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Integrin αIIb promoter-targeted expression of gene products in megakaryocytes derived from retrovirus-transduced human hematopoietic cells

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ABSTRACT **Megakaryocyte-specific expression of the platelet-adhesion receptor, integrin αIIbβ3, is caused by the presence of regulatory elements of the αIIb promoter that direct high-level, selective gene transcription early in megakaryocytopoiesis. To develop methods for targeted expression of transgenes, we transduced human CD34+ peripheral blood cells with a murine leukemia virus (MuLV) vector controlled by the human integrin αIIb promoter (nucleotides −889 to +35). A naturally occurring cDNA encoding the Pl^{A2} alloantigen form (Pro_{33}) of the integrin β3 subunit was subcloned into this construct (−889Pl^{A2}β3) and transduced into cells that endogenously synthesized Pl^{A1}β3 (Leu_{33}) as a marker for detection of provirus-derived β3. The ability of this vector to target expression of Pl^{A2}β3 to megakaryocytes was first examined in cell lines. Immunoblot analysis with human anti-Pl^{A2} alloserum detected synthesis of Pl^{A2}β3 in transduced promegakaryocytic cells; however, Pl^{A2}β3 protein was not detected in transduced epithelial cells. Human hematopoietic CD34+ cells were transduced with −889Pl^{A2}β3 virions and induced to differentiate with megakaryocyte growth and development factor. A hybrid αIIbβ3 complex was formed in progeny megakaryocytes where provirus-derived Pl^{A2}β3 was detected associated with endogenous αIIb subunit. Another αIIb promoter-driven MuLV vector (−889nlacZ) encoding *Escherichia coli* β-galactosidase was used to demonstrate that transgene expression was selectively targeted to the megakaryocyte progeny of transduced CD34+ cells. These studies demonstrate the feasibility of using αIIb promoter-driven MuLV vectors for gene transfer of hematopoietic CD34+ cells to target transgene expression in developing megakaryocytes and platelets and indicate potential applications toward human gene therapy for platelet disorders.**

During megakaryocytopoiesis, pluripotent hematopoietic progenitor and precursor cells differentiate to mature polyploid megakaryocytes that shed small anucleate platelets. This process is associated with three remarkable cellular events. First, megakaryocyte-specific adhesion receptors are expressed, namely integrin αIIbβ3 and the glycoprotein Ib-V–IX complex, which mediate platelet–platelet and platelet–extracellular matrix interactions. Second, cytoplasmic granules are formed that contain agonists, hemostatic mediators, and growth factors. Third, signaling pathways develop that produce cyclic nucleotides, inositol phosphates, and endoperoxides, which induce release of granule components and activation of membrane receptors. Interactions between membrane receptors, cytoplasmic granules, and signaling pathways induce platelets to bind to adhesive proteins exposed on damaged blood vessels, complex with plasma components, mediate blood coagulation, and release granule contents. This regulates blood clotting, stimulates wound healing, and mediates platelet aggregation to seal damaged vessels.

Megakaryocyte-specific expression of the major platelet-aggregation receptor, integrin αIIbβ3, is caused by the presence of regulatory elements of the αIIb promoter that direct high-level, selective gene transcription early in megakaryocytopoiesis. The αIIb promoter has been previously demonstrated to direct high-level, megakaryocyte-targeted gene transcription in human cell lines (1–3), rat cells (4), and transgenic mice (5, 6). An 800-nt fragment of the human αIIb promoter directed expression of the thymidine kinase gene in a megakaryocyte-selective manner in the transgenic mice studies (5, 6). This promoter fragment binds GATA and Ets factors to induce a high level of gene transcription (7), which is restricted to developing megakaryocytes because of an element localized to the immediate 5′ upstream region of the αIIb gene between nucleotides −80 and −130 (2–4).

This investigation uses murine leukemia virus (MuLV)-derived vectors driven by an 889-nt fragment of the promoter of the human αIIb gene to induce early and specific transgene expression during megakaryocytopoiesis of human cells. Human CD34+ hematopoietic cells transduced with MuLV-derived vectors encoding either β3 or β-galactosidase (β-gal) demonstrated lineage-specific transgene expression after differentiation along a megakaryocytic pathway (8). The platelet alloantigen 2 (Pl^{A2}) form of the integrin β3 subunit was used to distinguish provirus-derived protein from endogenous protein in cultured megakaryocytes. This result demonstrates the feasibility of lineage-specific gene expression in pluripotent hematopoietic stem cells and has implications for human gene therapy of hematopoietic disorders.

MATERIALS AND METHODS

Antibodies. Monoclonal antibody AP3 (9) and polyclonal antibodies specific for β3 were from Peter J. Newman (Blood Research Institute, Milwaukee, WI). Human anti-Pl^{A2} β3 alloserum was from Brian Curtis (Blood Center of Southeastern Wisconsin, Milwaukee, WI). Monoclonal antibody AP2 (10), which recognizes the αIIbβ3 complex was from Robert R. Montgomery (Blood Research Institute, Milwaukee, WI). A monoclonal antibody that recognizes the erythrocyte-specific protein, glycophorin A (GPA), was from Sigma.

Cell Lines. Dami and human 293 cell lines were from the American Type Culture Collection. CFT1 cells were previously described (11).

αIIb Promoter. Genomic DNA was isolated from the human promegakaryocyte cell line, Dami, and a fragment of the

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Abbreviations: β-gal, β-galactosidase; GPA, glycophorin A; rhIL, recombinant human IL; MuLV, murine leukemia virus; LTR, long terminal repeat; CMV, cytomegalovirus.

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human αIIb gene promoter was amplified by PCR using sense primer "−889" (5′-TTACGCGTCGACAGATC**TGTGCTCAATGCTGTGCC**-3′) from nucleotide −889 to −872 (boldface) of αIIb and antisense primer (5′-ATAGTTTAGCGGCCGCGCTCGCATCTTCCTTCTTCCAC-3′) encoding nucleotides +32 to +22 of β3 and nucleotides +35 to +19 of αIIb. The antisense primer positions the αIIb promoter in-frame with the translation start site of β3. An αIIb promoter used for β-gal gene transcription was constructed with the −889 primer and antisense primer (5′-ATTATTGGGCCCCATCTTCCTTCTTCCAC-3′) encoding nucleotides +4048 to +4058 of β-gal in pCMVnlac (12) and nucleotides +35 to +19 of αIIb. PCR products were cloned into plasmid vectors pGL3 or pGEM-7zf (+) (Promega), and sequence was confirmed by nucleotide analysis (13).

Retroviral Constructs. *p-889Pl^{A2}β3.* The −889 αIIb promoter for β3 expression was cloned 5′ of cDNA encoding the platelet alloantigen 2 (Pl^{A2}) form of β3 (14) (from Peter J. Newman) within Bluescript plasmid vector (Stratagene). Briefly, Pl^{A2}β3 cDNA in Bluescript was treated with restriction enzymes (*Not*I, *Nsi*I, and *Sal*I), and a three-way ligation was performed to join the *Not*I and *Sal*I αIIb promoter fragment and the two Pl^{A2}β3-Bluescript fragments. The −889Pl^{A2}β3 DNA cassette (Fig. 1) was removed from Bluescript by treatment with *Xba*I and ligated between the long terminal repeats (LTR) of the MuLV-derived retroviral vector, pHIT-SIN, to construct p−889Pl^{A2}β3. pHIT-SIN was derived from vector, pS3, which encodes a 3′-LTR sequence (from Estuardo Aguilar-Cordova, Baylor College of Medicine, Houston, TX) (15) lacking the viral enhancer/promoter so that the αIIb promoter could direct gene transcription.

p−889nLacZ. The −889 αIIb promoter for β-gal was cloned into plasmid vector, pCMVnlac (12) (from Jeffrey S. Bartlett, University of North Carolina, Chapel Hill, NC), encoding *Escherichia coli* β-gal. Briefly, pCMVnlac was treated with restriction enzymes (*Apa*I and *Sal*I), and the αIIb promoter (treated with enzymes *Apa*I and *Sal*I) was inserted 5′ to cDNA encoding β-gal. The −889nLacZ cDNA cassette was removed from pCMVnlac by treatment with *Bgl*II and *NaeI* and ligated between the LTRs of pHIT-SIN to construct p−889nLacZ.

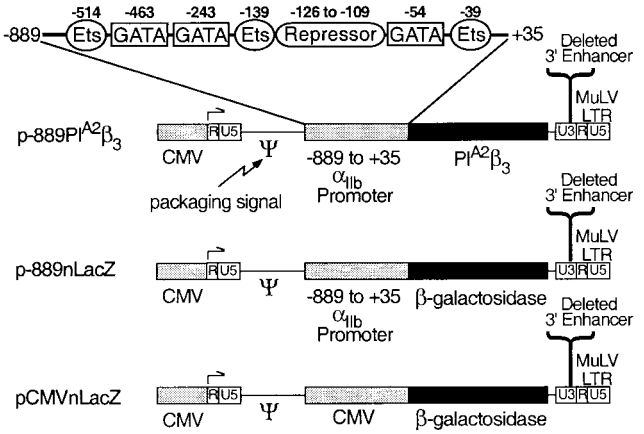

FIG. 1. Schematic diagram of the retroviral plasmid constructs. Transgene cassettes were inserted between the LTR-containing elements (U3, R, U5) of a MuLV-derived vector. The MuLV enhancer region was deleted from a sequence (U3) of the 3′ LTR (15), and the αIIb promoter (nucleotides −889 to +35) was used to direct expression of the Pl^{A2}β3 or β-gal (nLacZ) in transduced cells. This αIIb promoter segment contains sequences necessary for megakaryocyte-specific expression (7). The immediate-early enhancer/promoter region of the CMV drives gene transcription for retroviral production in the 293 cells (see *Materials and Methods*). As a positive control, the CMV promoter was used to drive nonspecific expression of β-gal in cells transduced with virions produced from vector pCMVnLacZ.

The sequence of p−889nLacZ was confirmed by nucleotide analysis.

pCMVnLacZ. pCMVnLacZ was constructed when a cassette encoding the cytomegalovirus (CMV) immediate early promoter and enhancer region and *E. coli* β-gal was removed from pCMVnlac with restriction enzymes (*NaeI* and *SamI)* and inserted between the LTRs of pHIT-SIN.

Retroviral Helper Plasmids. Plasmids pCI-GPZ and pCI-VSV-G were previously described (16).

Typing of Pl^{A} Alloantigen. All cell samples used for −889Pl^{A2}β3 transduction were confirmed homozygous for the Pl^{A1}β3 alloantigen as described (17).

Retrovirus Production. Human 293 cells were transiently transfected on 10-cm plates with 15 μg each of pCI-GPZ, pCI-VSV-G, and either p−889Pl^{A2}β3, p−889nLacZ, or pCMVnLacZ by using the calcium transfection system (Life Technologies, Gaithersburg, MD). After 12 h, cells were placed in fresh medium containing 10 mM *n*-butyric acid (Sigma) (18), and incubation continued at 37°C for 24 h. Virions were concentrated 500-fold, resuspended in Iscove's modified Dulbecco's Eagle's medium (IMDM), and stored at −80°C. Replication-competent virions were confirmed absent from viral preparations by using extended marker rescue assays as described (18).

Selection of CD34+ Cells. Peripheral blood cells were collected after obtaining written informed consent from normal healthy subjects enrolled in an Institutional Review Board-approved study. Subjects were given granulocyte colony-stimulating factor (Amgen Biologicals) at 10 $\mu g \cdot kg^{-1} \cdot d^{-1}$ subcutaneously for days 1–5, and peripheral blood cell collection was performed on days 5 and 6 by using the CS-3000 Plus cell separator (Baxter Diagnostics, McGaw Park, IL). CD34 antigen-positive cells were immunoselected (89% CD34+ purity) from the apheresis product on an Isolex 300i magnetic cell separator (Nexell Therapeutics, Irvine, CA, distributed through Baxter Health Care, Mundelein, IL) as described (19). Selected cells were suspended in X-Vivo 10 (BioWhittaker) containing 1% (wt/vol) human serum albumin, frozen in 10% (vol/vol) DMSO at 5–10 × 10^6 cells per ml, and stored in liquid nitrogen.

Transduction of Human Cell Lines. Dami and CFT1 cells (2.5 × 10^5) were transduced with 500 μl of −889Pl^{A2}β3 retroviral supernatant titered at approximately 1 × 10^6 infectious units/ml in the presence of 8 μg/ml polybrene (Sigma) in one well of a six-well plate at 37°C in 5% CO_2. After 2.5 h, retroviral supernatant was removed, and fresh medium was added. Twenty-six days after transduction, 7.0 × 10^6 cells were lysed from each sample, and 800 μg of each lysate (BCA protein assay, Pierce) was subjected to immunoprecipitation analysis.

Transduction of CD34+ Cells. Human CD34+ cells were transduced by a modified version of a previously described protocol (20). Briefly, cells were maintained in Iscove's modified Dulbecco's Eagle's medium containing 20% FBS, 10 units/ml recombinant human (rh) IL-3, 100 units/ml rhIL-6, 30 units/ml recombinant murine stem cell factor (Genetics Institute, Cambridge, MA) and 10 ng/ml flk2/flt3 ligand (R&D Systems) for 48 h at 37°C in 5% CO_2. Cells were transduced at 1 × 10^5 cells per well of a sterile, 24-well non-tissue culture-treated plate (Falcon-Becton Dickinson) coated with 20 μg/cm^2 RetroNectin (21, 22) (Takara Shuzo, Otsu, Japan) with an estimated virion titer of 1 × 10^6 infectious units/ml (−889Pl^{A2}β3, −889nLacZ, or CMVnLacZ) in Iscove's modified Dulbecco's Eagle's medium plus 20% FCS and rhIL-3, rhIL-6, recombinant murine stem cell factor, and flk2/flt3 ligand. Viral supernatant was removed and fresh supernatant added after 2, 4, and 6 h. This procedure was repeated after 24 h. Twenty-four hours after the final transduction, megakaryocyte formation was induced similar to a described method (8). Cells were resuspended at 2.5 × 10^5 per ml in

Iscove's modified Dulbecco's Eagle's medium containing 10% platelet-poor plasma and recombinant human IL (rhIL)-3, rhIL-6, recombinant murine stem cell factor, and flk2/flt3 ligand plus 100 ng/ml rhIL-11 (Genetics Institute) and 100 ng/ml recombinant human megakaryocyte growth and development factor (23) (Amgen Biologicals) for up to 17 days. Cells were solubilized in 1 ml of lysis buffer and stored at −80°C.

Immunoprecipitation Analysis. Immunoprecipitation analysis was performed as described (24). Precleared lysates were immunoprecipitated for 1 h at 25°C with either AP2 or AP3 coupled to Affi-gel Hz (Bio-Rad). Immunoprecipitates were electrophoresed on a SDS/PAGE gel under nonreducing conditions, and proteins were transferred to Immobilon-P (Millipore) and blocked in 10% FBS in Tris-buffered saline/Tween. Immunoblots were analyzed with human anti-Pl^{A2} alloimmune serum (1:1,000 dilution) and a peroxidase-conjugated $F(ab')_2$ fragment donkey anti-human IgG (H+L) (Jackson ImmunoResearch) at 1:20,000 dilution followed by detection by chemiluminescence. Some membranes were stripped by incubation in buffer (100 mM 2-mercaptoethanol/2% SDS/62.5 mM Tris·HCl, pH 6.7) at 50°C for 15 min, reblocked for 1 h, and reprobed with a rabbit polyclonal antibody specific for $\beta 3$ (4 μg/ml) and a peroxidase-conjugated $F(ab')_2$ fragment donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch) at 1:20,000 dilution followed by chemiluminescence detection.

Indirect Immunofluorescence and Histochemical Staining. Indirect immunofluorescence analysis was performed after 10 days of cytokine treatment to CD34+ cells transduced with −889nLacZ, CMVnLacZ, and untransduced cells. Cells (5×10^5) were blocked for 15 min in 2% BSA in PBS and incubated with 5 μg of AP2 or anti-GPA for 20 min at 25°C and then were treated with phycoerythrin-conjugated $F(ab')_2$ donkey anti-mouse secondary antibody (Jackson ImmunoResearch) for 20 min on ice. Cells were resuspended in 200 μl of 2% formaldehyde and 0.2% gluteraldehyde in PBS and fixed to single wells of a 24-well plate for 15 min at 25°C while centrifuging at 230 × *g*. Positive-staining cells were detected and photographed with a Zeiss Axiovert 10 fluorescence microscope at ×320 magnification. Histochemical staining of cells for β-gal activity was performed as described (12). Cells were photographed a second time, and staining results were tabulated.

RESULTS

Effect of Viral LTR on αIIb Promoter-Targeted Transgene Expression. Previous studies have demonstrated a small region of the αIIb promoter sequence (1.2 kilobase) extending from nucleotide −813 to +33 to control megakaryocyte-specific transgene expression in transfected cell lines (7, 25). To construct a retroviral vector with potential to direct megakaryocyte-targeted gene expression, the 5′ region of the human αIIb gene was amplified by PCR from nucleotide −889 to +35 (−889) by using genomic DNA from a promegakaryocyte cell line (Dami) and inserted between the LTR of a MuLV-derived vector (pHIT-SIN) lacking the 3′-viral enhancer/promoter. Integrin $\beta 3$ cDNA was subcloned 3′ of the αIIb promoter to construct plasmid p−889$Pl^{A2}\beta 3$ (Fig. 1), and −889$Pl^{A2}\beta 3$ virions were produced and confirmed replication-incompetent (see *Materials and Methods*). A rare form of $\beta 3$ cDNA encoding a single amino acid substitution ($Leu_{33} \rightarrow Pro_{33}$) that is recognized as the Pl^{A2} alloantigen of $\beta 3$ was used as a marker of transgene expression. This allowed the use of human alloimmune serum to distinguish the provirus-derived Pl^{A2} (Pro_{33}) form of $\beta 3$ in cells that endogenously synthesized the Pl^{A1} (Leu_{33}) form of $\beta 3$.

A promegakaryocyte cell line (Dami) and an epithelial cell line (CFT1) were first transduced with −889$Pl^{A2}\beta 3$ virions to determine whether viral LTR sequences adversely affect the ability of the αIIb promoter to target gene expression to cells where the endogenous αIIb promoter is active. Dami and CFT1 cells were chosen because they transduce with equally high efficiency by using a MuLV-derived vector, HIT-LZ (16), encoding the *E. coli* β-gal gene (LacZ) (D.A.W. and G.C.W., unpublished observation). On day 26 posttransduction, proviral DNA was detected by PCR analysis in −889$Pl^{A2}\beta 3$-transduced Dami and CFT1 cells (data not shown). Immunoblot analysis was performed to determine whether $Pl^{A2}\beta 3$ was synthesized in Dami and CFT1 cells transduced with −889$Pl^{A2}\beta 3$. Cellular lysates were immunoprecipitated with a $\beta 3$-specific monoclonal antibody (AP3) and provirus-derived $\beta 3$ was detected with human anti-Pl^{A2} alloimmune serum (Fig. 2). $Pl^{A2}\beta 3$ was present in the −889$Pl^{A2}\beta 3$-transduced promegakaryocyte cell line (Dami) but was not detected in −889$Pl^{A2}\beta 3$-transduced epithelial cells (CFT1)or LacZ-transduced and -untransduced (control) cells. In addition, $Pl^{A2}\beta 3$ was not detected after −889$Pl^{A2}\beta 3$ transduction of a lymphoblastic cell line (Raji) (data not shown). These results indicate that viral LTR sequences do not adversely affect the ability of the αIIb promoter to direct transgene expression in a tissue-specific manner. The additional band appearing in Fig. 2 at 97 kDa on the control and transduced immunoblots is likely nonspecific background resulting from chemiluminence detection using AP3 and human sera.

αIIb Promoter-Directed Expression of $Pl^{A2}\beta 3$ in Megakaryocytes Derived from Human CD34+ Hematopoietic Cells. To determine whether the αIIb promoter could target transgene expression to primary megakaryocytes, −889$Pl^{A2}\beta 3$ virions were transduced into mobilized, peripheral blood CD34+ cells from individuals homozygous for the Pl^{A1} form of $\beta 3$. The transduced cells were expanded *in vitro* and induced to undergo megakaryocyte differentiation with IL-3, IL-6, IL-11, recombinant murine stem cell factor, and megakaryocyte growth and development factor. After 17 days, the megakaryocyte-specific αIIb$\beta 3$ receptor was immunoprecipitated from cellular lysates with a complex-specific antibody (AP2), and provirus-derived $\beta 3$ was detected with human alloimmune serum specific for the Pl^{A2} form of $\beta 3$. As shown in Fig. 3 *Upper*, $Pl^{A2}\beta 3$ was synthesized in transduced cells but not in untransduced cells. As expected, $\beta 3$ was detected in the transduced and untransduced samples when the blot was reprobed with an anti-$\beta 3$ polyclonal antibody (Fig. 3 *Lower*). Thus, provirus-derived $\beta 3$ paired with the αIIb subunit to form the αIIb$\beta 3$ complex in the presence of endogenously derived $\beta 3$ in megakaryocyte progeny of CD34+ cells.

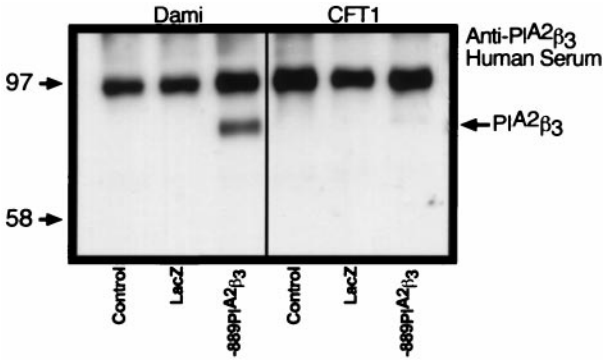

FIG. 2. Analysis of $Pl^{A2}\beta 3$ synthesis in transduced promegakaryocytic (Dami) and epithelial (CFT1) cell lines. Immunoblot shows $Pl^{A2}\beta 3$ synthesis in Dami but not CFT1 cells. After cells were transduced with −889$Pl^{A2}\beta 3$, 800 μg of each cell lysate (7.0×10^6 cells) was immunoprecipitated with a $\beta 3$-specific mAb (AP3). The complexed proteins were separated on a nonreduced SDS/ 8% PAGE gel. $Pl^{A2}\beta 3$ was detected (arrow) by using human anti-Pl^{A2} alloimmune serum and, chemiluminescence. Control represents untransduced cells and LacZ are cells transduced with a retroviral vector encoding β-gal. Molecular mass markers are shown in kDa (left).

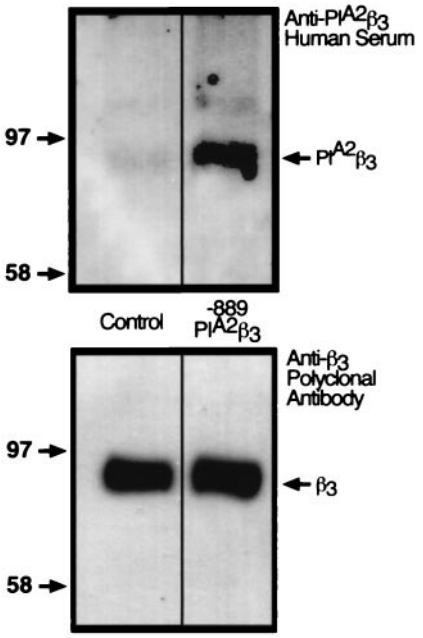

FIG. 3. Analysis of $Pl^{A2}\beta3$ synthesis after transduction of human CD34+ cells. Immunoblot showing $Pl^{A2}\beta3$ synthesis in megakaryocytes derived from transduced CD34+ cells. Seventeen days after transduction and differentiation, 850 μg of each cellular lysate was immunoprecipitated with a αIIbβ3 complex-specific mAb, AP2, and detected with human anti-$Pl^{A2}\beta3$ alloimmune serum (*Upper*). The blot reprobed with a polyclonal anti-$\beta3$ antibody demonstrates the presence of $Pl^{A1}\beta3$ in the untransduced Control sample (*Lower*). Molecular mass markers are shown in kDa (left).

Transgene Expression Was Selectively Targeted to Megakaryocyte Progeny of CD34+ Cells by the αIIb Promoter. To investigate the targeting specificity of the αIIb promoter to megakaryocytes, CD34+ cells were transduced with an αIIb promoter-driven construct (−889nLacZ) encoding the reporter gene, β-gal, and induced for 10 days to expand and undergo megakaryocyte differentiation. The result was a population of multilineage cells that included 20% megakaryocytes expressing αIIbβ3 (data not shown). Histochemical analysis for β-gal activity was performed on these cells to identify transgene expression, as illustrated in Fig. 4 *a–c*. The αIIb promoter directed detectable expression of β-gal in cells transduced with −889nLacZ (Fig. 4*b*), whereas a tissue-nonspecific promoter drove β-gal activity in a noticeably greater population of cells transduced with CMVnLacZ (Fig. 4*c*). As a negative control for histochemical staining, β-gal activity was not detected in untransduced cells (Fig. 4*a*). The −889nLacZ-transduced cells were simultaneously stained for β-gal activity and expression of the megakaryocyte-specific marker, αIIbβ3, to determine whether transgene expression was selectively targeted to megakaryocyte progeny (Fig. 4 *d–f*). Expression of β-gal was detected in 94 of 1,173 total cells (8%) transduced with −889nLacZ (Fig. 5, hatched bar 3), and 63 of 1,173 cells (5%) simultaneously expressed αIIbβ3 and β-gal (Fig. 5, solid bar 3). Thus, 63 of the 94 cells expressing β-gal (67%) were megakaryocytes in the −889nLacZ-transduced sample (Fig. 5, bar 3). In contrast, expression of β-gal was identified in 327 of 1,759 total cells (19%) transduced with CMVnLacZ (Fig. 5, hatched bar 1), whereas 105 of 1,759 total cell (6%) simultaneously expressed αIIbβ3 and β-gal (Fig. 5, solid bar 1). Thus, only 105 of the 327 cells expressing β-gal (32%) were megakaryocytes in the CMVnLacZ-transduced sample (Fig. 5, bar 1). This difference in the capacity of the αIIb promoter compared with the CMV promoter to selectively target expression of β-gal to megakaryocytes (67% vs. 32%) was statistically significant with the χ^2 test ($P = 0.001$). The αIIb promoter limited expression of β-gal to a lower percent of the total cell population than the CMV promoter (8% vs. 19%), although ≈5% of the total cells simultaneously expressed αIIbβ3 and β-gal in both CMVnLacZ and −889nLacZ-transduced samples, demonstrating that the promoters were equal in their ability to drive transgene expression in megakaryocytes (Fig. 5, solid bars 1 and 3).

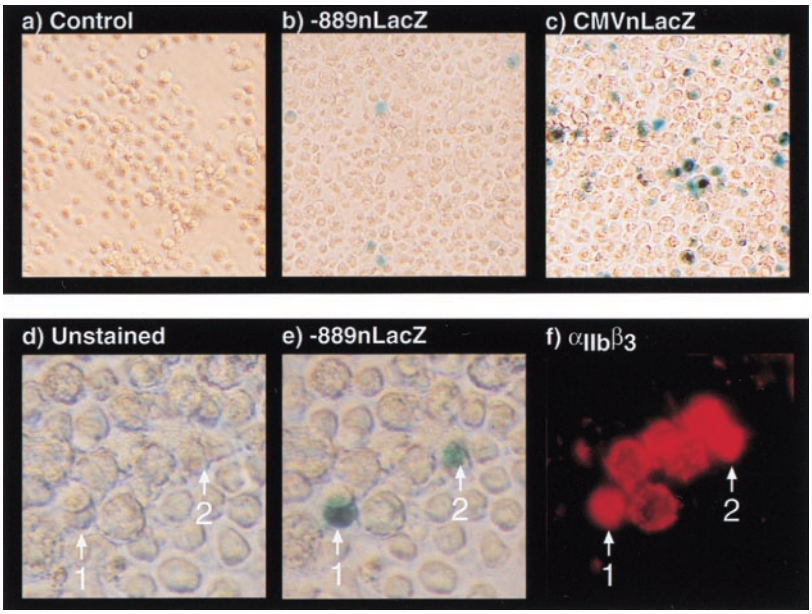

FIG. 4. β-gal targeted to megakaryocyte progeny of −889nLacZ-transduced human CD34+ cells. CD34+ cells were transduced with a MuLV-derived construct, −889nLacZ, encoding β-gal and then induced to form megakaryocytes in culture. Histochemical staining was performed after 10 days of differentiation to determine the percentage of the total cell population expressing β-gal. (*a–c*) β-Gal activity was detected in 0% of untransduced cells (*a*), 8% of cells transduced with −889nLacZ (*b*), and 19% of cells transduced with CMVnLacZ (*c*). (*d–f*) The −889nLacZ-transduced CD34+ cells were simultaneously stained for β-gal activity and αIIbβ3 expression to determine whether β-gal expression was selectively targeted to progeny megakaryocytes. Arrows point to the same two cells unstained (*d*), stained for β-gal activity (*e*), and stained for αIIbβ3 (*f*).

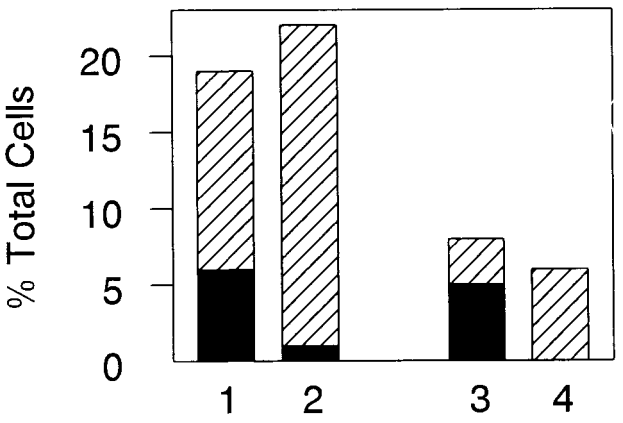

FIG. 5. A stacked bar graph demonstrating the difference in αIIb and CMV promoter-targeted transgene expression to megakaryocyte and erythrocyte progeny of transduced CD34+ cells. Human CD34+ cells were transduced with MuLV-derived constructs, CMVnLacZ (bars 1 and 2) and −889nLacZ (bars 3 and 4) and then induced to expand and differentiate into a multilineage cell population. Histochemical staining was performed after 10 days of differentiation to determine the percent of the total cell population expressing β-gal for each promoter (hatched bars). The cells were simultaneously stained for β-gal activity and expression of the megakaryocyte-specific marker, αIIbβ3 (solid bars 1 and 3) or the erythrocyte-specific marker, GPA (solid bars 2 and 4) and scored to determine whether β-gal expression was selectively targeted to megakaryocytes. In a negative control, β-gal was not detected in untransduced cells. The results are from a single experiment that is representative of two experiments.

The −889nLacZ-transduced cells were examined by simultaneously staining for β-gal activity and expression of the erythrocyte-specific marker, GPA, to determine whether the αIIb promoter directed transgene expression in erythrocyte progeny (Fig. 5, bars 2 and 4). Cells were induced for 12 days to expand and differentiate into a cell population of multilineages including 3% erythrocytes expressing GPA (data not shown). When the tissue-nonspecific CMV promoter was used to direct expression of β-gal, 167 of 761 total cells (22%) had detectable β-gal activity (Fig. 5, hatched bar 2), and nearly 1% of the total cells (4 of 761 cells) simultaneously expressed GPA and β-gal (Fig. 5, solid bar 2). Interestingly, this meant that 14% of all erythrocytes (4 of 29 cells) identified expressed β-gal in the CMVnLacZ sample (data not shown). Although β-gal was expressed in 81 of 1,422 total cells (6%) transduced with −889nLacZ (Fig. 5, hatched bar 4), none of these cells simultaneously expressed β-gal and the erythrocyte marker, GPA. Likewise, 0 of 38 erythrocytes detected expressed β-gal. This difference in β-gal expression in erythrocytes transduced with αIIb promoter versus the CMV promoter (0% vs. 14%) was statistically significant (Fisher's exact test $P = 0.031$). Thus, the αIIb promoter remained silent in erythrocyte progeny but active in the −889nLacZ-transduced sample, whereas the CMV promoter directed expression of β-gal in erythrocytes as well as megakaryocytes, demonstrating a difference in the ability of each promoter to drive β-gal expression in erythrocytes.

DISCUSSION

Lineage-specific expression of proteins is potentially important for gene therapy of hematopoietic disorders affecting distinct cell types (26). For example, indiscriminate expression of the platelet-specific integrin, αIIbβ3, in neutrophils, erythrocytes, monocytes, or lymphocytes might alter the adhesive properties of those cells, resulting in aberrant behavior that could be detrimental (27). As a prelude to tissue-specific human hematopoietic gene therapy, we used MuLV-derived vectors under control of the promoter from αIIb, a gene up-regulated during megakaryocyte differentiation (7), to target synthesis of the integrin β3-subunit and β-gal to megakaryocyte progeny of transduced CD34+ cells. Our results demonstrate that (*i*) MuLV LTR sequences do not adversely affect the ability of the αIIb promoter to direct transgene expression of β3 in a cell type-specific manner, (*ii*) the αIIb promoter targeted transgene expression to megakaryocyte progeny of transduced progenitor blood cells, as evidenced by the formation of a hybrid αIIbβ3 complex consisting of endogenously derived αIIb-subunit and provirus-derived β3, and (*iii*) the αIIb and CMV promoters equally directed expression of β-gal in megakaryocyte progeny of CD34+ cells; however, the αIIb promoter had a statistically increased ability to confine β-gal expression to megakaryocytes. Based on these data, we conclude that αIIb promoter-driven MuLV constructs selectively targeted transgene expression to megakaryocyte progeny of human CD34+ cells. Likewise, we speculate that αIIb promoter targeted expression of β3 in megakaryocytes derived from CD34+ cells could have therapeutic value for the platelet-bleeding disorder Glanzmann's thrombasthenia.

αIIb promoter driven synthesis of β-gal was detected primarily in megakaryocytes within a multilineage population derived from CD34+ progenitor cells. Because transduction of CD34+ cells with CMVnLacZ resulted in the expression of β-gal in 20% of the cell population, and transduction with −889nLacZ confined β-gal expression to 7% of the total cells, we reasoned that the nearly 3-fold decrease in expression may be explained by the CMV promoter's ability to direct expression of the transgene in a lineage-nonspecific manner, whereas β-gal expression under control of the αIIb promoter was achieved primarily in cells that differentiated into megakaryocytes. To test this hypothesis, we determined whether transgene expression was targeted to megakaryocyte progeny by simultaneously staining the cells for β-gal activity and expression of the megakaryocyte-specific marker αIIbβ3. Our results demonstrated that the αIIb promoter has an increased ability compared with the CMV promoter to target transgene expression to megakaryocytes because 67% vs. 32% of all cells expressing β-gal under control of the respective promoters also displayed αIIbβ3.

Evidence from *in vivo* studies with transgenic mice models (5, 6) demonstrate that the αIIb promoter was activated transiently in multilineage potential progenitor cells. Transgene expression was maintained in the megakaryocyte progeny and progressively turned off during erythroid and myeloid lineage differentiation. Their observations suggest that our detection of β-gal in cells not identified as megakaryocytes may be potentially explained by a low-level up-regulation of the αIIb promoter in multipotent hematopoietic progenitor cells or in very early differentiating megakaryocytic, erythroid, and myeloid cells. Based on this, we speculate that the 33% of cells expressing β-gal in the absence of αIIbβ3 in −889nLacZ-transduced samples are early megakaryocytes or multipotent progenitors that express αIIbβ3 at subdetectable levels with the relatively insensitive immunofluorescence analysis but stain positive for β-gal with a sensitive enzymatic assay.

Because megakaryocytes and erythrocytes are derived from a common precursor cell, we investigated αIIb promoter-driven β-gal expression in erythrocytes derived from transduced CD34+ cells by simultaneously staining the cells for β-gal activity and expression of the erythrocyte-specific marker GPA. β-Gal was not detected in erythrocytes after −889nLacZ transduction of CD34+ cells, providing support for the contention that retrovirus-mediated transgene expression driven by the αIIb promoter was not leaky. In contrast, transgene synthesis driven by the CMV promoter, a lineage-nonspecific promoter, was evident in megakaryocytes as well as erythrocytes.

The strategy to transduce CD34+ cells followed by their induction to differentiate along the megakaryocytic pathway *in vitro* was chosen to parallel strategies that might be applicable for human gene therapy *in vivo* (8). Because terminally differentiated megakaryocytes, leukocytes, and erythrocytes have limited lifespans, genetic material must be transferred into the self-replicating pool of stem cells to maintain long-term transgene expression. Small animals have been transplanted with an engrafting population of human CD34+ cells that have sustained long-term transgene expression into the progeny cells (28). Confusingly, only short-term transgene expression has been achieved when CD34+ cells are transplanted into humans. This may be partially because of an immune response to stem cells expressing a transgene under the control of a tissue-nonspecific promoter. Likewise, stem cells transduced with a MuLV construct under control of the αIIb promoter may avoid elimination by the immune system and sustain long-term transgene expression that is not activated until derived pluripotent progenitor cells commit to megakaryocytopoiesis. Short-term transgene expression in humans may also be due to a low efficiency of transduction into the stem cell population necessary for reconstituting the human hematopoietic system. We observed a moderate efficiency of transduction into CD34+ cells with the detection of αIIb promoter-driven transgene expression in 63 of 310 derived megakaryocytes (20%); however, the potential usefulness of this MuLV vector for human gene therapy remains to be determined *in vivo*. Although MuLV-derived vectors have a limited capability to transduce the slow-dividing pool of stem cells, the αIIb promoter may alternatively be used in other gene-transfer systems [i.e., adeno-associated virus type 2 (12) or lentivirus (29, 30)] that can transduce nondividing cells with greater efficiency than MuLV.

The capacity to target expression of heterologous gene products to megakaryocyte progeny of CD34+ cells presents a variety of possible applications. First, signaling and other molecules or their activators may be expressed in megakaryocytes to examine the effect on cytoskeletal development and receptor activation (31). Second, modulation of the activatability of multiple-subunit receptors like αIIbβ3 of megakaryocytes may be achieved by targeting expression of abnormal subunits (β3) that can complex with their normal counterparts (αIIb) to study the result on receptor function (32). Third, megakaryocyte-targeted expression of dominant-negative gene products may be performed to observe the consequence of inhibited synthesis of growth factors, membrane receptors, and signaling molecules on platelet development and function. These three applications may be studied *in vitro* and have the potential to be assessed *in vivo* by infusion of transduced cells into animal models to examine the effect of the altered platelets on physiological processes such as primary hemostasis and wound healing in addition to pathophysiologic events leading to atherogenesis, thrombogenesis, and thrombocytopenia. Finally, this technology was developed for its potential use for human gene therapy of platelet disorders and may allow platelets to deliver other therapeutic agents to the site of a vascular injury.

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