Transgene Insertion in Proximity to the *c-myb* Gene Disrupts Erythroid-Megakaryocytic Lineage Bifurcation[▽]

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The nuclear proto-oncogene *c-myb* plays crucial roles in the growth, survival, and differentiation of hematopoietic cells. We established three lines of erythropoietin receptor-transgenic mice and found that one of them exhibited anemia, thrombocythemia, and splenomegaly. These abnormalities were independent of the function of the transgenic erythropoietin receptor and were observed exclusively in mice harboring the transgene homozygously, suggesting transgenic disruption of a certain gene. The transgene was inserted 77 kb upstream of the *c-myb* gene, and c-Myb expression was markedly decreased in megakaryocyte/erythrocyte lineage-restricted progenitors (MEPs) of the homozygous mutant mice. In the bone marrows and spleens of the mutant mice, numbers of megakaryocytes were increased and numbers of erythroid progenitors were decreased. These abnormalities were reproducible *in vitro* in a coculture assay of MEPs with OP9 cells but eliminated by the retroviral expression of c-Myb in MEPs. The erythroid/megakaryocytic abnormalities were reconstituted in mice *in vivo* by transplantation of mutant mouse bone marrow cells. These results demonstrate that the transgene insertion into the *c-myb* gene far upstream regulatory region affects the gene expression at the stage of MEPs, leading to an imbalance between erythroid and megakaryocytic cells, and suggest that c-Myb is an essential regulator of the erythroid-megakaryocytic lineage bifurcation.

The nuclear proto-oncogene *c-myb* product (c-Myb) belongs to the Myb family of transcription factors (23) and is believed to be an important regulator of growth, survival, and differentiation of hematopoietic cells (5). The *c-myb* gene is expressed predominantly in hematopoietic tissues, especially in immature hematopoietic progenitors (12). The mutation in homozygously *c-myb*-null mice is embryo lethal by embryonic day 15 (E15) due to severe anemia, which is caused by a failure of fetal liver erythropoiesis (21). Primitive nucleated erythrocytes circulate in the *c-myb*-null mutant embryos, suggesting that the primitive hematopoiesis in the yolk sac is unaffected by the absence of c-Myb. In addition to the primitive erythrocytes, mature megakaryocytes and macrophages are present in the *c-myb*-null fetal liver (21, 32). While elucidation of the contribution of c-Myb to the definitive hematopoiesis is intriguing and important, the embryonic lethality of the *c-myb*-null mutation hampered the further study of the consequence of lost c-Myb function in definitive hematopoiesis.

In this regard, it is interesting that several lines of *c-myb* knockdown mutant mice were recently reported, providing important insights into c-Myb function. Whereas the *c-myb* knockout mutation is embryo lethal, mice with *c-myb* knockdown (i.e., reduced expression) are born alive, which enables us to perform various analyses in the adult stage. The first example is the *c-myb* knockdown mouse that exhibits increased megakaryocyte production but diminished production of erythroid and lymphoid cells (4). The second case is that of mice

bearing mutations in the KIX domain of p300, which is an interface of p300 interacting with c-Myb. This line of mice displays anemia, thrombocytosis, megakaryocytosis, B-cell deficiency, and thymic hypoplasia (11). In a large-scale suppressor screen with *N*-ethyl-*N*-nitrosourea (ENU) aiming to identify mutations capable of ameliorating thrombocytopenia in *Mpl*-null mice, two lines of c-Myb-dysfunctional mice harboring point mutations individually in the DNA-binding domain and leucine zipper domain were identified (3). Homozygotes of one of these mutant mouse lines displayed multilineage hematopoietic alterations, including anemia, lymphopenia, and eosinopenia, as well as elevated numbers of megakaryocytes, platelets, and hematopoietic stem cells (28). These lines of evidence suggest that c-Myb plays an important role in the erythroid and megakaryocytic differentiation of hematopoietic progenitors, but the precise mechanisms of regulation of expression of the *c-myb* gene, as well as the stage at which c-Myb functions, remain to be clarified.

Insertion mutagenesis is an inherent event in transgenic mouse studies and is relatively common (19). Approximately 5% of established transgenic mouse lines are found to carry insertional mutations (19). Conversely, an insertion mutagenesis screening procedure is a powerful technique for studying mammalian genome function. Analysis of transgene-induced insertional mutations has successfully led to the identification of new genes and new functions of known and unknown genes (37).

In this study we identified a transgene insertion mutation in proximity to the *c-myb* gene in one of the transgenic mouse lines harboring the erythropoietin receptor (EPOR) transgene. Homozygotes of this line suffered from anemia, thrombocythemia, and splenomegaly, but these hematological abnormalities

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were not linked directly to the transgenic expression of EPOR. The transgene insertion appeared instead to block the function of an important enhancer for *c-myb* gene expression. We found that the transgene is integrated approximately 77 kb upstream of the *c-myb* gene and disrupts an enhancer that directs *c-myb* gene expression in megakaryocyte/erythrocyte lineage-restricted progenitors (MEPs). The lack of c-Myb in MEPs perturbs the bifurcation of megakaryocyte-erythrocyte differentiation, which results in an increase in megakaryocyte levels and a decrease in erythroid cell levels. These results strongly suggest that c-Myb is an important regulator for the lineage specification of MEPs along either an erythroid or a megakaryocytic pathway.

MATERIALS AND METHODS

Transgenic mice and hematological analyses. Transgenic mice were generated by the microinjection of an 11-kb construct, containing mouse EPOR cDNA under the control of the *Gata1* gene-hematopoietic regulatory domain (*GI-HRD*), into fertilized mouse embryos from strain BDF1. PCR of genomic DNA was performed to identify transgenic mice for the amplification of endogenous EPOR alleles (387 bp) and the transgenic EPOR allele (303 bp) (33). Three independent lines of transgenic mice were generated, and the level of transgene-derived mRNA was determined by semiquantitative RT-PCR. Quantitative Southern blot analysis was performed to distinguish heterozygotes from homozygotes. Blood was collected from the retroorbital plexus, and complete blood counts were measured with an automatic counter (Nihon-Kohden). All animal work was carried out at the animal facility at the University of Tsukuba under approved animal protocols and in accordance with institutional guidelines.

Flow cytometry. Mononuclear cells (MNCs) from bone marrow (BM-MNCs) or spleen were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD71, anti-CD41, anti-Gr-1, or anti-CD4, phycoerythrin-conjugated anti-Ter119, anti-CD61, anti-CD11b (Mac-1), or anti-CD8, and allophycocyanin-conjugated anti-c-Kit antibodies. These antibodies were from Becton Dickinson Co. (BD). The samples were subjected to flow cytometry using a FACSCalibur (BD), and data were analyzed with the CellQuest program (BD).

Serum thrombopoietin levels and megakaryocyte ploidy. Serum thrombopoietin (TPO) concentrations were measured using sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (22). A double-staining technique and flow cytometry were used to measure megakaryocyte ploidy (35). Briefly, megakaryocytes were identified after labeling with an FITC-anti-CD41 antibody, and BM-MNCs were fixed with 70% ethanol for 30 min on ice. Cells were then washed with phosphate-buffered saline containing 2% bovine serum albumin and incubated for 30 min at 37°C with propidium iodide (50 µg/ml; Sigma). DNA contents were analyzed with a FACS Calibur.

In vitro progenitor assays. Erythroid colony assays were performed by standard procedures (24). For the CFU-erythroid (CFU-E) assays, 1×10^5 BM-MNCs were cultured in methylcellulose medium (MethoCult M3231; StemCell Technologies) containing 1 U/ml recombinant human EPO (generous gift from Chugai Pharmaceutical). After 3 days of culturing, cells were stained with benzidine, and positive colonies were counted as CFU-E. For counting the burst-forming unit (BFU)-E-derived colonies, 2×10^4 MNCs were cultured with 2 U/ml EPO and 100 ng/ml of stem cell factor (SCF; R&D Systems) for 7 days. For the CFU-megakaryocyte (CFU-Meg) assay, 1×10^5 MNCs were cultured with a MegaCult-C kit (StemCell Technologies) in the presence of 50 ng/ml TPO (generous gift from Kirin Brewery), 10 ng/ml interleukin 3 (IL-3; Kirin), 20 ng/ml IL-6 (Kirin), and 50 ng/ml IL-11 (R&D Systems). After 8 days, the colonies were stained for acetylcholine esterase (AChE) as described previously (8), and the number of AChE-positive colonies was counted.

Immunohistochemical staining. Spleens were fixed in 4% paraformaldehyde, followed by embedding in Tissue-Tek OCT compound (Sakura Finetechnical), and were quickly frozen. Cryosections were stained with hematoxylin-eosin (HE) for histological examination and with AChE, anti-B220, and anti-CD3 antibodies as described previously (20).

Identification of the transgene insertion site. Genomic DNA (200 µg) from homozygously transgenic mouse liver was digested with EcoRI. The DNA fragment containing the junction of genomic and transgenic DNA was identified by Southern blot hybridization analysis using the PstI-EcoRI fragment of *GI-HRD* as a probe. The DNA fraction containing the junction DNA was enriched by using sucrose gradient ultracentrifugation and Southern blot hybridization with

the same probe. A genomic DNA bookshelf library (i.e., a mini-genomic DNA phage library) was constructed by using the ZAP Express vector (Stratagene). Isolated DNA fragments were ligated into the ZAP Express vector and packaged with Gigapack III Gold packaging extract (Stratagene). The bookshelf library containing 10^7 independent PFU was screened using the same probe as that used in the Southern blot analysis. Plasmid clones were generated by the in vivo excision of the positive phage clones following the manufacturer's protocol. The inserts in these ZAP plasmid clones were sequenced with the sequencing primers T3 (5'-AATTAACCTCACTAAAGGG-3') and T7 (5'-GTAATACGACTCATATAGGCG-3'). A BLAST search of the DNA sequence utilized the Celera Discovery system.

c-Myb expression in hematopoietic cells. Total RNA (20 µg) extracted from bone marrow, spleen, and liver was electrophoretically separated. The RNA-blotted membrane was hybridized with radiolabeled probes corresponding to the genes *c-myb*, *G3PDH*, and *Hbs-1 like*. Ter119-, CD41-, CD19-, and CD3-positive cells were sorted using FACS Vantage (BD), and total RNA was extracted from sorted cells using an RNeasy Mini kit (QIAGEN). Reverse transcriptase PCR amplification of c-Myb mRNA was performed as previously described (16). For the immunoblotting analysis, BM-MNCs not expressing Mac-1, Gr-1, CD4, or CD8 were prepared by negative selection using a Vario magnetic cell-sorting (MACS) separator (Miltenyi Biotec), and 1 µg/ml anti-c-Myb antibody (clone 1-1; Upstate) was used to detect c-Myb.

MEP cell assay. MEPs were sorted using FACS Vantage by the previously described method (1) with minor modifications. Briefly, OP9 stromal cells were maintained in α -modified minimum essential medium (Gibco) supplemented with 20% fetal bovine serum (FBS; Gibco). MEPs (1,500 to 2,000 cells/well) were transferred onto the confluent OP9 stromal cells and cultured in the presence of 10 ng/ml SCF, 40 ng/ml Flt-3 ligand (R&D Systems), 20 ng/ml IL-11, 20 ng/ml IL-3, 10 ng/ml granulocyte-macrophage colony-stimulating factor, 10 ng/ml TPO, and 2 U/ml EPO. After 4 days of culture, MEPs were analyzed by flow cytometry and colony formation assays. Colonies were counted after cells were stained with benzidine (erythroid colonies) or AChE (megakaryocyte colonies).

Construction of retroviral vectors. Murine stem cell virus-internal ribosomal entry site-enhanced green fluorescent protein (MSCV-IRES-GFP) was kindly provided by A. Kume (Jichi Medical University). Murine c-Myb cDNA was ligated into the NotI site of MSCV-IRES-GFP to produce MSCV-*c-myb*-IRES-GFP. Phoenix-Eco packaging cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS. Phoenix-Eco cells on 6-cm dishes were transfected with 1 µg DNA using a FuGENE transfection kit (Roche). To establish retroviral packaging cell lines, the supernatants from transfected Phoenix-Eco cells were harvested and used to infect dual-potential PT67 cells (Clontech) in the presence of 8 µg/ml Polybrene (Sigma). After 2 to 4 days, GFP-expressing cells were sorted using FACS Vantage and expanded.

Infection of MEPs with retroviral constructs. Retroviral supernatants were collected at 72 h and used to infect MEPs by Magnetofection (OZ Biosciences) as described elsewhere (27). Briefly, sorted MEP cells were placed on OP9 cells for 1 h at 37°C in a 5% CO₂ incubator. Retroviral supernatant and CombiMag (3 µl CombiMag/ml supernatant; OZ Biosciences) were incubated for 20 min at room temperature. The resulting mixture was added to MEPs, and the cell culture plate was placed on a magnetic plate for 20 min at 37°C in a 5% CO₂ incubator. After the magnetic plate was removed, a dose of cytokines equal to that used in the MEP and OP9 coculture system was added to the cells. After 4 days, cultured MEPs were analyzed by flow cytometry, and benzidine- or AChE-stained colonies were counted.

Bone marrow transplantation (BMT). Transgenic mutant lines of mice were backcrossed with C57BL/6J-Ly-5.2 mice for six generations, and peripheral blood leukocytes obtained from the mice were detected with anti-mouse CD45.2 antibody. At this stage, the backcrossed mice were considered Ly5.2 mice. Eight- to 12-week-old recipient mice (C57BL/6J-Ly-5.1) were irradiated with a single lethal dose (9.2 Gy) and injected with 1×10^6 freshly isolated bone marrow cells obtained from the Ly5.2 homozygously transgenic mice. To assess the reconstitution of bone marrow, peripheral blood or bone marrow cells were analyzed for chimerism, expressed as the percentage of Ly5.2-expressed leukocytes determined by using anti-Ly5.2 antibody (BD).

RESULTS

Identification of a mouse line with a transgenic insertion mutation. In order to examine the domain function of EPOR, we generated transgenic mouse lines bearing EPOR cDNA ligated to the *Gata1* gene-hematopoietic regulatory domain

FIG. 1. Homozygously transgenic line B mice exhibit hematological abnormalities. (A) Three independent lines of transgenic mice were produced. Levels of transgene-derived mRNA were determined by semiquantitative RT-PCR. Reverse-transcribed cDNAs were subjected to 27, 30, 33, 36, and 39 cycles of amplification. (B) The integration levels of transgene (Tg) were analyzed by Southern blot hybridization analysis and compared with endogenous (endo) DNA levels. Tg/endo ratios are in arbitrary units. Phenotypes were anemia, thrombocythemia, and splenomegaly. (C to H) Wright-Giemsa staining of peripheral blood smears (C to E) and bone marrow smears (F to H). Photomicrographs show samples from wild-type mice (C and F), heterozygously transgenic mice (D and G), and homozygously transgenic mice (E and H). In panel E, large platelets and spindle-shaped platelets are indicated by black and white arrowheads, respectively. Original magnification, $\times 200$.

(*G1-HRD-EPOR*) (25, 33). We obtained three lines of transgenic mice, and the expression level of the EPOR transgene was determined by semiquantitative RT-PCR. Based on the expression level of the transgene, these three mouse lines were named lines A, B, and C (Fig. 1A). Founder transgenic animals (generation 0 [G0]) and heterozygous G1 transgenic animals were normal in appearance and were fertile. However, when heterozygous G1 transgenic mice were intercrossed to produce G2 mice, $\sim 25\%$ of the offspring from line B showed anemia, thrombocythemia, and splenomegaly, while homozygotes from lines A and C never showed such abnormalities. We genotyped the progeny of several crossings of line B heterozygous mice by Southern blotting, and the results demonstrated that the hematopoietic abnormalities were observed only in the mice harboring the transgene homozygously (Fig. 1B). These abnormalities were absent in heterozygous or nontransgenic animals from the same litter.

The hematological abnormalities did not appear to correlate directly to the transgenic expression of EPOR. Indeed, the expression level of the EPOR transgene was about 64 times higher in line C than in line B (Fig. 1A), yet mice of line C never showed such abnormalities. Upon intercrossing of line B homozygously transgenic mice, all offspring showed similar abnormal phenotypes (data not shown). Through several generations of mating, mice expressing the hematopoietic abnormalities were born in conformity with Mendelian expectation (data not shown). Thus, on the basis of the recessive genetics of the mutant, as well as the fact that none of the other mouse

lines prepared with the same transgene construct showed the abnormal phenotype belonging to line B, we concluded that the phenotype arose from a mutation caused by the integration of the transgene into the host genome.

To further investigate the influence of the genetic background, heterozygously transgenic mice were bred with C57BL/6 mice for eight generations, and then homozygously transgenic mice were generated by intercrossing of these heterozygously transgenic mice. The homozygously transgenic mice with the C57BL/6 background also exhibited anemia, thrombocythemia, and splenomegaly, consistent with the abnormality observed with the BDF1 background (data not shown). This result supports our contention that insertion of the transgene disrupted a gene essential for erythroid and megakaryocytic differentiation.

Hematological analysis of homozygously transgenic mice.

The significant traits of the homozygous line B mice are anemia and thrombocythemia. The hemoglobin (Hb) and hematocrit (Ht) levels of the homozygously transgenic mice were significantly lower than those of the heterozygously transgenic and wild-type mice (Table 1). In contrast, platelet counts in homozygotes were approximately three times higher than those in heterozygotes and wild-type mice. White blood cell counts did not differ significantly among these three genotypes.

Peripheral blood smears from homozygously transgenic mice showed that erythrocyte numbers decreased while the variation in the size of the erythrocytes increased, compared with results for heterozygously transgenic and wild-type mice (compare Fig. 1E

TABLE 1. Hematopoietic indices of mutant mice^a

Mouse (n)	Hb (g/dl)	Ht (%)	MCV (fl)	MCH level (pg)	WBC count (/μl)	Plt count (10 ⁴ /μl)
Wild type (24)	13.7 ± 0.8	43.7 ± 2.5	49.8 ± 2.5	15.8 ± 1.0	7,917 ± 2,773	63.2 ± 16.9
Heterozygously transgenic (25)	14.1 ± 1.0	46.2 ± 4.1	50.8 ± 2.1	15.4 ± 0.8	8,116 ± 3134	67.5 ± 15.3
Homozygously transgenic (25)	9.0 ± 1.8*	28.8 ± 6.0*	72.2 ± 4.7*	22.7 ± 2.2*	9,428 ± 3,027	180.0 ± 48.6*

^a Transgenic mice 6 to 12 weeks of age were used. Age-matched wild type BDF-1 mice were used as controls. Values are means ± standard errors of the means. *, *P* < 0.001 compared with wild-type and homozygously transgenic mice. Abbreviations: Hb, hemoglobin; Ht, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; WBC, white blood cell; Plt, platelet.

with 1C and D). Marked thrombocytosis was also observed (Fig. 1E) along with large platelets and spindle-shaped platelets. The bone marrow of homozygously transgenic mice showed megakaryocytic hyperplasia (Fig. 1H), in contrast to that of the wild-type mice (Fig. 1F) and heterozygously transgenic mice (Fig. 1G). Collectively, these results suggest that the transgene somehow disrupted a gene essential for the regulation of erythroid-megakaryocytic lineage bifurcation in line B mice.

Identification of transgenic insertion sites. To determine the chromosomal localization of the transgene, we prepared a genomic DNA library utilizing EcoRI DNA fragments from a line B homozygously transgenic mouse. To this end, we performed a series of sucrose density gradient centrifugation and Southern blotting analyses with the *G1-HRD-EPOR* fragment as a probe and isolated a genomic DNA fraction enriched with the portion containing the junction of genomic and transgene

DNA. This genomic DNA fraction was ligated into a lambda phage ZAP Express vector to generate a mini-genomic DNA library (or a genomic DNA bookshelf). We subsequently screened the bookshelf using the same *G1-HRD-EPOR* probe and isolated positive clones, with several phage clones being analyzed by standard procedures. An insertion site was finally identified by sequencing the genomic DNA clones and comparing the sequence with the transgene and mouse genome sequences obtained from the Celera Discovery system.

The transgene insertion site was localized on mouse chromosome 10qA3 (Fig. 2A), which corresponds to human chromosome 6q22-23. Although we could not find a gene directly interrupted by the transgene insertion (Fig. 2B to D), there are three known genes, *c-myb*, *G3PDH pseudo*, and *Hbs-1 like*, neighboring the integration site. We also found that the EST clone BF720900 mapped near the insertion site.

FIG. 2. Chromosomal localization and expression analysis of neighboring genes around the transgene integration site. (A) Karyogram of mouse chromosome 10. (B) A3 region. Known genes are shown. (C) Physical and transcript map of the genomic region around the integration site. Arrowheads in the boxes indicate the direction of transcription. (D) Precise map of the region around the integration site, shown with a vertical arrow. The combined thick and thin lines show an EST clone. (E) *c-Myb* mRNA expression levels determined by RNA blotting analyses. RNA samples were obtained from hematopoietic (bone marrow [BM] and spleen [Sp]) and nonhematopoietic (liver [Li]) tissues of wild-type mice (Wild) or homozygously transgenic mice (Tg homo). (F) *c-Myb* mRNA expression levels in Ter119⁺, CD41⁺, CD19⁺, and CD3⁺ cells. Semiquantitative RT-PCR was carried out using hypoxanthine phosphoribosyltransferase (HPRT) as a standard. (G) Immunoblotting analyses of BM-MNCs without Mac-1⁺, Gr-1⁺, CD4⁺, or CD8⁺ cells with antibodies against *c-Myb* and β -actin.

We therefore examined the expression of these genes in the homozygously transgenic mice and found significant changes in the expression of the *c-myb* gene in the mutant mice. The *c-myb* gene is located approximately 77 kb downstream of the transgene integration site. While *c-Myb* mRNA expression levels in the whole BM-MNCs and whole splenic cells were comparable to those in the wild-type mice (Fig. 2E), the *c-Myb* mRNA level was markedly decreased in Ter119⁺ erythroid and CD41⁺ megakaryocytic cells isolated by the fluorescence-activated cell sorting (FACS) procedure from homozygously transgenic mice (Fig. 2F). Interestingly, the *c-Myb* mRNA level was also decreased in CD19⁺ B cells but was increased in CD3⁺ T cells (Fig. 2F). In contrast, the expression levels of *Hbs-1 like*, *G3PDH pseudo*, and BF720900 did not change much in the spleens and livers of homozygously transgenic mice (data not shown).

To further examine the expression level of the *c-Myb* protein in homozygously transgenic mice, we enriched erythroid and megakaryocytic cells from BM-MNCs by depleting Mac-1⁺, Gr-1⁺, CD4⁺, and CD8⁺ cells using the MACS system (see Materials and Methods). Enriched BM-MNCs from homozygously transgenic mice were found to express *c-Myb* at approximately 15% of the level seen in an equivalent fraction from wild-type mice (Fig. 2G). These data indicate that insertion of the transgene 77 kb upstream of the *c-myb* gene affects the *c-myb* gene expression in erythroid- and megakaryocytic-lineage cells and suggest that the transgene insertion site is important for lineage-specific expression of the *c-myb* gene.

Homozygously transgenic mice exhibit defects in erythroid differentiation. To determine how erythroid differentiation was blocked in homozygously transgenic mice, we examined the status of erythropoiesis. Erythroid early progenitors at the BFU-E stage were significantly diminished in the bone marrow of homozygously transgenic mice (Fig. 3A). The BFU-E colonies from homozygously transgenic mice were significantly smaller than those from wild-type and heterozygously transgenic mice (Fig. 3A, inset). CFU-E colony numbers were decreased significantly in homozygously transgenic mice (Fig. 3B). These results indicate that erythroid differentiation was blocked in homozygous line B mice at or before the BFU-E stage.

We next examined erythroid cells in bone marrow by FACS. BM-MNCs were stained with anti-c-Kit, CD71 (transferrin receptor) and Ter119 antibodies. CD71 and Ter119 antibodies were selected, as two-color staining with these antibodies indicates the differentiation stage of erythroid cells (31); CD71 is expressed at a high level in proerythroblasts and early basophilic erythroblasts, whereas Ter119 is highly expressed in terminally differentiating erythroblasts. In the bone marrow of homozygously transgenic mice, c-Kit⁺ Ter119⁺ cells corresponding to proerythroblasts (34) were markedly decreased (Fig. 3C). Total Ter119-positive erythroid cells (regions 1 to 4 [R1 to R4]; R1, CD71^{high} Ter119^{low} proerythroblasts; R2, CD71^{high} Ter119^{high} basophilic erythroblasts; R3, CD71^{low} Ter119^{high} late basophilic and chromatophilic erythroblasts; and R4, CD71⁺ Ter119^{high} orthochromatophilic erythroblasts) were decreased in homozygously transgenic mice (Fig. 3D; 65.5% in wild-type mice, 55.4% in heterozygous mice, and 38.4% in homozygous mice). Especially, CD71^{low} Ter119^{high} cells in R3 were markedly reduced in homozygously transgenic

mice compared with those in wild-type mice. The intensity of the Ter119-positive signal was diminished in both homozygously and heterozygously transgenic mice. These data are thus indicative of a wide deduction in homozygously transgenic mice erythroid cells, from early erythroid progenitors to mature erythroid cells. Although heterozygously transgenic mice did not show apparent anemia or thrombocythemia, the results of colony assays and FACS analyses suggest that erythropoiesis in these mice is also affected.

Increase of megakaryocytes and platelets in transgene homozygotes. Megakaryopoiesis in the bone marrow of line B homozygously transgenic mice was also examined. In agreement with the observation that megakaryocytes were increased in homozygously transgenic mouse bone marrow (Fig. 1H), the numbers of CFU-Meg colonies obtained with cells from these mice were approximately threefold higher than those from wild-type and heterozygously transgenic mice (Fig. 4A). In contrast, the serum concentration of TPO, a cytokine stimulator of megakaryopoiesis, was not elevated in homozygously transgenic mice compared with wild-type and heterozygously transgenic mice (Fig. 4B). These findings imply that the increased megakaryopoiesis and platelet production were caused by a mechanism independent of the TPO pathway.

Our FACS analysis also revealed an increase in megakaryocytes in the bone marrow of homozygously transgenic mice, as the bone marrow contained many more CD41⁺ CD61⁺ cells than the marrow of wild-type and heterozygous mice (Fig. 4C). When megakaryocyte development was examined by quantifying the DNA content in CD41⁺ bone marrow cells (Fig. 4D), homozygously transgenic mice contained more immature megakaryocytes with a modal ploidy of 2N to 8N, while megakaryocytes with a modal ploidy of 16N to 32N were predominant in the wild-type mice.

Homozygously transgenic mice showed splenomegaly with megakaryocytosis. The spleens of homozygously transgenic mice were markedly enlarged compared with those of wild-type and heterozygously transgenic mice (Fig. 5A). The total cell counts in spleens from homozygously transgenic mice were three times ($[1.3 \pm 0.1] \times 10^8$) higher than those in wild-type mice ($[4.3 \pm 0.4] \times 10^7$). Histological examination of homozygous spleens revealed that the red pulp was significantly enlarged and the structure of the white pulp was disrupted (Fig. 5B to D). The number of megakaryocytes was significantly increased in the spleens of homozygously transgenic mice and were detectible as large cells (Fig. 5E to G) or AchE-staining cells in enlarged red pulps (Fig. 5H to J). Whereas the white pulp of spleen was filled with B220-positive B cells in wild-type and heterozygously transgenic mice, in homozygously transgenic mice the white pulp was disorganized and numbers of B220-positive cells were reduced (Fig. 5K to M). T-cell-marker-positive cells were relatively unchanged; the cells make up approximately 20% of the wild-type, heterozygously transgenic, and homozygously transgenic mouse spleen cells. Myeloid-marker-positive-cell levels were also similar in wild-type, heterozygously transgenic, and homozygously transgenic mouse spleen.

In FACS analyses of splenic cells, erythroid cell levels were found to be decreased in homozygously transgenic mice, and this was apparent in CD71^{high} Ter119^{high} cells (R2) and CD71^{med} Ter119^{high} cells (R3), corresponding to basophilic

FIG. 3. Numbers of erythroid-lineage cells decreased in line B homozygously transgenic mice. (A and B) In vitro hematopoietic colony formation using BM-MNCs of wild-type, heterozygously transgenic (Tg hetero), and homozygously transgenic (Tg homo) mice. Note that the production of erythroid progenitor cells, BFU-E (A) and CFU-E (B), is reduced in homozygously transgenic mice. The data are means plus standard errors of the means for experiments performed in triplicate. The colonies of BFU-E cells from homozygously transgenic mice were significantly smaller than those from wild-type mice (inset). (C and D) The expression of erythroid lineage markers was analyzed by FACS using BM-MNCs. The percentage in each quadrant is shown. (C) Numbers of c-Kit⁺ Ter119⁺ immature erythroid cells were decreased in homozygously transgenic mice. (D) R1, R2, R3, and R4 represent Ter119^{med} CD71^{high}, Ter119^{high} CD71^{high}, Ter119^{high} CD71^{med}, and Ter119^{high} CD71^{low} populations, respectively. The relative number in each region as a percentage of gated cells is given.

erythroblasts and chromatophilic erythroblasts, respectively (Fig. 5N). In contrast, levels of CD41⁺ CD61⁺ megakaryocytic cells were increased in the spleens of homozygously transgenic mice compared with wild-type and heterozygous mice (Fig. 5O). While the architecture of the spleen appeared to be disrupted to some extent in heterozygously transgenic mouse spleen, the number of megakaryocytes was comparable to that in the wild type.

Megakaryocyte-erythroid common progenitor cells are affected in homozygously transgenic mice. To determine the

mechanisms underlying the erythroid-megakaryocytic differentiation imbalance, we examined MEPs in the homozygously transgenic mice. MEPs are characterized as FcγR^{low} CD34⁺ c-Kit⁺ Sca-1⁻ IL-7Rα⁻ Lin⁻ cells, and when cultured in the presence of EPO and TPO, MEPs give rise to CFU-Meg, BFU-E, and CFU-E colonies (1). We found that c-Myb mRNA expression was significantly diminished in the MEPs of homozygously transgenic mice compared with that in cells of wild-type mice (Fig. 6A).

To elucidate the consequence of c-Myb down-regulation in

FIG. 4. Supraphysiological production of megakaryocytes in homozygous line B transgenic mice. (A) In vitro megakaryocytic colony formation examined using BM-MNCs. The data are means plus standard errors for triplicate experiments. (B) Serum TPO concentration measured with ELISA. (C) Expression of megakaryocyte lineage markers analyzed by FACS using BM-MNCs. The percentage in each quadrant is given. Numbers of CD41⁺ CD61⁺ megakaryocytes were increased in homozygously transgenic mice. (D) Ploidy analysis of megakaryocytes using FACS. Homozygously transgenic mouse megakaryocytes had a lower ploidy (2N to 8N) than wild-type megakaryocytes (16N and 32N).

MEPs, we designed an in vitro coculture method for MEPs (see Materials and Methods). In this experiment, MEPs isolated from homozygously transgenic mice and wild-type mice were cocultured with OP9 cells for 4 days. In excellent agreement with the mutant mouse analysis in vivo, the number of benzidine-positive erythroid colonies was decreased, while the number of AchE-positive megakaryocytic colonies was increased, in the coculture of MEPs from homozygously transgenic mice compared with MEPs from wild-type mice (Fig. 6B and C). FACS analysis further revealed that in the cocultured cells of homozygously transgenic mice, numbers of Ter119⁺ erythroid-lineage cells were sharply decreased, while numbers of CD41⁺ megakaryocytic cells were increased (Fig. 6D and E). These results support our contention that *c-Myb* down-regulation affects erythroid-megakaryocytic bifurcation at the MEP stage.

Retroviral complementation of *c-Myb* rescued mutant MEPs from abnormal differentiation. To clarify whether the reduction of *c-Myb* in MEPs was the direct cause of the he-

matopoietic abnormalities, we introduced *c-Myb* retrovirally into the mutant MEPs. For this purpose, mouse *c-Myb* cDNA was inserted into the MSCV-IRES-GFP vector, and this *c-Myb*-expressing virus was used to infect MEPs isolated from the homozygously transgenic or wild-type mice. Since the *c-Myb* virus-infected cells emit a GFP signal, these cells were easily identified by fluorescent microscopy (Fig. 7A). We found an increase in the frequency of benzidine-positive erythroid cells in *c-Myb* virus-infected mutant MEPs, but no such increase was observed in the control vector-infected cells (Fig. 7B). In contrast, the frequency of AchE-positive megakaryocytes was decreased in the *c-Myb* virus-infected mutant MEPs (Fig. 7C). This indicates that the mutant MEP from homozygously transgenic mice can reproduce in vitro the hematological abnormalities observed in homozygously transgenic mice in vivo. Furthermore, retroviral complementation of *c-Myb* effectively rescued the mutant MEPs from the differentiation imbalance. These data indicate that the transgene insertion upstream of the *c-myb* gene affects hematopoietic cells espe-

FIG. 5. Hematological analysis of spleens from homozygous line B transgenic mice. (A) Gross appearance of spleens. Note that massive splenomegaly was observed in homozygously transgenic mice. Bar, 1 cm. (B to M) Sections of spleens from wild-type (B, E, H, and K), heterozygously transgenic (C, F, I, and L), and homozygously transgenic (D, G, J, and M) mice, stained with HE (B to G), AchE (H to J), and

FIG. 6. MEPs analyses of homozygously transgenic mice. (A) Expression level of *c-Myb* mRNA in MEPs analyzed by RT-PCR. HPRT, hypoxanthine phosphoribosyltransferase. (B and C) In vitro erythroid (B) and megakaryocyte (C) colony formation by MEPs, examined using a coculture system with OP9. Erythroid colonies were stained with benzidine (B), and megakaryocyte colonies were stained with AchE (C). The numbers of total colonies and benzidine (Be)- and AchE-positive colonies are given. The data are means $\pm$ standard errors for experiments done in triplicate. (D and E) FACS analyses of MEPs after coculturing with OP9 cells for 4 days. Results for Ter119⁺ erythroid cells (D) and CD41⁺ megakaryocyte cells (E) are boxed.

cially at the stage of MEP and that the most essential function of *c-Myb* for the bifurcation of erythroid and megakaryocyte lineages exists during the stage of MEPs.

BMT reproduces the hematopoietic abnormalities of homozygously transgenic mice. To investigate whether the hematological abnormalities of homozygously transgenic mice are caused in a cell-autonomous manner, a long-term reconstruction experiment with lethally irradiated mice was performed with BMT. Whereas irradiated mice all died within 2 weeks, mice given a transplant of bone marrow cells from homozygously transgenic or wild-type mice survived for more than 4 months. By 4 months following transplantation, nearly all the leukocytes (>97%) of the recipient mice expressed Ly5.2 donor-type antigen, regardless of the transplantation with wild-type or homozygously transgenic mouse bone marrow cells (Fig. 8A). Importantly, mice given a transplant of bone marrow cells from homozygously transgenic mice showed hematological abnormalities similar to those observed in the original homozygously transgenic mice (Table 2).

FACS analyses further revealed that in mice given a transplant of homozygously transgenic mouse bone marrow, num-

bers of Ter119⁺ erythroid cells (especially CD71^{high} Ter119^{low} cells in R1) were decreased (Fig. 8B). In contrast, numbers of CD41⁺ CD61⁺ megakaryocytes were increased threefold in mice given a transplant of homozygously transgenic mouse bone marrow (Fig. 8C). These data demonstrate that the bone marrow cells from homozygously transgenic mice reconstructed their own hematopoiesis in the wild-type environment and that the hematological abnormalities observed in the homozygously transgenic mice are caused in a cell-autonomous manner.

DISCUSSION

We identified hematological abnormalities in mice of line B, one of three independent EPOR cDNA-transgenic mouse lines. We concluded that the abnormalities were brought about by insertion mutagenesis of an important gene, based on the following three observations: (i) transgenic line C mice expressing the EPOR transgene at a much higher level than line B mice did not show any such hematopoietic abnormalities; (ii) mice expressing the abnormal phenotype were born in confor-

B220 (K to M). Original magnifications, $\times 40$ (B to D and H to M) and $\times 200$ (E to G). (N) FACS analysis for expression of the erythroid lineage markers Ter119 and CD71 in spleen MNCs. R1 to R4 represent the same regions as in Fig. 3D. The relative number in each region is given as a percentage of gated cells. (O) FACS analysis for the expression of the megakaryocyte lineage markers CD41 and CD61 in spleen MNCs. The percentage in each quadrant is given.

FIG. 7. *c-Myb* regulates erythroid-megakaryocytic bifurcation at the stage of MEPs. (A) Virus-infected MEPs observed by bright and fluorescent microscopy. GFP expression was detected in *c-Myb*-infected MEPs but not in uninfected MEPs. (B and C) In vitro erythroid (B) and megakaryocyte (C) colony formation by MEPs supplemented with *c-Myb* examined by the OP9 coculture system. Columns labeled “wild” and “Tg homo” show numbers of colonies without viral infection, while those labeled “vector” and “*c-Myb*” show numbers of colonies with viral vector and *c-Myb* vector infection, respectively. The numbers of total colonies and benzidine (Be)- and AchE-positive colonies are indicated. The data are means ±

FIG. 8. Phenotype analyses of bone marrow transplant mice. (A) Chimerism of recipient BM-MNCs examined using FACS analysis. Ly5.1 indicates recipient origin, and Ly5.2 indicates donor origin. (B) Expression of erythroid lineage markers in recipient mouse BM-MNCs examined by FACS analyses. R1 to R4 are the same as in Fig. 3D. The relative number in each region is given as a percentage of gated cells. (C) Expression of megakaryocyte lineage markers examined by FACS analyses. The percentage in each quadrant is shown.

mity with Mendelian inheritance; and (iii) while activation of the EPO-EPOR pathway should increase numbers of both erythroid and megakaryocytic cells (14, 17, 18), line B mice actually showed a decrease in erythroid cells. We therefore attempted to determine the integration site of the transgene and determined that it is in proximity to the *c-myb* gene, suggesting that the transgene interrupted a certain regulatory

standard errors for experiments carried out in triplicate. MEPs used in these experiments (A to C) were obtained from homozygously transgenic mice.

TABLE 2. Hematopoietic indices of mice after BMT^a

Donor mouse (n)	Hb (g/dl)	Ht (%)	MCV (fl)	MCH level (pg)	WBC count (/μl)	Plt count (10 ⁴ /μl)
Wild type (6)	14.3 ± 1.0	45.1 ± 3.0	46.1 ± 2.6	14.6 ± 0.8	15,400 ± 2,053	59.2 ± 8.0
Homozygously transgenic (3)	11.2 ± 1.2*	34.4 ± 4.4*	51.7 ± 4.7	17.0 ± 1.9	6,833 ± 1,550§	164.5 ± 29.0§

^a Values are means ± standard errors of the means. *, *P* < 0.005; §, *P* < 0.001. Abbreviations are as in Table 1.

region for *c-myb* gene expression. Indeed, c-Myb mRNA and protein expression in MEPs, as well as in erythroid cells and megakaryocytes, was severely compromised in the mutant mice, whereas that in total BM-MNCs or splenic cells was rather well preserved. Therefore, as summarized in Fig. 9, we conclude that the transgene integration site encodes a specific enhancer for *c-myb* gene expression in erythroid and megakaryocytic lineages and that the transgene disrupted the function of this enhancer.

Genomic regions surrounding the *c-myb* gene have been reported to coincide with the region where integration sites of retroviruses in leukemias and lymphomas cluster. For example, insertion of a retrovirus near the *c-myb* gene locus has been found in myeloid leukemias and T- and B-cell lymphomas in chickens and mice (36). A cluster of retrovirus insertion sites has been mapped in close proximity (25 to 250 kb) to the mouse *c-myb* gene, but outside the transcription unit. Detailed physical mapping of the latter enabled placement of the retroviral integration sites at approximately 25 kb (*murine leukemia virus-induced myeloid leukemia 1* [*Mml1*]), 51 kb (*Mml2*), and 70 kb (*Mml3*) upstream of the *c-myb* locus (7). The *fit-1* locus, an integration site of feline leukemia virus in T-cell lymphoma, has been mapped to a location 100 kb upstream of the *c-myb* gene locus in human and mouse genomes (2). In cases of Abelson murine leukemia virus-induced lymphoma, the retrovirus integration site *Ahi-1* is located 35 kb downstream of the *c-myb* gene (9). It was recently reported that four lymphoma cell lines bearing the virus insertion at the *fit-1* or *Ahi-1* locus showed a high level of expression of c-Myb mRNA and protein compared to a panel of phenotypically similar cell lines lacking such insertions (6). These observations suggest that integration of the retrovirus in close proximity to the *c-myb* gene may cause activation or inactivation of the *c-myb* gene and that altered *c-myb* gene expression may be involved in leukemogenesis. However, a correlation between viral gene integration and *c-myb* gene expression was not clear from these analyses, and the molecular mechanisms of *c-myb* gene regulation largely remain to be elucidated.

In this regard, it should be noted that there are a few previous reports of cases in which transgene insertion into an important regulatory region indeed caused perturbation of gene expression. One salient example of such cases is *doubleridge*, a transgene-induced mutation resulting in mice displaying forelimb postaxial polysyndactyly. The transgene insertion site of *doubleridge* was identified as 150 kb downstream of the *dickkopf1* (*Dkk1*) gene, and because of the transgene integration, the expression level of *Dkk1* was reduced to less than 1% of the wild-type level in E13.5 mutant embryos (15).

As mentioned above, changes in c-Myb mRNA and protein expression were marginal when we examined total MNCs in the bone marrow and spleen. c-Myb mRNA expression was

reduced specifically in the MEPs, Ter119⁺ erythroid cells, and CD41⁺ megakaryocytes. Thus, in the present case the transgene insertion perturbed *c-myb* gene expression in a lineage-specific manner. In contrast, the expression level or activity of c-Myb was reduced fairly uniformly in the other recently reported *c-myb* knockdown mutant mouse cases (3, 4, 11). While the mechanisms leading to reduced *c-myb* gene expression differ among these mutant animals, the resulting phenotypes are similar, namely anemia, thrombocythemia, B-cell deficiency, and maturation impairment of T cells.

In line B homozygously transgenic mice, while B cells were depleted, thymus development and T-cell maturation were normal. We therefore carried out preliminary c-Myb expression analysis in lymphoid lineages and found that c-Myb mRNA expression was decreased in the B-cell fraction but increased in the T-cell fraction. Thus, the c-Myb expression in each hematological lineage correlated with the abnormalities in the mutant mice. These results suggest that there may be a T-cell-oriented regulatory domain in the *c-myb* gene, which is independent of the one currently disrupted with the transgene integration. Since normal T-cell maturation was not observed in the other *c-myb* knockdown mutant mouse cases, our mutant mouse line provides a unique mechanism for studying c-Myb reduction and phenotype in the lymphoid lineages as well.

Although line B homozygously transgenic mice suffer from anemia, thrombocytosis, and splenomegaly, these mice lived approximately 2 years in our specific-pathogen-free animal facility, showing a life span similar to that of wild-type mice (data not shown). In this long-term observation study, aged line B

FIG. 9. Hematopoietic abnormalities in homozygously transgenic mice. Broad and narrow lines indicate pathways that are enhanced or repressed, respectively. HSC, hematopoietic stem cell; CMP, common myeloid progenitor.

homozygously transgenic mice suffered from splenic fibrosis, but we did not observe significant occurrence of specific malignancies, such as lymphoma (data not shown). These data indicate that the transgene integration gives rise to perturbation in *c-myb* gene expression in line B mice, but the perturbation was not so serious as to affect the life span of the mice.

In the classical model of hematopoiesis, erythroid and megakaryocyte lineages arise from MEPs derived from common myeloid progenitors (1, 10). Although erythroid and megakaryocyte lineages are believed to share the common progenitors (1, 10), the mechanism regulating the final separation of these two lineages is not well defined. During hematopoietic differentiation from lineage commitment to terminal maturation, a multiple complex of transcription factors coordinately regulates the chromatin organization of lineage-specific genes and primes them for expression (29). Transcription factors whose targeted disruptions lead primarily to defects in the megakaryocyte and erythroid lineages, such as GATA-1 (26, 30) and NF-E2 p45 (13), are highly expressed in MEPs (1), and these transcription factors positively promote both erythroid and megakaryocytic lineages. In contrast, *c-Myb* has opposite effects on erythroid and megakaryocytic lineages; the present study clearly indicates that *c-Myb* is required to promote erythroid lineage proliferation and differentiation while suppressing megakaryopoiesis. This contrasting effect is a unique function of *c-Myb* in the development of erythroid and megakaryocyte lineages.

The erythroid/megakaryocytic abnormalities were reconstituted in vivo in sublethally irradiated mice through the transplantation of mutant mouse bone marrow cells, demonstrating that the hematological abnormalities of the mutant mice are cell autonomous. The coculture experiment of MEPs with OP9 stromal cells successfully reproduced the blockage of erythroid differentiation and promotion of megakaryocyte differentiation in vitro, further supporting this notion. A twofold reduction in the abundance of MEPs seen in our mutant mice was also found in mice with an ENU-induced *c-Myb* knockdown mutation (*c-Myb*^{M303V/M303V}) (28). Importantly, while levels of hematopoietic stem cells were increased 5- to 10-fold in *c-Myb*^{M303V/M303V} mice (28), those in our *c-Myb* mutant mice were unchanged or slightly decreased compared to those in wild-type mice (data not shown). These results support our contention that *c-Myb* contributes to the bifurcation of erythroid and megakaryocytic lineages during the stage of MEP. We surmise that further analysis of the *c-Myb* function in MEPs will clarify molecular mechanisms of erythroid-megakaryocyte divergence through MEPs.

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