

Overexpression of *HOXA10* in Murine Hematopoietic Cells Perturbs both Myeloid and Lymphoid Differentiation and Leads to Acute Myeloid Leukemia

UNNUR THORSTEINSDOTTIR,¹ GUY SAUVAGEAU,^{1†} MARGARET R. HOUGH,¹
WIESLAWA DRAGOWSKA,¹ PETER M. LANSDORP,^{1,2} H. JEFFREY LAWRENCE,³
COREY LARGMAN,³ AND R. KEITH HUMPHRIES^{1,2*}

Terry Fox Laboratory, British Columbia Cancer Agency,¹ and Department of Medicine, University of British Columbia,² Vancouver, British Columbia, Canada V5Z 1L3, and Department of Medicine, Veterans Affairs Medical Center, and University of California, San Francisco, California 94121³

Received 6 June 1996/Returned for modification 29 July 1996/Accepted 30 September 1996

Multiple members of the *A*, *B*, and *C* clusters of *Hox* genes are expressed in hematopoietic cells. Several of these *Hox* genes have been found to display distinctive expression patterns, with genes located at the 3' side of the clusters being expressed at their highest levels in the most primitive subpopulation of human CD34⁺ bone marrow cells and genes located at the 5' end having a broader range of expression, with downregulation at later stages of hematopoietic differentiation. To explore if these patterns reflect different functional activities, we have retrovirally engineered the overexpression of a 5'-located gene, *HOXA10*, in murine bone marrow cells and demonstrate effects strikingly different from those induced by overexpression of a 3'-located gene, *HOXB4*. In contrast to *HOXB4*, which causes selective expansion of primitive hematopoietic cells without altering their differentiation, overexpression of *HOXA10* profoundly perturbed myeloid and B-lymphoid differentiation. The bone marrow of mice reconstituted with *HOXA10*-transduced bone marrow cells contained in high frequency a unique progenitor cell with megakaryocytic colony-forming ability and was virtually devoid of unilineage macrophage and pre-B-lymphoid progenitor cells derived from the transduced cells. Moreover, and again in contrast to *HOXB4*, a significant proportion of *HOXA10* mice developed a transplantable acute myeloid leukemia with a latency of 19 to 50 weeks. These results thus add to recognition of *Hox* genes as important regulators of hematopoiesis and provide important new evidence of *Hox* gene-specific functions that may correlate with their normal expression pattern.

Hematopoiesis is an ordered process of differentiation and proliferation leading to the generation of mature blood cells from a small number of totipotent hematopoietic stem cells (HSC). From a number of recent studies, a variety of transcription factors have emerged as key components of the regulatory processes involved in lineage-specific development and in some cases proliferation of early hematopoietic cells (21).

Among such factors are the mammalian *Hox* homeobox gene family of transcription factors consisting of 38 members arranged in four clusters (*A*, *B*, *C*, and *D*) on four different chromosomes (1). These genes are structurally related by the presence of a 183-bp sequence, the homeobox, which encodes a helix-turn-helix DNA binding motif (15). During embryogenesis, these genes have been shown to play critical roles in pattern formation (thus, cell fate), where they are activated in a time- and site-specific manner that correlates with their 3'-to-5' chromosomal position (10).

Apparent stage- and lineage-specific expression of numerous *HoxA*, *-B*, and *-C* genes has now been demonstrated for both hematopoietic cell lines (14) and primary hematopoietic cells (6, 18, 26), suggesting that these genes are involved in the processes of hematopoietic differentiation and proliferation (14). This hypothesis has been supported by studies in which

the expression of specific *Hox* genes has been modulated in primary hematopoietic cells by either antisense oligonucleotides or by overexpression (23, 31, 37). Thus, for example, antisense oligonucleotides specific to *Hoxb-7* in murine bone marrow (37) or *HOXC6* in human bone marrow (31) lead to significant inhibition of granulocyte-macrophage CFU cells (CFU-GM) and suppression of CFU erythroid cells (CFU-E) for these two antisense constructs, respectively. Conversely, overexpression of *Hoxb-8* in murine bone marrow cells was found to enhance the proliferation of hematopoietic progenitor cells and predispose to leukemia (23).

Most recently, *Hox* genes have been directly implicated in the pathogenesis of human leukemias (2, 19). In the t(7;11)(p15;p15) translocation, which is recurrently observed in a subset of acute myeloid leukemias and in rare cases of chronic myeloid leukemias, the N-terminal half of the nucleoporin gene *NUP98* is fused in frame with most of the coding region of the *HOXA9* gene. Intriguingly, murine *Hoxa-9* and its near neighbor *Hoxa-7* have also been implicated in myeloid leukemias associated with retroviral insertional activation in the BXH-2 mouse line (20).

We have recently shown, with a reverse transcription-PCR-based technique, that members of the *HOXA* and *HOXB* cluster genes are preferentially expressed in the CD34⁺ fraction of human bone marrow cells, which contains most if not all of the hematopoietic progenitor cells (26). Further detailed analyses of the expression of several of these *Hox* genes in functionally distinct subpopulations of CD34⁺ cells revealed two patterns of expression. Specific genes, primarily located at the 5' end of

* Corresponding author. Mailing address: Terry Fox Laboratory, 601 West 10th Ave., Vancouver, B.C., Canada V5Z 1L3. Phone: (604) 877-6070. Fax: (604) 877-0712. E-mail: keith@terryfox.ubc.ca.

† Present address: Institute de Recherches Cliniques de Montreal, Montreal, Quebec, Canada H2W 1R7.

the clusters (e.g., *HOXA10* and *HOXB9*), showed essentially invariant expression in these subpopulations, whereas a second group of genes, located towards the 3' side of the clusters (e.g., *HOXB3* and *HOXB4*), were expressed at their highest levels in the subpopulation containing the most primitive hematopoietic cells and then were sharply downregulated in later cell populations (26). By retrovirally overexpressing in murine bone marrow one of the 3'-located genes, *HOXB4*, we have recently shown that *HOXB4*'s overexpression selectively enhances the proliferative potential of primitive hematopoietic cells, most profoundly HSC, without detectable effects on hematopoietic differentiation (27).

On the basis of these results, it was hypothesized that these expression patterns might reflect different functional activities of *Hox* genes in hematopoiesis. As an initial test of this hypothesis, we have examined both the *in vitro* and *in vivo* behavior of primary murine hematopoietic cells, which were engineered by retroviral gene transfer to overexpress *HOXA10*. Here, we demonstrate that overexpression of *HOXA10* profoundly perturbs differentiation of bone marrow progenitors with megakaryocytic and monocytic potentials, is not permissive for B-cell development, and enhances proliferation of hematopoietic progenitor cells. Furthermore, a significant proportion of recipients of *HOXA10*-transduced bone marrow cells eventually develop acute myeloid leukemia (AML).

MATERIALS AND METHODS

Animals. Seven- to 12-week-old male or female (C57BL/6J \times C3H/HeJ) F_1 [(B6C3) F_1] mice were used as recipients, and (C57BL/6Ly-Pep3b \times C3H/HeJ) F_1 [(PepC3) F_1] mice were used as donors. (B6C3) F_1 and (PepC3) F_1 mice are phenotypically distinguishable by their cell surface expression of different allelic forms of the *Ly5* locus; (B6C3) F_1 mice are homozygous for the *Ly5.2* allotype, and (PepC3) F_1 mice are heterozygous for the *Ly5.1/Ly5.2* allotypes. These mice were bred from parental strain breeders originally obtained from the Jackson Laboratories (Bar Harbor, Maine) and were maintained in microisolator cages and provided with sterilized food and acidified water in the animal facility of the British Columbia Cancer Research Center.

Retroviral generation and infection of primary bone marrow cells. The *HOXA10* cDNA region encompassing the complete coding sequence was isolated as an *EcoRI* fragment and subcloned upstream of the PGK-*neo* cassette at the *HpaI* site of the murine stem cell virus (MSCV) 2.1 retrovirus by standard procedures (5). High-titer helper-free recombinant retroviruses were generated and tested exactly as described in reference 27. The generation of the *HOXB4* and *neo* retroviruses has already been described elsewhere (27). Bone marrow cells obtained from (PepC3) F_1 (Ly5.1) mice injected intravenously 4 days previously with 150 mg of 5-fluorouracil (5-FU) per kg of body weight were pre-stimulated in Dulbecco's modified Eagle medium, containing 15% fetal calf serum (FCS), 6 ng of murine interleukin-3 (mIL-3) per ml, 100 ng of murine Steel factor (mSF) per ml, and 10 ng of human IL-6 (hIL-6) per ml for 48 h and then cocultivated on irradiated viral producer cells with identical medium with the addition of 6 μ g of Polybrene per ml for an additional 48 h (27). mSF, hIL-6, and mIL-3 were used as diluted supernatants from transfected COS cells as prepared in the Terry Fox Laboratory. Loosely adherent and nonadherent bone marrow cells were recovered from the cocultures by repeated washing of dishes and then were counted with a hemocytometer. All media, serum, and growth factors unless otherwise specified were obtained from StemCell Technologies, Inc. (Vancouver, British Columbia, Canada).

Transplantation of retrovirally transduced bone marrow. For bone marrow transplantation procedures, lethally irradiated (950 cGy, 110 cGy/min, ^{137}Cs gamma rays) (B6C3) F_1 (Ly5.2) recipients were injected intravenously with 2×10^5 bone marrow cells derived from (PepC3) F_1 (Ly5.1/Ly5.2) mice immediately after their cocultivation with *HOXA10* or *neo* viral producer cells. Donor-derived repopulation in recipients was assessed by the proportion of leukocytes in bone marrow, thymus, spleen, and peripheral blood which expressed the *Ly5.1* allelic form of the *Ly5* locus by flow cytometry and fluorescein isothiocyanate (FITC)-conjugated anti-*Ly5.1* (A20-1.7). For all mice analyzed, the donor-derived repopulation was found to be >85%.

In vitro clonogenic progenitor assays. For myeloid clonogenic progenitor assays, cells were cultured in 35-mm-diameter petri dishes (Greiner, Nürtingen, Germany) in a 1.1-ml mixture of 0.8% methylcellulose in alpha medium supplemented with 30% FCS, 1% bovine serum albumin (BSA), 10^{-4} M β -mercaptoethanol (β -ME), 3 U of human urinary erythropoietin (hEpo) per ml, and 2% spleen cell-conditioned medium (SCCM) in the presence or absence of 1.4 mg of G418 per ml. Bone marrow cells harvested after cocultivation with viral producer

cells or from *neo* or *HOXA10* mice were plated at a concentration of 1×10^3 to 2×10^3 or 4×10^4 cells/dish, respectively. Spleen cells from *HOXA10* or *neo* mice were plated at 3×10^5 to 10×10^5 or 3×10^6 to 10×10^6 cells/dish, respectively. Colonies were scored on days 10 to 12 of incubation as derived from macrophage CFU, granulocyte-macrophage CFU (CFU-GM), burst-forming unit erythroid (BFU-E), or granulocyte-erythrocyte-macrophage-megakaryocyte CFU (CFU-GEMM) according to standard criteria (9). In some experiments, identification of colony types was confirmed by Wright-Giemsa staining of cytospin preparations of colonies. For pre-B clonogenic progenitor assays, cells were plated in 0.8% methylcellulose in alpha medium supplemented with 30% FCS, 10^{-4} M β -ME, and 0.2 ng of IL-7 per ml with or without 1.4 mg of G418 per ml. Pre-B colonies were scored on day 7 of incubation.

CFU-S assay. *HOXA10*- or *neo*-transduced bone marrow cells were injected into lethally irradiated recipients either immediately after retroviral infection or after 1 week of culture, at an initial density of 1×10^5 to 5×10^5 cells/ml in medium containing 30% FCS, 1% BSA, 10^{-4} M β -ME, 3 U of hEpo per ml, and 2% SCCM with or without 1.4 mg of G418 per ml. The number of cells that each mouse received was adjusted to give 10 to 15 macroscopic spleen colonies. Untransplanted lethally irradiated mice were tested in each experiment for endogenous spleen CFU (CFU-S) surviving irradiation and consistently gave no spleen colonies. Twelve days after injection, animals were sacrificed by cervical dislocation, and the numbers of macroscopic colonies on the spleen were evaluated after fixation in Telleysniczky's solution. In certain cases, prior to fixation, well-isolated spleen colonies were excised with a scalpel blade and cut open, and then the cells were gently spread on a microscopic slide for cytological evaluation after Wright-Giemsa staining.

Purification and culturing of Sca1⁺Lin⁻WGA⁺ cells. Sca1⁺Lin⁻WGA⁺ cells were purified from the bone marrow of *neo*, *HOXA10*, and untransplanted (PepC3) F_1 mice, as previously described (25). For culturing of single cells, purified cells were resorted and deposited directly into wells of 96-well plates with an automatic cell deposition attachment to FACStar⁺ (Becton Dickinson). Single cells were cultured in serum-free medium as described previously (12) supplemented with the following growth factors: 20 ng of mIL-3 per ml, 10 ng of hIL-6 per ml, 5 ng of hIL-7 per ml, 25 ng of hIL-11 per ml, 3 U of hEpo per ml, 50 ng of mSF per ml, 10 ng of human granulocyte colony-stimulating factor (hG-CSF) per ml, and 1.4 mg of G418 per ml for selection of transduced cells. After G418 selection, 9 to 15 days later, the wells containing ≥ 10 cells were scored by visual inspection for the presence of megakaryocytes. Visual scoring criteria were validated by Wright-Giemsa staining of cytospin preparations and by flow cytometry for expression of the megakaryocyte-specific GPIIb/IIIa (CD41) surface antigen with the D9 monoclonal antibody (MAb) (K. A. Ault, Maine Medical Center Research Institute, South Portland).

Phenotyping of hematopoietic cell populations. At various times after transplantation, peripheral blood cell counts and hematocrits of *HOXA10* and *neo* mice were determined with a Coulter CBC5 counter. Differential counts of bone marrow, spleen, and peripheral blood cells from all *HOXA10* and *neo* mice that were sacrificed or that became terminally ill were performed with Wright-Giemsa-stained cytospin preparations. Various hematopoietic populations in peripheral blood, bone marrow, spleen, and thymus of *HOXA10* and *neo* mice were analyzed at various times posttransplantation by flow cytometry. Single-cell suspensions of 1×10^6 cells were incubated with phycoerythrin (PE) and FITC-conjugated MAb for 40 min. The cells were washed twice and then analyzed on a FACSort fluorescence-activated cell sorter (Becton Dickinson & Co., Mountain View, Calif.). The following MAbs to lineage-specific differentiation antigens were used: anti-Mac-1, purified from the M1/70.15.11 hybridoma (American Type Culture Collection, Rockville, Md.); anti-Gr-1 B220, anti-CD4, and anti-CD43 (S7) (Pharmingen, San Diego, Calif.); anti-immunoglobulin G (IgG) and anti-IgD (Southern Biotechnology, Birmingham, Ala.); and anti-CD8 (Boehringer Mannheim, Indianapolis, Ind.).

Southern, Northern, and Western blot analyses. Southern blot analyses to assess proviral integration were performed as previously reported (27) by standard techniques. High-molecular-weight DNA was digested with *KpnI* (in some cases, *SstI*), which cuts in the long terminal repeats (LTRs) to release the proviral genome, or with *HindIII*, which cuts the provirus once to release DNA fragments specific to the proviral integration site(s). Total cellular RNA was isolated, transferred, and hybridized as described previously (27). The probes used were a *XhoI/SalI* fragment of pMC1neo (32); a *KpnI/MseI* fragment of pXM(ER)-190, which releases the full-length erythropoietin receptor (*EpoR*) cDNA (kindly provided by A. D'Andrea); the 1.8-kb genomic *KpnI/HindIII* fragment of the murine SH2-containing inositol phosphatase (*SHIP*) gene (4); the 1.9-kb full-length human *HOXA10* cDNA; a 0.3-kb 3' homeodomain-free fragment from the murine *Hoxa-10* gene; and the 2.0-kb *PstI* fragment containing the β -actin gene. Western blot analyses were done with whole-cell lysates from *neo* and *HOXA10* viral producer cells or from bone marrow cells of normal untransplanted, *neo*, and *HOXA10* mice. After electrophoresis in a 10% polyacrylamide gel, protein was transferred to nitrocellulose and probed with a whole rabbit antiserum to a synthetic oligopeptide derived from the C-terminal, nonhomeodomain region of *HOXA10*. The blot was then treated with a goat anti-rabbit IgG second antibody, followed by incubation with the enhanced chemiluminescence (ECL) detection reagent (Amersham Corp., Arlington Heights, Ill.).

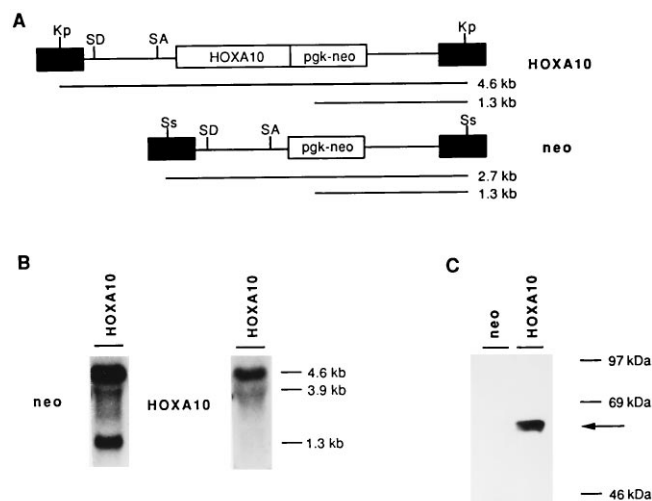


FIG. 1. Structure and expression of the *HOXA10* retrovirus used in this study. (A) Diagrammatic representation of the integrated *HOXA10* and *neo* proviruses. The expected sizes of the full-length viral transcripts and those initiated from the internal PGK promoter are shown. Two viral transcripts are generated from each virus due to the splice donor and acceptor sites in the MSCV 2.1 vector (B) Northern blot analysis of total RNA isolated from the *HOXA10* virus producer cells. The membrane was hybridized with a probe specific for *neo* that detects both viral transcripts (a 4.6-kb full-length RNA and 3.9-kb spliced RNA) and the 1.3-kb *neo* transcript initiated from the PGK promoter (left) and subsequently with a full-length *HOXA10* cDNA probe that detects both viral transcripts (right). (C) Western blot analysis of whole-cell lysate from *neo* and *HOXA10* virus producer cells. The blot was probed with polyclonal antisera directed against a *HOXA10* synthetic oligopeptide. The arrow indicates the band for the *HOXA10* protein migrating at the expected size of 55 kDa. Kp, *KpnI*; Ss, *SmaI*; SD, splice donor; SA, splice acceptor.

RESULTS

Retrovirus-mediated transduction of *HOXA10* to murine bone marrow cells. For this study, we used a full-length *HOXA10* cDNA isolated from the human myeloid cell line ML3, which encodes a 55-kDa protein (16). This *HOXA10* cDNA was chosen because it represents the most abundant *HOXA10* transcript found in human bone marrow cells (16). The *HOXA10* cDNA was inserted into the MSCV 2.1 retroviral vector (8) 5' to a phosphoglycerate kinase promoter (PGK)-driven *neo* gene such that *HOXA10* was expressed from the viral enhancer and promoter sequences within the LTR (Fig. 1A). The LTR sequences in this MSCV vector have been shown previously to give high and long-term expression in primitive murine hematopoietic cells and their mature progeny of both myeloid and lymphoid lineage (7, 22, 27). The integrity of the *HOXA10* retrovirus was verified by Northern and Western blot analyses, which detected the two expected *HOXA10*-containing viral mRNAs (due to the splice donor and acceptor sites in the MSCV 2.1 vector) (7) and the *HOXA10* protein in the viral producer cells, respectively (Fig. 1B and C). For some parts of the study, we also used an MSCV retroviral vector containing the *HOXB4* cDNA under the control of the viral LTR, which has previously been described (27).

To investigate the effects of *HOXA10* overexpression in primary hematopoietic cells, bone marrow cells from mice injected 4 days previously with 5-FU were cocultivated with *neo* or *HOXA10* viral producer cells for 48 h, and the subsequent effects of deregulated expression of *HOXA10* on the behavior of hematopoietic cells were analyzed both in vitro and in vivo.

Altered colony formation in vitro of myeloid progenitor cells overexpressing *HOXA10*. To test whether overexpression of

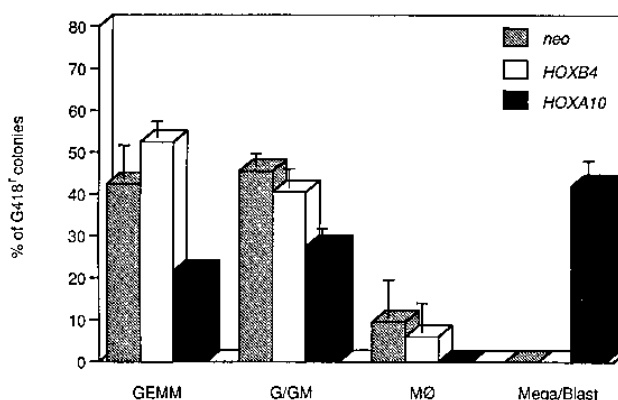


FIG. 2. Effects of *HOXA10* and *HOXB4* overexpression on the relative frequency of various colony types generated in vitro, immediately after retroviral infection of bone marrow cells. On days 12 and 13, well-isolated G418-resistant colonies were randomly picked ($n = 74$ for *neo*, $n = 76$ for *HOXB4*, and $n = 77$ for *HOXA10*) and examined after Wright-Giemsa staining. Results are expressed as means \pm standard deviations from two independent experiments. The various colony types (granulocyte-erythrocyte-macrophage-megakaryocyte [GEMM], granulocyte and granulocyte-macrophage [G/GM], and macrophage [MØ]) generated from *neo*- and *HOXB4*-transduced cells were not significantly different ($P > 0.5$). Megakaryocyte-blast (Mega/Blast) colonies were significantly increased in *HOXA10* cultures compared to in *neo* cultures ($P < 0.01$), and G/GM and GEMM colonies were significantly decreased in *HOXA10* cultures compared to in *neo* cultures ($P < 0.05$). Although no MØ colonies were detected in *HOXA10* cultures, they were not significantly decreased compared to those in *neo* cultures, because the numbers of MØ colonies in *neo* cultures were low and varied greatly between experiments (Student *t* test).

HOXA10 would affect the ability of committed myeloid progenitor cells to complete their differentiation in vitro, *neo*- and *HOXA10*-transduced bone marrow cells were plated, immediately after retroviral infection, in methylcellulose cultures for myeloid colony formation. The efficiencies of retroviral infection, as assessed by the proportion of G418-resistant clonogenic cells, were $50\% \pm 28\%$ and $30\% \pm 3\%$ for *neo* and *HOXA10* retrovirus, respectively (values are means \pm standard deviations from two independent experiments). The total numbers of colonies generated were similar for both types of infected cells (~ 80 colonies/1,000 cells). However, there was a striking difference between the cellular constituents of *neo*- and *HOXA10*-transduced colonies, as revealed by cytological examinations of Wright-Giemsa-stained G418-resistant colonies (Fig. 2). About 45% of the progenitors transduced with *HOXA10* generated large colonies containing megakaryocytes and blast cells (Fig. 3A), a colony type not detected among *neo*-transduced colonies. The generation of this unique colony type in *HOXA10* cultures was accompanied by a proportional reduction in multilineage GEMM, granulocyte-macrophage, and granulocyte colonies; moreover, no unilineage macrophage colonies could be detected among G418-resistant (i.e., *HOXA10*-transduced) colonies. In contrast to this altered myeloid differentiation mediated by *HOXA10*, overexpression of *HOXB4* did not alter the proportion of various colony types generated in vitro (Fig. 2).

***HOXA10* overexpression increases the maintenance of day 12 CFU-S in vitro.** In our previous study of the effects of overexpression of *HOXB4* in murine bone marrow cells, we observed a more than 2-log enhancement in the recovery of multipotent myeloid progenitor cells (day 12 CFU-S) after a 7-day culture period following retroviral infection with *HOXB4* (27). To assess whether *HOXA10* overexpression had a similar effect, the day 12 CFU-S contents of *neo*- and *HOXA10*-infected bone marrow cells were measured immediately after

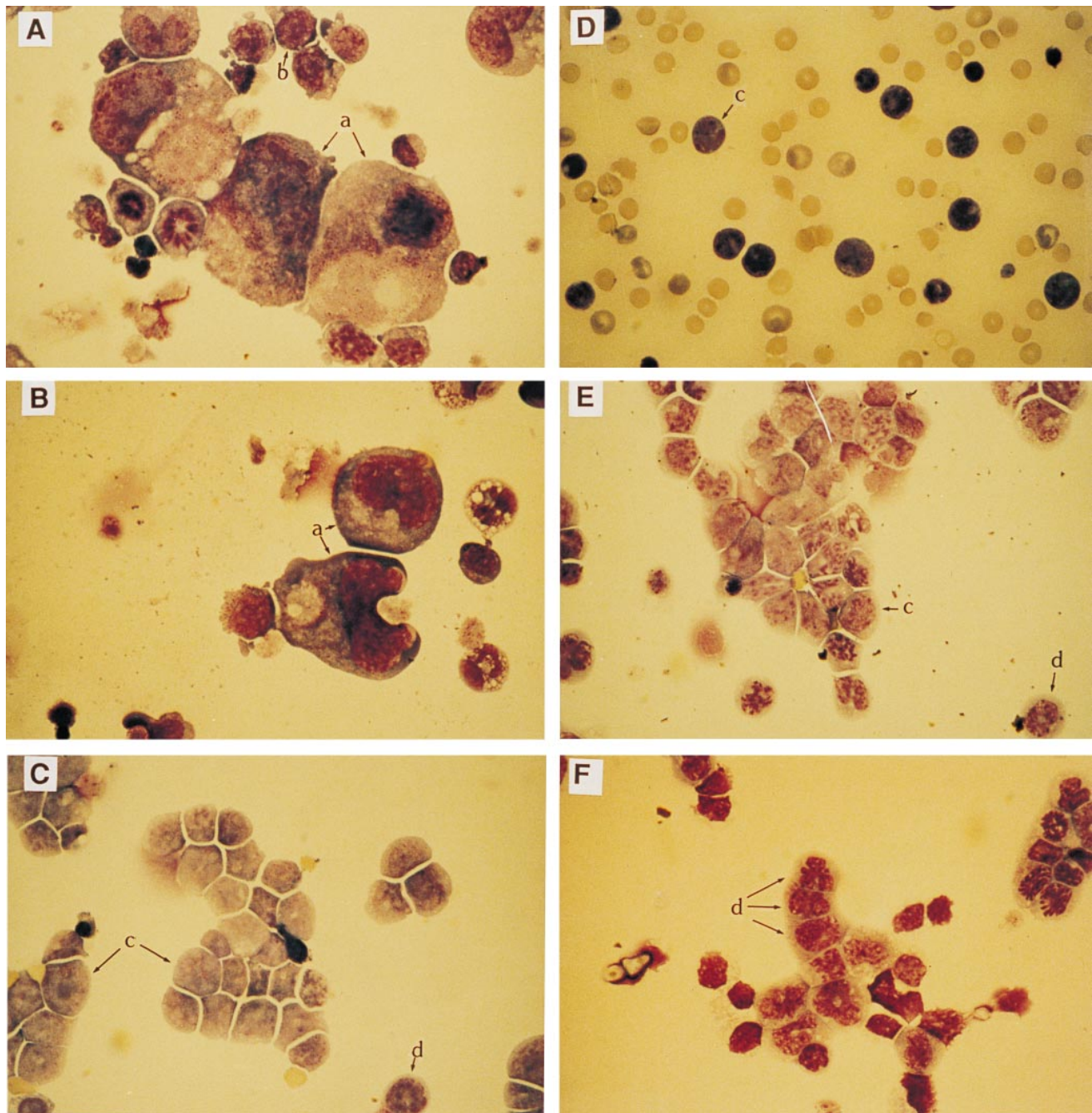


FIG. 3. Wright-Giemsa staining of cytospin preparations of *HOXA10*-transduced myeloid colonies (A to C) and of hematopoietic tissues from a representative leukemic *HOXA10* mouse (D to F). Representative megakaryocyte (a) and blast cell (b) colonies picked from methylcellulose cultures initiated with cells obtained either immediately after retroviral infection of 5-FU-treated bone marrow cells (A) or with cells recovered from bone marrow of *HOXA10* mice (B). (C) Smaller colony observed only in methylcellulose cultures initiated with bone marrow cells from *HOXA10* mice containing immature blast-like myeloid cells (c) together with few differentiated granulocytic elements (d). (D, E, and F) Peripheral blood, bone marrow, and lymph nodes, respectively, from a representative *HOXA10* mouse that developed AML. Note the presence of leukemic blasts together with a few mature granulocytic elements in each of these three organs. Magnification, $\times 600$.

retroviral infection and again after 7 days in liquid culture supplemented with growth factors and 1.4 mg of G418 per ml for selection of transduced cells. The day 12 CFU-S frequencies measured immediately after retroviral infection were similar for *neo*- and *HOXA10*-transduced bone marrow cells (Fig. 4, day 0). However, after maintenance of these cells for 7 days in liquid cultures, the day 12 CFU-S content of cultures initiated with *HOXA10*-transduced cells showed a net increase to

200% of input values, whereas levels in *neo* control cultures had decreased to $\sim 5\%$ of input (Fig. 4, day 7). Thus, *HOXA10* like *HOXB4* can reverse the decline in day 12 CFU-S numbers normally observed under these culture conditions, suggesting that overexpression of *HOXA10* can affect processes involved in the generation or maintenance of cells with day 12 CFU-S ability.

Cytological examination of Wright-Giemsa-stained cell

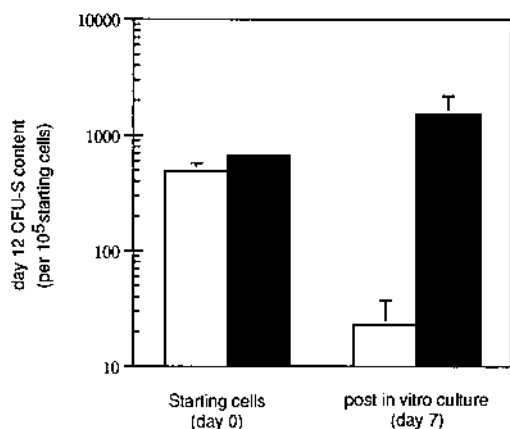


FIG. 4. Effect of overexpression of *HOXA10* on the recovery of day 12 CFU-S in vitro. The CFU-S content was assessed immediately after cocultivation (day 0) and also after 7 days in liquid culture. Results (means \pm standard deviations) are expressed as day 12 CFU-S number per 10^5 starting day 0 cells from two independent experiments, except for day 0 values for *HOXA10*, which represent one experiment. Open bars, *neo*; solid bars, *HOXA10*.

preparations from day 12 spleen colonies showed the same proportion of differentiated erythroid (>90%) and myeloid elements for both *HOXA10*- and *neo*-transduced colonies (data not shown), indicating that overexpression of *HOXA10* did not overtly alter the pattern of terminal differentiation of day 12 CFU-S during spleen colony formation in vivo.

Expansion in vivo of myeloid progenitor cells overexpressing *HOXA10* and their altered colony formation in vitro. To delineate further the effects of *HOXA10* overexpression on hematopoiesis, we analyzed long-term myeloid and lymphoid

reconstitution in lethally irradiated mice transplanted with *HOXA10*- or *neo*-transduced bone marrow cells (hereafter called *HOXA10* and *neo* mice, respectively). At 8 to 15 weeks posttransplantation, hematopoietic regeneration in either *neo* or *HOXA10* mice was essentially completely donor derived, because >85% of bone marrow, thymic, and peripheral blood leukocytes were of donor origin (Ly5.1⁺). Moreover, the intensities and patterns of proviral signals seen upon Southern blot analysis of DNA from bone marrow and thymus of these mice were consistent with high-level polyclonal reconstitution by transduced cells in both *neo* and *HOXA10* mice (Fig. 5). Expression of the transduced *HOXA10* cDNA was readily detected in total bone marrow cells of *HOXA10* mice, at both the RNA and protein levels by Northern and Western blot analyses, respectively (Fig. 6A and B, respectively). Endogenous *Hoxa-10* expression, in contrast, was below the detection level, by the same methods, in either *neo*, *HOXA10*, or normal untransplanted mice (Fig. 6).

When analyzed 8 to 15 weeks posttransplantation in contrast to *neo* control mice, *HOXA10* mice showed weight loss and signs of hematological abnormalities (Table 1). Although their peripheral blood and bone marrow nucleated cell numbers were within the normal range, *HOXA10* mice had moderate splenomegaly and mild anemia (Table 1).

The numbers of myeloid progenitor cells were significantly increased in *HOXA10* mice compared with those in *neo* control mice 15 weeks posttransplantation (Table 2). This was most pronounced in the spleen, with on average a ninefold increase over that found in *neo* mice (Table 2). Preferential expansion of *HOXA10*-transduced progenitor cells over that of nontransduced cells was indicated by a higher proportion of G418-resistant myeloid colonies detected from the *HOXA10* mice compared to that of *neo* control mice (Table 2), despite lower

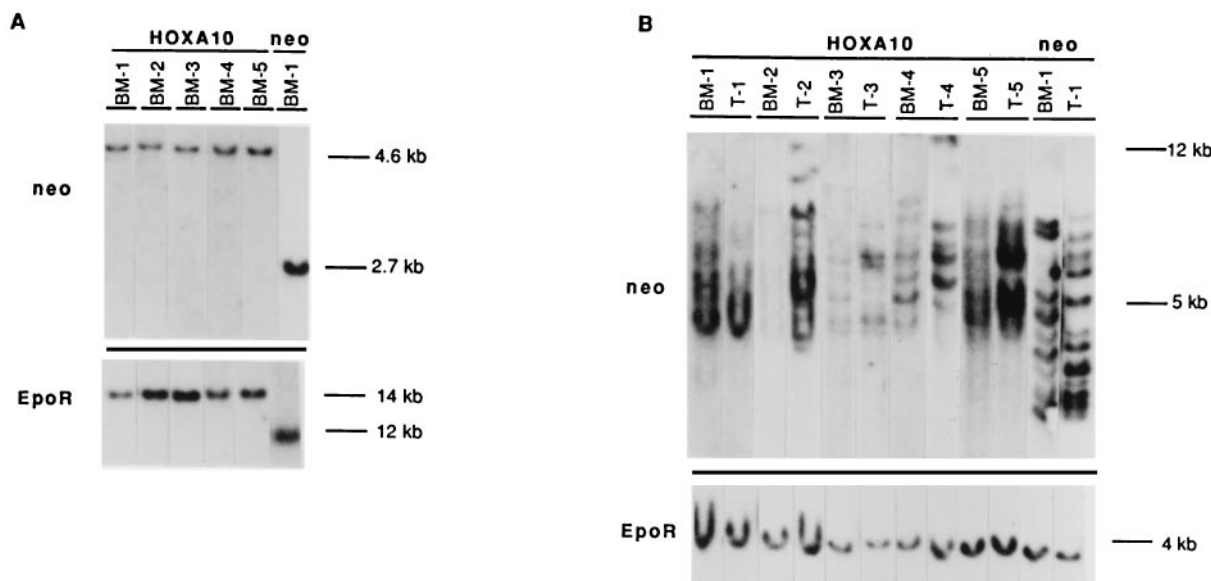


FIG. 5. Southern blot analysis of DNA isolated from hematopoietic tissue of *HOXA10* and *neo* mice. (A) Demonstration of the presence of intact integrated *HOXA10* and *neo* proviruses. DNA from bone marrow of *HOXA10* mice was digested with *KpnI* and that of the *neo* mouse was digested with *SstI* to release the integrated *HOXA10* (4.6 kb) and *neo* (2.7 kb) proviral fragments, respectively. The blots were hybridized with a *neo*-specific probe to detect the proviruses and then subsequently with a probe specific for murine *EpoR* to provide a single gene copy control for loading. The exposure times were equivalent for both probes (~35 h). (B) Analysis of proviral integration patterns in DNA isolated from bone marrow and thymic cells from the same *HOXA10* and *neo* mice. DNA was digested with *HindIII*, which cuts the integrated provirus once, generating DNA fragments unique to each integration site. The membranes were hybridized with a probe specific for *neo* to detect proviral fragments and subsequently with a probe specific for murine *EpoR* to provide a single gene copy control for loading. The exposure times were 72 h for the *neo* probe and 24 h for the *EpoR* probe. Each number assigned to a lane identifies a specific mouse. *HOXA10* mouse 1 was sacrificed 8 weeks posttransplantation, whereas the other *HOXA10* mice and the *neo* mouse were sacrificed 15 weeks posttransplantation. BM, bone marrow; T, thymus.

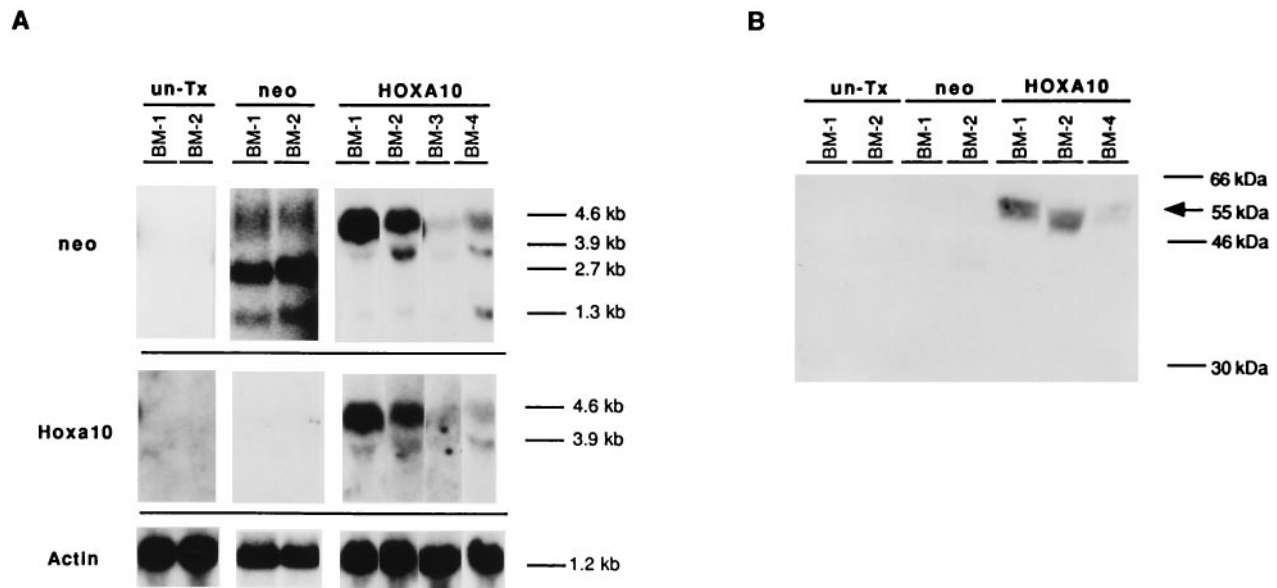


FIG. 6. Northern and Western blot analyses demonstrate high levels of virus-derived *HOXA10* messages and protein in reconstituted *HOXA10* mice. (A) Northern blot analysis of total RNA (10 μ g) isolated from the bone marrow of normal, *neo*, and *HOXA10* mice. The membranes were hybridized with a probe specific for *neo* that detects viral transcripts in both *neo* (2.7 kb) and *HOXA10* (4.6-kb full-length RNA and 3.9-kb spliced RNA) mice and the 1.3-kb *neo* transcript initiated from the PGK promoter, and subsequently the membranes were hybridized with a probe specific for the murine *Hoxa10* for detection of both the endogenous *Hoxa10* messages and the *HOXA10* viral transcripts. The endogenous 2.0- or 2.5-kb murine *Hoxa10* messages were not detected in any of the mice. As a control for loading, the membranes were also hybridized with a probe specific for actin. The exposure times were equivalent for both the *Hoxa10* and *actin* probes (\sim 48 h). (B) Western blot analysis of whole-cell lysates from bone marrow of normal, *neo*, and *HOXA10* mice. The blot was probed with a polyclonal antisera directed against a *HOXA10* synthetic oligopeptide. The arrow shows the band for the *HOXA10* protein migrating at the expected size of 55 kDa. Each number assigned to a lane identifies a specific mouse (not the same mice as in Fig. 5). *HOXA10* mice 1 and 2 were leukemic, whereas mice 3 and 4 were not. BM, bone marrow; un-Tx, normal untransplanted mouse.

gene transfer in the initial transplanted bone marrow inoculum for the *HOXA10* mice ($67.5\% \pm 4\%$ and $42.5\% \pm 18\%$ for *neo* and *HOXA10*, respectively). For some of these *HOXA10* mice, the splenic nucleated cell counts were also found to be elevated, which in all cases was due to an increase in cells expressing both the Mac-1 and Gr-1 antigens as determined by flow cytometry analyses (data not shown).

Myeloid clonogenic progenitors of *HOXA10* mice remained growth factor dependent for in vitro colony formation when analyzed 8 weeks posttransplantation. However, in the presence of added growth factors, $\sim 45\%$ of the *HOXA10*-transduced progenitor cells from bone marrow of these mice generated a unique colony type containing megakaryocytes and blast cells (Fig. 3B and Table 3), representing at least a 35-fold increase in the absolute numbers of this progenitor type compared to *neo* control mice. Curiously, despite this high frequency in bone marrow of *HOXA10* mice of a progenitor cell with potential to differentiate in vitro into megakaryocytes, inspection of bone marrow and peripheral blood smears from

HOXA10 mice revealed no gross increase in megakaryocyte or platelet counts (data not shown). In addition to a high proportion of megakaryocyte and blast cell colonies, another $\sim 20\%$ of the colonies from *HOXA10* mice were small (~ 100 cells), containing mainly highly granular blast cells and very few differentiated myeloid elements, a colony type not detected among the *neo*-transduced colonies (Fig. 3C and Table 3). Furthermore, no unilineage macrophage colonies could be detected among the *HOXA10*-transduced colonies, which in contrast represented about 30% of the colonies generated from the bone marrow of *neo* mice. Interestingly, macrophage colonies could be detected in the absence of G418 in cultures initiated with bone marrow cells from *HOXA10* mice (data not shown), indicating the presence of nontransduced macrophage progenitor cells capable of normal differentiation in these mice. Thus, the block in macrophage colony formation due to overexpression of *HOXA10* appears to be intrinsic to transduced cells rather than attributable to accessory cells. Similarly, the induction of megakaryocytic differentiation also ap-

TABLE 1. Body weight and hematological parameters in *neo* and *HOXA10* mice^a

Mouse group	Body wt (g) ^b	Spleen wt (g) ^c	No. of leukocytes (10 ⁷)/femur ^c	Peripheral blood parameter ^c		
				No. of erythrocytes (10 ⁹)/ml	Hemoglobin (g/dl)	No. of leukocytes (10 ⁶)/ml
<i>neo</i>	31.5 \pm 3.0	0.11 \pm 0.01	1.9 \pm 0.5	8.6 \pm 1.0	15.8 \pm 1.5	8.3 \pm 2.3
<i>HOXA10</i>	26.5 \pm 1.3 ^d	0.17 \pm 0.06 ^e	2.1 \pm 0.2	7.5 \pm 1.1 ^f	13.8 \pm 1.8	11.3 \pm 5.6

^a Results are given as means \pm standard deviations.

^b $n = 11$ mice sex and age matched 15 weeks posttransplantation.

^c $n = 7$ for *neo* mice and $n = 9$ for *HOXA10* mice 8 to 15 weeks posttransplantation.

^d Significantly less than *neo* control ($P < 0.03$).

^e Significantly greater than *neo* control ($P < 0.03$).

^f Significantly less than *neo* control ($P < 0.05$).

TABLE 2. *HOXA10* mice have increased numbers of myeloid clonogenic progenitor cells compared to *neo* control mice^a

Mouse group (n)	No. of myeloid CFC (10 ³)/femur (% G418 ^r)	No. of myeloid CFC (10 ³)/spleen (% G418 ^r)
<i>neo</i> (6)	44 ± 11 (53 ± 19)	4 ± 2 (62 ± 22)
<i>HOXA10</i> (8)	75 ± 37 ^b (62 ± 13)	37 ± 22 ^c (82 ± 20)

^a Results are given as means ± standard deviations of the number of myeloid clonogenic progenitor cells in bone marrow and spleen of *neo* and *HOXA10* mice 15 weeks posttransplantation. CFC, colony-forming cell.

^b Significantly higher than in *neo* control mice ($P < 0.05$).

^c Significantly higher than in *neo* control mice ($P < 0.005$).

appears to be intrinsic to *HOXA10*-transduced progenitor cells. When cells with the primitive phenotype Sca1⁺Lin⁻WGA⁺ (purified from bone marrow of *HOXA10*, *neo*, and untransplanted mice) were cultured as single cells in serum-free liquid cultures supplemented with various growth factors, *HOXA10*-transduced Sca1⁺Lin⁻WGA⁺ cells showed increased potential to generate megakaryocyte-containing colonies, compared to both *neo*-transduced (Table 4) and untransduced Sca1⁺Lin⁻WGA⁺ cells (data not shown).

Thus, overexpression of *HOXA10* in vivo, like that of *HOXB4* (27), induces expansion of myeloid progenitor cells, but in contrast to *HOXB4*, alters their normal differentiation.

Overexpression of *HOXA10* in vivo is not permissive for pre-B-lymphoid colony formation. While transduced myeloid progenitor cells were increased in numbers in *HOXA10* mice, total pre-B colony-forming cells were slightly reduced in number (~2-fold), and virtually none were derived from transduced cells (G418 resistant). Overall, transduced pre-B progenitors in *HOXA10* mice were some 22-fold lower in absolute number than those in *neo* control mice (Table 5). To further demonstrate the inability of *HOXA10*-transduced cells to contribute to B lymphopoiesis, the intensities of the proviral signals in DNA isolated from spleen, in which mature B cells normally constitute the majority of cells (approximately two of three), were compared to those from bone marrow and thymus. For that purpose, only the *HOXA10* mice ($n = 4$) in which the proportion of splenic B and myeloid cells were within normal range, as determined by flow cytometric analyses (B220⁺ cells, 60 to 62%; Mac-1⁺, 3 to 4%), were analyzed. In contrast to *neo* control mice, in which equal proviral signals were detected in all three tissues, the strengths of the proviral signal from spleen cells in the *HOXA10* mice were on average only one-third of that in bone marrow and thymus, consistent with minimal contribution of marked cells from the B-cell fraction (see Fig. 8A). Flow cytometric analysis of various B-cell populations in bone marrow and spleen of *neo* and

HOXA10 mice showed that the absolute numbers of pro-B, pre-B, and mature B cells were within the normal range in *HOXA10* mice (data not shown) and thus that the regeneration and differentiation of B cells in *HOXA10* mice from untransduced cells (estimated to be ≥50% of bone marrow cells in the transplant inoculum [described above]) appeared unaffected and compensated for impaired B-cell differentiation by transduced cells.

When analyzed 8 to 15 weeks posttransplantation, *HOXA10* mice had normal thymic size and, with antibodies against CD4 and CD8, the numbers and relative frequencies of thymocyte subpopulations in them were found to be normal (data not shown). Contribution of transduced cells to thymic regeneration in these *HOXA10* mice was evident by Southern blot analysis that detected proviral signals, which in most cases were at levels comparable to those seen in bone marrow (Fig. 5B and 8A). Overexpression of *HOXA10* thus appears to have no gross effect on T-lymphoid development.

AML arises in recipients of *HOXA10*-transduced bone marrow cells. In two independent experiments, a total of 23 mice were transplanted with *HOXA10*-transduced bone marrow cells. Of these, 11 animals were sacrificed 8 to 15 weeks posttransplantation, but the remaining 12 were monitored for a prolonged period of time to test whether overexpression of *HOXA10* would eventually lead to a disease state. Nine of these *HOXA10* mice (from both transplantation experiments) became terminally ill and died 19 to 50 weeks posttransplantation; three remained alive at 52 weeks posttransplantation (Fig. 7). The diminished survival of *HOXA10* mice contrasts with that of mice transplanted with *HOXB4*-transduced bone marrow cells, for which survival was not different from that of the *neo* control mice (Fig. 7).

In all cases in which documentation was possible ($n = 7$), AML was the cause of death of the *HOXA10* mice. These mice had elevated leukocyte counts (>50,000/μl) and were profoundly anemic, and some had hind limb paralysis, likely caused by leukemic infiltration of lumbo-sacral roots. Cytological examination revealed large numbers of blast cells in bone marrow, spleen, and lymph nodes (Fig. 3D to F). For the two mice tested, the leukemia was readily transplantable to both irradiated ($n = 5$) and nonirradiated ($n = 5$) recipients, which developed a fulminant leukemia 3 to 5 weeks after intravenous injection of 2×10^6 bone marrow cells. Southern blot analysis of hematopoietic tissue from leukemic *HOXA10* mice showed that the leukemic cells contained the *HOXA10* provirus and that the leukemia appeared mono- or biclonal (Fig. 8B). Similarly, Northern and Western blot analyses of bone marrow cells from two leukemic mice detected high levels of the full-length retrovirally driven *HOXA10* messages (4.6 and 3.9 kb)

TABLE 3. *HOXA10* mice have greatly increased numbers of bone marrow megakaryocyte-blast and blast colony-forming cells (CFC) and decreased numbers of unilineage macrophage CFC^a

Mouse group	No. of G418 ^r CFC (10 ³)/femur ^b					
	Total CFC	GEMM/BFU-E ^c	G/GM	MØ	Mega/Blast	Blast
<i>neo</i>	16.9 ± 0.8	5.5 ± 1.5	6.5 ± 2	4.7 ± 3	<0.3	<0.3
<i>HOXA10</i>	26.2 ± 1.3	3.8 ± 2.0	5.0 ± 2.5	<0.5 ^d	11.5 ± 1.0 ^e	5.5 ± 1.0 ^f

^a On days 12 and 13, well-isolated colonies were randomly picked and analyzed by Wright staining ($n = 50$ for *neo* mice and $n = 58$ for *HOXA10* mice). Results shown represent the means ± standard deviations of the number of various G418-resistant colony types generated from two mice in each group.

^b The fold differences between the *HOXA10* and *neo* groups were as follows: total CFC, 1.6; GEMM/BFU-E, 0.7; G/GM, 0.7; MØ, 0.1; Mega/Blast (megakaryocyte-blast), 36.5; and Blast, 16.0.

^c BFU-E colonies represented only 0.5% of the colonies analyzed and therefore were combined with GEMM.

^d Significantly lower than in *neo* control mice ($P < 0.05$).

^e Significantly higher than in *neo* control mice ($P < 0.005$).

^f Significantly higher than in *neo* control mice ($P < 0.05$).

TABLE 4. Sca1⁺Lin⁻WGA⁺ cells overexpressing *HOXA10* have increased potential to generate megakaryocyte-containing colonies in vitro^a

Mouse group	No. of Sca1 ⁺ Lin ⁻ WGA ⁺ cells (10 ³)/femur	% G418 ^r Sca1 ⁺ Lin ⁻ WGA ⁺ cells	No. of G418 ^r Sca1 ⁺ Lin ⁻ WGA ⁺ cells (10 ³)/femur generating megakaryocyte-containing colonies (% of total G418 ^r colonies)
<i>HOXA10</i>	30.0 ± 0.3	57.5 ± 9	6.6 ± 2.3 ^b (38 ± 6)
<i>neo</i>	29.0 ± 9.9	30.0 ± 10	1.3 ± 0.5 (15 ± 4)
Normal control	15.0 ± 4	<0.7	1.0 ± 0.3 (8 ± 1)

^a Sca1⁺Lin⁻WGA⁺ cells purified from bone marrow cells of *neo* (*n* = 2) and *HOXA10* (*n* = 2) mice 8 weeks posttransplantation and from untransplanted mice (*n* = 2) were seeded as single cells per well, and following G418 selection, 9 to 15 days later, the wells containing ≥10 cells (*n* = 296 for *HOXA10*, *n* = 186 for *neo*, and *n* = 141 for untransplanted) were scored for the presence of megakaryocytes. Results shown represent means ± standard deviations.

^b Significantly different from *neo* control (*P* < 0.05).

and the *HOXA10* protein (Fig. 6). The mono- or biconality of the leukemias, together with their delayed onset, suggests that overexpression of *HOXA10* is in itself not fully transforming and that an additional mutation or mutations are required for leukemic transformation.

Further evidence for the leukemogenic nature of overexpression of *HOXA10* was obtained by transplanting bone marrow cells from an apparently healthy primary *HOXA10* mouse (15 weeks posttransplantation) into lethally irradiated secondary recipients. By 8 to 12 weeks posttransplantation, all of the secondary recipients (*n* = 17) developed AML, which in all cases was found to be caused by the same *HOXA10*-transduced leukemic clone, as evident by Southern blot analysis (Fig. 8B). This clone was also found to be the dominant *HOXA10*-transduced clone in the bone marrow of the primary donor mouse (Fig. 8B), indicating the existence of a preleukemic state in this mouse. Interestingly, the primitive Sca1⁺Lin⁻WGA⁺ bone marrow fraction of the same primary *HOXA10* mouse was not as leukemogenic. Of the two secondary recipients which received *HOXA10*-transduced Sca1⁺Lin⁻WGA⁺ cells, the AML developed after a longer latency period (34 and 45 weeks) and, interestingly, for the one mouse analyzed, the leukemia originated from a different *HOXA10*-transduced clone (Fig. 8B).

Although overexpression of *HOXA10* in nonleukemic primary recipients did not render clonogenic cells growth factor independent, all three leukemic clones tested showed growth factor independence in cultures containing FCS.

DISCUSSION

In this study, we report that retroviral overexpression of one of the 5'-located *Hox* genes, *HOXA10*, perturbs the differentiation of both myeloid and B-lymphoid progenitor cells and eventually leads to the generation of AML. These effects are quite distinct from those we previously observed for the 3'-located *HOXB4* gene, whose overexpression enhanced the expansion of primitive hematopoietic cells but neither altered hematopoietic differentiation nor predisposed to leukemia (27). Taken together, these data add further functional evi-

dence for involvement of *Hox* genes in key hematopoietic developmental processes in a *Hox* gene-specific manner.

A striking effect of overexpression of *HOXA10* was the enhanced generation in vitro of colonies containing megakaryocytes and blast cells and the suppression of macrophage colony formation. This effect was seen both for the transduced progenitors recovered from the regenerated bone marrow of *HOXA10* mice and immediately following *HOXA10* infection of committed progenitor cells in the 5-FU bone marrow, thus arguing for a direct effect of *HOXA10* on differentiation of committed myeloid progenitor cells. The detection of non-transduced macrophage progenitors in bone marrow of *HOXA10* mice further supports the idea that the observed block in macrophage colony formation is an intrinsic property of *HOXA10*-transduced cells rather than attributed to accessory cells. The induction of megakaryocytic differentiation also appears to be intrinsic to *HOXA10*-transduced progenitor cells, since *HOXA10*-transduced Sca1⁺Lin⁻WGA⁺ cells when cultured as single cells in liquid culture showed a similar increase in megakaryocyte generation. Interestingly, although colonies containing megakaryocytes were generated in high frequency from *HOXA10*-transduced progenitor cells in vitro, we did not detect any gross increase in megakaryocyte or platelet numbers in *HOXA10*-reconstituted mice. This suggests that the probability of *HOXA10*-mediated megakaryocytic induction is increased when growth factor concentrations are above normal physiological levels, as is the case in vitro, or alternatively, that *HOXA10*-induced megakaryocytic differentiation could be inhibited in vivo by some unknown negative regulatory mechanisms either not present or not functional in vitro.

The transcriptional control of megakaryocytic development remains largely unknown. Of the lineage-specific transcription factors known to be expressed in the megakaryocytic lineage (e.g., *GATA-1* and -2, *Tal-1/SCL*, and *NF-E2*), only *NF-E2* has been shown to be essential for normal megakaryocytic differentiation, for which it is needed for completion of megakaryocytic maturation and platelet generation (24, 29, 30, 34). A functional role for *GATA-1* during early stages of megakaryocytic development has been inferred, since forced overexpression of *GATA-1* is able to reprogram both murine and avian myeloid cell lines to differentiate into the megakaryocytic lineage (11, 36). Although *HOXA10* expression has not been detected in limited studies of cell lines which have both megakaryocytic and erythroid differentiation potential (16, 17, 35), the results presented in this study implicate *Hox* genes as potentially important regulators of megakaryocytic differentiation. More detailed assessment of *Hox* gene expression patterns, particularly *HOXA10*, during megakaryocytic differentiation will now be of considerable interest, as will attempts to identify effects subsequent to *Hox* gene knockout.

TABLE 5. Overexpression of *HOXA10* is not permissive for lymphoid pre-B colony formation^a

Mouse group (<i>n</i>)	No. of pre-B progenitors (10 ³)/femur	% G418 ^r pre-B progenitors
<i>neo</i> (5)	10 ± 5.5	43 ± 10
<i>HOXA10</i> (5)	5.5 ± 4	4 ± 4.5 ^b

^a Results are expressed as means ± standard deviations of the number and percentage of G418^r colonies generated from bone marrow of *neo* and *HOXA10* mice 8 and 14 weeks posttransplantation.

^b Significantly less than in *neo* control mice (*P* < 0.0005).

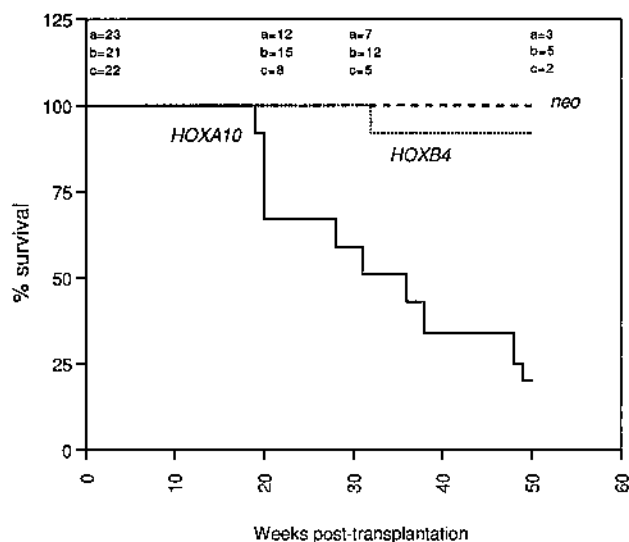


FIG. 7. Survival of *HOXA10* mice compared with that of *neo* control mice and mice transplanted with *HOXB4*-transduced bone marrow cells determined with Kaplan-Meier curves. Survival at various time points posttransplantation was calculated on the basis of the size of each group of mice when an animal died or became terminally ill. The size of each group at various time points posttransplantation is shown in the upper part of the graph, where a, b, and c represent the numbers of *HOXA10*, *HOXB4*, and *neo* mice, respectively. Survival of *HOXA10* mice was statistically different from that of *neo* control mice and *HOXB4* mice ($P < 0.05$).

Although *HOXA10* expression during megakaryocytic differentiation has not been assessed, its expression during other stages of myeloid differentiation has been analyzed in some detail (13, 26). *HOXA10* expression was found to be highest in

subpopulations of human bone marrow enriched for long-term culture initiating cells (LTC-IC), CFU-GM, and BFU-E, and then downregulated approximately sixfold with progression to bone marrow CD34⁺ cells (26) and undetectable in mature circulating monocytes and granulocytes (13). Thus, the suppression of macrophage colony formation and the appearance of colonies containing immature myeloid blast cells when *HOXA10* is overexpressed suggest that the observed down-regulation of *HOXA10* as myeloid cells mature, is a critical event for normal myeloid differentiation.

The second major effect of overexpression of *HOXA10* was an impairment in early B-cell development as reflected by virtual absence of *HOXA10*-transduced pre-B clonogenic progenitor cells in bone marrow of *HOXA10* mice. Nontransduced B cells in *HOXA10* mice appeared to develop normally and compensated for the absence of transduced B cells; thus the effect of *HOXA10* again appears to be intrinsic to transduced cells. This finding for *HOXA10* contrasts to the increase in pre-B progenitor cells induced by overexpression of *HOXB4* in mice transplanted with *HOXB4*-transduced bone marrow cells (27), thus indicating differential effects of *Hox* proteins on B lymphopoiesis. By using a reverse transcription-PCR-based approach, we could not detect expression of the murine *Hoxa-10* message in fluorescence-activated cell sorter-purified B-cell subpopulations from normal (B6C3)_{F1} mice, representing both early and late stages of B-cell development (28). These results are in agreement with those of earlier studies, in which *HOXA10* expression could not be detected in cell lines of pre-B- or mature B-cell origin nor in leukemic cells from patients with pre-B-cell acute lymphoid leukemia (ALL), B-cell ALL, or B-cell chronic lymphoid leukemia (CLL) (3, 13, 35). The observed block in B-cell development when *HOXA10* is retrovirally overexpressed suggests that *HOXA10* target genes may interfere with normal B-cell development and thus that

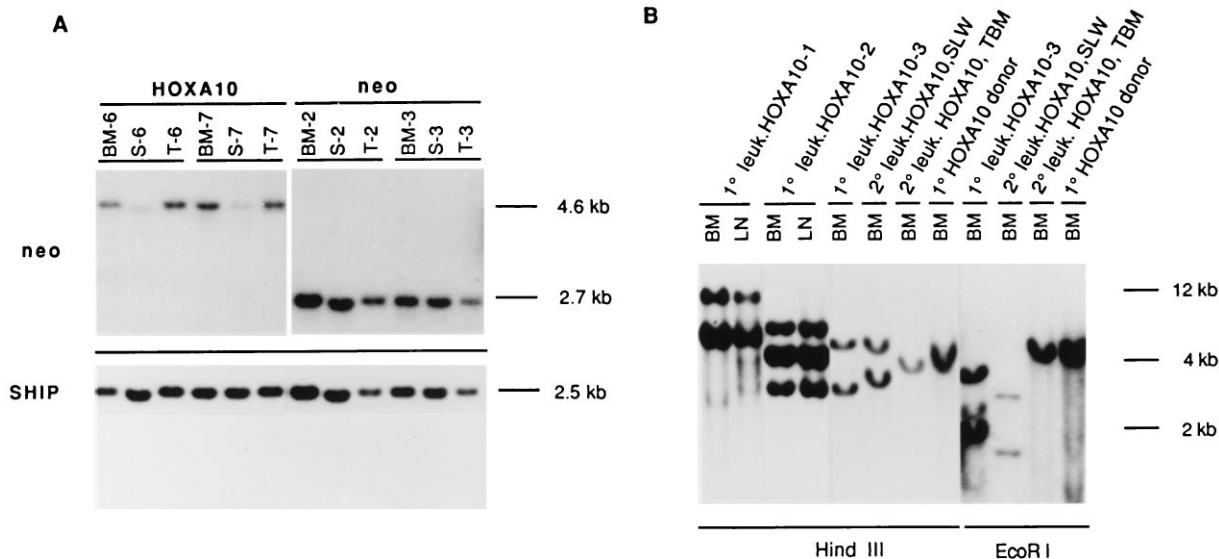


FIG. 8. (A) Southern blot analysis of the intensity of the proviral signal in bone marrow, spleen, and thymus of *HOXA10* and *neo* mice. DNA was digested with *KpnI*, which releases the integrated *HOXA10* (4.6-kb) and *neo* (2.7-kb) proviral fragments. The blot was hybridized to a probe specific for *neo* to detect the proviruses and subsequently to a probe specific for the murine *SHIP* gene as a single-copy gene control for loading. Digitized images of the autoradiograms were obtained by densitometric scanning with a Computing Densitometer (Molecular Dynamics), and the signal intensity for each lane was then analyzed by ImageQuant (4.1). Each number assigned to an individual lane identifies a specific mouse. *HOXA10* and *neo* mice were sacrificed 8 (mice 6 and 3) or 14 weeks posttransplantation. (B) Southern blot analysis of proviral integration sites in DNA isolated from leukemic *HOXA10* mice. DNA was digested with either *HindIII* or *EcoRI*, which each cut the integrated provirus once, generating DNA fragments unique to each integration site. The membranes were hybridized with a probe specific for *neo* to identify proviral fragments. BM, bone marrow; S, spleen; T, thymus; LN, lymph node; leuk., leukemic; 1°, primary mouse; 2°, secondary mouse; SLW, Sca1⁺Lin⁻WGA⁺ bone marrow cells; TBM, total bone marrow.

downregulation of *HOXA10* is important after commitment to the B-cell lineage. Since many *Hox* genes are expressed during hematopoiesis, it is also possible that *HOXA10* is mimicking the overexpression or blocking the action of another *Hox* gene normally involved in B-cell development.

A major consequence of *HOXA10* overexpression in transplanted mice was a high frequency of AML. The relatively long latency period (>19 weeks) and the mono- or biconality of the leukemias suggest that another or other complementing mutations are needed for dominant outgrowth of a single cell. The hallmark of leukemia is a block in the normal differentiation program coupled with unrestrained growth, which leads to clonal expansion of immature blast cells. Like *HOXA10*, overexpression of *HOXB4* clearly leads to expansion of primitive hematopoietic cells as evident by increased progenitor numbers in transplanted mice and enhanced recovery of day 12 CFU-S following in vitro culture. Overexpression of *HOXB4*, however, unlike that of *HOXA10* does not predispose to leukemia. This difference may relate to the absence of *HOXB4*-induced differentiation changes in contrast to the profound effects of *HOXA10* on differentiation. The effects of *HOXA10* are thus reminiscent of that reported for the nuclear oncoprotein *c-myc*, which like *HOXA10*, is normally predominantly expressed in immature hematopoietic cells and is thought to contribute to leukemogenesis by blocking differentiation but maintaining proliferation (33). Perkins et al., using experimental strategies similar to those applied here for *HOXA10*, have shown that *Hoxb-8* also has leukemogenic potential, and at least in some cases, the onset of the leukemias may have been triggered by autocrine growth factor production (23). Interestingly, some of the *HOXA10* leukemic clones were also found to be capable of factor-independent growth. The recently observed coactivation of the *PBX-1*-related *Meis-1* gene with *Hoxa-7* or *Hoxa-9* in myeloid leukemias in BXH-2 mice (20) raises the possibility that the leukemogenic effect of *HOXA10* may also involve *Hox* protein cofactors.

The results presented in this study, together with the extensive literature showing that *HOXA10* expression is largely confined to primitive cells of the myeloid lineage, suggest that under normal physiological conditions, *HOXA10* might be involved in processes of hematopoietic lineage commitment and differentiation, playing a positive role in megakaryopoiesis but negatively regulating monocytic and B-cell development. These results add to the recognition of *Hox* genes as important regulators of hematopoiesis and point to *Hox* gene-specific effects that likely reflect their regulation of different target genes during hematopoietic development. Further resolution of the *Hox* gene code and of the molecular processes that *Hox* genes affect during hematopoietic development remains an important challenge.

ACKNOWLEDGMENTS

We are grateful to Chantal Lacroix for expert assistance with statistical analysis, Patricia Rosten, Gayle Thornbury, and Sofia Rozenfeld for expert technical assistance, and K. A. Ault for the anti-mouse GPIIb/IIIa (CD41) MAAb (D9).

This work was supported by the National Cancer Institute of Canada with funds from the Canadian Cancer Society and the Terry Fox Foundation, the Medical Research Council of Canada, and grants from the Departments of Veterans Affairs (C.L. and H.J.L.). Antisera against *HOXA10* were produced under National Institutes of Health grant N44DK322 to the Berkeley Antibody Company and C.L. U.T. is the recipient of a University of British Columbia Graduate Fellowship, G.S. is the recipient of a Clinician Scientist Fellowship of the Medical Research Council of Canada, and H.J.L. is the recipient of a VA Career Development Award.

U. Thorsteinsdottir and G. Sauvageau contributed equally to this work.

REFERENCES

- Boncinelli, E., D. Acampora, M. Pannese, M. D'Esposito, R. Somna, G. Gaudino, A. Stornaiuolo, M. Cafiero, A. Faiella, and A. Simeone. 1989. Organization of human class I homeobox genes. *Genome* **31**:745-756.
- Borrow, J., A. M. Shearman, V. P. Stanton, Jr., R. Becher, T. Collins, A. J. Williams, I. Dube, F. Katz, Y. L. Kwong, C. Morris, K. Ohyashiki, K. Toyama, J. Rowley, and D. E. Housman. 1996. The t(7;11)(p15;p15) translocation in acute myeloid leukaemia fuses the genes for nucleoporin NUP98 and class 1 homeoprotein HOXA9. *Nature Genet.* **12**:159-167.
- Celetti, A., P. Barba, C. Cillo, B. Rotoli, E. Boncinelli, and M. C. Magli. 1993. Characteristic patterns of HOX gene expression in different types of human leukemia. *Int. J. Cancer* **53**:237-244.
- Damen, J. E., L. Liu, P. Rosten, R. K. Humphries, A. B. Jefferson, P. W. Majerus, and G. Krystal. 1996. The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase. *Proc. Natl. Acad. Sci. USA* **93**:1689-1693.
- Davis, L., M. Kuehl, and J. Battey. 1994. *Basic methods in molecular biology*, p. 277-302. Appleton and Lange, Norwalk, Conn.
- Giampaolo, A., P. Sterpetti, D. Bulgarini, P. Samoggia, E. Pelosi, M. Valtieri, and C. Peschle. 1994. Key functional role and lineage-specific expression of selected HOXB genes in purified hematopoietic progenitor differentiation. *Blood* **84**:3637-3647.
- Hawley, R. G., A. Fong, M. Lu, and T. S. Hawley. 1994. The HOX11 homeobox-containing gene of human leukemia immortalizes murine hematopoietic precursors. *Oncogene* **9**:1-12.
- Hawley, R. G., A. Z. C. Fong, B. F. Burns, and T. S. Hawley. 1992. Transplantable myeloproliferative disease induced in mice by interleukin 6 retrovirus. *J. Exp. Med.* **176**:1149-1163.
- Humphries, R. K., A. C. Eaves, and C. J. Eaves. 1981. Self-renewal of hemopoietic stem cells during mixed colony formation in vitro. *Proc. Natl. Acad. Sci.* **78**:3629.
- Krumlauf, R. 1994. Hox genes in vertebrate development. *Cell* **78**:191-201.
- Kulesa, H., J. Frampton, and T. Graf. 1995. GATA-1 reprograms avian myelomonocytic cells into eosinophils, thromboblats and erythroblast. *Genes Dev.* **9**:1250-1262.
- Lansdorp, P. M., and W. Dragowska. 1992. Long-term erythropoiesis from constant numbers of CD34+ cells in serum-free cultures initiated with highly purified progenitor cells from human bone marrow. *J. Exp. Med.* **175**:1501-1509.
- Lawrence, H. J., G. Sauvageau, N. Ahmadi, A. R. Lopez, M. M. LeBeau, M. Link, R. K. Humphries, and C. Largman. 1995. Stage- and lineage-specific expression of the HOXA10 homeobox gene in normal and leukemic hematopoietic cells. *Exp. Hematol.* **23**:1159-1165.
- Lawrence, H. J., G. Sauvageau, R. K. Humphries, and C. Largman. 1996. The role of HOX homeobox genes in normal and leukemic hematopoiesis. *Stem Cells* **14**:281-291.
- Levine, M., and T. Hoey. 1988. Homeobox proteins as sequence-specific transcription factors. *Cell* **55**:537-540.
- Lowney, P., J. Corral, M. M. LeBeau, L. Deaven, H. J. Lawrence, and C. Largman. 1991. A human Hox 1 homeobox gene exhibits myeloid-specific expression of alternative transcripts in human hematopoietic cells. *Nucleic Acids Res.* **19**:3443-3449.
- Magli, M. C., P. Barba, A. Celetti, G. De Vita, C. Cillo, and E. Boncinelli. 1991. Coordinated regulation of HOX genes in human hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **88**:6348-6352.
- Moretti, P., P. Simmons, P. Thomas, D. Haylock, P. Rathjen, M. Vadas, and R. D'Andrea. 1994. Identification of homeobox genes expressed in human haemopoietic progenitor cells. *Gene* **144**:213-219.
- Nakamura, T., D. A. Largaespada, M. P. Lee, L. A. Johnson, K. Ohyashiki, K. Toyama, S. J. Chen, C. L. Willman, I.-M. Chen, A. P. Feinberg, N. A. Jenkins, N. G. Copeland, and J. D. Shaughnessy, Jr. 1996. Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia. *Nature Genet.* **12**:154-158.
- Nakamura, T., D. A. Largaespada, J. D. Shaughnessy, Jr., N. A. Jenkins, and N. G. Copeland. 1996. Cooperative activation of Hoxa and Pbx1-related genes in murine myeloid leukaemias. *Nature Genet.* **12**:149-153.
- Orkin, S. H. 1995. Transcription factors and hematopoietic development. *J. Biol. Chem.* **270**:4955-4958.
- Pawliuk, R., and R. K. Humphries. Unpublished data.
- Perkins, A. C., and S. Cory. 1993. Conditional immortalization of mouse myelomonocytic, megakaryocytic and mast cell progenitors by the Hox-2.4 homeobox gene. *EMBO J.* **12**:3835-3846.
- Pevny, L., M. C. Simon, E. Robertson, W. H. Klein, S. F. Tsai, V. D'Agati, S. H. Orkin, and F. Costantini. 1991. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* **349**:257-260.
- Rebel, V. I., W. Dragowska, C. J. Eaves, R. K. Humphries, and P. M. Lansdorp. 1994. Amplification of Sca-1+ Lin- WGA+ cells in serum-free

- cultures containing steel factor, interleukin-6, and erythropoietin with maintenance of cells with long-term in vivo reconstituting potential. *Blood* **83**:128–136.
26. Sauvageau, G., P. M. Lansdorp, C. J. Eaves, D. E. Hogge, W. H. Dragowska, D. S. Reid, C. Largman, H. J. Lawrence, and R. K. Humphries. 1994. Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. *Proc. Natl. Acad. Sci.* **91**:12223–12227.
 27. Sauvageau, G., U. Thorsteinsdottir, C. J. Eaves, H. T. Lawrence, C. Largman, P. M. Lansdorp, and R. K. Humphries. 1995. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. *Genes Dev.* **9**:1753–1766.
 28. Sauvageau, G., U. Thorsteinsdottir, and R. K. Humphries. Unpublished data.
 29. Shivasani, R. A., E. L. Mayer, and S. H. Orkin. 1995. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature* **373**:432–434.
 30. Shivasani, R. A., M. F. Rosenblatt, D. Zucker-Franklin, C. W. Jackson, P. Hunt, C. J. M. Saris, and S. H. Orkin. 1995. Transcription factor NF-E2 is required for platelet formation independent of the action of thrombopoietin/MGDF in megakaryocyte development. *Cell* **81**:695–704.
 31. Takeshita, K., J. A. Bollekens, N. Hijiya, M. Ratajczak, F. H. Ruddle, and A. M. Gewirtz. 1993. A homeobox gene of the Antennapedia class is required for human adult erythropoiesis. *Proc. Natl. Acad. Sci. USA* **90**:3535–3538.
 32. Thomas, K. R., and M. R. Capecchi. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**:503.
 33. Thompson, M. A., and R. G. Ramsay. 1995. Myb: an old oncoprotein with new roles. *BioEssays* **17**:341–350.
 34. Tsai, F. Y., G. Keller, F. C. Kuo, M. Weiss, J. Chen, M. Rosenblatt, F. W. Alt, and S. H. Orkin. 1994. An early haematopoietic defect in mice lacking the transcriptional factor GATA-2. *Nature* **371**:221–226.
 35. Vieille, G. I., V. Roullot, and G. Courtois. 1992. Lineage and stage specific expression of HOX 1 genes in the human hematopoietic system. *Biochem. Biophys. Res. Commun.* **183**:1124–1130.
 36. Visvader, J. E., M. Crossley, J. Hill, S. H. Orkin, and J. M. Adams. 1995. The C-terminal zinc finger of GATA-1 and GATA-2 is sufficient to induce megakaryocytic differentiation of an early myeloid cell line. *Mol. Cell. Biol.* **15**:634–641.
 37. Wu, J., J.-Q. Zhu, D.-X. Zhu, G. Scharfman, G. Lamblin, and K.-K. Han. 1992. Selective inhibition of normal murine myelopoiesis in vitro by a Hox 2.3 antisense oligodeoxynucleotide. *Cell. Mol. Biol.* **38**:367–376.