bp double-stranded probe, containing the homologous human silencer region, failed to give rise to any significant complex formation (Fig. 2C). For the −135 to −101 bp double-stranded probe, varying salt conditions (from 20 to 100 mM), Mg²⁺ concentration (from 0 to 10 mM, Fig. 2C), amount of nonspecific competitor (from 1 to 3 μg of poly(dI-dC) per reaction), and buffering capacity (from 5 mM to 25 mM HEPEs, pH 7.8) did not lead to a detectable band.

To further localize the binding site of the complex, we tested a double-stranded DNA probe that spanned from −150 to −126 bp (Fig. 2A). This region formed a complex similar to that for the −150 to −116 bp probe, and these two probes can cross-
compete (data not shown). In contrast, a probe from −140 to−116 bp (Fig. AA) did not form a complex, nor could it compete away the band seen with the −150 to −126 bp probe (data not shown). We then further shortened the probe to −145 to −125 bp (Fig. 2A). We found that the −145 to −125 bp probe formed a similar mobility complex as the −150 to −116 bp and −150 to −126 bp probes (Fig. 3, lanes 2, 8, and 10, respectively) and that it can be cold competed away by itself and by the other two DNAs (Fig. 3, lanes 3-6).

Therefore, it appears by EMSA that we can identify a single complex in nuclear extracts from a number of different cell lines that bind specifically to the −150 to −101 bp region of interest. We further localized this DNA binding to a subregion between −145 and −125 bp upstream of the rat αIIb transcriptional start site.

Further Characterization of the Protein Binding Sequence in the −145 to −125 bp Region—To identify the base pairs that are important for protein binding in the −145 to −125 bp region, three CC → AA substitutions were made at −140/−139 bp (M1), −136/−135 bp (M2), and −131/−130 bp (M3) as shown in Fig. 2A. These mutated double-stranded DNAs were used in EMSA as cold competitors against wild-type −145 to −125 bp binding (Fig. 4, lanes 4–6) as well as probes to assay their own binding activity (Fig. 4, lanes 7–12). As a cold competitor, M1 (lane 4) showed moderate competition against the wild-type −145 to −125 bp probe, while M2 (lane 5) did not appear to be an effective competitor, and M3 (lane 6) appeared to compete as well as the wild-type cold competitor (lane 3). Consistent with these findings, labeled M1 formed a slight amount of complex (lane 8), M2 formed no observable complex (lane 10), and M3 readily formed a complex (lane 12) identical to the wild-type complex in intensity (lane 2).

It is of interest that these findings are consistent with the degree of sequence conservation at these three sites (Fig. 2A); M2 is conserved among all three species, while M1 is a CT rather than a CC sequence in the human αIIb promoter region. M3, while conserved between rat and human, is deleted in the mouse promoter region. Thus, the site that is most highly conserved across species appears to be the crucial point of complex binding.

Expression Studies with the Three CC → AA Mutations—We then asked whether the EMSA findings would be reflected in expression studies. We introduced the three CC → AA mutations into the wild-type rat 453bp-pGL3 construct, and tested these new constructs in HeLa, NIH 3T3, and HL60 cells. All three cell lines gave similar results (Fig. 4B). In agreement with the EMSA data, M1 had a modest increase in expression (3–16% of the 453Δ construct), M2 had a significant increase (40–80% of the 453Δ construct), and M3 had the same low level of expression as the wild-type 453 construct (0.12–1.2% of the 453Δ construct). Thus, elimination of binding of a single complex to this region appears to correlate with loss of silencing of the αIIb promoter.

EMSA with the Human Homologue of the Rat −145 to −125 bp Region—To examine whether this site was species-specific or more universally applicable, we then tested whether the human homologue to this region, which has 721 nucleotide substitutions, would also bind to the same complex in EMSA studies. As can be seen in Fig. 5A (lanes 2–6), this human homologue formed a similar size complex that can be effectively competed away by itself and by the rat −145 to −125 bp double-stranded DNA. Conversely, the human homologue was equally effective at competing away binding to the rat −145 to −125 bp probe (Fig. 5A, lanes 8–12). M1, M2, and M3 cold competition studies (Fig. 5B, lanes 7–12) were identical to those shown in Fig. 4A for the rat wild-type probe. These findings suggest that the same complex formed with both the human and rat sequence in this region.

Sp1-related Protein(s) Are Involved in the Binding of −145 to −125 bp Region—In an attempt to identify the silencer-binding protein, we searched the Transfac data base by Transcription Element Search Software (TESS),2 looking for known transcription factors that have consensus binding sequence homologous to our silencer region around bases −136/−135. The search suggested MAZ (Myc-associated zinc-finger protein) (24–26), Yi (27), and NF-1 (28) as potential candidates. Mela1 (24–26) and YiMT3 (27), known MAZ- and Yi-binding elements, respectively, and the NF-1 consensus binding sequence (Santa Cruz Biotechnology, Inc.), were used as cold competitors in EMSA for the binding to the −145 to −125 bp region using HeLa nuclear extract. While YiMT3 and NF-1 consensus did not show any effect on the binding, Mela1 did cold compete to the same extent as unlabeled wild-type −145 to −125 bp DNA, suggesting that a MAZ-related protein might be involved (data not shown).

To extend our characterization of the complex, a Southwestern blotting analysis was performed using HeLa nuclear extract to determine the molecular weight(s) of the proteins that bind to the −145 to −125 bp region. A single band at −110 kDa (Fig. 6, lanes 3 and 4, respectively) was recognized by both the rat −145 to −125 bp probe and its human homologue, while no protein appears to bind to the M2 mutant probe (Fig. 6, lane 5). MAZ is a 58-kDa protein, so that Southwestern blotting did not appear to support a role for this protein in binding to the silencer region. Nevertheless, when M1a1 was used as a probe, it bound to several bands including an −58-kDa band

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2 TESS software is available via the World Wide Web (http://www.cbil.upenn.edu/cbil-home/index.html).
and an -110-kDa band (Fig. 6, lane 2). It is known that ME1a1 binds Sp1 protein in addition to MAZ (29, 30), and that Sp1 has molecular mass of -105 kDa (31, 32). We, therefore, examined whether Sp1 was involved in the complex binding to the silencer element. In Fig. 7A, supershift studies were performed to see if an anti-Sp1 antibody could recognize the complex bound to the rat -145 to -125 bp probe. The addition of rabbit anti-human polyclonal Sp1 antibody (Santa Cruz Biotechnology, Inc.) supershifted the protein complex (lane 3). A control rabbit anti-YY1 antibody (Santa Cruz Biotechnology, Inc.) did not cause any shift in the band (lane 4). (Not shown is that on another EMSA gel, using a consensus YY1 DNA probe, the YY1 antibody resulted in a supershift band, while the rat -145 to -125 bp complex was not supershifted.) In Fig. 7A (lanes 13 and 14), the human homologue probe was used. The Sp1 antibody again formed a supershifted band.

Also shown in Fig. 7A, an Sp1 consensus binding sequence that contains a classic GC box Sp1 binding site from the SV40 promoter is a strong cold competitor for the complex binding to the rat -145 to -125 bp probe (lane 6). When this Sp1 consensus sequence was labeled, it formed a similar protein complex with HeLa nuclear extract as seen with the rat -145 to -125 probe (lanes 8 and 2, respectively). Sp1 antibody also supershifted the consensus complex (lane 9), while the control YY1 antibody had no effect (lane 10). Although not a strong cold competitor in comparison with Sp1 consensus sequence, the rat -145 to -125 did compete with Sp1 binding (lanes 11 and 12). Not shown is that the M2 substituted rat probe did not compete at all against the Sp1 consensus probe.

Fig. 7B shows that recombinant human Sp1 protein forms a complex with the rat -145 to -125 bp probe with the same mobility as seen with the HeLa nuclear extract (lane 10 versus lane 2). As predicted, M2 does not form a complex with the recombinant Sp1 protein (lanes 11 and 12).

We also tested whether the -145 to -125 bp region can bind Sp3, an Sp1 family member with the same binding sequence as Sp1, but which has been shown to act as a negative regulator of other genes (33–35). We used varying amounts of anti-Sp1 and anti-Sp3 specific antibodies. As can be seen in Fig. 7B (lanes 3–5), increasing the amount of anti-Sp1 antibody from 0.5 to 2 μg per sample virtually eliminated the initial band. In lanes 6–8, increasing the amount of anti-Sp3 had no effect on the intensity of the original band and did not supershift the band.

In lane 9, the addition of both 1 μg anti-Sp1 and 1 μg of anti-Sp3 was no better than the result when 1 μg of anti-Sp1 alone was used in lane 4.

Therefore, it appears that the complex that binds to both the
Further Confirmation of the Role of Sp1 in the Silencing of αIIb Gene Expression by Gal4/Sp1 Cotransfection Studies—To further confirm that Sp1 is important in αIIb silencing, we substituted a Gal4 binding sequence from nucleotides −145 to −125 in the 453-bp αIIb promoter region. As expected, this substitution in the silencer region (453Gal4-pGL3) markedly increased expression of the 453bp-pGL3, leading to levels of expression comparable to the SV40-driven positive control (40% of SV40-pGL3) and nearly twice as high as 453M2-pGL3 (Fig. 6A). More importantly, when this construct is coexpressed with a series of Sp1/Gal4 fusion proteins, containing the DNA binding domain of the yeast transcription factor Gal4 (21), silencing activity is seen with the two longest fusion proteins, pSG-Sp1WT and pSG-Sp1N (see “Materials and Methods”) (Fig. 6B). While pSG-Sp1A and pSG-Sp1A&B increased the expression of 453Gal4-pGL3 2.3- and 1.7-fold, respectively, pSG-Sp1N and pSG-Sp1WT both decreased its expression to 50% and 60%, respectively, compared with the pSG424 control (Gal4 only vector). In addition to providing further support for our findings that Sp1 is involved in silencing, these data suggest that near full-length Sp1 is necessary to achieve silencing of the α promoter and that other constructs that may not be able to interact in a specific fashion with regulatory domains in this promoter actually lead to a further increase in expression.

Ectopic Reinsertion of the −150 to −101 bp Region Immediately Upstream of the 453bpΔ-pGL3 Construct and Its Effect on Expression in HeLa Cells—To examine whether or not the Sp1-binding silencer element functions in a position- and orientation-independent fashion, we deleted the −150 to −101 bp region from 453 bp of rat αIIb promoter and reintroduced this 50-bp region back into the same promoter, but upstream from the 453-bp region. A single copy was inserted in two different constructs, one in forward and one in reverse orientation. These constructs were transfected into HeLa cells to examine the relocation effect on expression. As we have shown above, the 453A-pGL3 construct gave rise to a significant level of expression. However, the reintroduction of this 50-bp region into a different position in either forward or reverse orientation did not suppress expression. Instead, these constructs resulted in a 1.7 ± 0.4-fold and 2.4 ± 0.3-fold further increase in expression, respectively, when compared with the 453A-pGL3 construct. Therefore, it appears that the inhibitory activity of this silencer element is dependent upon its physical location in the αIIb promoter.

**DISCUSSION**

We have found that the previously described effect of deleting the −150 to −101 region upstream of the transcriptional start site of the rat αIIb gene (11) was not due to a physical disruption of the promoter region, but rather due to the loss of a cross-species conserved, Sp1-binding site. This site centers on nucleotides −136/−135, and we have named it the Sp1135 site. Complex binding to this site silences αIIb promoter-driven expression in both megakaryocytic and non-megakaryocytic cell lines.

Our data also suggest that this silencer site binds Sp1, a member of a multigene family of zinc finger transcription factors (36, 37). Sp1 is ubiquitously expressed in all tissues, and its ubiquity is consistent with our finding that Sp1135 complex suppresses αIIb expression in all tested cell lines. Sp1 binds to GC boxes and similar motifs (38−41), but also binds to a number of non-consensus sequences as well (42−44). The −145 to −125 bp region is a CT-rich region that is consistent with such non-consensus Sp1-binding sites.

Further confirmation that near full-length Sp1 is necessary to achieve silencing of the α promoter and that other constructs that may not be able to interact in a specific fashion with regulatory domains in this promoter actually lead to a further increase in expression.

**Fig. 5.** EMSA studies with the human homologue to the rat −145 to −125 region. A, EMSA studies with the human homologue and the rat probe are shown with cross-over cold competition studies. The arrow points to the complex of interest. Cold competition was done at 50- and 200-fold molar excess. B, the human homologue probe is cold competed with the wild-type and the three mutant rat DNAs. Cold competition was done at 50- and 200-fold molar excess.

**Fig. 6.** Southwestern blot of HeLa nuclear extract. M, prestained protein markers. Lane 1, Coomassie Brilliant Blue-stained HeLa nuclear extract. Lanes 2−5 are nitrocellulose strips from the same experiment as lane 1 hybridized to labeled probes: lane 2, Mel1; lane 3, rat −145 to −125 bp region; lane 4, the human homologue to the rat −145 to −125 bp region; lane 5, the M2 −145 to −125 bp region.

Further confirmation that near full-length Sp1 is necessary to achieve silencing of the α promoter and that other constructs that may not be able to interact in a specific fashion with regulatory domains in this promoter actually lead to a further increase in expression.
sequence (36), while Sp3 (36, 37) and Sp4 (37) have binding specificity and affinity similar to those of Sp1. Sp3 also appears to be ubiquitously expressed, while Sp4 appears to be limited in vivo to neural tissues (36). The zinc finger DNA-binding domains and glutamine- and serine/threonine-rich activation domains are highly conserved between Sp1, Sp3, and Sp4 (17, 45–47). However, while Sp1 and Sp4 appear to mostly promote transcription, Sp3 has often been shown to be a negative regulator of expression (17, 33, 47). The DNA-binding domains of Sp1 and Sp3 appear to be functionally interchangeable, but the activation domain of Sp3 is not functional when chimerically linked to the Sp1 DNA binding domain (17). This suggests that the negative regulatory activity of Sp3 may be due to its competition with Sp1 for a common binding site. Indeed, Sp3 suppression of Sp1-mediated transcriptional activation has been described in many genes, including both basal and Tat-activated expression of the human immunodeficiency virus promoter (33). Differential expression of Sp1 and Sp3 in different tissues and altered Sp1/Sp3 ratio during cell differentiation and transformation have been shown to be responsible for the regulation of several epithelial-specific promoters (44, 48). Therefore, Sp3 binding would have provided an explanation for the involvement of an Sp1-binding element in negative regulation of gene transcription.

We have examined whether or not Sp3 is involved in Sp1135 binding by EMSA studies using an anti-Sp3-specific antibody. Neither supershift nor blocking was observed. Furthermore, the anti-Sp1-specific antibody can supershift virtually all of the complex (Fig. 7B, lane 5), suggesting that the bound protein is Sp1. However, it is still possible that in the intact cell, the Sp1135 site, in the context of the entire proximal αIIb promoter region, binds Sp3. Perhaps the silencer elements proposed by others just upstream or downstream of this site (14, 15), interact with the Sp1135 site to allow it to bind specifically to Sp3 and inhibit transcription.

FIG. 7. EMSA studies with antibodies to Sp1 and recombinant Sp1 protein. A, labeled rat −145 to −125 bp, its human homologue, and an Sp1 consensus probe was used with and without the addition of an anti-Sp1 antibody (1 μg/lane). The rat and Sp1 consensus probes were also studied using an anti-YY1 (66) (control) antibody (1 μg/lane). Cold cross-competition studies between the rat probe and the Sp1 consensus probes were done at 200-fold molar excess. B, EMSA studies of the rat −145 to −125 bp wild-type and M2 probes. The wild-type probe was studied using anti-Sp1 antibody (lanes 3–5, 0.5 μg, 1.0 μg, and 2.0 μg, respectively) and anti-Sp3 antibody (lanes 6–8, 0.5 μg, 1.0 μg, and 2.0 μg, respectively) with HeLa nuclear extracts. In lane 9, 1.0 μg of anti-Sp1 and 1.0 μg of anti-Sp3 antibody were used together. Both probes were also used with recombinant Sp1 protein (0.5 footprint units/lane) indicated by an asterisk in lanes 10 and 12.
by the formation of inactive (non-DNA-binding) complexes between Sp1 and other nuclear proteins such as Sp1-I and p107 (52, 53). However, interference with Sp1 activation, an established mechanism by which gene transcription can be altered, obviously cannot explain the activity of Sp1135 site in \( \alpha_{IIb} \) promoter, because the silencing function of this site correlates directly rather than inversely with its binding of Sp1-related proteins.

Therefore, our study raises the possibility that Sp1 may itself decrease transcription when bound to certain Sp1 elements. Several recent reports have suggested a similar negative regulatory role for bound Sp1 (53–55). How this occurs is unclear, but one mechanism may involve its interactions with other nuclear elements. Sp1 has been shown previously to interact with other nuclear factors such as GATA-1 and Ets proteins (57–60). Sp1 is also described to be a tethering factor to recruit the transcription initiation complex to TATA-less promoters by physically interacting with components of general transcriptional machinery (61, 62).

Interestingly, we have shown previously that the Sp114 site is a positive regulator of \( \alpha_{IIb} \) expression (13). The complex at this site appears to interact with the complex bound to the Ets35 site. It is suggested that the Sp114 complex promotes transcription by tethering the transcriptional initiation complex to this TATA-less promoter. Our present finding is that another Sp1-binding site is a negative regulator of \( \alpha_{IIb} \) expression. Therefore, depending on which site Sp1 is bound to, this nuclear factor appears to have both a positive and negative role in the regulation of \( \alpha_{IIb} \) expression. A similar dual effect of Sp1 has been described in the proximal promoter of human adenine nucleotide translocase 2 (ANT2) gene (56). For the TATA-containing ANT2 gene, the more proximal Sp1 site (from −7 bp to −2 bp) partially inhibits transcription, probably by disrupting the recruitment and assembly of transcriptional initiation complex. A more distal site, containing two adjacent Sp1-binding sites at −87 bp to −58 bp, activates expression.

Given the fact that the effect of Sp1 on transcriptional activity is context-dependent, it is not surprising that only specific Gal4/Sp1 fusion protein shown in Fig. 8 inhibit expression, and that other fusion constructs actually increase expression. The context dependence of Sp1 binding in this region is also consistent with what was seen following ectopic reinsertion of the −150 to −101 bp region, which did not return silencer activity, but rather resulted in increased expression. These finding support the idea that the complex bound to the Sp1135 site interacts with other bound nuclear factors in the \( \alpha_{IIb} \) promoter region. Physical disruption of the structural organization between Sp1 and these other regulators, therefore, may alter the regulatory activity of Sp1135 in \( \alpha_{IIb} \) transcription.

Silencer elements have been proposed in the regulated expression of several other megakaryocyte-specific genes. A silencer element was identified for the rat platelet factor 4 (PF4) promoter (23). However, whether this region actually contains a silencer element was questioned in studies of the human PF4 promoter, which suggest that the homologous region is actually a strong promoter of PF4 expression (63). The \( \alpha_2 \) integrin gene is also TATA-less, and its regulated expression in megakaryocytic cell lines has also been suggested to involve a silencer region (64). In addition to a core promoter (−92 bp to the transcriptional start site) that is active both in megakaryocytic and non-megakaryocytic cells, a silencer element (−92 to −351 bp) region was defined that showed tissue specificity. It is inactive in non-hematopoietic cells but active in megakaryocytic cells. Located further upstream is a strong megakaryocyte-specific enhancer that overcomes the silencer effect and restores megakaryocyte-specific expression of the \( \alpha_2 \) gene. The tissue specificity of the \( \alpha_2 \) gene silencer contrasts with the Sp1135 site, which appears to be an active silencer of \( \alpha_{IIb} \) expression in all cell lines tested. However, the two genes share a common mechanism in that the silencer element is overcome by a distal megakaryocyte-specific enhancer, leading to tissue-specific expression.

Two studies of the human \( \alpha_{IIb} \) gene have defined a silencer domain (14, 15). The exact site(s) of the involved silencer element varied between the two studies. EMSA studies by Fang and Santoro (14) suggested that there may be two sites involved in the silencer effect seen in the human \( \alpha_{IIb} \) promoter, −198 to −178 bp and −124 to −99 bp. Their studies focused on K562 cells and suggested that PMA induction markedly increased \( \alpha_{IIb} \) expression and led to a inverse decrease in EMSA complex formation at both sites. Preliminary data that was not shown by the authors suggested that the protein that bound to the silencer element had a molecular mass of −30 kDa. Prandini et al. (15) also detected a silencer element that was in part consistent with the above studies, suggesting that there were two silencer elements at −120 to −116 bp and at −102 to −93 bp, whose mutation increased promoter activity of the human promoter region 4- and 8-fold, respectively, and 20-fold in combination. No EMSA studies were done, but DNase I footprinting showed a protected region between −120 bp and −116 bp. Thus, these two studies suggest a common silencer site centered at human −120 to −116 bp with the sequence 5’-AT-
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GAG-3' (corresponding to rat -113 to -109 bp) (Fig. 2A).

Our studies suggest another site as being critical for the observed silencing, -30 bp upstream from the proposed human silencer site. Prandini et al. (15) suggested that the sequence of 5'-ATGAG-3', which is found in the 5'-flanking region of several platelet-specific promoters is a common silencer element for all of these genes. However, this region is not conserved in the rat and mouse αIIb promoter regions, being 5'-GTGGTG-3' in the rat and 5'-G-ACG-3' in the mouse, thereby having 2/5 and 4/5 mismatches (Fig. 2A). Indeed, the rat homologue (probe -114 to -88 in Fig. 2A) of the human αIIb silencer region did not form a complex in EMSA studies with HeLa cell nuclear extract. We focused our analysis on the -145 to -125 bp region that did form a complex. It turned out that this region has a well localized site that binds Sp1 in both the rat and human sequences, and that the human homologue of this region is protected on the DNase I footprinting studies by Prandini et al. (15). It may be that silencing of the αIIb gene is more complex than any of these studies suggest. Interactions between a number of sites may be necessary to achieve αIIb gene silencing. Perturbations of any of these sites may then have the same effect of relieving the silencer effect.

In summary, we present two important new findings: 1) that there is an important Sp1,35 silencer domain involved in the regulation of αIIb gene expression, and 2) that despite the presence of the Sp1,35 silencer domain in megakaryocytes, sequences further 5' to the -453 bp can overcome this silencer effect in the developing megakaryocytes. Our previous studies suggest that this upstream megakaryocyte-specific element involves the GATA,454 site. Thus, we present in Fig. 9 a simplified model of how the αIIb gene is regulated in agreement with our data. In non-megakaryocytic tissues, a silencer complex forms and with studies by us and others on other regulatory elements 5' to the αIIb gene is that they are all based on analysis of relatively short stretches of the 5'-flanking region of this gene. Whether or not a ubiquitous silencer element will be demonstrated in studies with the intact gene and whether the upstream enhancer elements maintain their role as a dominant, tissue-specific regulator remain to be tested. It is possible that a targeted mutation of the Sp1,35 site in transgenic mice may have a number of different outcomes, varying from having no effect on αIIb expression to having an effect only in megakaryocytes to having a wider effect of expression on other hematological or non-hematological lineages.

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