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Overexpression of *HOXB3* in Hematopoietic Cells Causes Defective Lymphoid Development and Progressive Myeloproliferation

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Summary

HOXB3 **mRNA levels are high in the earliest CD34$^+$ lineage$^-$ bone marrow cells and low to undetectable in later CD34$^+$/CD34$^-$ cells. To gain some insight into the role this gene may play in hematopoiesis, *HOXB3* was overexpressed in murine bone marrow cells using retroviral gene transfer. Thymi of *HOXB3* marrow recipients were reduced in size compared with control transplant recipients, with a 24-fold decrease in the absolute number of CD4$^+$CD8$^+$ cells and a 3-fold increase in the number of CD4$^-$CD8$^-$ thymocytes that contained a high proportion of γδ TCR$^+$ cells. B cell differentiation was also perturbed in these mice, as indicated by the virtual absence of transduced IL-7-responsive pre-B clonogenic progenitors. Recipients of *HOXB3*-transduced cells also had elevated numbers of mature granulocyte macrophage colony-forming cells in their bone marrow and spleen. Together these results suggest roles for *HOXB3* in proliferation and differentiation processes of both early myeloid and lymphoid developmental pathways.**

Introduction

Every day billions of mature specialized cells found in the peripheral blood of a normal human adult need to be replaced (Abkowitz et al., 1995). The burden of the life-long production of these mature elements is carried out by a small number of primitive bone marrow cells constituting less than 0.01% of the total marrow (Harrison et al., 1993; Szilvassy et al., 1990). These primitive cells have the key properties of being able to undergo self-renewal, i.e., divide and give rise to progeny cells with essentially the same biological properties, or they can differentiate and give rise to "committed progenitors," which progressively acquire the specialized characteristics of one of the ten lineages that constitute human hematopoiesis (Jordan and Lemischka, 1990; Keller and Snodgrass, 1990).

While much progress has been made in identifying cytokines that can regulate the cycling status of primitive hematopoietic cells, little is known about the genetic mechanisms directing self-renewal and differentiation outcomes of these early cells. Accumulating evidence, however, points to transcription factors such as *AML-1* (Okuda et al., 1996), *Ikaros* (Georgopoulos et al., 1994), *SCL/Tal-1* (Shivdasani et al., 1995), *Rbtn-2* (Warren et al., 1994), *PU-1* (Scott et al., 1994), *GATA-2* (Tsai et al., 1994), and *HOX* homeobox genes (Sauvageau et al., 1995) as important regulators of these processes.

The *HOX/HOM-C* family of homeobox genes was initially described in Drosophila as master regulators of embryonic cell proliferation and differentiation. In mammals, at least 39 different *HOX* genes are grouped on four separate chromosomes (i.e., four clusters A to D). As each *HOX* cluster is believed to have originated by duplication, it is possible to align individual members by homology. Genes belonging to the same homology group are named paralogs (n = 13), and they share a similar pattern of expression in the developing embryo. More precisely, paralogs found in the 3′ region of each of the *HOX* clusters, such as *HOXA1*, *B1*, and *D1* (i.e., first paralogs) are expressed in the anterior structures of the embryo, whereas paralogs located in the 5′ region (i.e., *HOXA13*, *B13*, *C13*, and *D13*) are expressed in more posterior structures.

HOX genes are not only expressed in embryonic tissues. Their expression is also well documented in normal and neoplastic adult cells (Barba et al., 1993; Cillo et al., 1992; Friedmann et al., 1994; Manohar et al., 1993, 1996; Peverali et al., 1990), including cells of the hematopoietic system (reviewed by Lawrence and Largman, 1992; Lawrence et al., 1996). By using a semiquantitative reverse transcriptase–polymerase chain reaction (RT–PCR) technique, we have recently shown that at least 22 of the 39 known *HOX* genes are expressed at various levels in five different CD34$^+$ human bone marrow cell subpopulations (Sauvageau et al., 1994), thus suggesting that *HOX* genes might play critical roles in regulating the proliferation or differentiation (or both) of the earliest hematopoietic cells. In support of this are our recent findings that overexpression of *HOXB4* in murine bone marrow cells markedly increased the regenerative potential of long-term repopulating cells and caused an expansion in clonogenic progenitor cell numbers, but did not alter their ability to differentiate normally into mature myeloid, erythroid, and lymphoid cells or cause abnormal end cell output (Sauvageau et al., 1995).

Although all *HOX* genes analyzed to date were predominantly expressed in CD34$^+$ bone marrow cells, the expression of *HOXB3* was restricted to phenotypically primitive hematopoietic cells (i.e., CD34$^+$ and lineage marker negative) representing less than 1% of all nucleated cells found in the bone marrow (Giampaolo et al., 1994; Sauvageau et al., 1994).

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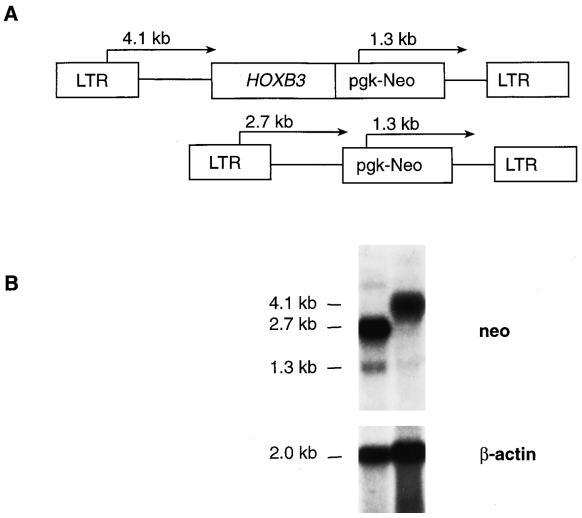

Figure 1. Structure and Expression of the *HOXB3* and *neo* Control Retroviruses Used in This Study

(A) Diagrammatic representation of the integrated *HOXB3* (upper) and *neo* (lower) proviruses.

(B) Northern blot analysis of 5 μg of total RNA extracted from splenocytes of mice 18 weeks after transplantation with *neo*- (left lane) or *HOXB3*-transduced (right lane) bone marrow cells. The membrane was sequentially hybridized to a probe specific for *neo* and β-actin and exposed for 48 hr. Two signals were obtained with the *neo* probe. The upper band represents long terminal repeat (LTR)-derived message of 2.7 and 4.1 kb for the *neo* and *HOXB3* provirus, respectively, and the lower band common to both constructs represents a 1.3 kb signal originating from the internal pgk promoter.

To explore further the function of *HOXB3* in hematopoiesis, we analyzed the regeneration of various hematopoietic compartments in mice transplanted with bone marrow cells manipulated to overexpress the human *HOXB3* cDNA. In contrast with findings for *HOXB4*, overexpression of *HOXB3* impaired both B and T lymphoid differentiation and enhanced proliferation of myeloid restricted cells.

Results

cDNA Cloning of a *HOXB3* Transcript from Hematopoietic Cells and Generation of a *HOXB3* Retroviral Vector

As is observed with many homeobox genes (Magli et al., 1991), *HOXB3* has several transcripts, some of which may be tissue specific (Sham et al., 1992). In an effort to identify a *HOXB3* transcript that is normally expressed in primitive blood cells, a 3.2 kb human *HOXB3* cDNA was isolated from a $CD34^+$ bone marrow expression library. The predicted coding region of this *HOXB3* cDNA (GenBank accession number U59298) shares >97% identity at the protein level over the coding region to the mouse *Hoxb-3* previously isolated from embryonic tissue (Sham et al., 1992). The *HOXB3* coding sequence was subcloned into the MSCV 2.1 retroviral vector 5′ to a phosphoglycerate kinase promoter (pgk)-driven *neo* gene such that *HOXB3* expression was under the control of the regulatory elements in the viral long terminal repeat (LTR) (Figure 1A). Integrity of this virus was shown both by the expression of the expected 4.1 kb LTR-derived full-length *HOXB3* message in reconstituted hematopoietic tissue of mice transplanted with *HOXB3*-transduced bone marrow (Figure 1B) and by the presence of an unrearranged 4.1 kb proviral fragment detected by Southern blot analysis of genomic DNA isolated from bone marrow and thymus of such mice (see Figure 3B).

Hematopoietic Reconstitution of Recipients of *HOXB3*-Transduced Bone Marrow Cells

High titer, helper-free viral producer cell lines for either the *HOXB3* or a *neo* control retrovirus were used to infect murine bone marrow cells as summarized in Figure 2. Immediately following retroviral infection, a proportion of the bone marrow cells was plated under G418 selection in methylcellulose cultures supplemented with hematopoietic growth factors that support proliferation and differentiation of myeloid and erythroid progenitors (Figure 2A). The gene transfer efficiencies as assessed by recovery of G418-resistant clonogenic progenitors were from 15% to 50% in the three experiments. Analyses of the cellular content of *HOXB3*-transduced colonies (i.e., G418 resistant) showed all types of myeloid or erythroid colonies (or both), suggesting that *HOXB3* overexpression was permissive for in vitro differentiation of already committed myeloid progenitor cells (data not shown).

To explore in more detail the possibility that *HOXB3* might affect hematopoiesis, we examined reconstitution of lymphoid and myeloid populations in lethally irradiated mice transplanted with *HOXB3*-transduced bone marrow cells. In three separate transplantation experiments, recipient mice ($Ly5.2^+$) were injected with a life-sparing dose (200,000 cells) of congenic $Ly5.1^+$ bone marrow cells exposed to *HOXB3* or *neo* retroviruses as detailed in Figure 2. Based on our previous work using competitive repopulation assays, it was estimated that each recipient received between 40 and 100 long-term repopulating cells (Sauvageau et al., 1995), of which

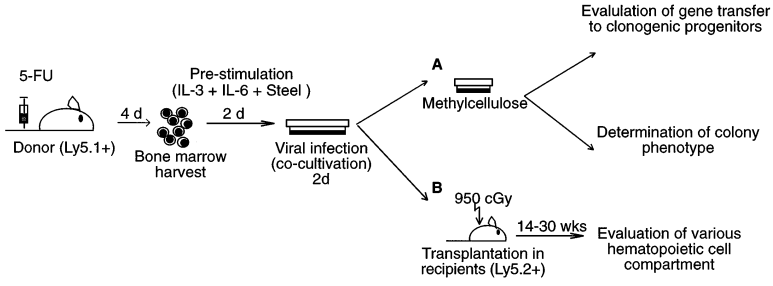

Figure 2. Overview of Experimental Design

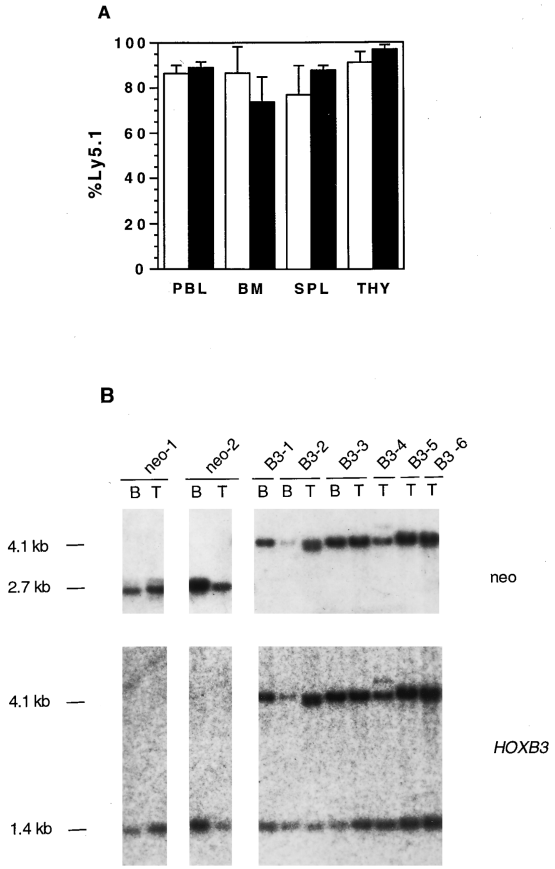

Figure 3. Donor-Derived Hematopoietic Reconstitution of *HOXB3* and *neo* Mice

(A) FACS analysis of proportion of donor-derived (Ly5.1$^+$) cells in various hematopoietic tissues of *HOXB3* (open bars) and *neo* (closed bars) mice. Results shown are mean ± SD for n ≥ 6 mice.

(B) Southern blot analysis of DNA isolated from bone marrow and thymi of *HOXB3* (B3-1 to B3-6) or *neo* (neo-1 and neo-2) mice, sacrificed 18–25 weeks after transplantation. DNA was digested with KpnI, which cuts once in each LTR of the integrated *neo* or *HOXB3* proviruses to release proviral fragments of 2.7 kb (*neo*) or 4.1 kb (*HOXB3*). Donor-derived reconstitution (i.e., percent Ly5.1) of the mice shown in this figure varied between 86%–98% for *HOXB3* mice and 78%–94% for *neo* controls. The membrane was sequentially hybridized to probes specific for *neo* (specific activity ~30 × 10^6 dpm; 70 hr exposure time) and *HOXB3* (specific activity not measured; 24 hr exposure time). The endogenous *HOXB3* signal is detected at 1.4 kb. PBL, peripheral blood leukocytes; BM or B, bone marrow; SPL, spleen; THY or T, thymus.

approximately 20%–50% were estimated to be transduced given the gene transfer efficiency to clonogenic progenitor cells in the transplant inoculum (see above).

Donor-derived (i.e., Ly5.1$^+$) reconstitution of hematopoietic tissues was assessed 14–30 weeks after transplantation by fluorescence-activated cell sorting (FACS) analysis (Figure 3A). Hematopoietic regeneration in either *HOXB3* or *neo* mice was essentially completely donor derived, since ≥80% of splenic, thymic, and peripheral blood leukocytes were of donor origin (Ly5.1$^+$; Figure 3A). The slightly lower levels that were detected in bone marrow of *neo* mice presumably reflects the presence of cells of the erythroid lineage, which are negative for the expression of Ly5.1, rather than incomplete chimerism (Figure 3A). Furthermore, Southern blot analysis of DNA isolated from the bone marrow and thymus of these mice indicated a significant contribution by transduced cells to this regeneration, as evidenced by detection of the expected *neo* or *HOXB3* proviral signals at levels comparable with that of the endogenous *HOXB3* gene (Figure 3B).

Recipients of *HOXB3*-Transduced Cells Show Increased Granulopoiesis in Their Bone Marrow and Spleen

When analyzed 18–27 weeks after transplantation, age- and sex-matched *neo* or *HOXB3* mice had similar peripheral blood values (hemoglobin and white blood cells; data not shown) and bone marrow cellularity (Figure 4A). *HOXB3* mice, however, showed a significant reduction in thymic cellularity and a gradual increase in spleen size over time (Figures 4A and 4B). FACS analysis (Figure 5) and cytological examination (data not shown) of spleen and bone marrow cells documented a consistent increase in mature granulocytes in these tissues. Increased numbers of peripheral blood granulocytes were also observed in some *HOXB3* mice (Figure 5D, e.g., *HOXB3-1* versus *HOXB3-2*). The absolute number of in vitro myeloid colony-forming cells in bone marrow and spleen of *HOXB3* mice was increased 3-fold (Figure 4C), consistent with the increase in bone marrow and spleen mature granulocytes in these two tissues. Significantly, between 50%–100% of these progenitors were G418 resistant, indicating that retrovirally transduced cells contributed substantially to these cell pools (Figure 4C).

Of the G418-resistant myeloid progenitors present in the bone marrow of these mice, between 10% and 100% generated small colonies (<100–200 cells) containing a mixture of greater than 95% mature and immature granulocytic cells, a colony type rarely detected in cultures initiated with cells from *neo* mice. The cellular content of larger G418-resistant colonies generated from bone marrow of *HOXB3* mice was similarly evaluated. Although all expected colony types were found (i.e., CFU-GEMM, CFU-GM, CFU-M, CFU-G, CFU-basophils-eosinophil, and BFU-E), the majority of these colonies were either restricted to the granulocytic or macrophage lineage (or both) (46 of 54 large colonies examined from three mice). Thus, only 1 colony out of 54 was of mixed lineage (CFU-GEMM), in contrast with 8 of the 21 such colonies from *neo* mice.

Together, these data suggest that *HOXB3* caused the expansion of more mature unilineage or bilineage myeloid progenitors, but had minimal effect on the proliferation of earlier, multipotent cell types. This possibility was further assessed by limit dilution analysis to quantitate the number of long-term repopulating cells present in *HOXB3* mice using the competitive repopulating unit (CRU) assay (Szilvassy et al., 1990). By 18 weeks post-transplantation, CRU frequencies in recipients of *neo* or *HOXB3* transduced marrow were similar and, as expected for experiments using transduced marrow, had only regenerated to approximately 5% of normal levels (frequencies of 0.3–1.0 or 0.3–2.0 per 10^5 bone marrow

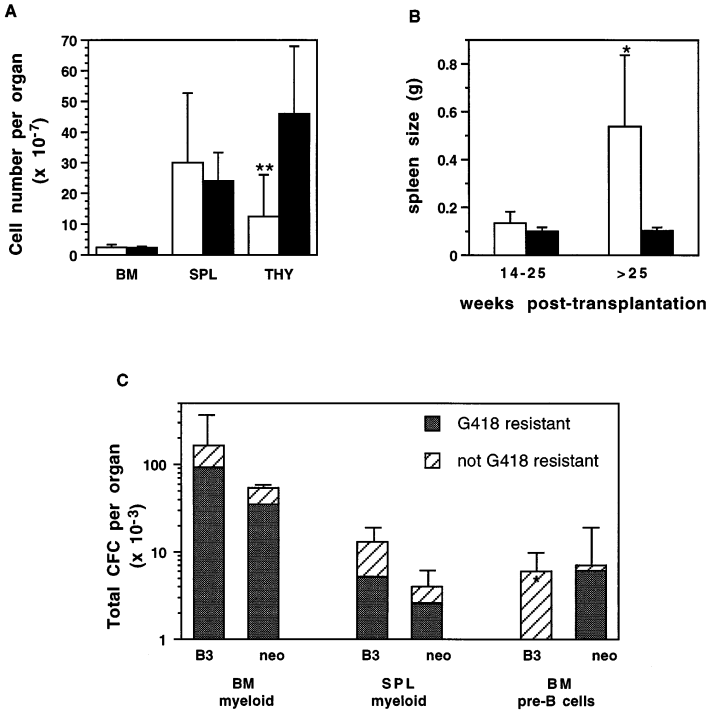

Figure 4. Hematopoietic Parameters of *neo* and *HOXB3* Mice

(A) Bone marrow, spleen, and thymic cellularity of *HOXB3* (open bars; n = 13) and *neo* mice (closed bars; n = 8) 18–27 weeks after transplantation.

(B) Increments in spleen weight in *HOXB3* mice (closed bars) with time.

(C) Myeloid and pre-B lymphoid clonogenic progenitor content in bone marrow and spleen of *neo* (n = 5) and *HOXB3* (n = 8) mice. All results shown in (A)–(C) represent mean values ± SD. Double asterisk, significantly decreased compared with *neo* control, $p < 0.005$; single asterisk, significantly increased compared with *neo* control, $p < 0.05$ (two-tailed Student's t test with unequal variance). BM, bone marrow; SPL, spleen; THY, thymus.

cells for *neo* or *HOXB3* mice, respectively). Thus, *HOXB3* overexpression, in contrast with that of *HOXB4* (Sauvageau et al., 1995), does not appear to enhance the regenerative potential of primitive long-term repopulating cells.

Recipients of *HOXB3*-Transduced Cells Have Defective T Cell Development

HOXB3 mice had significantly smaller thymi than age- and sex-matched *neo* control recipients (Figure 4A), with corresponding abnormalities in the numbers and proportions of various subpopulations detected by flow cytometry (Figure 6). All recipients of *HOXB3*-infected cells had abnormal CD4/CD8 profiles as evidenced by a 24-fold decrease in the proportion of $CD4^+CD8^+$ (double positive) cells and a 6-fold decrease in a single positive cell population ($CD4^-CD8^+$; Figure 6). In contrast, the proportions of $CD4^-CD8^-$ (double negative) and $CD4^{lo}CD8^-$ thymocytes were increased 3- and 2-fold, respectively (Figure 6; data not shown). Although there was some heterogeneity among mice analyzed, in most mice (11 of 14) the majority of thymocytes were either $CD4^-CD8^-$ or $CD4^{lo}CD8^-$. These cells expressed little or no B220 or Mac-1 (in general <3% each) and, morphologically, were free of contaminating myeloid cells as assessed by cytospin preparations (data not shown). The dominant thymic $CD4^-CD8^-$ and $CD4^{lo}CD8^-$ populations in these mice were heat-stable antigen–positive (HSA^{hi}) and expressed variable levels of CD25 (interleukin-2 receptor α [IL-2Rα]; Figure 7; data not shown). Curiously, the majority of these cells were $\gamma\delta$ TCR^+, whereas few were of the $\alpha\beta$ lineage (Figure 7). There was a tendency for mice analyzed at later times (i.e., >25 weeks posttransplantation) to have a more severe thymic phenotype, with one mouse analyzed at 14 weeks posttransplantation having over 90% of $CD4^-CD8^-$ or $CD4^{lo}CD8^-$ thymocytes (data not shown). *HOXB3* overexpression was thus associated with an apparent block in thymocyte maturation from the double negative to the double positive stages coupled with expansion of the $CD4^{lo}CD8^-\gamma\delta$ TCR^+ and $CD4^-CD8^-\gamma\delta$ TCR^+ thymocytes (Figure 7).

Although the intensity of the proviral signal seen on Southern blot analysis of DNA extracted from whole thymi of *HOXB3* mice was consistent with high level reconstitution by transduced cells (Figure 3B; all thymi >80% $CD4^{lo/-}CD8^-$ T cells), RT–PCR analysis was also performed to assess proviral expression in various thymic subpopulations of *neo* and *HOXB3* mice. In contrast with *neo* control mice, in which strong to moderate retroviral-derived signals were detected in all subpopulations, for *HOXB3* mice retroviral-derived *HOXB3* mRNA were only detected at high levels in double negative thymocytes, at low to moderate levels in $CD4^+CD8^-$ (mostly $CD4^{lo}CD8^+$), and were not detectable in $CD8^+$ $CD4^+$ and $CD8^+CD4^-$ cells (Figure 8). These results thus suggest that *HOXB3* overexpression is not compatible with progression from double negative to the double positive stage of thymic development and further indicate that the few double positive thymocytes and their progeny found in some *HOXB3* mice were derived from either untransduced cells or *HOXB3*-transduced cells in which proviral expression was down-regulated.

To assess whether the perturbations in thymic development in *HOXB3* mice extended into the periphery, T cells in peripheral blood, spleen, and lymph nodes were analyzed by flow cytometry (Figure 5). In all *HOXB3* mice analyzed (n = 6), there were normal distributions of $CD4^+$ and $CD8^+$ single positive cells and essentially no $CD4^+CD8^+$ T cells. Although these single positive cells

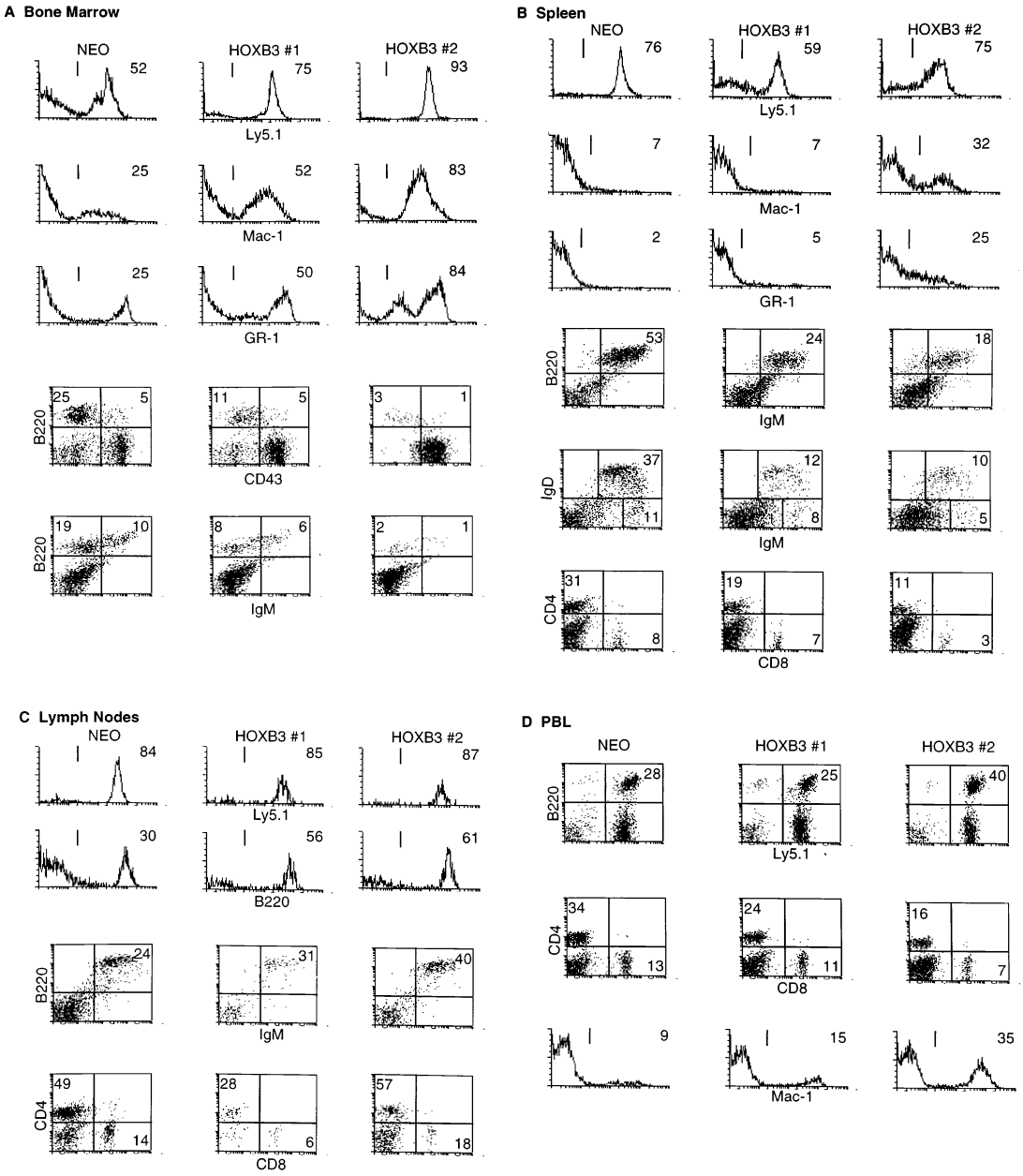

Figure 5. Flow Cytometry Analysis of Cells Isolated from Hematopoietic Organs of *HOXB3* or *neo* Control Mice

Bone marrow (A), spleen (B), lymph nodes (C), and peripheral blood leukocytes (D) were isolated from mice transplanted 18 weeks earlier with *HOXB3*- or *neo*-transduced cells. *HOXB3-1* and *HOXB3-2* are two representative mice with moderate and severe phenotypes, respectively. Numbers in boxes represent percentages of live cells found in this region. Lower numbers of cells were collected for analysis of lymph node populations of the *HOXB3-1* mouse (C).

were donor derived, it was not ascertained whether they arose from transduced or nontransduced cells.

Altered B Cell Development by Overexpression of *HOXB3*

To detect possible effects of *HOXB3* overexpression on B cell development, bone marrow IL-7-responsive B lymphoid clonogenic progenitor numbers were measured. While the absolute numbers of these progenitors in *HOXB3* mice were within the normal range (~7000 per femur; Figure 4C), only a small proportion were G418 resistant. This contrasts with both the high proportion of transduced myeloid progenitors in these same *HOXB3* mice and that of pre-B progenitors in *neo* control mice (Figure 4C). Furthermore, the rare G418-resistant colonies derived from the *HOXB3* mice were only approximately one tenth of normal size. Together, these results suggest that *HOXB3* overexpression inhibits early B cell development.

To characterize further possible B cell alterations in *HOXB3* mice, phenotypically distinct B cell populations were evaluated by flow cytometry using a combination

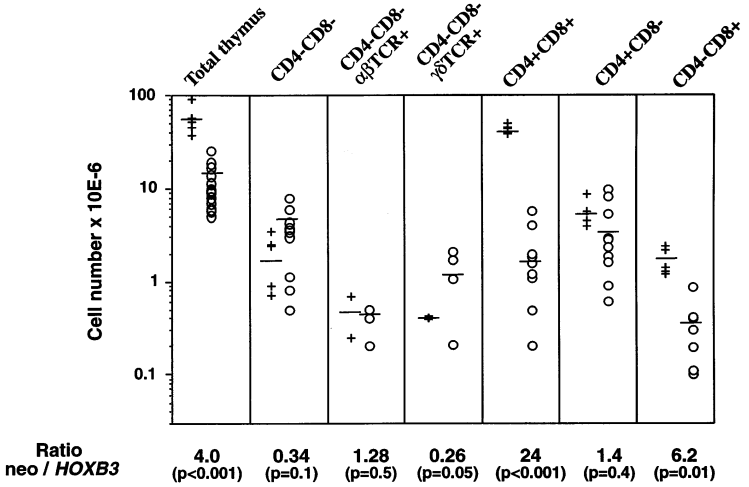

Figure 6. Cellularity of Thymi and Their Subpopulations in Individual *neo* and *HOXB3* Mice Sacrificed 18–27 Weeks after Transplantation

neo and *HOXB3* mice are indicated by crosses and circles, respectively. Mean values are shown as horizontal lines. Ratios of mean values for each thymic population in *neo* and *HOXB3* mice are indicated below with p values (two-tailed Student's t test with unequal variance). One *HOXB3* recipient was omitted from this figure because it had excessive values including 5.5×10^7 $\gamma\delta$ T cells (see Discussion). Three mice analyzed at 25–27 weeks posttransplantation had >80% $\gamma\delta$ T cells in their thymus, but since their exact thymic cellularity is not available these mice were not included in this figure. Contaminant Mac-1$^+$ or B220$^+$ cells were below 5% in all thymic samples used for these analyses.

of monoclonal antibodies (anti-CD45R, anti-CD43, anti–immunoglobulin M [IgM], and anti-IgD) (Figure 5). Normal proportions of the B cell subpopulations were seen in the peripheral blood and lymph nodes of *HOXB3* mice (Figure 5; profiles from two of five *HOXB3* recipients analyzed 18 weeks after transplantation); their proportions were somewhat reduced in the spleen, but considering the increase in splenic cellularity, the absolute number of B cells in this organ was within the normal range. Abnormalities were apparent, however, in the bone marrow, where total B cells were consistently reduced (Figure 5A) with marked variations (moderate phenotype, *HOXB3-1*, and severe phenotype, *HOXB3-2*). *HOXB3* mice manifested a 2- to 10-fold reduction in total bone marrow B cells (B220$^+$), with all B cell populations affected in those mice showing a more severe phenotype (*HOXB3-2*, Figure 5).

Discussion

Overexpression of *HOXB3* had multiple effects on hematopoiesis as assessed in a bone marrow transplantation model. Specifically, overexpression of *HOXB3* lead to an almost complete block in the thymic production of CD4$^+$CD8$^+$ T lymphocytes, accompanied by an expansion of $\gamma\delta$ TCR$^+$ thymocytes, impaired B lymphoid development, and enhanced myelopoiesis leading to a myeloproliferative disorder.

HOXB3-induced alterations were particularly prominent in thymocyte development. The generation of mature $\alpha\beta$ T cells was blocked by overexpression of *HOXB3*, as evidenced by reductions in double positive and single positive thymocytes. Furthermore, the few double positive thymocytes and their progeny found in thymi of *HOXB3* mice were negative for retroviral-derived *HOXB3* expression, indicating either selection for transduced cells in which there was spontaneous down-regulation of the LTR-driven expression or contributions from untransduced cells. In contrast with the block in $\alpha\beta$ T cell maturation, the $\gamma\delta^+$ thymocytes in *HOXB3* mice were expanded (on average ~4-fold). This reached the extreme in one *HOXB3* mouse that had a large thymus containing >97% HSA$^+$ $\gamma\delta$ T cells. Since we did not rule out the possibility that these cells had acquired pre-neoplastic properties as a result of additional somatic mutations, this mouse was not included in

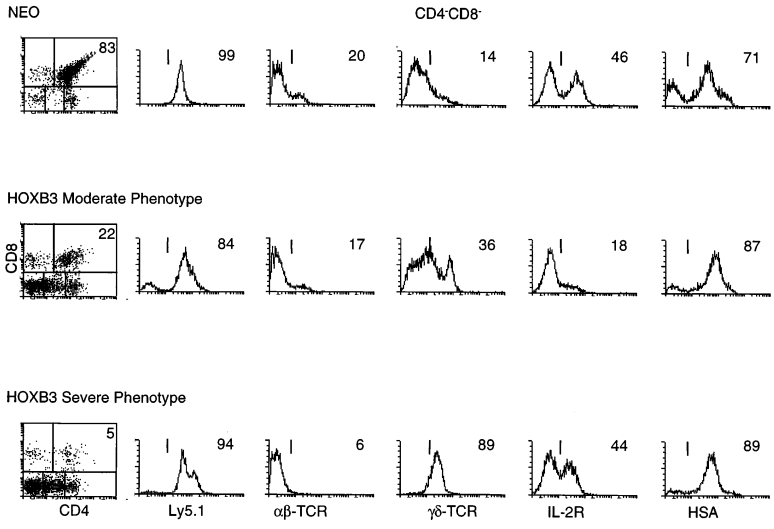

Figure 7. Flow Cytometry Analysis of Thymi Isolated from Recipients of *neo*- or *HOXB3*-Transduced Cells

One representative *neo* mouse and two representative *HOXB3* mice with moderate and severe thymic phenotype are shown. CD4 and CD8 profiles of total thymocytes are shown in the scatterplot (left). Each of the histograms (right) illustrates a FACS profile of 5000 CD4$^-$CD8$^-$ (double negative) cells analyzed for their expression of $\alpha\beta$ and $\gamma\delta$ TCR, IL-2 receptor (CD25), and HSA. Results for all *neo* and *HOXB3* mice analyzed (including these) are presented in Figure 6.

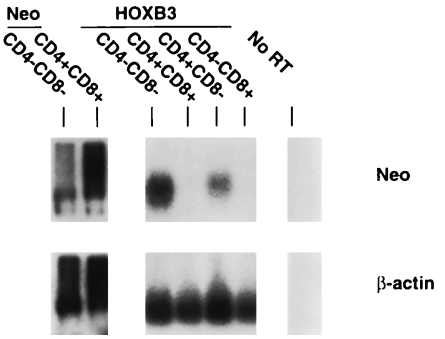

Figure 8. Expression by RT–PCR of Retroviral-Derived Genes in Purified Subpopulations of Thymocytes

Total RNA was isolated from FACS-purified thymic subpopulations (10,000 cells per sample) of *HOXB3* or *neo* mice (n = 4 separate mice; only one representative of each is shown). The blot was sequentially hybridized with a probe specific for β-actin (lower panel) and *neo* (upper panel). Hybridization to a probe specific to *HOXB3* gave results superimposable to those obtained with the *neo* probe (data not shown). Note that double positive ($CD4^+CD8^+$) and single positive $CD4^-CD8^+$ cells from *HOXB3* recipients do not express the retroviral-derived transcripts. Exposure times are 30 min for *neo* and 1 hr for β-actin.

Figure 6. The expanded γδ TCR^+ thymocytes in *HOXB3* mice were distributed equally between $CD4^-CD8^-$ and $CD4^{lo}CD8^-$ populations and consisted predominantly of cells expressing high levels of HSA, suggesting that they represent an immature subset of γδ T cells (Suda and Zlotnik, 1993).

Together these results indicate that overexpression of *HOXB3* has different effects on precursors of the αβ TCR lineage than on those of the γδ TCR lineage, blocking the maturation of the former while inducing expansion of the latter. Whether these different effects reflect normal *HOXB3* expression patterns in these cells is currently unknown. While we have shown using an RT–PCR approach that the murine *HOXB3* is normally expressed at very low levels in FACS-purified double negative, double positive, and mature single positive thymocytes (G. S., unpublished data), its expression pattern in subsets of double negative cells is currently unknown. Further characterization of *HOXB3* expression in these subsets will be of interest since this population contains both mature γδ T cells and precursors of both γδ and αβ lineages (Dudley et al., 1995).

It is curious that the defect in thymocyte development is not compensated by the nontransduced donor cells. One possible explanation for this is that *HOXB3*-transduced progenitors have a superior thymus repopulating ability and therefore occupy the majority of the limited number of thymic niches (Spangrude and Scollay, 1990). Preferential niche occupation could also occur as a result of other mechanisms, such as self-renewal advantages of *HOXB3*-transduced cells or active suppression of normal thymocyte development by *HOXB3*-transduced cells. This thymic defect does not appear to be due to replacement by myeloid cells, since no myeloid infiltration is seen cytologically and fewer than 5% of Mac-1^+ cells are seen on FACS analysis of the thymi isolated from *HOXB3* mice.

B lymphoid development was also impaired by *HOXB3* overexpression. This was apparent from the virtual absence of transduced pre-B colony-forming cells in bone marrow of *HOXB3* mice and small colony size from the few such progenitors detected. However, in contrast with the findings in the thymus, the absolute numbers of pre-B colony-forming cells in *HOXB3* mice were within normal range, presumably reflecting competitive repopulation from nontransduced cells. Some overall reduction in B cell content of the bone marrow was seen, however, likely reflecting infiltration and displacement by the expanding granulocytic populations (Figure 5).

The defects in B and T cell development described in this paper have not been reported with any other molecules, including overexpression of another homeobox gene *Hlx*, which caused a release of immature $CD4^+CD8^+$ T cells in the periphery (Allen et al., 1995).

HOXB3 mice also developed a myeloproliferative disorder as evidenced by splenomegaly, a marked accumulation of myeloid clonogenic progenitors with granulocyte or macrophage differentiation potential (or both), and by granulocytic infiltration of bone marrow and other hematopoietic organs. For all *HOXB3* mice analyzed (n = 4) this myeloproliferative disorder was readily transplantable to secondary recipients (data not shown). Most *HOXB3* mice were sacrificed before 30 weeks and, in this time frame, progression of the myeloproliferative disorder to frank leukemia was not observed except for one mouse. Bone marrow clonogenic progenitor cells from this mouse were all G418 resistant and showed growth factor independence in the presence of fetal calf serum. Studies to assess characterization of this leukemia and further leukemic transformations that might develop in *HOXB3* mice are currently underway. Although incomplete, these data nonetheless support the accumulating evidence of *HOX* gene involvement in both murine and human leukemic transformation (Borrow et al., 1996; Nakamura et al., 1996a, 1996b; Perkins et al., 1990).

The results presented in this report stand in contrast with our previous findings that overexpression of *HOXB4* did not detectably perturb myeloid, B cell, or T cell differentiation, but did induce expansion of myeloid and lymphoid progenitor cells and enhanced up to 47-fold the regeneration of the most primitive hematopoietic repopulating cells (Sauvageau et al., 1995). These results also differ from our most recent findings that overexpression of *HOXA10* in murine bone marrow cells leads to enhanced formation of megakaryotic progenitors in vivo and in vitro, diminished numbers of macrophage and B lymphoid progenitors, and generation of myeloid leukemias in a significant proportion of mice 5–8 months after transplantation (Thorsteinsdottir et al., 1996). These findings demonstrate that very distinctive effects result from the enforced expression of different *HOX* genes in marrow cells (summarized in Figure 9). These observations, combined with our previous studies showing that *HOX* mRNA levels differ significantly among various purified subpopulations of bone marrow cells, suggest that, as in embryonic development, *HOX* genes are important regulators of early hematopoietic developmental processes.

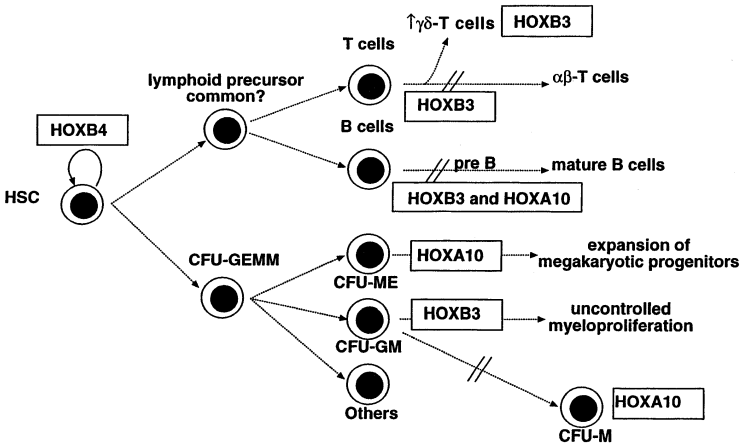

Figure 9. Schematic Representation of the Effects Observed on Various Hematopoietic Lineages with Overexpression of *HOXB3*, *HOXB4*, or *HOXA10*

While *HOXB4* selectively enhanced the regenerative potential of primitive hematopoietic cells without altering their normal differentiation, *HOXB3* selectively blocked differentiation of αβ T cells and expanded cells of the γδ lineage. Overexpression of *HOXB3* also lead to myeloproliferation (granulocytic) and precluded the generation of IL-7-responsive pre-B clonogenic progenitor cells. Overexpression of *HOXA10* caused profound expansion (37-fold) of megakaryocytic progenitors and impeded the generation of unilineage macrophage or pre-B progenitor cells.

Experimental Procedures

Mice

Recipients of bone marrow cells were 7- to 12-week-old male or female (C57Bl/6J × C3H/HeJ)F1 ((B6C3)F1) and donors were (C57Bl/6Ly-Pep3b × C3H/HeJ)F1 ((PepC3)F1). Hybrid F1 mice were bred from parental strain breeders originally obtained from the Jackson Laboratories (Bar Harbor, ME) and maintained in microisolator cages and provided with sterilized food and acidified water in the animal facility of the British Columbia Cancer Research Center. For bone marrow transplantation procedures, lethally irradiated (950 cGy, 110 cGy/min, ^{137}Cs γ-rays) (B6C3)F1 mice were injected intravenously with 2×10^5 bone marrow cells from (PepC3)F1 mice immediately after their cocultivation with *HOXB3* or *neo* viral producer cells as described previously (Sauvageau et al., 1995). This number of cells was sufficient to spare 100% of the recipients, while death normally occurred at approximately 14 days in the absence of donor cells.

Retroviral Generation and Infection of Primary Bone Marrow Cells

The *HOXB3* cDNA region encompassing the complete coding sequence was isolated as a KpnI fragment and subcloned upstream of the pgk–*neo* cassette at the HpaI site of the MSCV 2.1 retroviral vector (provided by Dr. Robert Hawley, Sunnybrook Research Institute, Toronto, Canada) using standard procedures (Davis et al., 1994). High titer helper-free recombinant retroviruses were generated and tested as previously described (Sauvageau et al., 1995). The generation of the *neo* retrovirus has already been described elsewhere (Sauvageau et al., 1995). Bone marrow cells obtained from (PepC3)F1 (Ly5.1) mice injected intravenously 4 days previously with 150 mg per kilogram of body weight of 5-fluorouracil were prestimulated and cocultivated on irradiated viral producer cells in the presence of IL-3, steel, IL-6, and polybrene as described previously (Sauvageau et al., 1995). Loosely adherent and nonadherent cells were recovered from the cocultures and injected into a lateral tail vein of recipient mice or directly cultured in vitro as described below.

In Vitro Clonogenic Progenitor and CRU Assays

For myeloid clonogenic progenitor assays, cells were plated on 35 mm petri dishes (Greiner, Federal Republic of Germany) in a 1.1 ml culture mixture containing 0.8% methylcellulose in α medium supplemented with 30% fetal calf serum (GIBCO BRL), 1% bovine serum albumin, 10^{-4} M β-mercaptoethanol (β-ME), 3 U/ml human urinary erythropoietin, and 2% spleen cell–conditioned medium in the presence or absence of 1.4 mg/ml G418 as described. Bone marrow cells harvested from the cocultivation with virus producer cells or from reconstituted *neo* or *HOXB3* mice were plated at a concentration of 1×10^3 to 2×10^3 cells per dish or 4×10^4 cells per dish, respectively. Spleen cells from *HOXB3* or *neo* mice were plated at 1×10^6 cells per dish. Colonies were scored on days 10–14 of incubation as derived from CFU-GM, BFU-E, or CFU-GEMM according to standard criteria (Humphries et al., 1981). In two experiments, identification of colony types was confirmed by Wright staining of cytospin preparations of colonies. For pre-B clonogenic progenitor assays, cells were plated in 0.8% methylcellulose in α medium supplemented with 30% FSC, 10^{-4} M β-ME, and 0.2 ng/ml IL-7 (Suda et al., 1989). Pre-B colonies were scored on day 7 of incubation. Evaluation of frequency of cells with long-term repopulating potential was done using the CRU assay (Szilvassy et al., 1990). Technical modification of the procedures that were applied in this study have been reported previously (Sauvageau et al., 1995).

Antibodies, Flow Cytometry, and Cell Sorting

A single cell suspension of bone marrow was prepared by injecting Hanks–HEPES-buffered salt solution containing 2% fetal calf serum and 0.1% sodium azide (HFN) into femurs to flush out cells, followed by gentle disaggregation through a 21-gauge needle. Cells were released from the thymus, spleen, and lymph nodes by disruption through a fine steel mesh. To lyse erythrocytes, cell suspensions were treated with 0.165 M NH_4Cl and washed once with HFN. Cells were stained with primary antibodies in HFN on ice for 30 min, washed twice with HFN, and resuspended in HFN containing 1 μg/ml propidium iodide. Flow cytometric analysis was performed using a FACSort or FACStar flow cytometer equipped with PC LYSISII software. For cell sorting, 10,000 cells were harvested and a proportion was reanalyzed for purity (>95%). Monoclonal antibodies (MAbs) were titered and used as described previously (Hough et al., 1994, 1996). FITC-labeled CD4, CD8, γδ TCR, CD43 (S7), GR-1; PE-labeled IL-2Rα, CD4, CD8, B220; and biotinylated αβ TCR MAbs were purchased from Pharmingen. HSA was detected by a cyanine 5-succinimidylester (Cy5)-labeled M1/69 MAb purified from the TIB125 hybridoma (American Type Culture Collection). The Mac-1 MAb was purified from the M1/70.15.11 hybridoma (American Type Culture Collection) and labeled with FITC. FITC-labeled mouse IgM and PE-labeled mouse IgD were purchased from Southern Biotechnology. PE- and FITC-conjugated streptavidin were purchased from Jackson ImmunoResearch Laboratories.

For mice analyzed at 14 weeks after transplantation (n = 6), staining was performed as described elsewhere (Hugo et al., 1993). In brief, 10^6 cells prepared in PBS, 2% fetal calf serum, 0.1 % NaN_3 (PBSWB) were deposited in microculture wells. After centrifugation, the pellet was resuspended in 10 μl of a blocking cocktail containing 5 μg/ml of human γ-globulins (Sigma) diluted in supernatant from the hybridoma 2.4G2, which produces a MAb against the FcRIIb/III (Unkeless, 1979) and incubated for 5 min at 23°C. A cocktail of 40 μl of PBSWB containing FITC-conjugated anti-TCR (GL3-1.4; Gorski et al., 1993), PE-conjugated anti-CD4 (GK.15; GIBCO BRL), red 613–conjugated anti-CD8 (GIBCO BRL), and biotinylated-anti-TCRα (H57-597; Kubo et al., 1989) MAbs at the appropriate concentrations was added. After a 25 min incubation at 4°C, the cells were washed three times and resuspended in 50 μl of PBSWB with streptavidin-conjugated red 670 (GIBCO BRL). The cells were subsequently incubated for an additional 30 min period, washed, and resuspended in

PBSWB to allow analysis on a Coulter XLTM flow cytometer equipped with a 488 nm laser beam and fluorescence detectors at 525, 575, 620, and 670 nm. For all cytofluorometric analyses done at 14 weeks posttransplantation, a minimum of 150,000 events were acquired.

cDNA Generation, Amplification, and Analysis

Reverse transcription and amplification of total messenger RNA isolated from the purified subpopulation was done exactly as previously reported (Sauvageau et al., 1994). In brief, 10,000 thymocytes purified by cell sorting were pelleted and lysed in 100 μl of 5 M guanidinium isothiocyanate solution. Nucleic acids were then precipitated and subsequent synthesis of the cDNA was done with a 60-mer primer containing a 3′ poly thymidine stretch as described (Brady et al., 1990). A short poly-adenosine tail was added to the 3′ end of the first strand cDNA using terminal deoxynucleotidyl transferase, and the second strand synthesis and subsequent PCR amplification were done by PCR with the same primer (but at a higher concentration) as used during the reverse transcription (Sauvageau et al., 1994). Amplified total cDNA was size fractionated on a 1% agarose gel, transferred to nylon membranes and hybridized as described below.

DNA and RNA Analyses

Southern blot analyses to assess proviral integration were performed using standard techniques (Pawliuk et al., 1994). High molecular weight DNA was digested with KpnI, which cuts in the LTRs to release the proviral genome. Total cellular RNA was isolated, transferred, and hybridized as described (Sauvageau et al., 1995). Probes used were a XhoI–SalI fragment of pMC1neo (Thomas and Capecchi, 1987), a 307 bp cDNA fragment 3′ to *HOXB3* homeobox obtained by ApaI–BamHI restriction digestion, and a 2.0 kb PstI–PstI chicken β-actin fragment. Probes were labeled as described with [α-^{32}P]dCTP and random primers (Sauvageau et al., 1994).

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