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A 2.7-kb Portion of the 5′ Flanking Region of the Murine Glycoprotein α_{IIb} Gene Is Transcriptionally Active in Primitive Hematopoietic Progenitor Cells

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The continuous generation of mature blood cells from primitive multipotent progenitor cells requires a highly complex series of cellular events that are still largely unknown. To examine the molecular events associated with the commitment of these hematopoietic progenitor cells to the megakaryocytic lineage, the α subunit of the platelet integrin $\alpha_{IIb}\beta_3$ was used as marker. Despite an abundance of information regarding the role of this integrin in platelet adhesion and aggregation, the mechanisms that control the expression of the genes that code for these proteins are poorly understood and the earliest hematopoietic cell capable of expressing them has not been clearly identified. Thus, a strategy was developed to eradicate, using a conditional toxigene, all the hematopoietic cells capable of expressing the α_{IIb} gene in mice. This was achieved by targeting the expression of the gene encoding the herpes simplex virus thymidine kinase (tk), specifically to these cell types, using a 2.7-kb fragment of the 5′-flanking region of the murine α_{IIb} gene. Three transgenic lines having 1, 3, and 4 copies of the transgene, respectively were produced and analyzed. Administration of ganciclovir (GCV) to these mice induced a severe thrombocytopenia, which was due to the depletion of the entire megakaryocytic lineage, as shown by bone marrow (BM) culture and electron microscopy analysis. The time required to attain a severe thrombocytopenia was dependent on the level of the expression of the transgene and varied from 7 to 11 days. This condition was completely reversed when GCV treatment was discontinued. Progenitor cell assays showed that the α_{IIb} promoter was active in primitive hematopoietic progenitor cells possessing myeloid, erythroid, and megakaryocytic potential and that the transcriptional activity of the promoter decreased progressively as differentiation proceeded towards the erythroid and myeloid lineages.

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PLATELET GLYCOPROTEIN α_{IIb} is known to be a marker of the megakaryocytic lineage. It constitutes the alpha subunit of the platelet integrin $\alpha_{IIb}\beta_3$, which functions as a receptor for adhesive proteins and plays a critical role in the formation of the platelet plug at the site of vessel injury.[1] The gene encoding the human α_{IIb} glycoprotein has been cloned[2,3] and analysis of its 5′-flanking region has been performed using primarily transient transfection assays in permanent cell lines exhibiting megakaryocytic features. This approach has allowed the identification of *cis*-acting elements within the first 820 bp of the human 5′-flanking sequence, including an erythromegakaryocytic enhancer, composed of GATA and Ets binding sites, and a negative regulatory element.[4-6] The negative regulatory domain has been shown to downregulate the expression of the α_{IIb} gene in permanent cell lines with nonmegakaryocytic features and also to diminish the transcriptional activity of the α_{IIb} promoter during megakaryocytic differentiation of K562 cells. The rat α_{IIb} promoter has also been cloned and comparisons with the human gene have shown that the position and the nucleotide sequence of most regulatory domains are similar suggesting that the molecular mechanisms that control the expression of this megakaryocytic gene may be highly conserved. Although significant differences in gene expression were observed between the rat and human α_{IIb} promoters when using primary cultures of bone marrow (BM) cells or immortalized cell lines, respectively, these differences were attributed to the in vitro expression system used.[7]

To date, reports in the literature directed at delineating the first hematopoietic cell capable of expressing the glycoprotein α_{IIb} have been inconsistent. Antisera against purified glycoproteins α_{IIb} and β_3 were found to inhibit colony-forming unit (CFU)-mix and spleen colony formation, indicating that these proteins may be present in a totipotent stem cell.[8,9] In contrast, using a similar approach, Levene et al[10] could not detect its presence in uncommitted progenitor cells. The α_{IIb} message has been detected in human $CD34^+$ enriched cell populations, however, this may only suggest that the gene is expressed at an early stage of megakaryocytic differentiation.[11] Thus, further studies are required to address this issue. Its unequivocal definition is of considerable importance, as this could specify, at the genetic level, the mechanism(s) by which the megakaryocytic phenotype is established during the differentiation of hematopoietic cells.

To avoid the limitations inherent to in vitro cell culture systems, we have developed an in vivo transgenic model to monitor the transcriptional activity of megakaryocytic promoters. In an earlier report, the feasibility of such a transgenic mouse model was demonstrated by using a 0.8-kb promoter fragment of the human platelet glycoprotein α_{IIb} gene to drive the expression of the herpes simplex virus thymidine kinase (tk) gene.[12] The strength of this model resides in the fact that administration of an antiherpetic drug such as ganciclovir (GCV), selectively eradicates cycling cells that are capable of expressing the tk gene, hence, identifying in this manner the precise stage at which the promoter is active during the developmental process. When these

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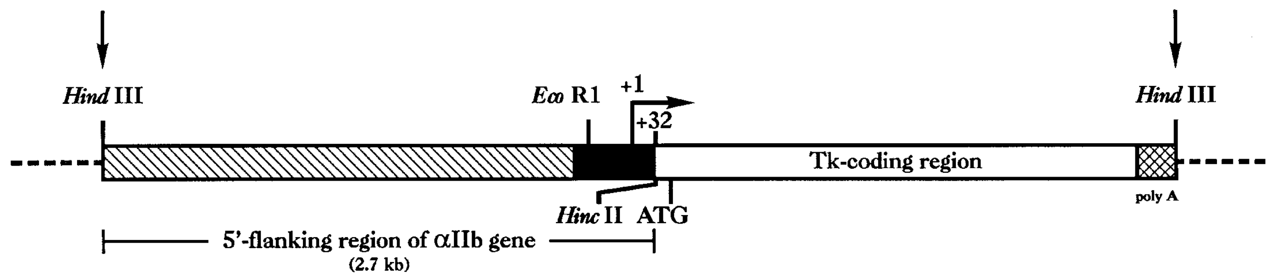

Fig 1. Schematic representation of the MαIIbtk transgene. The proximal promoter region of the murine α_{IIb} gene (up to the *Eco*R1 site) was amplified by PCR to mutate the endogenous α_{IIb} ATG initiation codon (at position +32) to ATC. The distal 5′-flanking region was then added to replace the PGK promoter in pPNT plasmid. The tk gene starts at site *Hinc*II (open box). The 4.2-kb *Hind*III fragment excised from the plasmid for the production of transgenic animals is represented by arrows.

transgenic animals were exposed to GCV, an acute on demand cessation of megakaryocytopoiesis was obtained, which could be reversed by discontinuing the GCV treatment.[12] While this response was predictable, a coincident suppression of erythropoiesis during GCV exposure was an unexpected finding given previous in vitro evidence showing that the same 0.8-kb promoter fragment was specific for permanent cells with megakaryocytic features and was not active in the erythroid cell line K562.[13] Results obtained with this transgenic model suggested that the regulatory elements contained within 0.8 kb of the 5′ flanking region of the human α_{IIb} gene were active in vivo in megakaryocytic, as well as in erythrocytic cells. The question remained, however, whether these findings were the result of species differences or an absence of *cis*-acting elements involved in the regulation of the expression of the α_{IIb} gene. These possibilities were therefore examined by using a 2.7-kb fragment of the murine α_{IIb} 5′-flanking region in a homologous system. These new transgenic lines reported here, appear to present a similar phenotype to that observed with mice transgenic for the human α_{IIb} promoter,[12] consequently allowing us to show that both the murine and the human α_{IIb} promoters are transcriptionally active in multipotent primitive hematopoietic cells. This activity was maintained in monopotent progenitor cells of all lineages at very low levels and was progressively turned off during erythroid and myeloid lineage differentiation.

MATERIALS AND METHODS

Cloning of the promoter for murine α_{IIb} and transgene construction. The λ fix II phage 129SVJ mouse genomic library (Stratagene, La Jolla, CA) was screened for clones containing the 5′-flanking regulatory region of the α_{IIb} gene with a 548-bp fragment of the previously isolated murine (M) α_{IIb} proximal promoter.[14] Three different overlapping clones were identified, following four rounds of screening, one of which was selected for further analysis. Partial restriction mapping of this clone indicated that a 6.5-kb *Hind*III fragment contained up to 2.7 kb of the 5′ region of the α_{IIb} gene, which was inserted 5′ of the HSV1-tk gene in pPNT plasmid.[15] The endogenous ATG initiation codon situated in the first exon (position +32) was mutated to ATC by the polymerase chain reaction (PCR) method.[16] For the production of transgenic mice a 4.9-kb fragment composed of the tk cDNA under the control of 2.7 kb of 5′-α_{IIb} putative regulatory sequences was excised from the plasmid by *Hind*III digestion (Fig 1). The product was electrophoresed on an agarose gel and further purified on Elutip-d columns (Schleicher and Schuell, Dassel, Germany). The DNA fragment was diluted in 10 mmol/L Tris-HCl, pH 7.5, 0.1 mmol/L EDTA and used to produce the transgenic lines.

Production and screening of mice transgenic for Mα_{IIb}tk. The hybrid gene, named MαIIbtk, was microinjected into fertilized eggs resulting from mating (C57Bl/6 × DBA2) F1 pronuclei using established procedures.[17] Transgenic offsprings were identified by Southern blot analysis of genomic DNA (10 μg) extracted from tail samples. Transgenic mice harboring the tk gene under the control of the human α_{IIb} promoter, named Hα_{IIb}tk, described previously,[12] were used for comparison purposes in this study.

RNA isolation and reverse transcription (RT)-PCR amplification. BM cells of transgenic and nontransgenic littermates were flushed from the femoral cavity with phosphate-buffered saline (PBS) supplemented with prostaglandin E1, using a syringe fitted with a 25-gauge needle. Cells were separated from the core matrix by manual pipeting, washed twice, recovered in PBS, and counted. Total RNA was extracted from these cells using an isolation kit based on the thiocyanate method (5′ Prime -3′ Prime, Inc, Boulder, CO).

For the RT-PCR amplification, 5 μg of total RNA was first treated with 1 U of RQ1 DNase (Promega Corp, Madison, WI). After inactivation of the enzyme (90°C for 5 minutes), RNA was denatured for 10 minutes at 70°C and used as a template in a 50-μL cDNA synthesis reaction using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) and random hexanucleotides (Pharmacia Uppsala, Sweden). A 5-μL aliquot of the RT mixture was amplified by PCR in a Techne PHC-3 thermocycler (Techne Corp, Cambridge, MA). PCR reactions were performed under standard Perkin Elmer-Cetus conditions, for 30 cycles (94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute), followed by 3 minutes at 72°C. The number of PCR cycles corresponded to the high end of the range in which a linear increase in products could be detected. PCR products were run through a 1.5% agarose gel and transferred to Hybond N+ nylon membrane (Amersham, Little Chaffont, England). Filters were hybridized with end-labelled internal specific oligonucleotides. Routine controls performed in each experiment included a cDNA reaction mixture without addition of RT as a check against genomic DNA contamination and a PCR control without any template added. The sequences of the sense primer (A), the antisense primer (B), and the internal oligonucleotide (I) were as follows:

α_{IIb} A: 5′-CAGAGCCTCCTGCGCATGGC-3′
α_{IIb} B: 5′-GCAACGGGTTGCACTTGCC-3′
α_{IIb} I: 5′-CTAGGACCCAAGAACAGTGGTG-3′
tk A: 5′-CCCCTGCCATCAACACGCG-3′
tk B: 5′-CGATGGGGATGGCGGTCGAAG-3′
tk I: 5′-GGCCGCGAGAACGCGCAGCCTGG-3′

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The tk primer sequences were deduced from previously published work,[18] whereas the α_{IIb} primer sequences were derived independently.

In vivo GCV administration. The nucleoside analog GCV (Cymevan, Roche, Basal, Switzerland) was administered to mice daily by intraperitoneal (IP) injection. The dose of GCV was 0.1 mg/day/g body weight. The duration of the treatment varied between experiments as specified in the text.

Cell counting procedures. Blood was collected from tail vein bleeds or by cardiac puncture into an anticoagulant consisting of acid citrate dextrose (ACD) and prostaglandin E1 (10 μmol/L) (10 vol blood:1 vol anticoagulant). Automated blood counts were performed with a Coulter T660 cell counter (Coulter, Miami, FL). The mean values for control nontransgenic animals (n = 10) were: platelets, $1.0 \pm 0.2 \times 10^6/\mu L$ (standard deviation [SD]); leukocytes, $11.2 \pm 6.1 \times 10^3/\mu L$ (SD); erythrocytes, $10.8 \pm 0.1 \times 10^6/\mu L$ (SD) and were within the normal range.[19,20]

Hematopoietic progenitor assays. For megakaryocyte progenitors (CFU-M_k) assays, unfractionated BM cells (5×10^4 cells per well) were cultured in triplicate in plastic dishes (Nunclon Roskilde, Denmark) in 1 mL of Iscove's modified Dulbecco's medium (IMDM) (Gibco, BRL) containing 0.3% agar (Difco Laboratories, Detroit, MI) and supplemented with various other ingredients used in serum-free cultures, as previously reported.[21] The number of megakaryocytic colonies was evaluated in situ on dried agar discs stained by the acetyl cholinesterase (AchE) method.[22] For erythroid progenitors (burst-forming unit-erythroid [BFU-E] and CFU-E) assays, BM cells (5×10^4 cells per well) were cultured in triplicate in plastic dishes (Nunclon, Roskilde, Denmark) as described.[12] In vitro cultures of late erythropoietic progenitor cells (CFU-E) were performed essentially the same as for BFU-E cultures, except that 0.5 U/mL of recombinant murineerythropoietin (rMuEPO) was used and rMuinterleukin 3 (rMuIL3) was omitted from the medium. Colonies were counted after 48 hours of incubation under the conditions indicated. Granulomonocytic-erythroid–megakaryocytic colony forming units (CFU-GEMM$_k$), mixed progenitors (CFU-MIX: CFU-GMM$_k$, CFU-GME, CFU-EM$_k$), and granulomonocytic colony forming cells (GM-CFC) assays were performed as described.[12]

Assessment of BM cellularity and morphology. To perform ultrastructural analysis, BM was removed from the femur of mice, being careful not to disturb its native structure, and fixed in 1.25% (vol/vol) glutaraldehyde (Fluka AG, Buchs, Switzerland). Samples were then prepared for electron microscopy as previously described.[23] Ultrathin sections were cut with an Ultracut E ultramicrotome (Reichert, Vienna, Austria) and subsequently examined at 80 KV using a Jeol JEM-1010 transmission electron microscope (Croissy-sur-Seine, France). A minimum of two BM samples was analyzed for each of the following groups of animals: (1) nontreated nontransgenic mice; (2) GCV-treated nontransgenic mice; (3) GCV-treated HαIIbtk mice containing one copy of the transgene, and (4) GCV-treated MαIIbtk mice harboring several copies of the transgene. The BM samples of transgenic HαIIbtk and MαIIbtk mice were analyzed when their platelet counts were approximately 50,000/μL.

RESULTS

Generation of mice transgenic for MαIIbtk. Mice transgenic for HSV-tk under the control of a 2.7-kb DNA fragment of the 5′-flanking region of the murine α_{IIb} gene were created. Six transgenic founder mice carrying the fusion gene were identified by Southern blot analysis of tail DNA using a tk probe. The number of copies of the transgene possessed by each founder mouse was determined by hybridizing these blots with an α_{IIb} probe followed by analysis with a phosphorimager. By comparing the intensity of the band corresponding to the transgene with that for the endogenous α_{IIb} gene, it was determined that founder animals contained 1, 3, 4, 8, 11, and 14 copies of the MαIIbtk transgene (data not shown). Females carrying 1, 3, and 4 copies were subsequently used to establish breeding lines, as males having 8, 11, and 14 copies were found to be sterile. Sterility of mice harboring HSV-tk as a reporter has been described by others and has been correlated to tk expression in the testes from a cryptic promoter located within the coding region of the gene.[24]

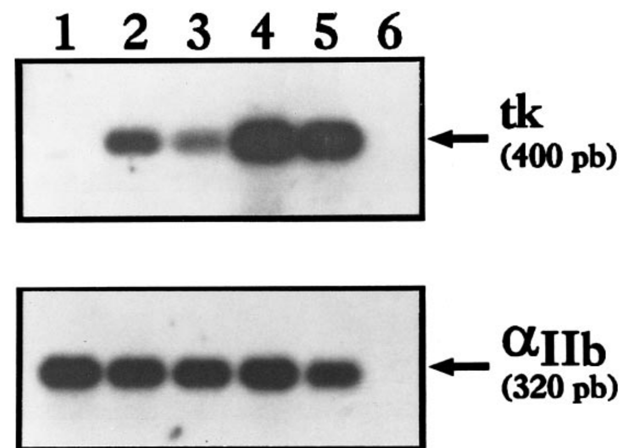

Fig 2. RT-PCR analysis of the expression of the tk gene driven by the α_{IIb} promoter. A 5-μL aliquot of the cDNA derived from BM cells was amplified with a pair of specific primers for 30 cycles. One fifth of the product was applied to 1.5% agarose gel for electrophoresis. Panels show the results of Southern hybridization of RT-PCR products with the internal probe of each gene. Each experiment was checked for reproducibility. Lane 1, nontransgenic mouse; lane 2, HαIIbtk (1 copy); lane 3, MαIIbtk (1 copy); lane 4, MαIIbtk (3 copies); lane 5, MαIIbtk (4 copies); lane 6, PCR performed without RT as control against contamination. Amplification of endogenous α_{IIb} transcripts were performed with α_{IIb}-specific oligonucleotides on the same samples.

To test for the expression of the chimeric gene, total RNA prepared from BM of MαIIbtk transgenic animals was analyzed for tk-specific transcripts by semiquantitative RT-PCR. The integrity of the RNA samples was verified by ethidium bromide staining following gel electrophoresis. As shown in Fig 2, the predicted amplification product of 400 bp corresponding to the size of the tk message was found in all MαIIbtk transgenic lines, indicating that the transgene was functional. The level of tk transcripts was higher in mice carrying several copies of the transgene compared with mice carrying only one copy. No tk transcripts were found in nontransgenic littermates. This was confirmed by Northern blot analysis (data not shown). The endogenous α_{IIb} message was amplified using α_{IIb} specific primers as a control for reproducibility. In this case, the levels of RT-PCR products were similar between all the mice, suggesting that the differences seen in tk expression were significant (Fig 2). Several other tissues besides BM were also tested for the presence of tk message to verify the tissue-specific expression of the tk reporter gene driven by the α_{IIb} promoter. Because tk

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Table 1. GCV-Induced Toxicity on Blood Cell Counts

	Platelets ($\times 10^6$)		Erythrocytes ($\times 10^6$)		Leukocytes ($\times 10^3$)	
	D0	D9	D0	D9	D0	D9
Nontransgenic	1.10 ± 0.20	1.25 ± 0.4	10.26 ± 1.4	8.00 ± 0.75	11.53 ± 3.2	10.27 ± 3
HαIIb-tk (1)	1.15 ± 0.25	0.3 ± 0.25	10.2 ± 2.7	8.42 ± 1.59	10.6 ± 2.6	9.53 ± 2.65
MαIIb-tk (1)	1.02 ± 0.17	0.35 ± 0.13	9.95 ± 2.5	10.4 ± 0.6	9.26 ± 1.14	8.5 ± 1.6
MαIIb-tk (3)	1.25 ± 0.65	0.15 ± 0.12	10.9 ± 1.30	4.2 ± 1.9	11.4 ± 3.6	5.05 ± 3.75
MαIIb-tk (4)	1.05 ± 0.25	0.12 ± 0.41	10.45 ± 2.45	3.1 ± 1.3	11.27 ± 5.5	2.85 ± 2.6

Mean peripheral blood cell counts (±SD) of control nontransgenic, HαIIb-tk, and MαIIb-tk mice (n = 5), before (D0) and after 9 days (D9) of GCV administration (0.1 mg/day/g body weight). The counts shown are per μL of blood volume. The number in brackets refers to the integrated copies of the transgene.

expression was found in blood (not shown), animals were cannulated and perfused with PBS before removal of tissue samples to eliminate any blood from the vasculture that could potentially yield false positive results. BM and spleen showed the highest levels of expression of the tk gene followed by the testes and adrenal glands. It should be noted, however, that message detected in the spleen may have been due to the presence of megakaryocytes in this tissue, whereas the expression detected in the testes may be due to the cryptic promoter present within the tk coding region, which can drive its expression in this organ regardless of the upstream promoter used. This same pattern of expression had been observed previously in HαIIbtk transgenic animals[12] and shows that both, the 2.7-kb fragment containing murine α_{IIb} sequences and the 0.8-kb of the human α_{IIb} promoter appear to exhibit similar transcriptional specificity.

Effect of GCV treatment on blood cell counts. To test for the function of the tk protein produced by the transgene and to measure the eventual in vivo toxic effect of GCV administration on blood cellularity, 5-week old transgenic mice were treated with 0.1 mg of GCV/day/g body weight. This concentration was determined on the basis of pilot studies in which the effects of different concentrations of GCV were evaluated. No apparent toxic effects were observed in normal mice at the concentration chosen. After 9 days of daily IP drug administration, a thrombocytopenic state was observed in all MαIIbtk transgenic mice and the degree of platelet eradication was dependent on the level of transgene expression (Table 1). Mice harboring only one copy of the MαIIbtk transgene exhibited a moderate thrombocytopenia (approximately 30%) with no significant effect on erythrocyte or leukocyte counts. This coincides with previous results using mice transgenic for tk directed by the human α_{IIb} promoter, which also had a single integrated copy of the transgene. In contrast, mice harboring three and four copies were more sensitive to the toxic effect of GCV. Greater than 85% inhibition of thrombopoiesis was induced in these animals in the same period of time, which was accompanied by a decrease in circulating erythrocyte and leukocyte counts (Table 1). Blood cell counts of the nontransgenic littermates were unaffected by the treatment. When GCV administration was interrupted, the process was reversed in the transgenic animals and the circulating cell counts returned to normal values 10 to 12 days following withdrawal of the drug. From these results we concluded that all of the transgenes produced an active tk protein, which eradicated platelets in the presence of GCV and toxicity was dependent on the level of tk synthesized. Higher levels of tk synthesis lead to a reduction in the number of erythrocytes and leukocytes suggesting that the enzyme was probably synthesized at an early stage of the differentiation process.

Primitive multipotent progenitor cells are sensitive to GCV. To characterize the precise stage of hematopoietic differentiation at which the expression of the hybrid gene was initiated, the toxic effect of tk was examined both in vivo and in vitro using progenitor cell assays. In the first set of experiments, BM was extracted from GCV-induced thrombocytopenic transgenic mice and cultured in vitro on methylcellulose. This allowed us to monitor the clonogenic capacity of myeloid multipotent progenitor cells remaining in BM of mice after in vivo administration of GCV. The results showed a dramatic inhibition of multipotential CFU-GEMM colonies, as well as all other CFU-mix colonies in BM cultures of the new transgenic MαIIbtk lines (Table 2). The growth of these progenitor colonies was also inhibited when BM of HαIIbtk transgenic mice having received the same treatment were assayed in the same manner. These progenitor cells were easily detectable in BM cultures of control GCV-treated or untreated nontransgenic mice. Thus, similar in vivo inhibition of primitive hematopoietic cells was found when the expression of tk was driven by either

Table 2. Number of CFU-M_K, BFU-E, CFU-E, GM-CFC, CFU-mix, and CFU-GEMM Colonies Derived From BM Cells of Control Nontransgenic, HαIIb-tk, and MαIIb-tk Mice Treated With GCV

	CFU-M_K	BFU-E	CFU-E	GM-CFC	CFU-mix	CFU-$GEMM_K$
Control	6 ± 2	10 ± 3	52 ± 12	62 ± 9	10 ± 3	7 ± 2
HαIIb-tk	1 ± 1	0 ± 1	46 ± 8	60 ± 6	0 ± 1	0
MαIIb-tk	1 ± 1	1 ± 1	37 ± 12	59 ± 4	0 ± 1	0 ± 1

Marrow cells from control, HαIIb-tk (one copy of the transgene integrated) and MαIIb-tk (several copies integrated) mice treated with 0.1 mg of GCV/day/g body weight were obtained by femoral aspiration cultured as described in Materials and Methods. Each number (±SD) is the mean of three wells in three different experiments. CFU-mix colonies consisted of erythroid and megakaryocytic component, granulocytic, macrophagic, and erythroid component, or granulocytic macrophagic megakaryocytic without an erythroid component and multipotent CFU-$GEMM_K$ (granulocytic, erythrocytic macrophagic and megakaryocytic components).

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Table 3. Analysis of the GCV Sensitivity of Committed and Mixed Progenitor Cells Derived From BM Cells of Control, HαIIb-tk, and MαIIb-tk Mice in Individual Semisolid Assays

	GCV	CFU-M_K	BFU-E	CFU-E	GM-CFC	CFU-mix	CFU-$GEMM_K$
Control	0	20 ± 2	24 ± 2	108 ± 7	98 ± 5	16 ± 2	7 ± 1
	0.5	18 ± 3	24 ± 2	98 ± 11	86 ± 2	13 ± 1	5 ± 1
	1	12 ± 5	22 ± 2	84 ± 6	70 ± 4	9 ± 1	7 ± 1
	5	13 ± 4	24 ± 7	77 ± 8	71 ± 9	9 ± 1	6 ± 1
HαIIb-tk (1)	0	17 ± 3	22 ± 2	98 ± 6	88 ± 13	9 ± 2	4 ± 1
	0.5	ND	ND	90 ± 4	ND	ND	ND
	1	2 ± 2	1 ± 1	62 ± 10	46 ± 8	0	0
	5	1 ± 1	0	50 ± 10	3 ± 1	0	0
MαIIb-tk (1)	0	24 ± 4	17 ± 2	70 ± 9	70 ± 2	11 ± 1	7 ± 1
	0.5	3 ± 2	5 ± 1	43 ± 7	45 ± 3	3 ± 1	2 ± 1
	1	5 ± 3	3 ± 1	53 ± 11	28 ± 1	3 ± 1	1 ± 1
	5	3 ± 2	1 ± 1	56 ± 8	10 ± 1	1 ± 1	0 ± 1
MαIIb-tk (3 or 4)	0	20 ± 2	23 ± 4	93 ± 17	116 ± 7	6 ± 1	3 ± 1
	0.5	0 ± 1	0 ± 1	34 ± 4	5 ± 1	0	0
	1	0	0	11 ± 2	0	0	0
	5	0	0	3 ± 1	0	0	0

Marrow cells (5×10^4 per well) were cultured in 0.33 mL complete IMDM supplemented with predetermined optimal concentrations of cytokines as described in Materials and Methods.

Abbreviation: ND, not determined.

the human or the murine α_{IIb} promoters, indicating that no significant species differences exist, and that the addition of 1.9 kb of regulatory elements to the promoter did not change the pattern of expression.

To further determine the sensitivity of these early multipotent progenitor cells to GCV, a separate set of experiments was performed in vitro in which intact BM cells from untreated MαIIbtk, HαIIbtk, and nontransgenic animals were cultured in methylcellulose in the absence or presence of various concentrations of GCV. CFU-GEMM and CFU-mix colonies from all transgenic animals were determined to be sensitive to the drug and their growth was inhibited at the lowest GCV dose used of 0.5 μmol/L, whereas early progenitors derived from nontransgenic animals were insensitive to the drug (Table 3). Thus, similar toxic effects due to GCV administration were observed both in vivo and in vitro, consistent with the expression of the tk gene in primitive multipotent progenitor cells.

Expression of the transgene in committed progenitors. To determine whether the expression of the tk gene directed by the α_{IIb} 5′-flanking region detected in multipotent progenitors persisted in megakaryocytic and nonmegakaryocytic progenitor cells following differentiation, BM of transgenic and nontransgenic littermates treated with GCV for a period of 9 days were cultured using individual semisolid progenitor cell assays. The development of CFU-M_k and BFU-E was drastically inhibited in these cultures and colonies were barely detectable. Surprisingly, CFU-E colonies were only moderately affected and the number and size of granulomonocytic colonies were in the control range (Table 2). These unexpected results appeared to be in contrast with the absence of mixed and early progenitor colonies. It was rationalized that if the expression of tk in these cell types is very low, it should be possible to inhibit their growth by increasing the concentration of GCV within the culture. Thus, in vitro cell assays were performed in which BM cells of untreated MαIIbtk and HαIIbtk transgenic animals, as well as their nontransgenic littermates were plated on methylcellulose in the presence of different concentrations of GCV. This showed a drastic inhibition of the growth of transgenic CFU-M_k and BFU-E colonies at the lower concentration used. Higher doses of GCV were needed to inhibit the growth of GM-CFC and CFU-E colonies compared with CFU-M_k colonies (Table 3), consistent with a higher level of tk expression in megakaryocytes. From these results, we concluded that either 2.7 kb of murine or 0.8 kb of human α_{IIb} promoter sequences were active in primitive totipotent progenitors and that there was a progressive loss of their transcriptional activity during the course of differentiation towards the erythroid and the myeloid lineages.

Changes in the cellular composition of BM following GCV administration. Electron microscopy analysis of BM extracted from thrombocytopenic transgenic animals was performed to evaluate morphologic modifications induced in BM following GCV administration. Any possible ultrastructural changes in the composition of the BM due to the administration of GCV per se was first evaluated. No detectable structural differences were observed in the organization of the BM of normal mice that were treated with GCV (not shown). In contrast, major morphological changes were observed within the BM of the thrombocytopenic transgenic animals following GCV treatment. The number of cells of the megakaryocytic lineage was dramatically reduced in the BM of HαIIbtk transgenic animals. Residual polyploid megakaryocytes were occasionally observed (data not shown) and the number of erythroblastic islets was also decreased. The granulocytic cells were apparently unaffected as shown by the presence of normal numbers of maturing neutrophils and eosinophils (Fig 3A). Furthermore, an increased level of large cells with an intense phagocytic activity was observed. These cells contained a higher number of phagocytic vacuoles within their cytoplasm as shown in Figs

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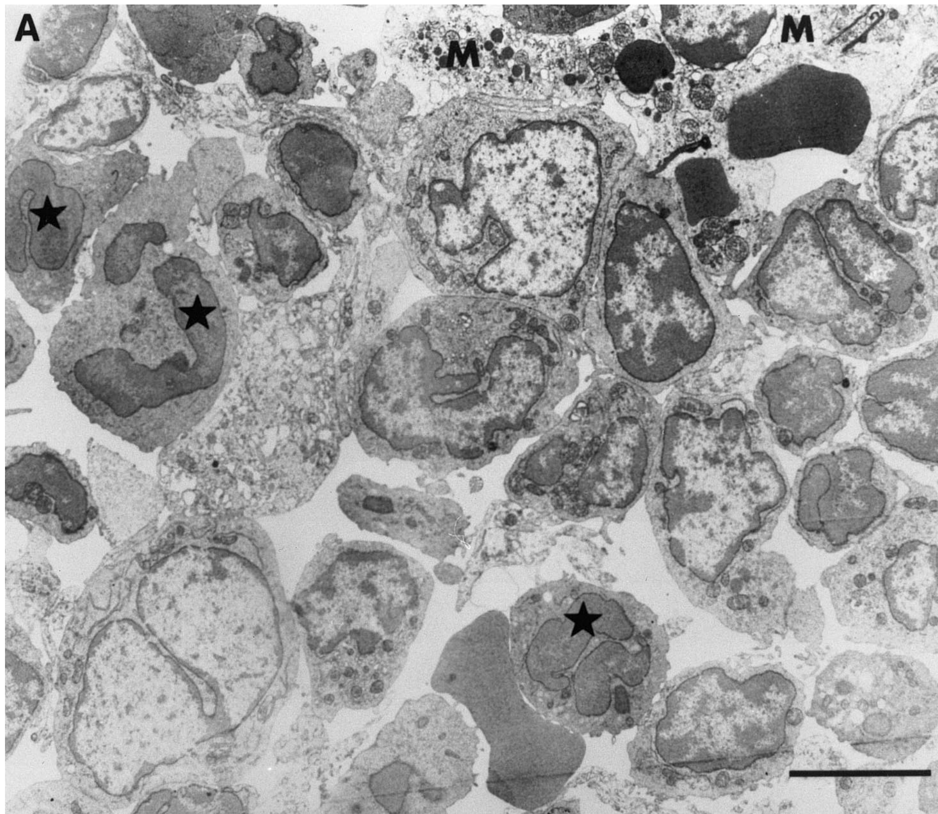

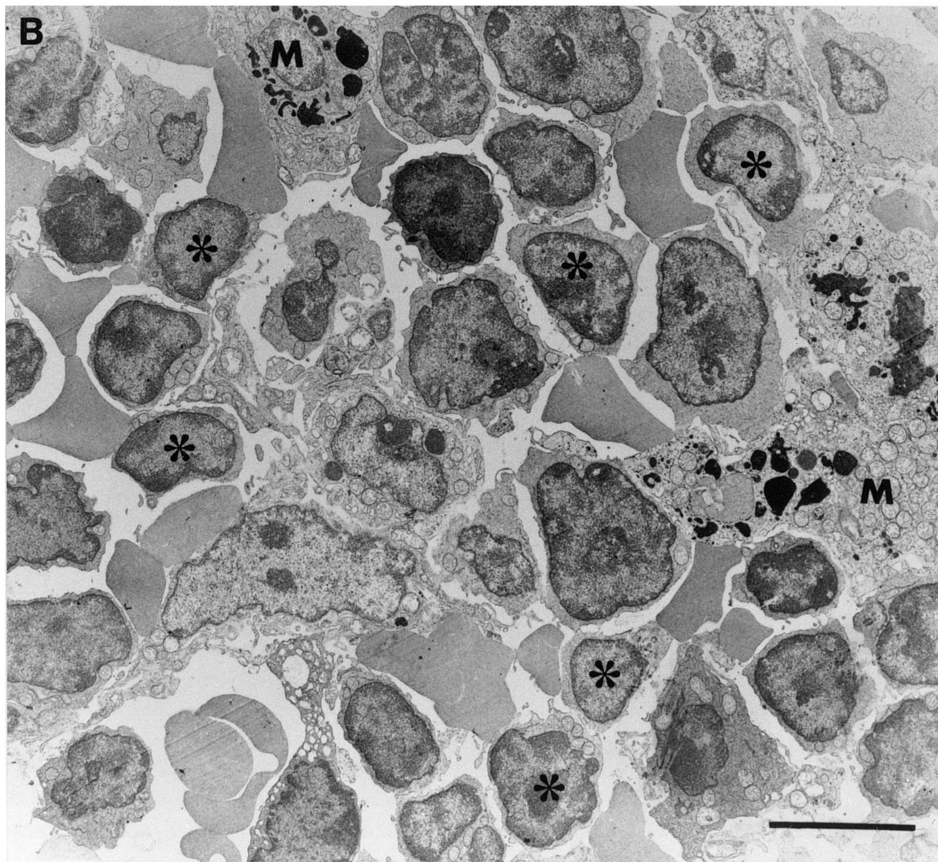

Fig 3. Morphologic changes in BM of GCV-induced thrombocytopenic mice. (A) BM of a HαIIbtk transgenic mouse. No megakaryocytes are present and cells from the erythrocytic lineage are decreased. Granulocytic cells such as neutrophiles (⋆) predominate in this BM compartment showing that the granulocytic lineage is unaffected. Large increase on the amount of macrophage-like cells (M) showing phagocytic activity in their cytoplasm was seen. Bar = 5 μm. (B) Section of BM obtained from a GCV-induced thrombocytopenic MαIIbtk mouse having several copies of the transgene integrated. Mature cells of the megakaryocytic, erythrocytic, and granulocytic lineage are severely decreased. Large macrophage-like cells with dense bodies suggesting phagocytic activity invade the BM. Cytoplasmic processes extend around the few resting hematopoietic cells. Poorly differentiated mononuclear cells (*) with high nucleocytoplasmic ratio, having the morphology of progenitor cells, seem to be present in higher amounts. Bar = 5 μm.

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Table 4. GCV Sensitivity of tk Nonexpressing Cells in the Presence of tk Expressing Cells

	M_K			GM			BFU-E		
GCV	−	+	±	−	+	±	−	+	±
0	19 ± 2	10 ± 1	16 ± 2	36 ± 3	32 ± 4	28 ± 4	11 ± 2	15 ± 2	13 ± 2
5 μmol/L	17 ± 2	0	8 ± 1	26 ± 1	0	25 ± 3	10 ± 1	0	6 ± 1

BM cells (1×10^5) of transgenic (+) mice or nontransgenic littermates (−) were plated in the presence of 5 μmol/L GCV or in its absence and cultured for 6 days. For the mixed-cultures (±), 75,000 cells/mL of nontransgenic BM cells were cultured in the same well as 75,000 cells/mL of transgenic cells.

3A and B. The MαIIbtk mice harboring several copies of the transgene showed even more dramatic changes in their BM morphology. Megakaryocytic, erythroid, and granulocytic lineages were all extremely depleted. The BM was essentially aplastic and considerably disorganized with very few mature cells present. In contrast, the number of two different cell types was found to be increased. Hematopoietic spaces were invaded by large cells with extending processes containing dense bodies suggesting an important phagocytic activity (Fig 3B), and with poorly differentiated mononuclear cells with a high nucleocytoplasmic ratio, having the same morphologic features of stem cells. The results of these ultrastructural studies show that all myeloid lineages were affected and are therefore in agreement with the changes observed in peripheral blood counts.

The toxic effect of GCV is restricted to transgene-expressing cells. A possible explanation for the GCV sensitivity of early nonmegakaryocytic progenitors could be that nonspecific toxicity occurred as a result of the general release of toxic phosphorylated GCV from dying cells specifically expressing tk. Although this bystander effect has been described in other systems,[25,26] it would seem to be an unlikely event, as most cells are impermeable to phosphorylated nucleotides. Nonetheless, this possibility was tested by culturing a mixture of BM cells obtained from transgenic mice possessing one copy of the transgene and control animals. These two populations of cells were mixed in equal proportions and were grown in the presence of GCV and specific cytokines that stimulate the development of erythrocytic, megakaryocytic, and granulomonocytic colonies. Colony development in these cultures was compared with cultures of separately grown non-tk–expressing or tk-expressing BM cells under identical conditions. Coculturing BM cells of nontransgenic mice in the presence of transgenic BM cells in a 1:1 ratio did not reduce the number of colonies expected by culturing the same number of nontransgenic BM cells under megakaryocytic or erythrocytic conditions (Table 4). In GM-CFC plates, although the transgenic cells were found to be eliminated as expected, another cell type appeared that was morphologically distinct from either the initial transgenic or nontransgenic cells. The proliferation of these cells could be induced by the secretion of cytokines or growth factors from the dying transgenic cells. Other than this one unexpected finding, in all cases the presence of transgenic tk expressing cells did not inhibit the growth of neighboring nontransgenic bystander cells. To eliminate the possibility that the results obtained on semisolid media were not due to the inability of the phosphorylated GCV to diffuse on methylcellulose, liquid cocultures were also performed leading to identical results (data not shown).

DISCUSSION

Stem cell commitment and differentiation is governed by the regulated transcriptional activation and repression of a host of genes. The cellular and molecular analysis of these processes has been hampered by the difficulty in obtaining homogeneous stem cell populations, by a low representation of these cells in BM, and the limited availability of in vivo assays. In particular, the analysis of the transcriptional regulation of the α_{IIb} gene has been limited to studies of the human and rat promoters in immortalized cell lines or BM cells transfected in vitro, which fail to take into account the dynamics and complexity of the hematopoietic process as it occurs in vivo.

With this in mind, we have developed a methodology based on the generation of transgenic mice to monitor the transcriptional activity of specific promoters during hematopoietic differentiation. In the initial model, the expression of the tk gene was directed by a 0.8-kb 5′-flanking region of the human α_{IIb} gene. In these mice, a severe and reversible thrombocytopenia could be produced on demand by administration and withdrawal of the antiherpetic drug GCV.[12] There was, however, evidence that the toxic effect was not limited to cells committed to megakaryocytopoiesis. Although less pronounced, GCV administration was found to induce suppression of erythropoiesis suggesting that, in this system at least, the activity of the α_{IIb} promoter was not limited to cells of the megakaryocytic lineage. The question arose whether this was, in fact, due to species differences between the promoter and the host animal or to the absence of *cis*-acting elements involved in the regulation of the expression of the α_{IIb} gene.

In this study, we have developed novel transgenic mice in which the expression of tk is driven by 2.7 kb of the 5′-flanking region of the murine α_{IIb} gene. This allowed the analysis of the promoter activity in a homologous system. Three transgenic lines carrying 1, 3, and 4 copies of the MαIIbtk transgene were established. All three lines expressed tk within their BM. The level of tk transcripts was found to be higher in mice having several integrated copies of the transgene compared with those having only one copy. This may however only reflect differences in the sites of integration of the transgenes within the genome. To derive a statistically significant correlation between copy number and expression level, more data are needed from high-copy–number lines to validate copy number dependence. The dif-

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ferences in tk levels synthesized is correlated with the level of thrombocytopenia induced in vivo following injection of GCV into these mice. In fact, greater than 90% platelet reduction could be induced after 9 days of drug administration in mice with the highest level of tk expression. This was accompanied by a concomitant reduction in erythrocytes and to a lesser extent of leukocytes. This was in contrast to mice synthesizing lower levels of tk in which only platelets were reduced in the same period of time. There was no apparent evidence of atrophy of spleen, adrenals, or testes following GCV administration.

To determine the earliest hematopoietic cell expressing the transgene, GCV was administered to mice until a severe thrombocytopenia was obtained. Detailed examination of the composition of methylcellulose colonies derived from cells remaining in BM of GCV-induced thrombocytopenic MαI-Ibtk mice showed a dramatic inhibition of CFU-GEMM$_k$ and CFU-mix colonies in all transgenic lines. BM cellularity of nontransgenic littermates was not affected by the GCV treatment, as highly proliferative progenitor cells, capable of giving rise to CFU-GEMM$_k$, CFU-mix progenitors, and individual colonies of myeloid, erythroid, and megakaryocytic lineages could develop in culture. The absence of the primitive progenitors from the BM of the GCV-induced thrombocytopenic mice suggested that the transgenes were being expressed in an early progenitor cell before commitment to the megakaryocyte progenitor, CFU-M$_k$.

Whether the expression of the α_{IIb} gene begins in hematopoietic stem cells or in committed megakaryocytic progenitors has long been a source of controversy. The results reported in this study using 2.7 kb of the 5′-flanking region of the α_{IIb} gene are in agreement with earlier publications[8,9] and those described by other investigators who have shown the presence of the $\alpha_{IIb}\beta_3$ complex on multipotent progenitor stem cells. In fact, Basch et al[27] showed through immunocytochemical staining that in addition to platelets and megakaryocytes, antibodies raised against purified glycoprotein β_3 or platelet membrane glycoprotein $\alpha_{IIb}\beta_3$ react with other BM cells, albeit less intensely. On the basis of light scattering properties, these cells appeared to be immature cells and monocytes. Immunocytochemical staining of monocytes by $\alpha_{IIb}\beta_3$-specific monoclonal antibodies has also been reported by others.[28-29] In addition, Murray et al[30] have shown that fetal BM CD34$^+$ and CD41$^+$ positive cells have high proliferative potential in vitro and give rise to cells of megakaryocyte, granulocyte, macrophage, and erythrocyte lineages.

The finding that a marker previously presumed to be specific for cells of a particular lineage is also expressed in other cell types is not without precedent, as exemplified by the *c-mpl* gene. Initially, the presence of *c-mpl* and the response to thrombopoietin (TPO) was thought to be strictly limited to cells of the megakaryocytic lineage.[31-33] However, using both normal and myelosuppressed mice, Kaushansky et al[34] found that erythroid, granulocyte-macrophage, and megakaryocytic progenitors are also sensitive to TPO treatment concluding that the effect of TPO administration may be wider than initially anticipated.[35] This conclusion is consistent with the expansion of primitive progenitor cells with TPO found by independent investigators.[36] Similarly, the hematopoietic defect in null *mpl* mice is not confined to cells of the megakaryocytic lineage, as they were found to be deficient in progenitor cells of multiple lineages.[37,38]

Stem cell commitment to a monopotent lineage implies that the expression of particular genes persist in a specific hematopoietic route with a concomitant decline in other lineages. To determine whether the expression of the tk gene directed by the α_{IIb} 5′-flanking region persisted in separate monopotent progenitors, individual CFU-M$_k$, BFU-E, CFU-E, and GM-CFC monopotent progenitors were assayed. Results of methylcellulose cultures showed that the growth of CFU-M$_k$ and BFU-E colonies was drastically inhibited in the presence of GCV and that of CFU-E and GM-CFC was moderately affected showing that there is a progressive decrease of tk production as the course of differentiation proceeds towards the erythrocytic and myeloid lineages. The possibility that tk expression in nonmegakaryocytic cells resulted from leakage of tk phosphorylated GCV from lysis of the primary tk-expressing cells following cell death was considered and accounted for by coculturing BM cells of transgenic and nontransgenic animals. The results obtained provide strong evidence that this is not the case, as only cells expressing tk were affected.

The toxicity induced by GCV on early erythroid progenitors is in agreement with previous studies showing that megakaryocytic and erythroid lineages are closely related in ontogeny and both cell types appear to derive from a common progenitor cell.[39-41] Moreover, transcription factors including NF-E2 and GATA-1 appear to regulate genes within these two lineages supporting the possibility of common programs or mechanisms of activation.[42-44]

The presence of CFU-E and GM-CFC colonies obtained in methylcellulose by culturing BM of GCV-treated mice was unexpected considering the absence of most of the multipotent progenitors. However, a similar result was obtained in *mpl* −/− mice, in which the reduction of the number of neutrophil, granulocyte, macrophage, erythroid, multipotential, and CFC-mix was not reflected in the mature cell populations.[37] Moreover, peripheral blood hematocrit and white blood cell counts were normal in these *mpl* −/− mice despite the reduction of early progenitors. This raises the interesting possibility of the existence of compensatory mechanisms during the final stages of maturation or the emergence of alternative differentiation pathways to produce myeloid cells in pathological conditions. While the molecular basis for such a proposal has yet to be elucidated, there is growing support that distinct developmental routes may exist in normal and pathological conditions, as already suggested for the erythroid and the B-cell lineages,[45,46] including unilineage route of differentiation from hematopoietic early progenitors.[47,48] Taken together, the establishment of the new transgenic line presented in this report supports the following conclusions: first, a high degree of similarity exists in the mechanisms controlling hematopoiesis in mammals. This is based on the similar phenotypes obtained with transgenic mice possessing the tk gene driven by either the human or the mouse α_{IIb} promoters along with the high conservation of regulatory elements found in these two promoter sequences.[14] Second, the 1.9-kb nucleotide sequence located

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adjacent to and upstream of the initial 800 bp sequence of the 5′-flanking region of the α_{IIb} gene does not appear to contain any other significant transcriptional regulatory domains involved in the tissue-specific expression of the α_{IIb} gene in early hematopoietic cells. Third, the toxic effect arising following GCV administration is restricted to those cells that express the transgene and the severity of the toxic response is dependent on the level of tk synthesized. Finally, primitive hematopoietic progenitor cells possessing myeloid, erythroid, and megakaryocytic potential appear to possess the transcriptional capacity required to activate the promoter used in this study. This potential tapers off, however, as differentiation proceeds further towards the erythroid and myeloid lineages. This is consistent with the repressor element identified within the rat and human α_{IIb} promoters[5,6] playing a role in this process. This new and evolving transgenic technique used to study the regulation of the α_{IIb} gene shows the necessity of studying gene regulation in a dynamic system and offers tremendous potential in providing new insights into the molecular basis of platelet production.

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