Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 1521–1525, February 1991
Cell Biology

Selective targeting of gene products with the megakaryocyte platelet factor 4 promoter

(platelet targeting)

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Communicated by Oscar D. Ratnoff, November 27, 1990

ABSTRACT **We have used the 1.1 kilobases of the 5′ upstream region of the platelet factor four (PF4) gene coupled to the prokaryotic β-galactosidase gene to generate two lines of transgenic mice that express this construct. Studies of blood, bone marrow, spleen, and thymus reveal that platelets are the only circulating blood cells and megakaryocytes are the only hematopoietic precursor cells that possess the prokaryotic enzyme. The lack of transgene expression in brain, heart, intestine, kidney, liver, lung, and skeletal muscle was established by *in situ* staining of tissue sections as well as kinetic assay of tissue homogenates. These data suggest that this domain of the PF4 promoter contains most, if not all, of the tissue-specific region of the gene. Unexpectedly, the adrenal gland exhibits ≈2% of the levels of β-galactosidase possessed by megakaryocytes and the distribution of the prokaryotic enzyme corresponds to the location of mineralocorticoid-secreting cells. This result implies that either the PF4 gene is transcribed at low levels in specialized adrenal cells or that these specialized endocrine cells possess trans-acting factors similar to those that control the megakaryocyte promoter. The selective high-level expression of transgenes linked to the PF4 promoter should allow us to augment or suppress the *in vivo* levels of critical components in megakaryocytes and platelets and subsequently ascertain the effects of these modifications.**

Pluripotent bone marrow stem cells give rise to the 2N megakaryoblast, which subsequently matures into a polyploid megakaryocyte. These transformations are associated with three striking cellular alterations. First, specialized membrane receptors such as GpIb and GpIIb/IIIa arise, which can interact with specific plasma components and adhesive macromolecules (1). Second, cytoplasmic α granules appear, which contain hemostatic mediators such as platelet factor 4 (PF4) and growth factors such as platelet-derived growth factor (2–6). Third, signaling pathways develop, which generate cyclic nucleotides, inositol phosphates, as well as endoperoxides, whose summed effect induces release of granule constituents as well as activation of membrane receptors (1). The mature polyploid megakaryocytes then fragment into small anucleate platelets, which possess all of the above cellular machinery and circulate in the blood.

Blood platelets play a central role in normal hemostasis and wound healing. The complex interactions between specific receptors, signaling pathways, and cytoplasmic granules allow platelets to bind to adhesive proteins exposed on damaged blood vessels, complex with plasma components that accelerate blood coagulation, release α granule constituents, which modulate blood clotting as well as stimulate wound healing, and take part in plasma protein-mediated platelet–platelet aggregate formation, which seals damaged areas of the vascular tree (1, 7). Hyperactivity of this system is believed to initiate thrombogenesis and atherogenesis.

The events that govern *in vitro* conversion of stem cells to mature megakaryocytes and platelets have been thoroughly investigated but the *in vivo* regulation of this process is poorly understood (8). Extensive biochemical studies have uncovered a wealth of details about how platelets are able to carry out their biologic functions under *in vitro* conditions but less is known about the importance of these mechanisms under *in vivo* conditions. We have recently isolated a full-length rat PF4 cDNA and have defined the structure of the megakaryocyte gene (9). In the sections below, we describe the use of the PF4 promoter in transgenic mice to selectively target prokaryotic β-galactosidase to megakaryocytes and platelets. This approach should allow us to alter the levels of critical megakaryocyte and platelet components and subsequently determine the *in vivo* effects of these modifications. Thus, this system should be useful in uncovering the *in vivo* pathway by which stem cells produce platelets as well as ascertaining the importance of given receptors, signaling pathways, and granule components to normal and abnormal *in vivo* platelet function.

MATERIALS AND METHODS

Plasmid Constructions and Generation of Transgenic Mice. The plasmid pPF4lacZ was constructed by inserting a 1.1-kilobase (kb) rat PF4 *Nde* I/*Ban* II genomic fragment, which includes 1104 bases of 5′ upstream sequence as well as the cap site to +20 into a PUC 19-based plasmid designated as pSDKlacZpA (a generous gift from Janet Rossant, Mount Sinai Research Institute, Toronto, Canada). This latter plasmid contains the 3.34-kb prokaryotic β-galactosidase gene without its upstream regulatory region flanked by a short oligonucleotide containing a Kozak sequence with a unique *Hin*dIII site at the 5′ end and a unique *Kpn* I site at the 3′ end. The construction of pPF4lacZ was accomplished by ligating the rat genomic fragment to *Hin*dIII linearized pSDKlacZpA with synthetic linkers, which creates a unique *Apa* I site at the 5′ end of the megakaryocyte promoter. The PF4 promoter/β-galactosidase gene of 4.4 kb was removed from pPF4lacZ by cutting with *Apa* I and *Kpn* I, isolated by agarose gel electrophoresis, and injected into the pronuclei of fertilized eggs from superovulated female mice (FVB/NTacfBR; Taconic Farms) as described (10). The resultant embryos were reimplanted into pseudopregnant outbred females (CD-1; Charles River Breeding Laboratories). The offspring were screened for transgene integration by Southern blot analysis of tail DNA purified as described (10) and probed with a ^{32}P-labeled 3.34-kb prokaryotic β-galactosidase gene.

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Abbreviations: PF4, platelet factor 4; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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Detection of β-Galactosidase Expression in Different Tissues. Transgenic mice as well as their normal littermates were anesthetized with ether prior to sacrifice. Tissue samples were immediately fixed for 1 hr at 4°C with 2% (wt/vol) paraformaldehyde in 0.1 M Pipes buffer (pH 6.9) supplemented with 2 mM $MgCl_2$ and 1.25 mM EGTA, incubated for 3 hr at 4°C with phosphate-buffered saline (PBS) (136 mM NaCl/8 mM Na_2HPO_4/2.6 mM KCl/1.4 mM KH_2PO_4, pH 7.4) supplemented with 2 mM $MgCl_2$ and 30% (wt/vol) sucrose, embedded in the embedding medium OCT (Miles), and frozen on dry ice. Serial 6-μm sections were cut with a Cryostat and placed on glass slides. Alternate serial cuts were stained with hematoxylin and eosin (12) or fixed for 5 min with 0.5% glutaraldehyde in PBS at 4°C; washed with PBS containing 2 mM $MgCl_2$; incubated at 4°C for 10 min in the presence of PBS containing 2 mM $MgCl_2$, 0.02% Nonidet P-40, and 0.01% sodium deoxycholate; and stained for β-galactosidase activity at 37°C with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) solution [35 mM $K_3Fe(CN)_6$/35 mM $K_4Fe(CN)_6$/2 mM $MgCl_2$/0.02% Nonidet P-40/0.01% sodium deoxycholate/1 mg of X-Gal per ml (Bethesda Research Laboratories)]. Prolonged staining for the enzyme (>8 hr) generates positive reactions in nontransgenic tissue unless a eukaryotic β-galactosidase inhibitor such as chloroquine is used. Therefore, all studies were carried out for 3–4 hr in the absence of chloroquine as well as for 10 hr in the presence of the inhibitor at a concentration of 0.3 mM.

Blood samples were obtained by cardiac puncture. Peripheral blood cells were isolated by centrifugation for 8 min at 800 × *g*, platelets were harvested by centrifugation for 15 min at 3160 × *g*, and femoral bone marrow cells were collected as described (13). Blood and bone marrow cells were stained with Wright's stain or fixed in suspension for 15 min with 0.5% glutaraldehyde in PBS, washed with PBS containing 2 mM $MgCl_2$, and stained at 37°C with modified X-Gal solution [7 mM $K_3Fe(CN)_6$/7 mM $K_4Fe(CN)_6$/2 mM $MgCl_2$/0.02% Nonidet P-40/1 mg of X-Gal per ml] in the presence and absence of chloroquine as outlined above.

Bone marrow cells of different DNA content that exhibit β-galactosidase activity were isolated by fluorescence-activated cell sorter (FACStar Plus, Becton Dickinson), and the populations obtained were identified with respect to cell type. To this end, the cells were cultured for 2 hr with 18 μM Hoechst dye added for quantification of DNA content per cell, washed with PBS, and then prepared for measurement of β-galactosidase with the fluorescent substrate fluorescein di-β-D-galactopyranoside (14). The cell populations of differing ploidy that exhibited β-galactosidase activity were sorted and then stained for the lineage-specific megakaryocyte marker acetylcholinesterase (15).

The various organs, bone marrow cells, and platelets were quantitatively assayed for β-galactosidase activity. The cells and organs were solubilized by adding 0.1–0.4 g of tissue to 2–3 ml of 1 mM EDTA/5 mM dithiothreitol/7% (vol/vol) glycerol in 20 mM Tris buffer (pH 7.4) and then homogenizing the mixtures on ice with a Polytron homogenizer (PCU-2; Brinkmann Instruments). The homogenates were centrifuged at 4°C for 20 min at 110,000 × *g*. Alternatively, bone marrow cells or platelets were lysed with PM solution (60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1 mM $MgCl_2$/50 mM 2-mercaptoethanol/0.02% Nonidet P-40) and centrifuged at 4°C for 15 min in a microcentrifuge. The supernatants were collected and then assayed for protein content (16). The amount of enzymatic activity present in the samples described above was determined with *o*-nitrophenyl β-D-galactopyranoside as described (17). For each time point, the value obtained for a given nontransgenic tissue homogenate was subtracted from the value obtained for the same transgenic tissue extract. The concentration of β-galactosidase in megakaryocytes was calculated by multiplying whole bone marrow values by 120. This correction factor is derived from the relative frequency of megakaryocytes within the bone marrow (0.05–0.1%) and the relative size of this polyploid cell compared with other cell types (20–70 μm versus 10–15 μm). No attempt was made to directly quantitate the levels of β-galactosidase in purified megakaryocytes because of the small amounts of bone marrow available from each mouse, the low frequency of this cell type within the bone marrow, and the relative insensitivity of the assay for the prokaryotic enzyme. The initial rates of substrate turnover were used to estimate the levels of prokaryotic enzyme with 1 unit of activity defined as an increase of 0.1 absorbance unit/hr at 420 nm. Prior experiments demonstrated that the homogenization procedure has a minimal effect on β-galactosidase activity since the calculated recovery of purified enzyme added during the initial solubilization is >90% (data not shown).

RESULTS

Generation of Transgenic Mice Containing the PF4 Promoter Linked to the Prokaryotic β-Galactosidase Gene. The construct used to produce transgenic mice contains 1104 base pairs of the 5′ upstream region of the rat PF4 gene linked to the prokaryotic β-galactosidase gene (PF4lacZ) (Fig. 1*A*). This segment of the PF4 gene was selected because transient expression assays with primary bone marrow cells suggested that the tissue-specific regulatory elements are located within this domain (18). PF4lacZ was microinjected into the pronuclei of fertilized mouse eggs, injected embryos were implanted into pseudopregnant outbred females, and offspring were screened for transgene integration. Two founder mice (founders 10 and 12) were identified of 12 mice produced. Both founders were mated to FVB females to generate F_1 transgenic heterozygous mice, which were then intercrossed to produce F_2 transgenic heterozygous and homozygous mice. Founder 10 transmitted the transgene to its progeny in a Mendelian fashion. Founder 12 was presumed to be mosaic as only 1 of 22 offspring was transgenic but subsequent inheritance was as predicted for a normal autosomal locus. Southern analyses of founders as well as F_1 progeny (data not shown) and F_2 offspring (Fig. 1*B*) utilizing *Eco*RI showed a single band of 4.4 kb. Heterozygous and homozygous F_2 mice were easily identified by gene dosage (Fig. 1*B*, lanes 6–8 versus lanes 1 and 2). Homozygosity was confirmed in founder 10 offspring by mating homozygotes to nontransgenic mice and demonstrating that all offspring were transgenic. Founder 10 exhibited two to four copies of PF4lacZ

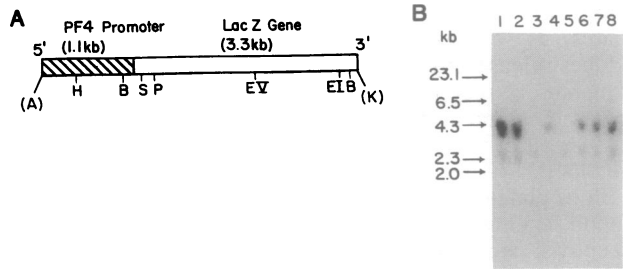

FIG. 1. (*A*) The PF4lacZ construct used for production of transgenic mice. The construct contains 1.1 kb of the 5′ upstream region of the rat PF4 promoter linked to the 3.34 kb of the prokaryotic β-galactosidase (*lacZ*) gene without its regulatory region. A, *Apa* I cohesive ends; B, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn* I cohesive ends; P, *Pst* I; S, *Sph* I. (*B*) Southern blot analyses of 10 μg of mouse DNAs prepared from F_2 offspring of founder 10 and fractionated by 1% agarose gel electrophoresis. Lanes: 1 and 2, homozygotes; 3 and 5, nontransgenic mice; 4 and 6–8, heterozygotes. *Hin*dIII markers are indicated by arrows.

integrated into a single chromosomal site in a head-to-tail fashion as judged by comparison with diluted linearized control DNAs (data not shown). F_1 progeny of founder 12, which inherited the transgene, possessed one copy of PF4lacZ integrated into a single chromosomal site (data not shown).

Selective Expression of the PF4lacZ Transgene in Platelets and Megakaryocytes but Not in Other Hematopoietic Cell Lines. The offspring of the founder mice were investigated for expression of β-galactosidase in peripheral blood cells, bone marrow progenitor cells, splenic cells, and thymic cells. The results described below were obtained with line 10 transgenic mice but identical data were generated with line 12 transgenic mice (data not shown). Blood smears were examined by *in situ* staining for β-galactosidase with X-Gal used as substrate. Fig. 2*A* shows that small, anucleate platelets of transgenic animals but not their normal littermates stain for β-galactosidase, whereas erythrocytes and leukocytes from both types of mice do not contain the prokaryotic enzyme. To pinpoint transgene expression, blood from the two types of mice was processed to obtain fractions containing either platelets or erythrocytes and nucleated leukocytes. Fig. 2 *B* and *C* reveals that platelets from transgenic mice but not from normal littermates stain intensely for β-galactosidase. Examination of the erythrocyte/nucleated leukocyte fractions from both types of mice showed no staining for the enzyme (data not shown). To ascertain whether transgene expression specifically takes place in megakaryocytes, bone marrow

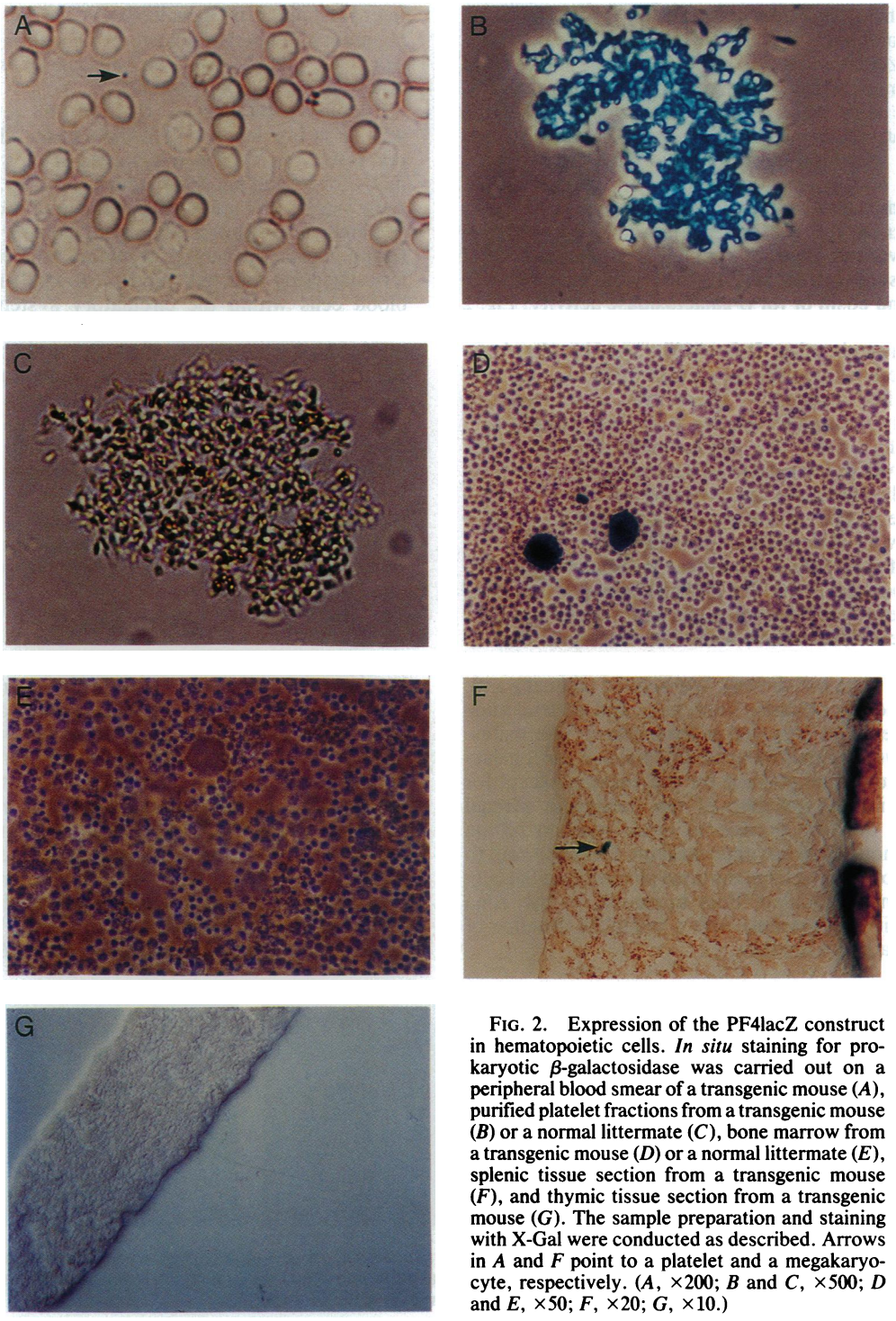

FIG. 2. Expression of the PF4lacZ construct in hematopoietic cells. *In situ* staining for prokaryotic β-galactosidase was carried out on a peripheral blood smear of a transgenic mouse (*A*), purified platelet fractions from a transgenic mouse (*B*) or a normal littermate (*C*), bone marrow from a transgenic mouse (*D*) or a normal littermate (*E*), splenic tissue section from a transgenic mouse (*F*), and thymic tissue section from a transgenic mouse (*G*). The sample preparation and staining with X-Gal were conducted as described. Arrows in *A* and *F* point to a platelet and a megakaryocyte, respectively. (*A*, ×200; *B* and *C*, ×500; *D* and *E*, ×50; *F*, ×20; *G*, ×10.)

cells were assayed by *in situ* staining for β-galactosidase. Fig. 2 *D* and *E* indicates that 0.05–0.10% of the nucleated bone marrow cells of transgenic mice but not their normal littermates stain positive for the enzyme and exhibit diameters that range from ≈20 to ≈70 μm (normal hemopoietic cell diameters are 10–15 μm). The frequency and size of these β-galactosidase-positive cells are typical of bone marrow megakaryocytes.

Transgenic bone marrow cells were also assayed for β-galactosidase with the fluorescent substrate fluorescein di-β-D-galactopyranoside as well as for DNA content with Hoechst dye and then subjected to flow cytometry. Based on two separate experiments, we demonstrated that 0.1% of the transgenic bone marrow cells contained prokaryotic β-galactosidase with 26.9% of the total enzymatic activity in the 2N peak, 16.8% of the total enzymatic activity in the 4N peak, 10.1% of the total enzymatic activity in the 8N peak, 43.8% of the total enzymatic activity in the 16N peak, and 2.4% of the total enzymatic activity in the 32N peak. The β-galactosidase-positive cells were sorted and then stained for the rodent megakaryocyte-specific enzyme acetylcholinesterase (15). All cells, including those obtained from the 2N peak, possessed this lineage-specific marker, which confirms that bone marrow megakaryocytes selectively express PF4lacZ.

To demonstrate the presence or absence of transgene expression in various types of lymphocytes, alternate serial tissue sections of spleen and thymus were stained with hematoxylin and eosin or for β-galactosidase activity. Fig. 2 *F* and *G* depicts the results obtained with the spleen and thymus of transgenic mice. It is readily apparent that large β-galactosidase-positive cells are present within the spleen at an extremely low frequency. These cells were identified morphologically as megakaryocytes in sections stained with hematoxylin and eosin. The β-galactosidase-positive cells were not observed in the spleens of nontransgenic mice (data not shown). The thymuses of transgenic mice exhibited no reaction for the prokaryotic enzyme, which was identical to that observed with their normal littermates.

Expression of the PF4lacZ Transgene in Other Organs. We examined the possibility that the PF4lacZ transgene might be expressed in cells other than platelets and megakaryocytes. The results described below were obtained with line 10 transgenic mice but identical data were generated with line 12 transgenic mice (data not shown). We isolated adrenal gland, brain, heart, intestine, kidney, liver, lung, and skeletal muscle from transgenic mice as well as from normal littermates. These tissues were examined by *in situ* staining for β-galactosidase and showed no staining for the enzyme except for the consistent intense reaction of the transgenic adrenal cortex (Fig. 3) and the occasional weak reaction of the transgenic and nontransgenic endocardium. It is important to note a gradient of staining from the zona glomerulosa (strongest) to the zona fasciculata (less apparent) to the zona reticularis (minimally present) with a complete absence of reaction in the adrenal medulla.

The relative levels of PF4lacZ transgene expression in the various organs were quantitated by kinetic (initial rate) assay of tissue homogenates for β-galactosidase activity per given amount of protein utilizing *o*-nitrophenyl β-D-galactopyranoside as substrate. Based on two separate experiments with line 10 mice, we determined that megakaryocytes contain 6.00 units of activity/30 μg of protein, whereas adrenal glands contain 0.11 unit of activity/30 μg of protein. The brain, heart, intestine, kidney, liver, lung, skeletal muscle, spleen, and thymus possess no detectable amounts of prokaryotic enzyme. Thus, transgene expression in megakaryocytes is ≈50-fold greater than in adrenal gland, and at least 200-fold above other organs. The quantitative results outlined above are in excellent agreement with the more qualitative observations made with *in situ* staining. For example, not the intense staining of megakaryocytes (Fig. 2*C*), the readily apparent but greatly reduced *in situ* staining of adrenal gland (Fig. 3*A*), and the complete absence of *in situ* staining for other organs (Fig. 2 *F* and *G*).

The above data suggest that the PF4 gene might be transcribed at low levels within the adrenal gland. To examine this issue, we carried out Northern blot analyses (11) on mouse or rat adrenal gland, which revealed a faint band of the same size as PF4 mRNA. Examination of other organs such as kidney also showed a barely detectable band in the same region. Northern analyses of various concentrations of platelets suggested that the trapping of small numbers of these blood cells within the circulatory system of the various organs could explain the above observations. The use of other megakaryocyte-specific DNA probes such as GpIIb to correct for potential platelet contamination proved fruitless because of the relatively low levels of expression of these genes vis-a-vis PF4. Thus, we have been unable to prove that the PF4 gene is actively transcribed within the adrenal gland but suspect that the megakaryocyte message is produced at low levels similar to that contributed by platelets trapped within the circulatory system of this endocrine organ. Alternatively, the adrenal gland may express trans-acting factors similar to those that control the megakaryocyte promoter.

DISCUSSION

PF4, located within the α granules of platelets and megakaryocytes, is believed to represent a tissue-specific marker (5). This polypeptide neutralizes the anticoagulant activity of endothelial cell heparan sulfate; induces chemotaxis of neutrophils, monocytes, and fibroblasts; suppresses the activity of collagenase as well as enhances the potency of elastase; serves as an immunomodulatory substance; and inhibits angiogenesis (19–25). Thus, liberation of this substance during the platelet-release reaction accelerates blood clot formation at the site of injury and then sets in motion many of the cellular processes that result in wound healing.

We have used a 1.1-kb fragment of the 5′ upstream region of the rat PF4 gene coupled to prokaryotic β-galactosidase to

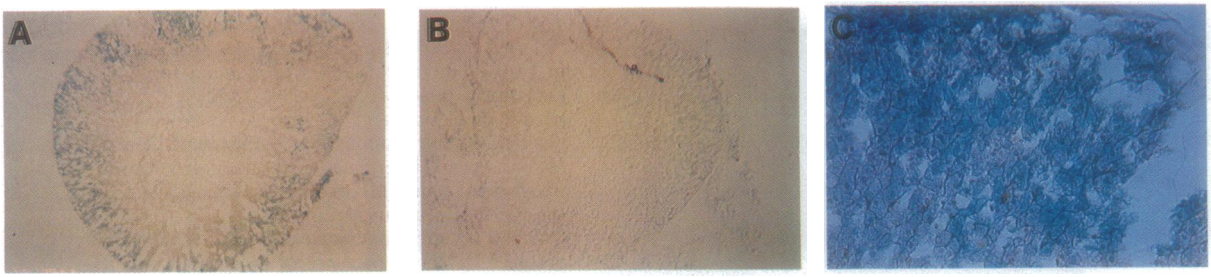

FIG. 3. Expression of the PF4lacZ construct in adrenal glands. *In situ* staining of prokaryotic β-galactosidase was carried out on tissue sections of adrenal gland from a transgenic mouse (*A* and *C*) and a normal littermate (*B*). *C* shows a region in the zona glomerulosa. The sample preparation and staining with X-Gal were conducted as described. (*A* and *B*, ×15; *C*, ×160.)

produce two lines of transgenic mice that express the construct. These studies were undertaken to ascertain whether the domain described above contains the tissue-specific elements of the gene. The data showed that transgene expression in hematopoietic cells was restricted to the megakaryocyte lineage and to platelets. The selective expression of the construct in megakaryocytes was confirmed by sorting all bone marrow cells that possess the prokaryotic enzyme and then demonstrating that these cells stained for the rodent megakaryocyte-specific marker acetylcholinesterase. These results constitute a stringent test of the fidelity of transgene expression since megakaryocytes represent only ≈0.1% of nucleated bone marrow cells. We also note with interest that the 2N megakaryocyte contains β-galactosidase and that the prokaryotic enzyme is expressed in all ploidy classes. Thus, the construct appears to be transcribed in a developmentally accurate fashion. The absence of transgene expression in lymphocytes was confirmed by showing that the spleen and thymus exhibited no staining for the enzyme except for rare megakaryocytes.

Based on *in situ* staining of organ sections for β-galactosidase, and kinetic assay of tissue homogenates for the prokaryotic enzyme, we determined that PF4lacZ is weakly transcribed in adrenal gland but expression of the construct is not detectable in brain, heart, intestine, kidney, liver, lung, and skeletal muscle. The kinetic assay data allow us to state that transcription of PF4lacZ in adrenal gland is 50-fold less than that of megakaryocytes, and expression of the transgene in other organs must be at least 200-fold less than that of megakaryocytes. The gradient of β-galactosidase staining in the adrenal gland corresponds to the position of mineralocorticoid-secreting cells and is distinct from the location of the endocrine organ vascular system (26). Given that the same observations were made in two independently derived transgenic lines, we believe that transcription of the transgene in the adrenal gland must be due to PF4 regulatory sequences linked to the prokaryotic enzyme rather than integration of the construct near a strong endogenous endocrine gland regulatory domain. This unexpected finding could be due to normal low level activity of the PF4 gene in the above locale. Northern analyses of the adrenal gland revealed small amounts of PF4 mRNA, which could not be definitively distinguished from that contributed by trapped platelets in the endocrine organ microvasculature. Alternatively, it is possible that mineralocorticoid-producing cells of the adrenal gland express trans-acting factors similar to those that control the PF4 gene. These trans-acting factors could be involved in the synthesis or secretion of salt-retaining hormones and would provide a link between platelet function and blood pressure regulation.

The selective high-level expression of the transgene in bone marrow progenitors and peripheral blood cells suggests a wide range of potential applications. For example, these transgenic mice could be used to devise simple *in vitro* and *in vivo* bone marrow assay systems for monitoring the transition of hematopoietic stem cells to megakaryocyte precursors, which may allow purification of postulated but elusive regulatory substances such as thrombopoietin. This approach could also be used in transgenic mice to selectively target gene products to megakaryocytes and platelets. Overexpression of protooncogenes such as c-*myb*, which appear to effect the *in vitro* transition of stem cells to megakaryocytes (27), could be evaluated under *in vivo* conditions. Suppression of megakaryocyte-specific gene products such as platelet-derived growth factor, which require assembly of homopolymers or heteropolymers for biologic activity, should be attainable by overexpressing an abnormal subunit that complexes with the normal subunit to generate a biologically inactive component (28). The construction of these dominant negative mutations could allow us to inhibit the synthesis of specialized membrane receptors, signaling molecules, and growth factors in a tissue-specific manner and then evaluate the *in vivo* effects of the circulating altered platelets on physiologic processes such as hemostatic function and wound healing as well as pathophysiologic mechanisms such as thrombogenesis and atherogenesis.

We are indebted to Stuart Conner for expert assistance in flow cytometry and cell sorting, S. Sotnikov for histologic consultations, D. Kuter and J. Plutzky for helpful discussions, and Monty Krieger for a critical reading of the manuscript. This work was supported by National Institutes of Health Grants HL72410 and HL71165.

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