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Transcriptional and epigenetic regulation of the integrin collagen receptor locus *ITGA1-PELO-ITGA2*

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SUMMARY

The integrin collagen receptor locus on human chromosome 5q11.2 includes the integrin genes *ITGA1* and *ITGA2*, and the cell cycle regulation gene *PELO*, embedded within *ITGA1* intron 1. *ITGA1* contains a CArG box that is bound by serum response factor (SRF), while *PELO* contains two Sp1 binding elements. A comparison of mRNA levels in megakaryocytic (MK) and non-megakaryocytic (non-MK) cell lines and an analysis of the transcriptional activity of promoter-LUC reporter gene constructs in transfected cells revealed that *ITGA1* is selectively suppressed in the MK lineage. Sodium bisulfite genomic sequencing established that a CpG-rich *ITGA1* promoter region (−209/+115) is fully methylated at 19 CpG sites in MK cells that do not express α1β1, but completely demethylated in expressing cells. *In vitro* methylation of *ITGA1* suppresses transcription, while treatment of megakaryocytic cells with 5-aza-2′-deoxycytidine, but not Trichostatin A, resulted in *de novo* expression of *ITGA1*. During thrombopoietin-induced i*n vitro* differentiation of primary human cord blood mononuclear cells into megakaryocytes, we observed rapid, progressive CpG methylation of *ITGA1*, but not *PELO* or *ITGA2*. Thus, selective CpG methylation of the *ITGA1* promoter is a specific feature of α1β1 regulation that coincides with the initiation of megakaryocyte differentiation.

Keywords

integrin; pelota; collagen; megakaryocyte; gene; differentiation

INTRODUCTION

The human genes for integrin subunits α1 (*ITGA1*) and α2 (*ITGA2*) are located adjacent to each other on chromosome 5 (5q11.2), where *ITGA1* lies 32 kb upstream from the 5′ regulatory region of *ITGA2* (Figure 1A). Together with phylogenetic evidence [1], the tandem location

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of these genes and a nearly perfect conservation of exon boundaries suggest that *ITGA2* was derived from the ancestral *ITGA1* by duplication. No other pair of integrin subunit genes is more closely associated within the human genome. Both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are receptors for collagen and laminin that are present in most tissues, but are expressed in a cell-specific manner. A prominent exception are cells of megakaryocyte lineage, in which $\alpha_1\beta_1$ is not expressed.

The transcriptional regulation of *ITGA1* has been characterized only in the chicken [2,3] where serum response factor (SRF) is a prominent enhancer recognizing a CArG box sequence within the proximal promoter. In this report, we show that this CArG-like sequence is highly conserved and the most prominent similarity between human *ITGA1* and chicken *Itga1* 5′ regulatory region sequences. *In vitro* experiments using lucirease reporter constructs indicate that it likely plays a significant role in the enhancement of *ITGA1* transcription.

The human gene *PELO* is entirely embedded within the large intron 1 (69 Kb) of *ITGA1* (Figure 1B). It consists of two translated exons: the first exon (958 base pairs) begins approximately 12.1 kb downstream from the beginning of *ITGA1* intron 1 and contains the ATG start codon; the second exon (626 base pairs) ends 47.4 kb upstream from the beginning of *ITGA1* exon 2. The relationship, if any, of *PELO* to *ITGA1* and *ITGA2* is unknown. *PELO*, which encodes the protein pelota, was originally discovered in *Drosophila melanogaster* [4]. The gene is also expressed in mice [5] and humans [6], and homologs have been identified in Archaebacteria [7], yeast [8] and plants (*Arabidopsis thaliana*) [9]. In *Drosophila*, *pelo* mutations result in cell cycling abnormalities, reflected in spermatogenetic arrest, female sterility, and disturbances in the patterning of the eye [4]. The mutation of the *PELO*-related yeast gene *dom34* results in disturbances of mitotic and meiotic cell cycling [8]. These findings suggest that pelota is involved in cell cycle regulation. In this study, we show that *PELO* transcription is largely controlled by *Sp1*, which is a ubiquitous transcription factor.

The selective suppression of *ITGA1* within the megakaryocyte lineage must result from locally-restricted modifications that specifically affect *ITGA1* transcription, since the physically proximal genes *ITGA2* and *PELO* are not affected. Methylation of cytosines within the dinucleotide sequence CpG is a common mechanism of transcriptional suppression in vertebrates [10], which can be involved in the establishment and maintenance of cell type-specific gene expression [11], since methylation patterns of tissue-specific genes are unequivocally different from tissue to tissue [12]. In this study, we show that CpG methylation plays a role in coordinated *ITGA1* regulation during human megakaryocyte differentiation.

METHODS

Sequence Analysis

DNA sequences were obtained using an Applied Biosystems ABI Prism Model 377 DNA Sequencer (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA) by personnel in the DNA Core Laboratory of the Department of Molecular and Experimental Medicine, The Scripps Research Institute.

Monoclonal Antibodies

The gene *PELO*, obtained by polymerase chain reaction (PCR) using Hela cDNA as template, was cloned into the pET28a expression vector (Novagen, San Diego, CA) that allows fusion of the protein with an N-terminal 6XHis tag using NdeI and EcoRI restriction sites. This plasmid was used to transform *Escherichia coli* BL21 (DE3) (Invitrogen, Carlsbad, CA). Purification of the protein was accomplished by affinity chromatography with Ni-NTA agarose resin (Qiagen Corporation, Valencia, CA) following the manufacturer's instructions. The eluate was then dialyzed against phosphate-buffered saline (PBS) and pH 7.4, for

approximately 20 hours at 4°C. Protein in the final preparation was quantified by the Bradford method. Immunization of mice and hybridoma fusion were performed by the Antibody Core Lab of The Scripps Research Institute. Briefly, four Balb/C mice were immunized intraperitoneally with 50 μg of the recombinant protein pelota mixed with RIBI's adjuvant system (Monophosphoryl-lipid A + Trehalose dicorynomycolate adjuvant (Sigma Aldrich, St. Louis, MO)). This was followed by three intraperitoneal injections of pelota mixed with RIBI's adjuvant system. Three days before fusion, the mouse with the highest titer of serum antibodies specific for pelota, by an indirect ELISA, was boosted with 20 μg of the protein intravenously. Primary hybridomas were screened for specific antibodies by indirect ELISA with the immunogen as antigen. Supernatants showing the highest titer were screened ability to bind to pelota by western blot. Positive hybridomas were then subcloned twice by limiting dilution.

Western blot assays

Nuclear and cytoplasmic extracts were prepared as described by Geneste and al. (1996). Total cells lysate was prepared in 0.04 M Tris HCl (pH 7.5), 0.15 M NaCl, 1 % Triton X-100 (Sigma Aldrich), and the protease inhibitor cocktail Complete EDTA free (Roche Applied Biosystems, Indianapolis, IN). Briefly, 20 μg of nuclear or cytoplasmic protein were loaded into the wells of a 4–12 % linear gradient polyacrylamide gel and subjected to electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), as previously described [13]. The separated proteins were then transferred electrophoretically to a nitrocellulose membrane. Membranes were blocked with 5 % (w/v) bovine serum albumin (Sigma Aldrich) in 1.5 M NaCl, 0.2 M Tris, 0.1 % (v/v) Tween 20, pH 7.6 (TBS-T) and immersed in a solution containing 0.8 μg/ml monoclonal mouse anti-pelota antibody 1A3 or GAPDH (Ambion, Austin, TX) for 2 hours at room temperature. After three washes of 15 min each in TBS-T with gentle agitation, the membranes were immersed in a solution containing the secondary Horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Invitrogen) diluted 1/3500 and incubated for 1 hour at ambient temperature. After three additional washes of 15 min each in TBS-T, bound antibody was visualized by chemiluminescence.

Cultured Human Cell Lines

The Dami cell line (a subclone of HEL) was a generous gift from Dr. David Wilcox (Medical College of Wisconsin, Milwaukee, WI). The megakaryocyte cell line CHRF-288-11 [14] was a gift from Dr. M.A. Lieberman (Cincinnati, OH). The human cell lines K562, HEL, C8161, HeLa and HEK293 were purchased from the American Type Culture Collection (Manassas, VA). For treatment with 5-aza-2′-deoxycitidine (5-aza-dC; Sigma, St. Louis, MO), cells were split to 10^5 cells/ml and cultured in the presence of 10μM 5-aza-dC in DMSO for 48 hours. The final concentration of DMSO was 0.1 % (v/v). Control cells were cultured in the presence of an identical 0.1% DMSO alone for 48 h. For treatment with Trichostatin A (TSA; Sigma), cells were split to 10^5 cells/ml and cultured in the presence of 50 nM TSA for 48 h. Control cells were cultured in the presence of an equivalent volume of buffer alone.

RT-PCR

RNA was extracted using the TRIZOL reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Total RNA was reverse transcribed using Superscript II reverse transcriptase (GIBCO BRL), and random hexanucleotides as primers at 48°C for 50 min. After reverse transcription, samples were subjected to amplification with Taq DNA polymerase(Qiagen), using pairs of oligonucleotide primers as follows:

ITGA1 forward, 5′-GCTGGCTCCTCACTGTTGTT -3′;

ITGA1 reverse, 5′-CTCCATTTGGGTTGGTGACT -3′;

ITGA2 forward, 5′-TGCTGGTTGAAAGACGTTCACATG -3′;

ITGA2 reverse, 5′-TATAACTCCTGTTGGTACTTCGGC-3′;

PELO forward, 5′-GCGCCACATACACTTTGATG -3′;

PELO reverse, 5′-GGCTAGCCACAGTAGGGTCA-3′;

HPRT forward, 5′-AGTGATGATGAACCAGGT-3′;

HPRT reverse, 5′-GGCTTTGTATTTTGCTTTTC-3′

Primer extension

10 picomoles of the primer GSP8 (ACGCTTTTCACATCCCAGAC) were end labeled and purified using QIAquick Nucleotide Removal Kit (Qiagen). 15μg of total RNA was incubated with 10^6cpm of GSP8 in a 25μl reaction containing 0.1M KCl, 40mM Tris-HCl (pH 8.3), and 1mM EDTA at 90 °C for 10min and then 65 °C for 30 min and, finally, 37°C for 18hr. The hybridization mixture was added to 25μl of 0.8mM dNTPs, 15mM $MgCl_2$, 20mM dithiothreitol, and then extended with 200 units of Superscript II reverse transcriptase at 42°C for 60min. The cDNA was precipitated, resuspended in the loading buffer, resolved in a 6% acrylamide/7M urea gel, and visualized with autoradiography.

5′ RACE

5′RACE was performed on 5μg samples of total RNA using the GIBCO BRL 5′RACE System according to the manufacturer's instructions. In the case of *ITGA1* mRNA, reverse transcription was primed with A1GSP4 (CTGACGTCAGAACAGATTCCAGT). The product of this first strand synthesis was tailed with dCTP and amplified using the abridged anchor primer and the nested primer GSP5 (CTCCATTTGGGTTGGTGACT). The product obtained was re-amplified using the abridged universal amplification primer and further nested primer GSP8 (ACGCTTTTCACATCCCAGAC). The reaction product was gel-purified and cloned into pGEM-T easy (Promega). Eleven different plasmid clones containing the desired product were sequenced. In the case of *PELO* mRNA, the same procedure was employed using the PELO3 (CCTCTTCAGAACTGGAATCACCC) to prime reverse transcription, PELO 4 (CCCCAGTCAACTGGCTGAGCTG) as the first nested primer, and PELO 7 (CTTCCCCAGCAGCTTTAGTGTC) as the last nested primer. Six different plasmid clones containing the desired product were sequenced.

Plasmid constructions and transfection of cell lines

To study the transcription of the *ITGA1* gene, we constructed a series of reporter plasmids containing segments of the 5′-flanking region of the *ITGA1* gene extending from −981, −397, −216, −53, +315 through +455. These fragments were amplified by polymerase chain reaction with XhoI sites at 5′-and 3′-ends using genomic DNA as template. These products were ligated into the polylinker site upstream of the luciferase reporter gene in the pGL2-basic plasmid. The same approach was used to generate a series of reporter pGL2b plasmids containing segments of the 5′-flanking region of the *PELO* gene extending from ′811, −433, −172, −119 and +138 to +180. Dami cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM; GIBCO/BRL, Burlington, ON, Canada) supplemented with 10% horse serum at 37° C under 5% CO_2. Transfections were performed with the Effectene transfection reagent (Qiagen, Mississauga, ON, Canada) according to the manufacturer's indications.

EMSA

An optimal number of Dami cells (1×10^8) were washed twice in PBS (centrifugation at 1800 × *g* for 10 minutes) and then lysed in 0.5 ml of 0.5% (vol/vol) Nonidet P-40 in 25 mM HEPES, 50 mM KCl, pH 7.9, containing 1 mM phenylmethylsulfonylfluoride (PMSF), 100 mM dithiothreitol (DTT), 10 mg/ml leupeptin, and 20 mg/ml aprotonin (lysis buffer). The nuclei were pelleted and rinsed once in lysis buffer without Nonidet P-40 (centrifugation at 10,000 × *g* for 1 minute). By vigorous micropipetting, nuclei were then physically disrupted in 25 mM HEPES, 500 mM KCl, pH 7.9, containing 10 % (vol/vol) glycerol, 1 mM PMSF, 100 mM DTT, 10 mg/ml leupeptin, and 20 mg/ml aprotonin (extraction buffer). When nuclei were sufficiently emulsified, the mixtures were centrifuged at 10 000 × *g* for 5 minutes, and the supernatants were collected. The concentration of nuclear proteins in the supernatants was determined by the method of Bradford [15]. Double-stranded DNA probes (Table 1) were end-labeled with α^{32}P–deoxycytidine triphosphate using Klenow DNA polymerase [16]. Five to ten mg nuclear protein were mixed with labeled DNA (5×10^4 cpm) in 10 ml of 25 mM HEPES, 50 mM KCl, 0.5 mM EDTA, pH 7.9, containing 10% (vol/vol) glycerol, 0.5 mM PMSF, and 0.5 mM DTT (binding buffer) and incubated at ambient temperature for 1 hour. In reactions using competitor DNA, a molar excess (as indicated) of the unlabeled competitor DNA fragment was used. In antibody-based supershift experiments, 3 mg of rabbit polyclonal anti-Sp1 or anti-Serum Response Factor (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were preincubated with the nuclear extract for 15 minutes at ambient temperature before addition of the α^{32}P-labeled DNA probe. The reaction products were separated by polyacrylamide gel electrophoresis using 4% acrylamide/Bis (19:1) in 10.5 X Tris-borate-EDTA buffer (TBE; Invitrogen, Carlsbad, CA). Protein complexes were visualized by autoradiography.

Bisulfite treatment of DNA samples

One μg genomic DNA was denatured in 50 μL 0.2 M NaOH at 37°C for 20 minutes and then mixed with 30 μL 10 mM hydroquinone (Sigma, St Louis, MO) and 520 μL 3 M sodium bisulfite (pH 5.0) (Sigma). Reactants were incubated at 55°C for 16 hours. Bisulfite-modified DNA was purified using the Wizard purification resin and a Vacuum Manifold (Promega) and eluted into 50 μL water. After addition of 5.5 μL of 3 M NaOH (final 0.3 M), samples were let stand at 37°C for 20 minutes, then precipitated in ethanol and dissolved in 20 μL of water. A fragment of genomic DNA encompassing the CpG island of *ITGA1* (−235 to +152) was amplified by PCR using the following primers: forward, 5′-GGATGGAAGGGGGGATT-3′; and reverse, 5′-TCTCTAAAATCTCACTAACAA-3′. In the case of *ITGA2*, the CpG island −251 to +35 was amplified with the primer pair: forward, 5′-CAGGGCAGGAAAGCCTGCCAGG-3′; and reverse, 5′-AAGCTGTCCAGAGGGCTTCTCTCC-3′. For *PELO*, the CpG island −424 to +57 was amplified with the primer pair: forward, 5″-GGGCCAAATCTGCACTTCTGAGAG-3; and reverse, 5′-AGCACTTCCTCTATCCTTTCCTCTCTCAGTCTCC-3′. In the case of established cell lines, PCR products were directly sequenced; when primary mononuclear or CD41-enriched cells were studied, the PCR products were gel purified using QIAquick Gel Extraction kit (Qiagen) and cloned into pGEM-T easy vector (Promega).

In vitro methylation of plasmid DNA

ITGA1 promoter reporter constructs (15 μg) were treated with 20 units of *Sss* I methylase (New England Biolabs, Inc., Beverly, MA) for 3 h with 160 μM S-adenosylmethionine (New England Biolabs). Methylation status was verified by digestion with *Hpa* I or *Msp* I. Methylated constructs were transfected into Dami cells and assayed for luciferase activity.

Serum-free liquid culture system for *in vitro* analysis of megakaryocytopoiesis

Culture was carried out as previously described, with modification [17]. Briefly, venous cord blood from the umbilical vessels of healthy, full-term infants was collected immediately after delivery and mixed with one quarter volume of 6% hydroxyethyl starch. Erythrocytes were allowed to settle at room temperature for 30 to 45 min. Mononuclear cells (MNC) in the supernatant were then isolated using a Ficoll-Hypaque density gradient, washed, and resuspended for culture, as previously described [18]. The culture system consisted of 1×10^6 MNC/ml in Iscove's modified Dulbecco's Medium (Irvine Scientific, Irvine, CA), supplemented with 1% BSA, 75 μM α-thioglycerol, 40 μg/ml each of linoleic acid, lecithin, and cholesterol, 1% Nutridoma Hu (Roche Boehringer Mannheim, Indianapolis, IN), and 30ng/ml of recombinant human thrombopoietin (rhTPO) (288-TPN-005, R&D Systems, Minneapolis, MN). The total number of suspension cells in culture was determined using a CELL-DYN®1600 multi-parameter hematology analyzer (Abbott Laboratories, Abbott Park, IL). The day 8 cells were used for the $CD41^+$ cell purification.

Flow cytometric analysis of human megakaryocytes

The yield of megakaryocytic cells in culture was quantified by a 2-color flow cytometric technique. The day-8 cultured cells were harvested, washed with cold Ca^{++}- and Mg^{++}-free PBS containing 3% bovine serum albumin (BSA) (fraction V, Sigma Chemical) and 5 mM ethylenediaminetetraacetic acid (EDTA) to block nonspecific binding. After washing, 1×10^6 cells from each aliquot were labeled with 1 μg IgG1 FITC-labeled antihuman CD41 (P2) mAb diluted in 100 μL PBS with BSA and EDTA. Nonspecific antibody binding was monitored with the use of an FITC-labeled murine IgG1 isotype control. After incubation in the dark for 30 minutes at 4°C with gentle mixing, cells were washed with PBS, resuspended in 0.1% sodium citrate containing50 μg/mL propidium iodide (PI) plus 100 μg/mL DNAse-free RNAse (Sigma Chemical), and incubated again on ice in the dark for 20 minutes. Following staining, cells were immediately analyzed by FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems). At least 50 000 cells were acquired for each sample and analyzed by CellQuest software. $CD41^+PI^+$ cells were considered to be megakaryocytic cells. Selected cell samples were also stained with another murine anti-CD41 mAb (5B12) to verify the results obtained with the use of mAb P2. Megakaryocyte buffer [19] containing sodium citrate, BSA, and PGE_1 was also used in selected samples to verify that the CD41 staining results were not buffer dependent.

Isolation of $CD41^+$ cells using Dynabeads Pan Mouse IgG and CD41

CD 41^+ cells were isolated using Dynabeads Pan Mouse IgG (Dynal Biotech, Inc., Lake Success, NY) and murine anti-CD41 mAb (clone 5B12, DAKO Corporation, Carpentaria, CA), according to the manufacturer's instruction (Dynal Biotech, Inc.). Briefly, Dynabeads Pan Mouse IgG and murine anti-CD41 mAb (0.5–l μg anti-CD41 mAb/$l0^7$ beads) were incubated at 4°C for 30 min and washed. 4×10^7 cultured cells were then incubated for 30 minutes at 4° C with the anti-CD41coated beads, and bead-rosetted cells separated magnetically.

Isolation of DNA from mononuclear cells and enriched $CD41^+$ cells

DNA from cord blood MNC or $CD41^+$-enriched cells was purified using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN), according to the manufacturer's instruction.

RESULTS

Characterization of the *ITGA1* and *PELO* 5′ regulatory region

Using 5′RACE, we determined the transcription start site of *ITGA1* and *PELO* in Dami cells. Sequencing of eleven *ITGA1* cDNA clones revealed several closely spaced sites, with the major

product starting 400bp upstream from the translation initiation site (FIGURE 2). To confirm these results, we performed primer extension analysis with the antisense oligonucleotide GSP8 that starts 195b upstream from the translation initiation site. Primer extension identified a product whose size matched the transcription initiation sites determined by 5′RACE.

Sequencing of six *PELO* cDNA clones indicates the presence of a transcription start site 231 bp upstream from the ATG start codon, in close agreement with the findings of Shamsadin et al. [6] (FIGURE 3). Within the proximal 5′ regulatory/promoter region, there are two Sp1 binding elements (CCCCGCCTC at −136/−128; and CCCCGCCCC at +130/+139).

CpG-rich islands were distinguishable in the proximal 5′-regulatory region of each of the three genes relevant to this study: *ITGA1*, *PELO*, and *ITGA2*. In *ITGA1* (FIGURE 2), the segment −210/+105 (relative to the transcription start site) contains 19 CpG dinucleotides (bold and numbered). This segment was amplified from genomic DNA with flanking PCR primers (underlined) ranging from −235/+152. The CpG rich region of the *PELO* gene was more extensive (FIGURE 3), with 33 CpG dinucleotides spanning the segment from −354/+23. This CpG-rich region was amplified within a larger segment ranging from −424/+57.

Deletion analysis of the 5′-flanking sequences of *ITGA1*

Since the human *ITGA1* promoter has not yet been characterized, we completed a basic analysis of the 5′-flanking region that is required for promoter activity. Luciferase reporter constructs were generated in pGL2-basic (pGL2b), and transient transfections were performed in Dami cells. Baseline activity was established by transfection of the pGL2b vector without an insert (FIGURE 4A). The full-length *ITGA1* 5′-regulatory sequence (−981 to +455) generated a 42-fold increase over baseline activity, while the reverse of this sequence (−981 rev) was inactive, as expected. Truncation of the regulatory region at −397 resulted in approximately a 30% increase in activity, suggesting the presence of a modest suppressor region between −981 and −397. Further truncation at −216 or −53 generated only a modest decrease in activity relative to the full-length construct (9% and 13%, respectively). The greatest difference was observed with truncation at +315 whereupon activity decreased by 84%. These results suggest that basal *ITGA1* promoter elements reside within the sequence from −53 to +315.

Analysis of the 5′-flanking sequences of *PELO*

The transcriptional activity of the 5′ regulatory region of *PELO* was analyzed next (FIGURE 4B). Maximal activity was obtained with the sequence −172 through +180. The upstream sequence from −172 to −811 had a modest suppressive influence reflected by 25–30% decrease in activity. Further truncation at −119 and +138 resulted in significant decreases in activity of 67% and 88%, respectively. These results indicate that two separate regions, −172 to −120 and −119 to +137, make significant contributions to basal promoter activity. Each of these regions contains an Sp1 recognition sequence (Cf. FIGURE 3).

EMSA

In a comparison of the 5′ regulatory region sequences (−1000/+400) of human *ITGA1* (NM_181501) and chicken *Itga1* [2], the most conserved sequence (25/28 bp or 89% identity) is that at *ITGA1* +25/+52, which encompasses the CArG box element at +35/+43, a *cis* element that is bound by serum response factor (SRF). The EMSA probe with *ITGA1* CArG box that we designed for this study (Table 1) represents that full homologous sequence. It is not certain that SRF or any of its analogs is expressed in cells of the megakaryocyte lineage. Thus, we utilized a nuclear extract from HEK293 cells, known to express SRF, as the source. To represent a *bona fide* CArG box sequence, we employed an *ACTA1* promoter sequence containing a CArG box (CCAAATATGG) (Table 1). In a representative gel mobility shift assay (FIGURE 5A), it is clear that SRF is bound to the CArG box at +24/+43 in human *ITGA1* (lane 1) and

supershifted by anti-SRF (lane 3), but not anti-Sp1 (lane 2). Complex formation can be blocked by an excess of the unlabelled *ACTA1* promoter CArG box sequence as an inhibitor, in a dose dependent manner (lanes 4–6). Lanes 2 and 3 in (A) are inserted slices from separate experiments, each of which included identical positive and negative controls.

In the case of *PELO* (FIGURE 5B), it is clear that Sp1 does bind to both the Sp1 site A and Sp1 site B promoter sequences. In a representative gel mobility shift assay, using a nuclear extract from Dami cells, three major complexes are formed in the presence of either Sp1 A (lane 1) or Sp1 B (lane 2) and are designated I, II and III, from the slowest to fastest mobility. As described in our previous study [20] and based on differential precipitation with polyclonal anti-Sp1 or anti-Sp3, complexes I and III are formed predominantly by the binding of Sp3, with some contribution of Sp1, while complex II is formed predominantly by Sp1. In prior studies of the *ITGA2* Sp1/Sp3 binding element at −52 [20], we showed that complex II was predominantly supershifted by anti-Sp1 antibodies. As seen in lanes 3 and 4, complex II is once again predominantly supershifted by the presence of anti-Sp1 antibodies. As a negative control, polyclonal anti-SRF failed to shift the mobility of either the Sp1 A sequence (lane 5) or the Sp1 B sequence (lane 6). Additional results (not shown) confirmed that anti-Sp3 antibodies bind to and shift the mobility of complexes I and III, and to a lesser extent, complex II. Lane 5 in (B) is an inserted slice from separate experiment, performed with identical positive and negative controls.

Cell line-specific expression of *ITGA, ITGA2* and *PELO*

To determine whether the activity of the *ITGA1-PELO-ITGA2* locus can be modified in the erythromegakaryocytic (MK) lineage, we compared the levels of corresponding mRNAs, by semi-quantitative RT-PCR, in MK cell lines (HEL, K562, Dami, and CHRF-288-11) versus non-MK cell lines (C8161 and HeLa), before and after induction with phorbol-myristate (PMA). As shown visually in FIGURE 6 and summarized graphically in FIGURE 7, the expression of *ITGA1*, *ITGA2* and *PELO* mRNAs is different for each cell line studied. Generally, the levels of *ITGA1* or *ITGA2* mRNA were markedly higher in the non-MK cell lines C8161 and HeLa (FIGURE 6) compared to the MK cell lines. At the opposite end of the spectrum was the MK cell line CHRF-288-11, in which there is no detectable *ITGA1* mRNA.

Additional differences were seen in response to PMA treatment. In C8161 or HeLa, PMA induction had a negligible effect on the levels of *ITGA1* mRNA, while *ITGA2* mRNA increased 2–fold and 3–fold, respectively. The same treatment increased the levels of both transcripts in the three of the MK cell lines, HEL, K562 and Dami (FIGURE 7). In HEL, the increase in *ITGA1* mRNA was nearly 12-fold; for K562 or Dami, 2-fold. The increase in ITGA2 mRNA was also greatest in HEL (9-fold), and substantial but less in K562 (4-fold) or Dami (3-fold). Once again, CHRF-288-11 cells were unique in that PMA treatment had absolutely no effect on *ITGA1* mRNA level, while *ITGA2* mRNA was increased by 5-fold.

The level of *PELO* transcripts was more consistent between the various cell lines studied. There seemed to be no real distinction between MK and non-MK cells, in that the levels of *PELO* mRNA were practically identical in all six cell lines, and PMA treatment had little if any effect in Dami, CHRF-288-11, C8161 or HeLa. Only in HEL and K562 was there a marginal increase in *PELO* mRNA (roughly 2-fold in each case).

Relative pelota content

The relative amount of pelota protein in representative cell lines was determined by western blot using our murine anti-pelota monoclonal antibody (FIGURE 8). Using equal amounts of total cell protein as the source, a comparable amount of pelota was detected in Dami, CHRF288-11, K562, and HeLa cells (FIGURE 8A). The relative level of pelota protein, by

visual inspection (highest to lowest), was K562 > HeLa >Dami > CHRF288-11. In additional assays, pelota protein was partitioned nearly equally between cytoplasmic and nuclear extracts from K562, Dami or CHRF288-11 cells (FIGURE 8B).

Repression of *ITGA1* promoter activity by *in vitro* methylation

To determine whether methylation of the 5′ regulatory region of *ITGA1* is sufficient to silence its expression, the *ITGA1* promoter inserts within reporter constructs were methylated in vitro using *Sss* I, an enzyme that methylates every CpG dinucleotide. As shown in FIGURE 9, the luciferase activity in Dami cells transfected with an unmethylated construct was 32.3- fold higher than that in cells transfected with the *in vitro* methylated counterpart. This result confirms that methylation of CpG dinucleotides in the proximal 5′-regulatory region of the *ITGA1* gene can repress transcriptional activity even in cells that express endogenous *ITGA1*.

Induction of *ITGA1* expression by 5-Aza-dC in CHRF-288-11 cells

If the methylation of the *ITGA1* promoter is the cause of inactivation, treatment with a demethylating reagent should reverse this inhibition. When CHRF-288-11 cells were treated with 10 μM 5-aza-dC for 24 or 48 hours, the *de novo* expression of ITGA1 mRNA by RT-PCR became evident **(**FIGURE 10A). This result was confirmed by flow cytometry **(**FIGURE 10B), using FITC-labeled murine monoclonal anti-human CD49b (integrin α1) (BD Pharmingen, Franklin Lakes, NJ). To the contrary, treatment of CHRF-288-11 cells with 50 nM TSA for 48 hours had no effect on the repression of *ITGA1* mRNA (FIGURE 10C). These results would argue that the more accessible chromatin architecture favorable to transcription that would result from treatment with TSA does not overcome the inhibition of *ITGA1* transcription and that methylation of DNA per se is the cause of the repression of this gene.

Methylation status of the CpG dinucleotides in the promoter region of *ITGA1, PELO, ITGA2, and GP6* in established cell lines

Since the 5′ regulatory region of *ITGA1* contains 19 CpG sites (FIGURE 2), we next used bisulfite sequencing to analyze the methylation status of these CpG sites in region −235/+105 (the underlined sequence in FIGURE 1). Primers were designed that are complementary to sequences flanking this region and lacking CpG dinucleotides, in order to permit amplification of all genomic sequences, regardless of the status of methylation. **G**enomic DNA from Dami cells is completely unmethylated at all 19 CpG sites, while genomic DNA from CHRF-288-11 cells is fully methylated at all 19 CpG sites. The specificity of the CpG methylation status of *ITGA1* is further highlighted by an absolute lack of CpG methylation of either *PELO* (−354/+23; underlined in FIGURE 2) or *ITGA2* (−228/+9; not shown) in all cell lines studied, including CHRF-288-11 cells.

These results establish that the expression of *ITGA1* correlates with the CpG methylation status of the proximal 5′-regulatory region of the gene. Established cell lines, however, are not always an accurate model for cell differentiation, and aberrant methylation of CpG islands in genomic DNA from established cell lines has been encountered. To obtain a more accurate evaluation of a role for CpG methylation in expression of these genes during megakaryocyte differentiation, we turned to the study of primary human megakaryocytes.

CpG methylation in primary human megakaryocytes

To investigate the methylation status of the *ITGA1* promoter in primary cells, we isolated mononuclear cells (MNCs) from human cord blood, selected the CD41+ cells, and cultured these in the presence of recombinant human TPO (rhTPO) for 8days. The methylation status

of *ITGA1*, *PELO* and *ITGA2* CpG-rich promoter regions was then compared between the rhTPO-stimulated CD41+ cells and the MNC from which they were derived.

In the case of established cell lines, the bisulfite sequencing approach is simplified by the fact that the genomic DNA is homogeneous, therefore direct sequencing of PCR amplified DNA is possible. With primary human megakaryocyte differentiation *in vitro*, this is not the case. In the initial MNC population, megakaryocytes and progenitors may represent no more than 5 % of the total cell population. Even after induction for 8 days with rhTPO, the CD41-enriched population contains a significant proportion of CD41-negative or CD41-low cells that can constitute up to 30–50% of the population. Consequently, we first subcloned the PCR amplified DNA prior to sequencing. Upon sequence analysis, the percentage of clones with methylated CpG sites is statistically a reflection of the percentage of cells with methylated sites. This permits us to estimate the frequency of methylation at each CpG site in the initial genomic DNA prior to cloning.

The results a representative comparison are summarized in tabular form in FIGURE 11. With respect to the *ITGA1* status in genomic DNA from cord blood mononuclear cells (MNC) (FIGURE 11A), only 3 of 26 clones (11.5%) showed significant methylation at any of the 19 CpG sites. Assuming that this is a reasonable reflection of the proportion of cells with methylated DNA, these results would argue that no more than 11.5% of cells within the MNC population are committed to the MK lineage and that the *ITGA1* promoter in these cells has been CpG methylated. The results of flow cytometry assays determined the number of CD41 + cells in the MNC samples to be 2 +/− 0.7 % (n = 4). The contrasting results in the CD41-enriched cell population were remarkable. After induction with rhTPO, (FIGURE 11B), a majority of DNA clones (13 of 22, or 59.1%) were now methylated at at least 16 of 19 CpG sites. This proportion reflects very well the proportion of CD41+ cells in these enriched populations, based on flow cytometry (63 +/− 3.8%; n = 4). In the case of either *PELO* (21 clones) or *ITGA2* (24 clones), ≥3% of the promoter region CpG sites in any single clone were methylated, and these were randomly distributed in either the MNC population or the CD41-enriched population (not shown).

These results confirm that megakaryocyte differentiation coincides with an increased and selective CpG methylation of the *ITGA1* promoter.

DISCUSSION

Compared to other members of the integrin family, there is a good deal of information regarding the structure of the integrin *ITGA2* 5′-regulatory region and the nature of the elements most responsible for transcriptional control of this gene [13,20–24]. Transcription is largely driven by Sp1 which binds to two tandem recognition sequences in the proximal promoter. On the other hand, surprisingly little has been published regarding the transcriptional control of *ITGA1* in mammalian cells. However, the promoter of chicken IT*G*A1 has been characterized [2,3], and serum response factor (SRF) binding to a CArG box was shown to be essential for transcriptional activation in differentiated smooth muscle cells.

Our study represents the first characterization of the human *ITGA1* promoter. We find that transcription starts 400bp upstream from the translation initiation site, which is located 33bp upstream from a CArG box (CCCTTTAAGG) that is identical in sequence to that found in chicken *Itga1*. Promoter analysis reveals that the bulk of the promoter activity is present in the sequence between −53 and +315, which includes this highly conserved sequence at +25/+52 (89% identity between humans and chickens) that encompasses this CArG box. A functional CArG box is defined as one in which the 10-bp consensus can deviate by no more than 1 bp across the CArG element (e.g., CCSWWWWWGG) yielding 1216 potential sequences that

can be bound by SRF [25]. Since it is not yet established whether cells of the megakaryocyte lineage (such as Dami cells) express SRF, we employed a nuclear extract from HEK293, known to express SRF [26], as its source. By EMSA and supershift assays, we confirmed that SRF does bind to the CArG box sequence in *ITGA1*. Moreover, the binding of SRF to the *ITGA1* CArG box sequence is inhibited by the unrelated and evolutionarily conserved CArG box sequence from the human skeletal muscle α-actin gene *ACTA1*. These results suggest that mechanisms governing the expression of *ITGA1* are comparable in chicken and human cells. The same may be true of other integrin genes that bear a functional CArG box. Indeed, the murine integrin α5 gene *Itga5* also bears a functional CArG box in its 5′ regulatory region, as validated by EMSA [25].

The gene *PELO*, originally discovered in *Drosophila melanogaster* [4], is embedded entirely within the very large intron 1 (69 Kb) of *ITGA1*, by inspection of the compiled human chromosome 5 genomic sequence NT_023081. Our findings in this study represent the initial characterization of the *PELO* 5′ regulatory region and promoter in any species. The notable presence of two Sp1 binding sites and our validation that these sites able to bind to Sp1 are findings that are consistent with the role of Sp1 in the regulation of genes relevant to cell cycle controls.

Embedded genes, such as *PELO*, are not frequently encountered in the human genome. Other examples are the factor VIII-associated gene located within intron 22 of human coagulation factor VIII [27], the G-protein-coupled receptor gene *U16* situated within intron 17 of the human retinoblastoma susceptibility gene [28], and the three genes *EV12A*, *EV12B*, and *Omgp* located within intron 27b of the gene *NF1* [29–31]. The biological relationship between such embedded genes and the “host” genes remains an area of active investigation, but little is known about differential expression of the embedded and host genes. Since there is a nuclear localization signal in human and mouse pelota, it has been suggested that pelota could act as transcription factor or a carrier to transport other transcription factors into the nucleus. The influence of the embedded gene *PELO* on the expression of *ITGA1*, and vice versa, warrants closer scrutiny.

Methylation at CpG dinucleotides in genomic DNA is one of the predominant epigenetic mechanisms of transcriptional regulation in vertebrates [32]. We have analyzed the methylation status of CpG islands in the proximal 5′-regulatory region of *ITGA1* and find that genomic DNA from expressive cells, such as Dami, is devoid of methylated CpG sites, whereas genomic DNA from non-expressive cells, such as CHRF-288-11, is methylated at all CpG sites. The relationship between CpG methylation and silencing of *ITGA1* in CHRF-288-11 cells was very intriguing because the CpG-rich segments within the 5-regulatory regions of the proximal genes *PELO* and *ITGA2* are completely unmethylated. This indicates that *ITGA1* methylation is not a simple consequence of global hypermethylation but a specific event that may be restricted to cells of the megakaryocytic lineage.

Since CHRF-288-11 cells are considered to bear a phenotype that is more like mature megakaryocytes than any other erythro-megakaryocytic cell line [33], we hypothesized that *ITGA1* is specifically methylated as a requisite step in the differentiation of progenitors into megakaryocytes. However, it is also evident that in some cases CpG methylation can be a feature of established cell lines without functional counterpart in primary cell of the same lineage. For example, although CpG methylation of the proximal human Toll-like receptor (TLR)2 promoter in cell lines such as U937 correlated with TLR2 repression, the promoter was completely unmethylated in primary cells, regardless of the transcriptional activity of the gene[34].

A better test of our hypothesis would derive from an analysis of CpG methylation status in primary human megakaryocytes in culture. To address this question, we employed our previously reported *in vitro* culture method, in which the differentiation of human cord blood MNC into megakaryocytes is driven by recombinant thrombopoietin. Comparing the precursor MNC population to the CD41-enriched (megakaryocyte-enriched) population obtained after 8 days in culture, we monitored the methylation status of the CpG islands within the 5′-regulatory regions of *ITGA1*, *PELO* and *ITGA2*. The results confirmed our hypothesis and showed convincingly that *ITGA1* becomes hypermethylated and repressed during the differentiation of primary human megakaryocytes. In a previous study, we have reported that the converse is true of the megakaryocyte-specific gene *GP6*, which becomes progressively hypomethylated and expressed under identical differentiation conditions [35].

In some cases, CpG methylation per se is not the direct cause of transcriptional repression. For example, when methylated in vitro, the osteocalcin (OC) promoter DNA is still recognized by the key regulators Runx/Cbfa and the vitamin D receptor complex [36]. In that case, CpG methylation does not affect either basal or vitamin D-enhanced OC promoter activity in transfected cells. However, we confirmed that direct CpG methylation of *ITGA1* promoter constructs resulted in repression of transcriptional activity when transfected into expressive cells, such as Dami. Moreover, the treatment of non-expressive cells, such as CHRF-288-11, with the demethylating agent 5-aza-dC resulted in *de novo* expression of *ITGA1* by these cells, a finding that supports a causal role for CpG methylation in the suppression of *ITGA1*.

One of the common mechanism by which CpG methylation can causes suppression involves proteins that bind to methylated CpG sites, such as the MBD protein family, including MBD1, MBD2, MBD3, and MeCP2. These proteins recruit and associate with histone deacetylases (HDAC) to promote hypo-acetylation of histones in the immediate vicinity, resulting in increased packing of chromatin and transcriptional repression. Inhibition of HDAC with Trichostatin A (TSA) can be used to evaluate the contribution of such MeCP2-like proteins to transcriptional regulation initiated by CpG methylation. For example, Schwab and Illges [37] found that TSA treatment induces *de novo* expression of CD21 in early B lymphocytes, although the CD21 CpG island remains fully methylated and transcription would otherwise be repressed. In our studies, TSA treatment failed to reverse the suppression of *ITGA1* transcription in CHRF-288-11 cells, a finding that argues for a direct CpG methylation-mediated suppression of transcription by alternate mechanisms. There is precedent for such direct effects of CpG methylation on promoter activity without then influence of histone acetylation and binding. For example, CpG methylation inhibits proenkephalin gene expression by directly interfering with the binding of the positively acting transcription factor, AP-2, essential for maximal basal activity [38]. Moreover, methylation of E2F elements derived from the dihydrofolate reductase, E2F1, and cdc2 promoters prevents the binding of all E2F family members, including E2F1 through E2F5 [39]

A role for CpG methylation in the differentiation of other cell lineages has been documented. During differentiation of primary diploid rat osteoblasts in culture, expression of the osteocalcin (OC) gene increases proportionally to the decrease in CpG methylation of the OC promoter [36]. As already mentioned, in immature B lymphocytes (pro-, pre- and intermediate B lymphocytes), CD21 is not expressed, and a CpG island within the CD21 promoter is fully methylated. In CD21-expressing mature B lymphocytes, plasma B lymphocytes and non-lymphoid cells, the CD21 CpG island is demethylated [37]. With the differentiation of CD4+ T lymphocytes into the Th1 and Th2 lineages, DNase I hypersensitive sites (DHS) appear at *IL-4* and *IL-13*, and CpG demethylation ensues exclusively at the Th2-specific DHS [40]. Lastly, certain genes with restricted expression to hematopoietic cells, such as the genes for myeloperoxidase [41], globin [42], c-fms [43], and the G-CSF receptor [44]are regulated by methylation in a lineage- and differentiation –dependent manner.

The *ITGA1-PELO-ITGA2* locus on chromosome 5 is unique in that it represents the closest proximity of two integrin genes in the human genome and a rare example of an embedded gene, *PELO*, which happens to play a pivotal role in cell cycle regulation. Despite their proximity, these genes are regulated by diverse mechanisms. *ITGA1* is transcriptionally activated by SRF or SRF-like proteins, while *PELO* and *ITGA2* are positively regulated predominantly by Sp1. During differentiation into the megakaryocyte lineage, the expression of *ITGA1* is completely suppressed by a mechanism that results in the full methylation of CpG sites within the proximal promoter region. At the same time, *PELO* and *ITGA2* proximal promoters remain completely unmethylated, and the genes continue to be expressed. Future studies will be directed at the characterization of the precise mechanisms involved in this lineage-restricted and highly selective gene silencing.

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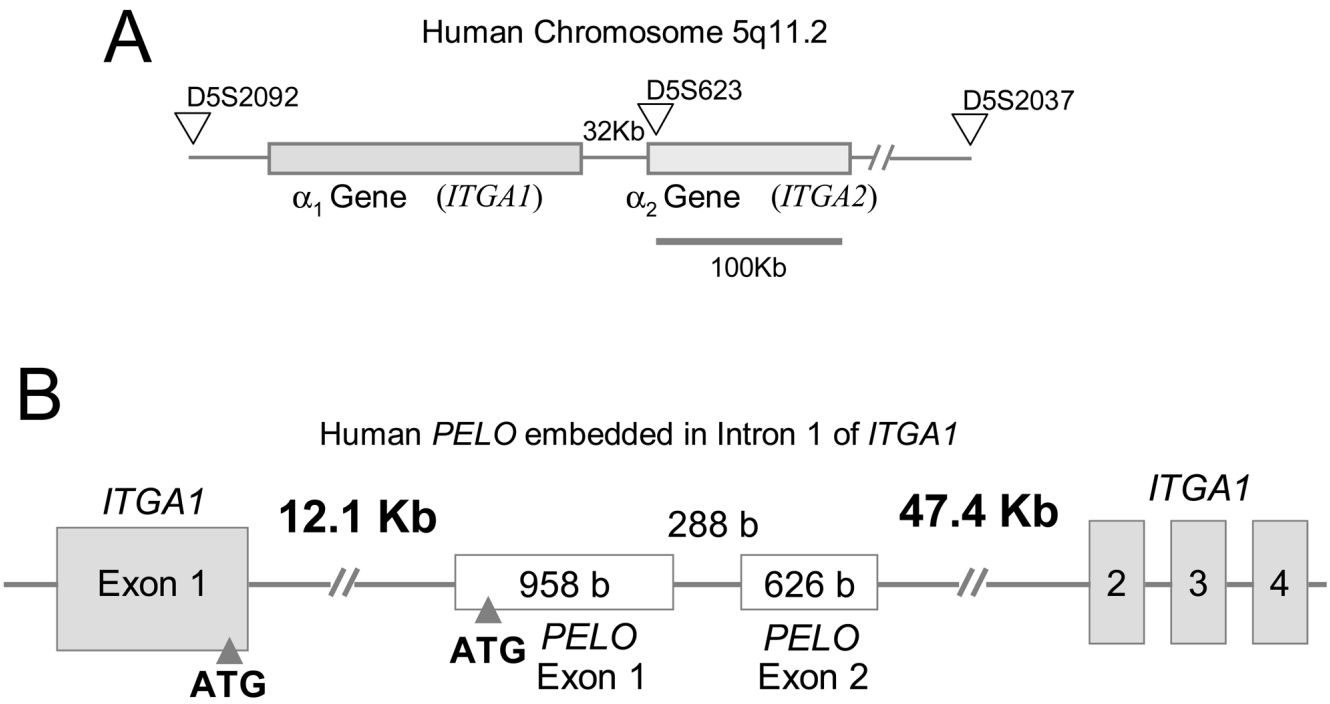


FIGURE 1.

(A) The human *ITGA1-PELO-ITGA2* locus is situated on chromosome 5q11.2 *ITGA1* is separated from *ITGA2* by only 32 Kb. The locus is demarcated by the expressed sequence tag (EST) D5S202 at the 5′ end, D5S623 in the center and D5S2037 at the 3′ end. **(B)** The human *PELO* gene is embedded within intron 1 of *ITGA1*. Human *PELO* consists of two translated exons (Exons 2 and 3) that encode the 385 amino acid protein, pelota. An untranslated Exon 1 starts 11.1kb downstream from the beginning of *ITGA1* intron 1.

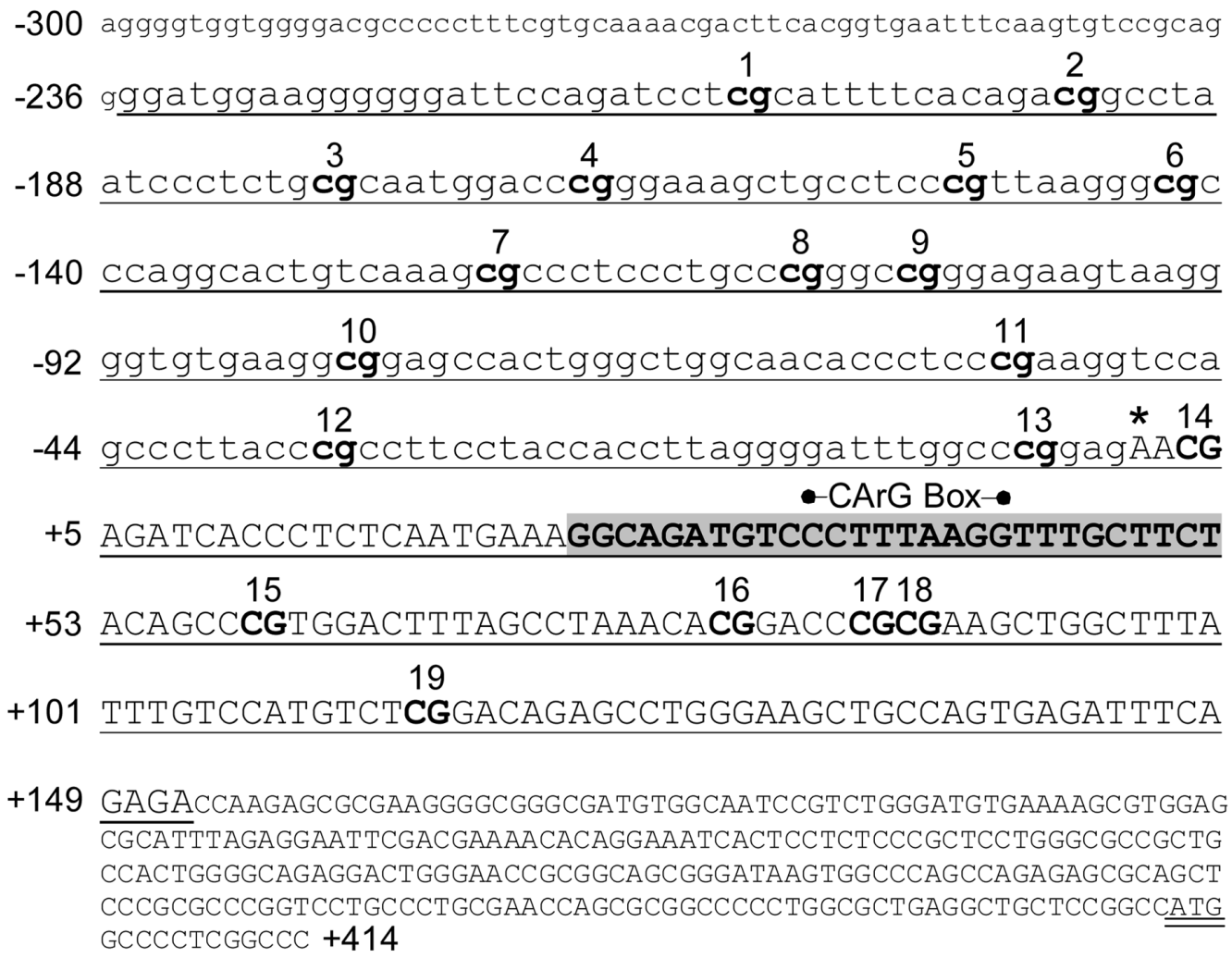


FIGURE 2.

ITGA1 5′ regulatory region and proximal promoter sequence (−300/+414). Nucleotide sequence numbering (from NM_181501) is shown to the left of text lines relative to the transcription start site (*). Exon 1 is indicated by uppercase lettering, and the ATG start codon is double underlined. 5′-regulatory, noncoding sequence is indicated by lowercase lettering. The segment employed for bisulfite DNA sequencing experiments is underlined, and each CpG site within that segment is indicated by bold text and numbered consecutively above the text line. A sequence (+25/+52) that is nearly identical (89%) between chicken [2] and human DNA is highlighted in gray, and the position of a CArG box within that sequence is indicated above the text line.

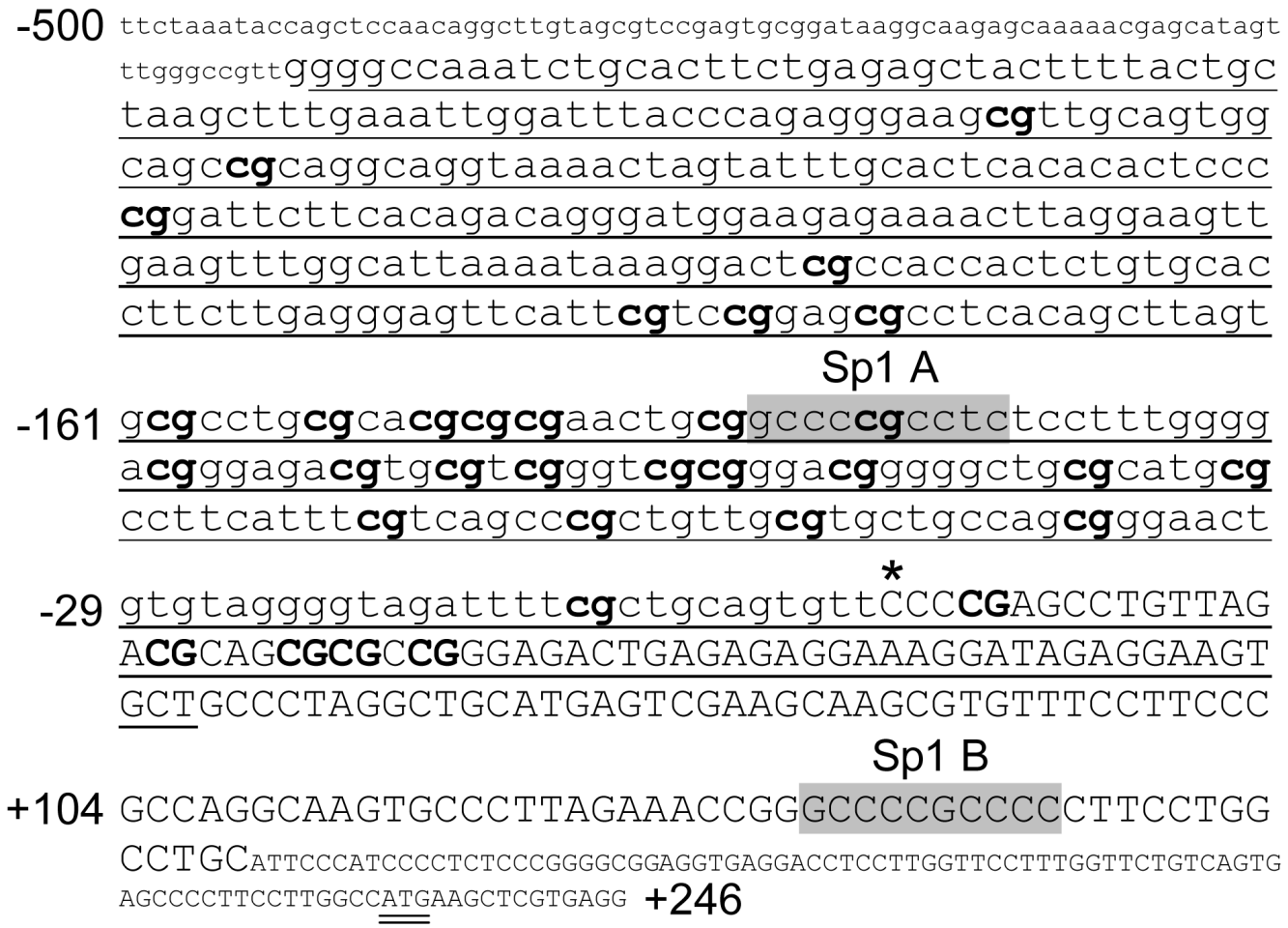


FIGURE 3.

PELO 5′ regulatory region and proximal promoter sequence (−500/+246). Nucleotide sequence numbering (from NM_015946) is shown to the left of text lines relative to the transcription start site (*). Exon 1 is indicated by uppercase lettering, and the ATG start codon is double underlined. 5′-regulatory, noncoding sequence is indicated by lowercase lettering. The segment employed for bisulfite DNA sequencing experiments is underlined, and each of 33 CpG sites within that segment is indicated by bold text. Two Sp1 binding sites are highlighted in gray: Sp1 A at −137/−128 and Sp1 B at +130/+139.

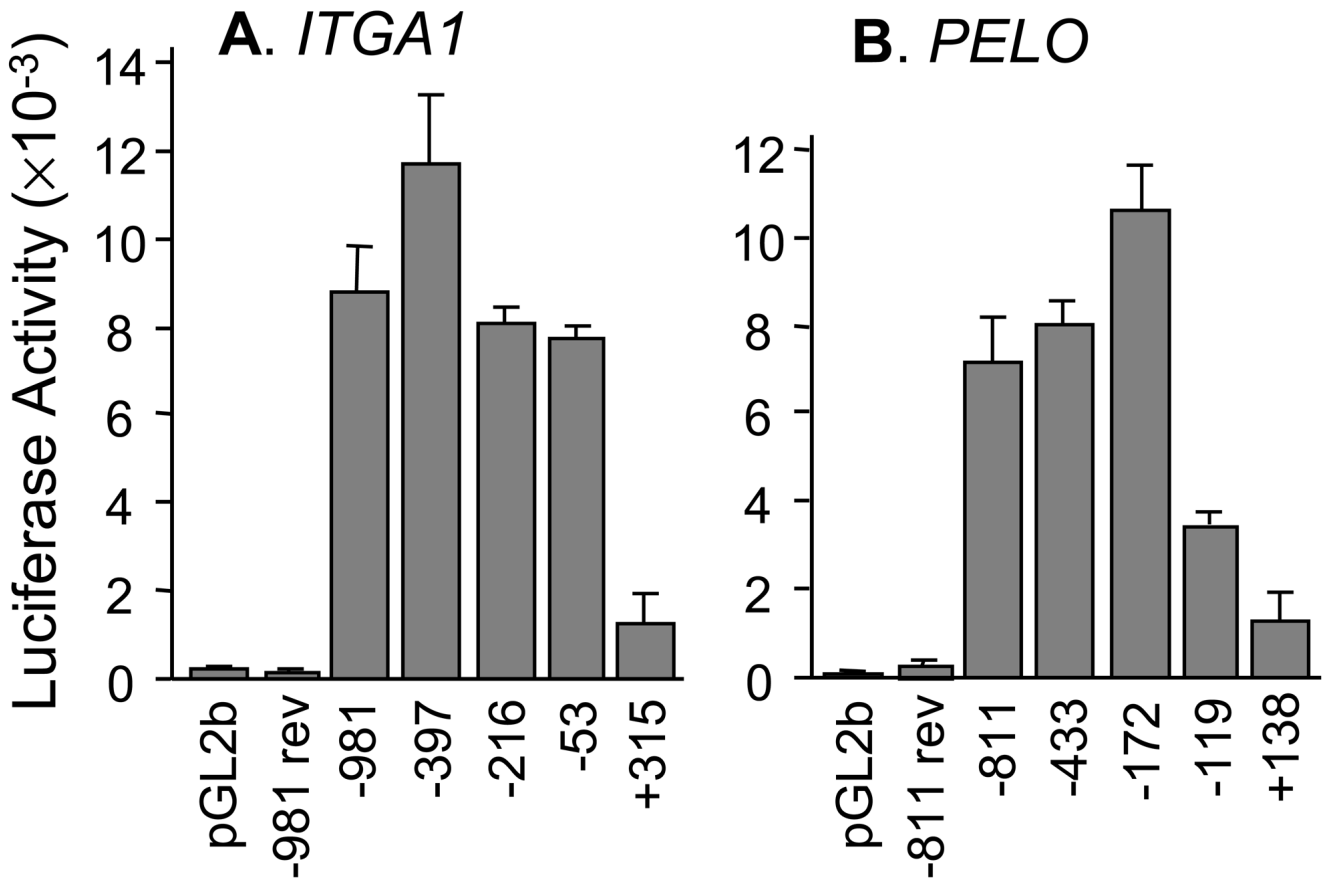


FIGURE 4.

Deletion analysis of the proximal 5′-regulatory region of **A)** *ITGA1* or **B)** *PELO*.

A) A series of reporter plasmids containing fragments of the 5′-region of human *ITGA1* extending from −981, −397, −216, −53, or +315 and through +455 were inserted upstream from the LUC reporter gene in pGL2basic (pGL2b) and transfected into Dami cells. Baseline activity was measured with the pGL2basic (pGL2b) vector alone. Co-transfection of the PRL-TK plasmid was used to normalize for transfection efficiency. Each vertical bar represents the mean +/− 1 SD for three independent experiments. For each construct depicted on the abscissa, relative luciferase activity is indicated on the ordinate. −981 rev represents the entire 5′-sequence from −981 to +455 inserted in the reverse orientation.

B) A series of reporter plasmids containing fragments of the 5′-region of human *PELO* extending from −811, −433, −172, −119, or +138 and through +180 were inserted upstream from the LUC reporter gene in pGL2basic (pGL2b) and transfected into Dami cells. Baseline activity was measured with the pGL2basic (pGL2b) vector alone. Each vertical bar represents the mean +/− 1 SD for four independent experiments. For each construct depicted on the abscissa, relative luciferase activity is indicated on the ordinate. −811 rev represents the entire 5′-sequence (−811/+180) inserted in the reverse orientation.

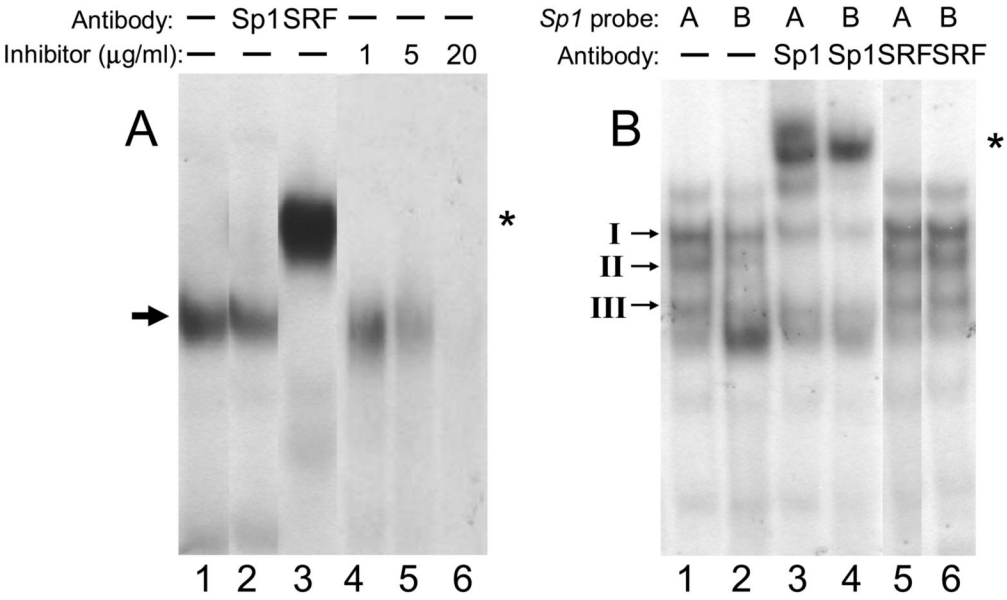


FIGURE 5.

Binding of nuclear proteins to oligonucleotides containing **(A)** the *ITGA1* CArG-like site (residues +35/+43) or **(B)** the *PELO* Sp1 site A (residues −146/−119) or site B (residues +121/+148). **(A)** Nuclear extracts were prepared from HEK293 cells and incubated with the radiolabeled *ITGA1* CArG box oligonucleotide probe (Table 1) either in the absence of antiserum (lanes 1, 4, 5 and 6) or following incubation with anti-Sp1 (lane 2) or anti-SRF (lane 3). Additional aliquots of the extract were incubated with the radiolabeled *ITGA1* CArG box oligonucleotide probe in the presence of 1, 5 or 20 μg/ml of unlabelled *ACTA1* CArG oligonucleotide (Table 1), as a competitor oligonucleotide (lanes 4–6, respectively). Arrow to the left of the gel indicates SRF complex; asterisk to the right of the gel denotes the supershifted complex. **(B)** Nuclear extracts were prepared from Dami cells and incubated with radiolabeled *PELO* oligonucleotide probes (Table 1) corresponding to Sp1 site A (lanes 1, 3 and 5) or Sp1 site B (lanes 2, 4 and 6) either in the absence of antiserum (lanes 1 and 2) or following incubation with anti-Sp1 (lanes 3 and 4) or anti-SRF (lanes 5 and 6). The positions of Sp1/Sp3 complexes I, II, and III [20] are indicated to the left of the gel. Asterisk to the right of the gel denotes supershifted complexes. Lanes 2 and 3 in (A) and lane 5 in (B) are inserted slices from separate experiments, each of which included identical positive and negative controls,

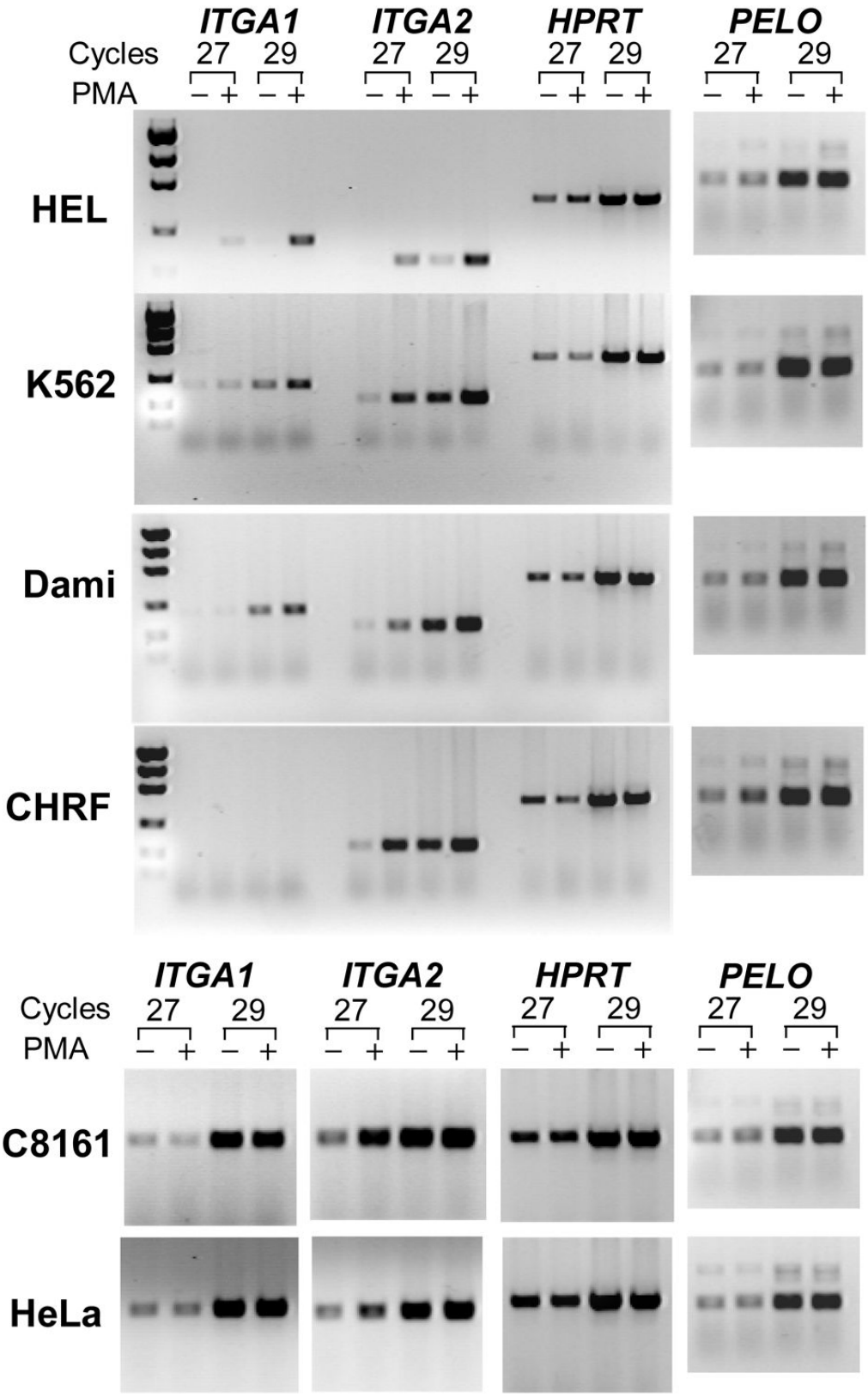


FIGURE 6.

The expression of *ITGA1*, *ITGA2*, *PELO* and the housekeeping gene *HPRT* in human cell lines. **(A)** The level of mRNA in each cell line was measured semi-quantitatively using limiting cycle RT-PCR. For each of the four mRNAs, the cDNA product obtained after 27 or 29 cycles was separated by agarose gel electrophoresis and visualized with ethidium bromide. Comparisons were made between cells cultured in the presence (+) of 5 nmol/L phorbol myristate (PMA) or an equal volume of DMSO (−) for 24 hours. Total RNA was reverse transcribed and the cDNA obtained was used for PCR amplification. Both the megakaryocytic (MK) cell lines HEL, K562, Dami, and CHRF-288-11 and the non-MK cell lines C8161 and HeLa were studied.

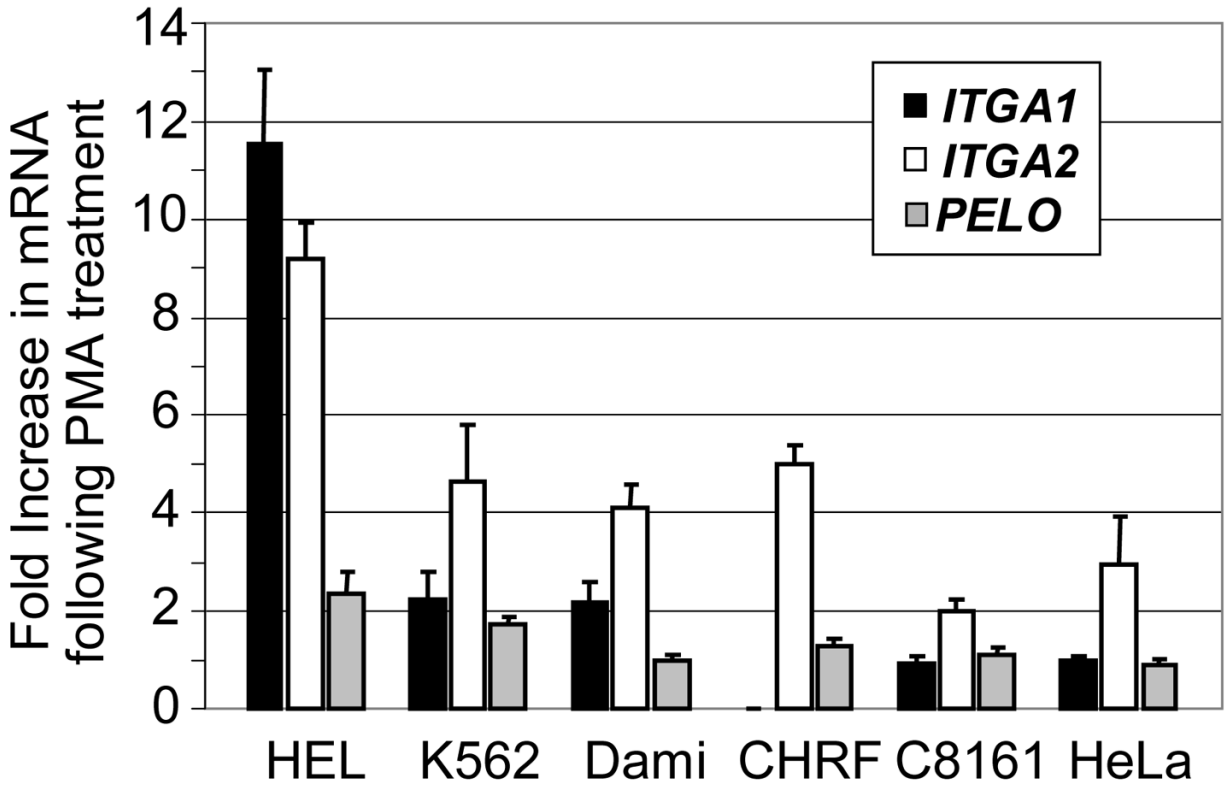


FIGURE 7.

Quantitation of the fold-increase in mRNA expression induced by PMA. From the results depicted in FIGURE 6 for each of the six cells lines indicated on the abscissa, the density of ethidium-bromide stained DNA bands was measured by optical scanning, and the fold-increase in density in PMA-treated samples relative to non-PMA treated samples was calculated. The results are the mean +/− 1 SD of three independent experiments. The increase in mRNA (band density) following PMA treatment is plotted on the ordinate. A value of 1 represents no increase; while a value of 2 represents a one hundred percent increase or doubling of the density. Levels of *ITGA1* mRNA are indicated by black vertical bars; *ITGA2* mRNA, by white bars, and *PELO* mRNA by gray bars.

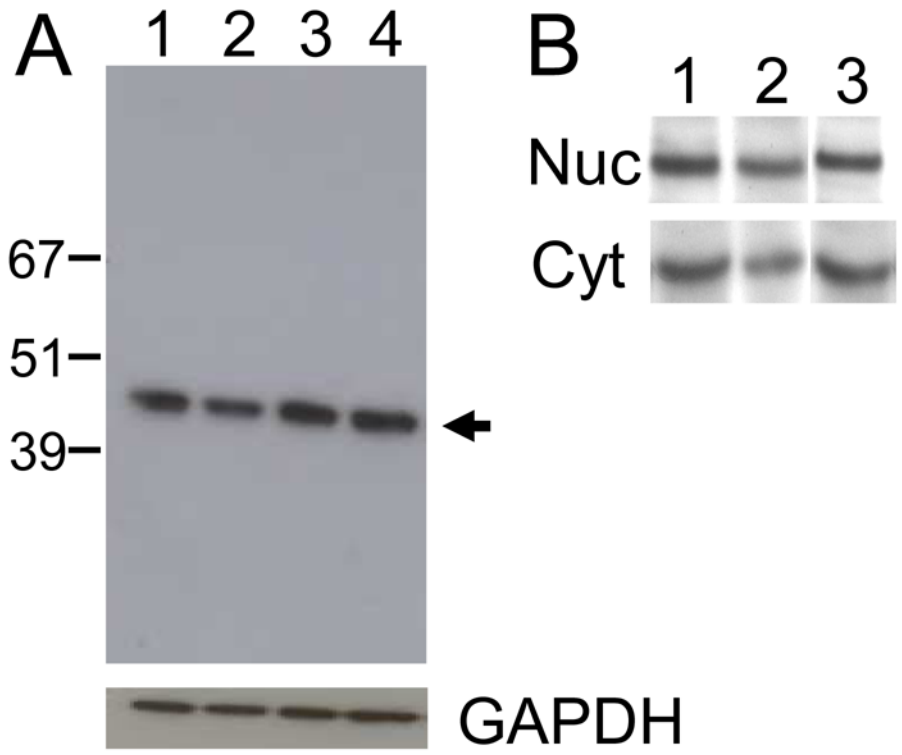


FIGURE 8.

Cellular content of pelota. (**A**) The relative total cell content of pelota was determined by western blot. Equal amounts of total cell protein (15 μg) from (lane 1) Dami cells, (lane 2) CHRF288-11 cells, (lane 3) K562 cells, or (lane 4) HeLa cells were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The position of representative molecular weight marker proteins is indicated to the left of the gel. Pelota was visualized by the binding of murine monoclonal anti-pelota antibody 1A3, and its position is indicated by the arrow to the right of the gel. (**B**) Legend as in A except that nuclear protein (Nuc) and cytoplasmic protein (Cyt) were separated prior to SDS-PAGE. The cell lines employed were: (lane 1) Dami, (lane 2) CHRF288-11, and (lane 3) K562.

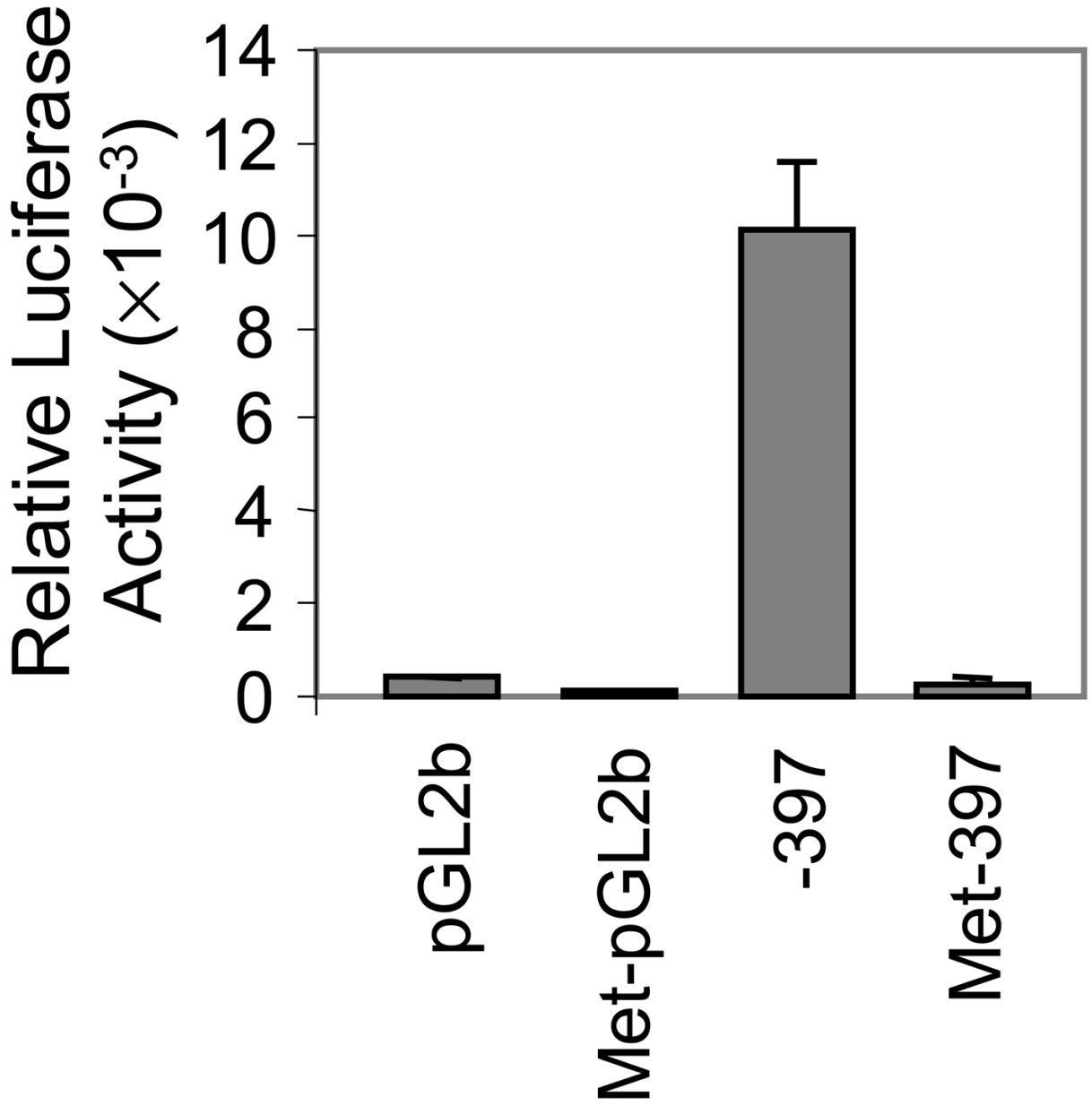


FIGURE 9.

Inhibition of *ITGA1* promoter activity by *in vitro* methylation. The *ITGA1* promoter construct (−397/+455) and pGL2b vector alone (control) were methylated in vitro with SssI methylase and transfected into Dami cells. Co-transfection of PRL-TK plasmid was used to normalize for transfection efficiency. The relative Luciferase activity for each transiently transfected cell preparation was measured and is plotted on the ordinate The activity of each of four constructs (abscissa) is compared: pGL2b; methylated pGL2b (Met-pGL2b); *ITGA1* promoter (−397); and methylated *ITGA1* promoter (Met-397).

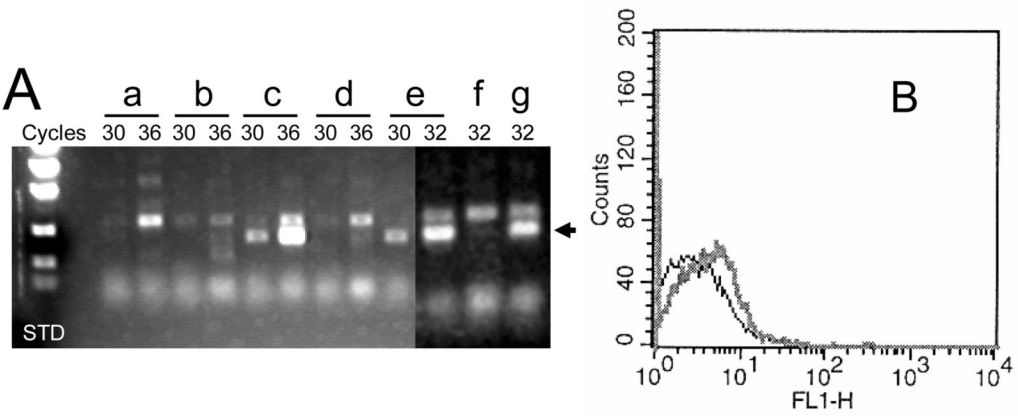
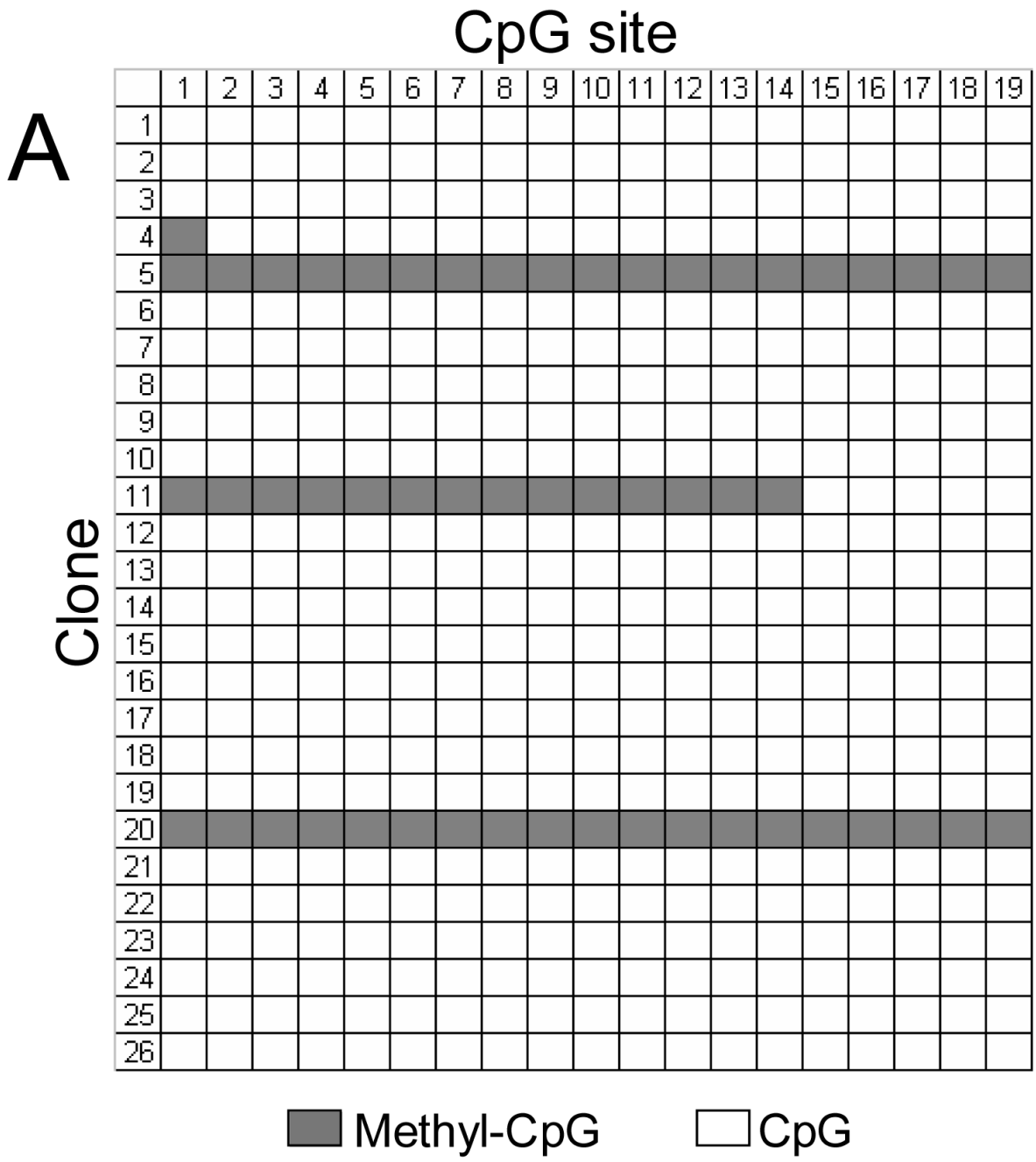


FIGURE 10.

Expression of *ITGA1* mRNA in CHRF288-11 cells after the treatment with 5-aza-deoxyCytidine (5-AZA) or Trichostatin A (TSA). **(A)** The cDNA product amplified by RT-PCR after 30, 32 or 36 cycles is depicted for cells were (a) untreated or treated with (b) 0.01 % (v/v) DMSO for 24 hrs; (c) 5μM 5-AZA for 24 hrs; (d) DMSO for 48 hrs; (e) 5-AZA for 48 hrs; (e) TSA for 48 hrs; or (f) 5-AZA plus TSA for 48 hrs. The arrow in the right hand margin denotes the position of the expected *ITGA1* cDNA product. **(B)** The surface expression of integrin α1 at 48 hours after treatment with 5-AZA (gray curve) is shifted to the right relative to that obtained for cells treated with DMSO (black curve), as measured by flow cytometry using monoclonal anti-α 1 antibody.



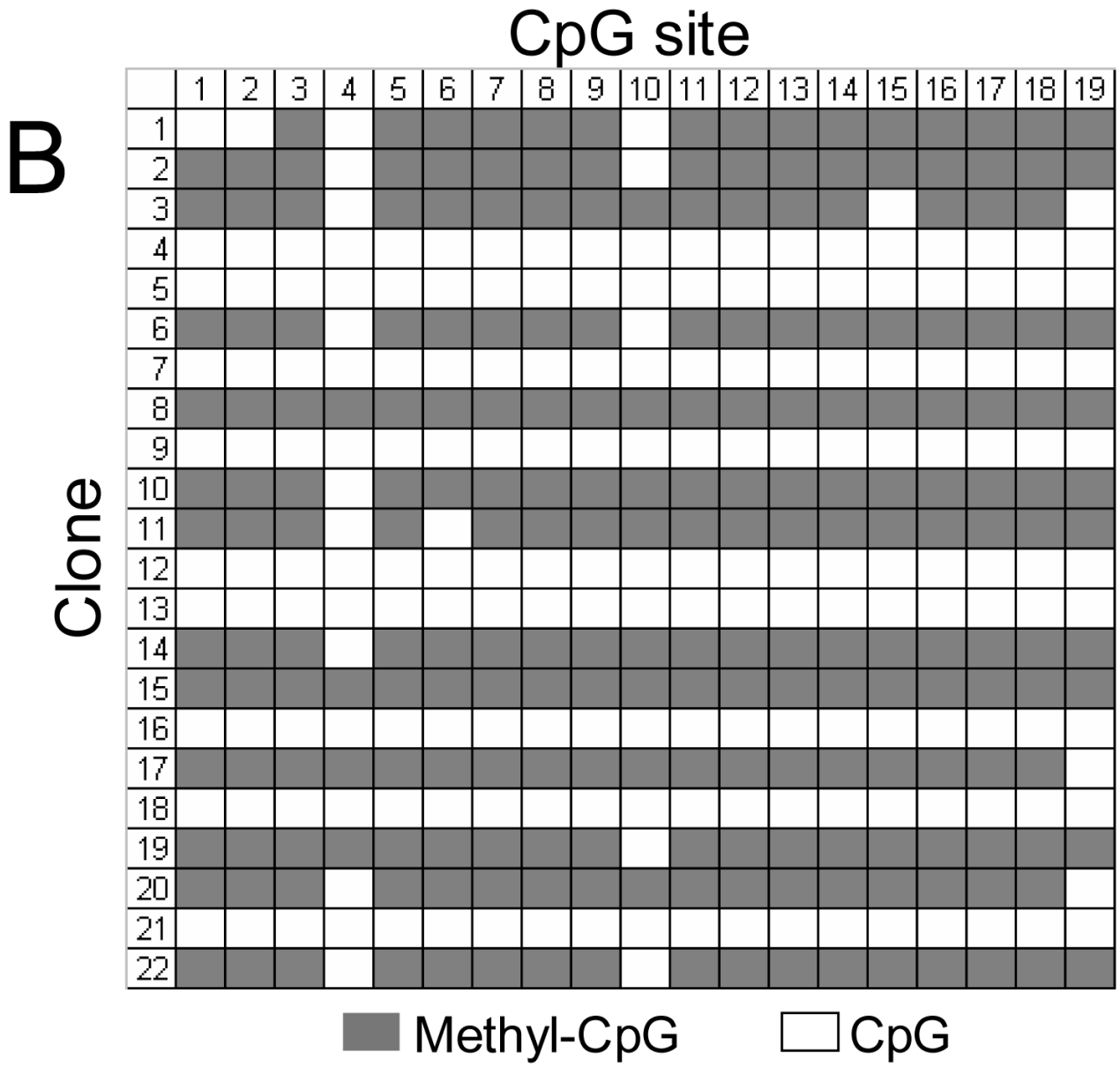


FIGURE 11.

Methylation status of the *ITGA1* promoter CpG-rich region of **(A)** cord blood mononuclear cells (MNC) or **(B)** CD41+ cells, enriched by culture in the presence of recombinant human thrombopoietin (rhTPO). In each grid, each CpG site is represented by a column that is numbered at the top, while individual cloned DNA sequences are represented by a row that is numbered to the left. Methylated CpG sites are indicated as filled squares, and unmethylated sites as open squares. Numbers at the top correspond to the same CpG sites shown in Figures 2 or 11. Mononuclear cells (MNCs) from human cord blood were isolated and cultured in the presence of rhTPO for 8days. CD41+ cells were selected, and genomic DNA was isolated, bisulfite treated, and amplified by PCR, targeting the *ITGA1* CpG-rich region. In MNC (**A**), 2 of 26 clones (#5 and #20) showed complete methylation of each of 19 sites, while a third clone (#11) showed methylation at sites 1 through 14 (3/26 = 11%); in the CD41+ enriched cells

derived from these progenitors (**B**), 13 of 22 clones (59%) showed methylation of at least 16 of the 19 sites.

Table 1

EMSA Oligonucleotide Probes

GENE	SITE	SEQUENCE	START	END	REFERENCE
ITGA1	CArG	GGCAGATGTCCCTTTAAGGTTTGCTTCT	+25	+52	NM_181501
ACTA1	CArG	GCCCAACACCCAAATATGGCTCGAGAAG	−104	−77	NM_001100
PELO	Sp1 A	CGAACTGCGGCCCCGCCTCTCCTTTGGG	−146	−119	NM_015946
PELO	Sp1 B	AGAAACCGGGCCCCGCCCCCTTCCTGGC	+121	+148	NM_015946