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The Oncoprotein E2A-Pbx1a Collaborates with Hoxa9 To Acutely Transform Primary Bone Marrow Cells

UNNUR THORSTEINSDOTTIR,[1] JANA KROSL,[1] EVERT KROON,[1] ANDRÉ HAMAN,[1]
TRANG HOANG[1,2] AND GUY SAUVAGEAU[1,3,4]*

*Laboratory of Molecular Genetics of Hemopoietic Stem Cells, Clinical Research Institute of Montréal,
Montréal, Québec, Canada H2W 1R7,[1] and Departments of Pharmacology[2] and Medicine[3] and
Division of Hematology Maisonneuve-Rosemont Hospital,[4] Université de Montréal,
Montréal, Québec, Canada H3C 3J7*

A recurrent translocation between chromosome 1 (*Pbx1*) and 19 (*E2A*) leading to the expression of the E2A-Pbx1 fusion oncoprotein occurs in ~5 to 10% of acute leukemias in humans. It has been proposed that some of the oncogenic potential of E2A-Pbx1 could be mediated through heterocomplex formation with Hox proteins, which are also involved in human and mouse leukemias. To directly test this possibility, mouse bone marrow cells were engineered by retroviral gene transfer to overexpress *E2A-Pbx1a* together with *Hoxa9*. The results obtained demonstrated a strong synergistic interaction between *E2A-Pbx1a* and *Hoxa9* in inducing growth factor-independent proliferation of transduced bone marrow cells in vitro and leukemic growth in vivo in only 39 ± 2 days. The leukemic blasts which coexpress *E2A-Pbx1a* and *Hoxa9* showed little differentiation and produced cytokines such as interleukin-3, granulocyte colony-stimulating factor, and Steel. Together, these studies demonstrate that the Hoxa9 and E2A-Pbx1a gene products collaborate to produce a highly aggressive acute leukemic disease.

Homeodomain (HD)-containing *Hox* gene products, regulators of pattern formation and tissue identity during embryogenesis (15), have also been identified as potential regulators of hemopoietic cell proliferation and differentiation (17). Several lines of evidence now also directly implicate *Hox* genes in human and murine leukemias. These include (i) the expression of Hoxa9 as a fusion protein Hoxa9-NUP98, in a subset of human myeloid leukemias (2, 21); (ii) the activation of *Hoxa7* and *Hoxa9* by retroviral insertional mutagenesis in myeloid leukemias in BXH-2 mice (22), and (iii) the development of leukemias in mice transplanted with bone marrow cells engineered to retrovirally overexpress *Hoxb8*, *Hoxb3*, *Hoxa10*, or *Hoxa9* (13, 25, 32, 39).

A number of studies have demonstrated that Hox proteins collaborate in the in vitro DNA binding with a group of HD-containing proteins comprising Pbx and Meis families (34–36). This cooperative interaction between Pbx and Hox proteins is important since genetic and molecular studies in mice and in *Drosophila* have shown that Pbx1 (or its *Drosophila* homolog exd) is required for some of the biological functions of Hox proteins (1, 4, 19, 29, 30). The relevance of this Hox-Pbx interaction for malignant transformation has been demonstrated, as we recently showed that *Hoxb3*- or *Hoxb4*-induced transformation of Rat-1 fibroblasts is dependent on the presence of endogenous Pbx1 (14). An oncogenic collaboration between Hox and Meis proteins has also been established both by proviral insertion (22) and by retroviral overexpression studies (13). The Hox-Pbx interacting surfaces have been the focus of a number of studies and include, in addition to the HDs of both proteins, a tryptophan-containing motif located N terminal to the HD of Hox (found in several Hox proteins) and a region of 20 to 25 conserved amino acids called HCM (Hox

cooperativity motif) located C terminal to the HD of Pbx1 (6, 24, 26, 27). The structure of the Hoxb1-Pbx1 complex bound to DNA was recently solved by crystallographic studies and confirmed the importance of these motives for Hox-Pbx interactions (28). Interestingly, these studies also demonstrated that the HCM motif of Pbx1 is part of its HD giving rise to a fourth α helix (28).

One member of the Pbx family, Pbx1, is also involved in human malignancy. In the t(1;19)(q23;p13.3) chromosomal translocation (11, 23), found in 10 to 20% of human pediatric pre-B acute lymphoblastic leukemias (3), most of the *Pbx1* coding sequence, including the segment encoding the HD, is fused to the 5′ half of the *E2A* gene, which encodes two transcription activation domains but lacks both the DNA binding and dimerization domains of *E2A* (11, 23). In addition to the involvement of the *E2A-Pbx1* fusion gene in human acute lymphoblastic leukemia, various transformation assays have clearly demonstrated that the E2A-Pbx1 fusion protein is oncogenic. Transgenic mice expressing the *E2A-Pbx1* cDNA in lymphoid cells developed T-cell lymphoblastic lymphomas (8), and mice reconstituted with bone marrow cells engineered by retrovirus-mediated gene transfer to overexpress *E2A-Pbx1* developed growth factor-dependent acute myeloid leukemias (AML) (10).

The in vitro cooperative DNA binding properties of Pbx1 and E2A-Pbx1 with Hox proteins are not significantly different (18). This has lead to one current hypothesis, that at least in part, the transforming capacity of E2A-Pbx1 could be mediated by Hox gene products. In agreement with this possibility, deletion of all of the Pbx1 sequence from the E2A-Pbx1 fusion protein completely abrogates transformation of NIH 3T3 cells, indicating that the Pbx1 half of the fusion protein is essential for its transforming abilities (12, 20). Furthermore, the HCM of the Pbx1 half of the E2A-Pbx1 fusion protein is essential for cellular transformation induced by E2A-Pbx1 (5). This finding is very interesting in light of the recent crystallographic studies mentioned above which have redefined the HCM as part of an

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* Corresponding author. Mailing address: Institut de Recherches Cliniques de Montréal, 110 Pine Ave. West, Montréal, Québec, Canada H2W 1R7. Phone: 514-987-5797. Fax: 514-987-5718. E-mail: sauvagg@ircm.qc.ca.

extended HD in Pbx1 (28). The concept that E2A-Pbx1-induced transformation is Hox dependent was challenged by studies which showed that the helices 1 to 3 of the HD in E2A-Pbx1 are dispensable for the capacity of this fusion protein to transform NIH 3T3 cells and T lymphocytes (12, 20). However, it was recently shown that the HD of E2A-Pbx1 is necessary to block cellular differentiation of myeloid progenitor cells, a process central to leukemic transformation (12). Together, these studies thus suggest that cellular transformation induced by *E2A-Pbx1* may involve more than one pathway (mechanisms).

In contrast to E2A-Pbx1, the Pbx proteins lack inherent transforming potential (12–14, 20), and fusion with E2A is essential for the transforming ability of E2A-Pbx1. Structure-function and mutagenesis experiments have demonstrated that the two transcriptional activation domains in E2A are essential for mediating both the malignant transformation of NIH 3T3 cells (20) and blocking the differentiation of myeloid progenitor cells (12). Furthermore, using a Pbx-responsive sequence which allows cooperative DNA binding between Hox and Pbx1 (or E2A-Pbx1), it was shown in a reporter assay that E2A-Pbx1, but not Pbx1, could induce significant transcriptional activity of the reporter gene and that Hox proteins had the capacity to modulate the transactivating activity of E2A-Pbx1 (18). Together, these observations suggest that the oncogenic potential of E2A-Pbx1 is directly linked to the transcriptional activating function of the chimeric protein, and that this activity can be modulated by Hox gene products.

To examine whether Hox proteins and the E2A-Pbx1a fusion protein could collaborate to transform primary hematopoietic cells, we engineered mouse bone marrow cells, by retroviral gene transfer, to cooverexpress *E2A-Pbx1a* with *Hoxa9*, and the oncogenic collaboration between these two genes was directly tested in vitro and in vivo following transplantation of retrovirally transduced cells.

MATERIALS AND METHODS

Animals. All mice were originally bought from The Jackson Laboratory (Bar Harbor, Maine) and then bred and maintained in the specific-pathogen-free animal facility of the Clinical Research Institute of Montreal (IRCM). Donors of primary bone marrow cells were over 12-week-old (C57BL/6Ly-Pep3b × C3H/HeJ)F_1 [(PepC3)F_1] mice, and recipients were 7- to 12-week-old (C57BL/6J × C3H/HeJ)F_1 [(B6C3)F_1] mice. All animals were housed in ventilated microisolator cages and provided with sterilized food and acidified water.

Generation of recombinant retroviruses. The MSCV-Hoxa9-pgk-neo retroviral vector was described before (13). The human *E2A-Pbx1a* cDNA (a kind gift of Mark Kamps, San Diego, Calif.) was subcloned into the *Eco*RI site of MSCV-pgk-PAC retroviral vector (which confers puromycin resistance). Both MSCV vectors were kindly provided by R. Hawley (The Toronto Hospital Research Institute, Toronto, Ontario, Canada). High-titer helper-free retroviral producer cells were generated from GP+E-86 and BOSC-23 viral packaging cells and tested as reported previously (13).

Retroviral infection of primary murine bone marrow cells. Bone marrow cells obtained from (PebC3)F_1 mice injected 4 days earlier with 5-fluorouracil (50 mg/kg of body weight; Sigma) were prestimulated in the presence of interleukin-6 (IL-6), IL-3, and Steel factor and cocultivated on GP+E-86 viral producer cells engineered to produce the MSCV-Hoxa9-pgk-neo or the MSCV-E2A-Pbx1a-pgk-puro recombinant retrovirus. The procedure for cell harvesting, prestimulation, cocultivation, and bone marrow transplantation were performed as described elsewhere (13, 31). For double infections of bone marrow cells with *Hoxa9* and *E2A-Pbx1a*, the respective viral producer cells were counted and seeded at equal numbers 24 h prior to the addition of bone marrow cells. Cells were cocultured for 48 h, with a medium change after 24 h. In an effort to enhance the efficiency of retroviral transduction of bone marrow cells, viral supernatants from *Hoxa9*- and *E2A-Pbx1a*-transfected BOSC-23 cells were added to the respective cocultures. The gene transfer efficiencies to primitive hemopoietic cells, as assessed by the in vitro colony formation of G418-resistant (containing the *neo* control or *Hoxa9* retrovirus) or puromycin-resistant (containing the *puro* control or *E2A-Pbx1a* retrovirus) myeloid clonogenic progenitors, were 50, 40, 72, and 20% for the *neo*, *puro*, *Hoxa9*, and *E2A-Pbx1a* retroviruses, respectively. The efficiency of double infection of myeloid progenitor cells with the *Hoxa9* and *E2A-Pbx1a* retroviruses was 4% as assessed by resistance of progenitor cells to both G418 and puromycin. All growth factors were used as diluted supernatants from appropriately transfected COS cells, as prepared at the IRCM. All reagents used in this study including media and serum were purchased at GIBCO Life Technologies.

Transplantations. The primary mice used in this study were generated by injecting intravenously into lethally irradiated (900 cGy, 179 cGy/min, ^{137}Cs gamma rays; J. L. Shepherd, San Fernando, Calif.) 7- to 12-week-old (B6C3)F_1 mice 2×10^5 bone marrow cells derived from the (PepC3)F_1 mice immediately following their harvesting from the cocultivation with viral producer cells. To determine the transplantability of the diseases that developed in some of the primary mice, lethally irradiated or nonirradiated secondary (B6C3)F_1 recipients were injected with 10^6 bone marrow and/or spleen cells obtained from the primary mice. For some of the leukemias that developed in the *Hoxa9 + E2A-Pbx1a* mice, the frequency of the leukemia repopulating cells (LRC) in bone marrow of these mice was determined by injecting various numbers of bone marrow cells (5 to 10^6 cells per mouse) into lethally irradiated (B6C3)F_1 mice, along with a life-sparing dose of 10^5 normal bone marrow cells derived from (B6C3)F_1 mice. LRC frequency in primary recipients was estimated by applying Poisson statistics to the proportion of negative recipients at different dilutions as described previously (37).

In vitro cultures. For myeloid clonogenic progenitor assays, cells were plated on 35-mm-diameter petri dishes (Corning, Fisher) in a 1.1 ml culture mixture containing 0.8% methylcellulose in alpha medium supplemented with 10% fetal calf serum, 5.7% bovine serum albumin, 10^{-5} M β-mercaptoethanol, 1 U of human urinary erythropoietin (Epo) per ml, 10% WEHI-conditioned medium (tested to contain 50 ng of IL-3 per ml), 2 mM glutamine, and 200 mg of transferrin per ml, in the presence or absence of 1.3 mg of G418 per ml and/or 1.5 μg of puromycin per ml. To test the ability of the retrovirally infected myeloid progenitor cells to form colonies in the absence of added growth factors, cells were plated in the above-described methylcellulose medium lacking both WEHI-conditioned medium and Epo. Bone marrow cells harvested from the cocultivation with virus-producing cells or recovered from reconstituted mice were plated at a concentration of 2×10^3 to 8×10^3 or 3×10^4 cells/ml, respectively. Spleen cells from *neo* control mice were plated at a concentration of 3×10^6 cells/ml, whereas those of the other reconstituted mice were plated at 10^5 cells/ml. Bone marrow or spleen cells from the reconstituted mice were plated at these same concentrations in the methylcellulose cultures lacking WEHI-conditioned medium and Epo. Colonies were scored on days 12 to 14 of incubation as derived from CFU–granulocyte-macrophage, burst-forming unit erythroid, or granulocyte-erythrocyte-macrophage-megakaryocyte CFU according to standard criteria (9). For some of the experiments, identification of colony types was confirmed by Wright staining of cytospin preparations of colonies. Bone marrow and/or spleen cells from the transplanted recipients were cultured in liquid cultures of Iscove's medium containing 10% fetal calf serum, 10^{-5} M β-mercaptoethanol, 2 mM glutamine, and 200 mg of transferrin per ml, in the presence or absence of 5 ng of IL-3 or 0.5 ng of granulocyte-macrophage colony-stimulating factor (GM-CSF) per ml.

Titration of growth factors. The presence of bioactive IL-3, G-CSF, and Steel present in conditioned medium obtained from 4-day-old cultures of cells obtained from the spleens of various mice and seeded at 10^6 cells/ml was tested by [^{3}H]thymidine incorporation assay to measure the proliferation of Ba/F3 (IL-3-responsive), NFS-60 (G-CSF- and IL-3-responsive) and TF-1 (Steel-responsive) cells. Titration of the bioactive substances was performed with COS cell-derived IL-3, G-CSF, and Steel as described above. The specificity of each growth factor to induce [^{3}H]thymidine incorporation into the DNA of the appropriate cell line was verified by using antisera specific to IL-3 (50% neutralizing dose [ND$_{50}$] = 0.0015 to 0.025 μg/ml), G-CSF (ND$_{50}$ = 0.4 to 0.8 μg/ml), and GM-CSF (ND$_{50}$ = 0.05 to 0.15 μg/ml) (all from R&D). Complete and specific neutralization of [^{3}H]thymidine incorporation was obtained for each supernatant tested except for that derived from 4.27 cells, where antibodies to both IL-3 and G-CSF were required for complete inhibition of proliferation of NFS-60 cells.

DNA and RNA analyses. To assess proviral integration, Southern hybridization analyses were performed as described elsewhere (13). Total cellular RNA was isolated with the TRIzol reagent, and Northern blot analysis was performed as described previously (7). The probes used were an *Xho*I/*Sal*I fragment of pMC1*neo* (*neo*) (38), a *Hind*III/*Cla*I fragment of MSCV-pgk-PAC (*puro*), or the full-length 1.4-kb *Hoxa9* and 2.9-kb *E2A-Pbx1a* cDNAs and were labeled with ^{32}P by random primer extension as described elsewhere (16). To assess the relative amounts of total RNA loaded, membranes were probed for 18S RNA by using end-labeled oligonucleotide 5'- ACG GTA TCT GAT CGT CCT CGA ACC-3'.

Nuclear extract preparation. A total of 2×10^7 cells were washed twice in phosphate-buffered saline, incubated for 10 min at 0°C in 600 μl of lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA), and then disrupted by passage through a 26-guage needle. Nuclei were collected by centrifugation at 500 rpm for 5 min and resuspended in 60 μl of extraction buffer (20 mM HEPES [pH 7.9], 400 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 5% glycerol) to allow elution of nuclear proteins by vigorous shaking for 1 h at 4°C. Nuclei were spun down by 2 min of centrifugation at 12,000 rpm, and the recovered supernatant was further cleared by 20 min of centrifugation at 40,000 rpm at 4°C. The protease inhibitors phenylmethylsulfonyl fluoride (2 mM), aprotinin (10 μg/ml), leupeptin (1 μg/ml), pepstatin A (10 μg/ml), and antipain (10 μg/ml) were added to both lysis and extraction buffers. Protein concentration
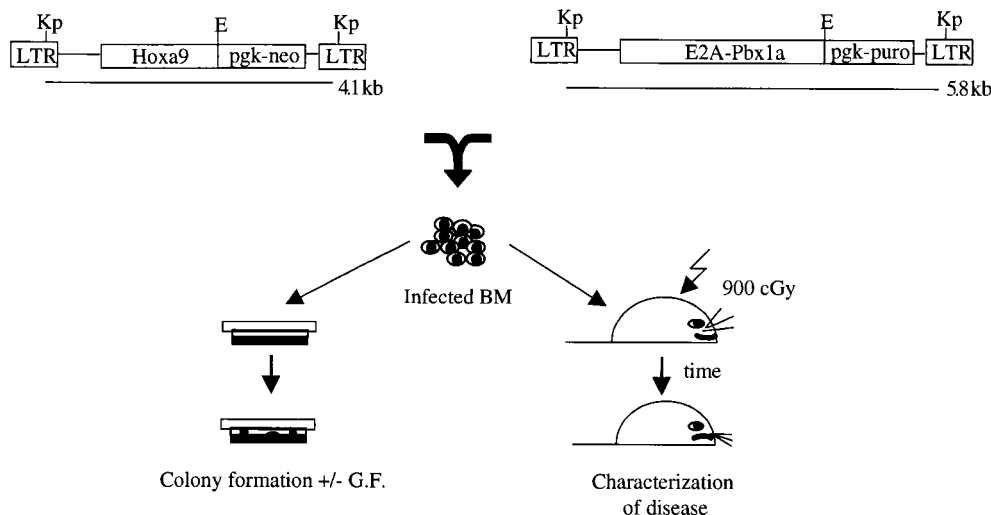
FIG. 1. Structures of the retroviruses and overview of the experimental strategy used in this study. Diagrammatic representation of the integrated *Hoxa9* and *E2A-Pbx1a* proviruses and the experiments described in this study. The expected sizes of the full-length viral transcripts are shown. Restriction sites indicated are *Kpn*I (Kp) and *Eco*RI (E). LTR, long terminal repeat. G.F., growth factor.

was determined by the MicroBCA assay as recommended by manufacturer (Sigma, St. Louis, Mo.).

Western blot analysis. Thirty-microgram aliquots of proteins separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis were transferred to Immobilon P membranes (Millipore, Bedford, Mass.). Membranes were blocked with 5% nonfat milk in TBST (20 mM Tris-Cl [pH 7.6], 140 mM NaCl, 0.05% Tween 20) and then subjected to Western blot analysis with anti-E2A (E47; Santa Cruz Biotechnology Inc, Santa Cruz, Calif.) and anti-Hoxa9 (generously provided by H. Jeffrey Lawrence, San Francisco, Calif.). Bound antibodies were detected by using horseradish peroxidase-conjugated anti-rabbit antibody (Sigma) followed by enhanced chemiluminescence (Amersham, Buckinghamshire, England).

RESULTS

Altered colony formation and growth factor dependence of myeloid progenitor cells overexpressing *Hoxa9* together with *E2A-Pbx1a*. The *Hoxa9* and *E2A-Pbx1a* cDNAs were introduced downstream of the long terminal repeat of either the MSCVneoEB (*Hoxa9*) or MSCVpac (*E2A-Pbx1a*) retroviral vector (Fig. 1). Immediately following the infection of mouse bone marrow cells with the *Hoxa9*, *E2A-Pbx1a*, or *Hoxa9* and *E2A-Pbx1a* retroviruses, the frequency of transduced myeloid colony-forming cells (CFC) was determined in methylcellulose cultures in the presence and absence of hematopoietic growth factors (Fig. 2A and B).

The in vitro growth of CFC engineered to overexpress *Hoxa9* or *E2A-Pbx1a* alone remained dependent on the presence of added growth factors (Fig. 2A and B), whereas ~20% of the bone marrow CFC which cooverexpressed both *Hoxa9* and *E2A-Pbx1a* were capable of colony formation in the absence of added growth factors, thus demonstrating the collaboration between these two genes in inducing autonomous growth in vitro (Fig. 2B).

In the presence of added growth factors, transduced (i.e., drug-resistant) CFC overexpressing *Hoxa9* or *E2A-Pbx1a* alone were capable of generating all types of colonies normally present in such cultures, as revealed by cytological examinations of Wright-stained G418- or puromycin-resistant colonies (Fig. 2C). In contrast, about half of the CFC transduced with *Hoxa9* and *E2A-Pbx1a* generated colonies which contained only immature blast cells, a colony type rarely detected in control cultures (Fig. 2C). The generation of these blast colonies was accompanied by the absence of primitive GEMM (myeloid-erythroid) colonies, whereas the proportion GM and M (myeloid) colonies were within normal range (Fig. 2C), indicating that the cooverexpression of *Hoxa9* and *E2A-Pbx1a* can inhibit the in vitro differentiation of primitive (i.e., CFU-GEMM) but not mature (i.e., CFU-GM or CFU-M) CFC. Together, these data suggest that the cooverexpression of *Hoxa9* and *E2A-Pbx1* could be sufficient to directly transform

TABLE 1. Absolute numbers of myeloid CFC and LTRC resistant to G418 (transduced with *neo* or *Hoxa9*) and/or puromycin (*puro* or *E2A-Pbx1a*) transplanted per mouse

Virus	No. of mice transplanted	No. of transduced CFC injected/mouse[a]			Estimated no. of transduced LTRC injected/mouse[b]		
		G418^r	Puro^r	G418^r Puro^r	G418^r	Puro^r	G418^r Puro^r
neo	6	2,100			21		
puro	4		2,340			23	
Hoxa9	7	2,232			22		
E2A-Pbx1a	6		650			7	
Hoxa9 + E2A-Pbx1a	6	1,764	954	252	18	10	3

[a] Determined as follows: (number of bone marrow cells injected per mouse) × (CFC frequency in injected bone marrow inoculum) × (percentage of CFC resistant to puromycin and/or G418).
[b] Estimated based on our previous results that determined the frequency of LTRC at 1 per 100 CFC (31) and estimation of gene transfer to LTRC equal to that of CFC (40).
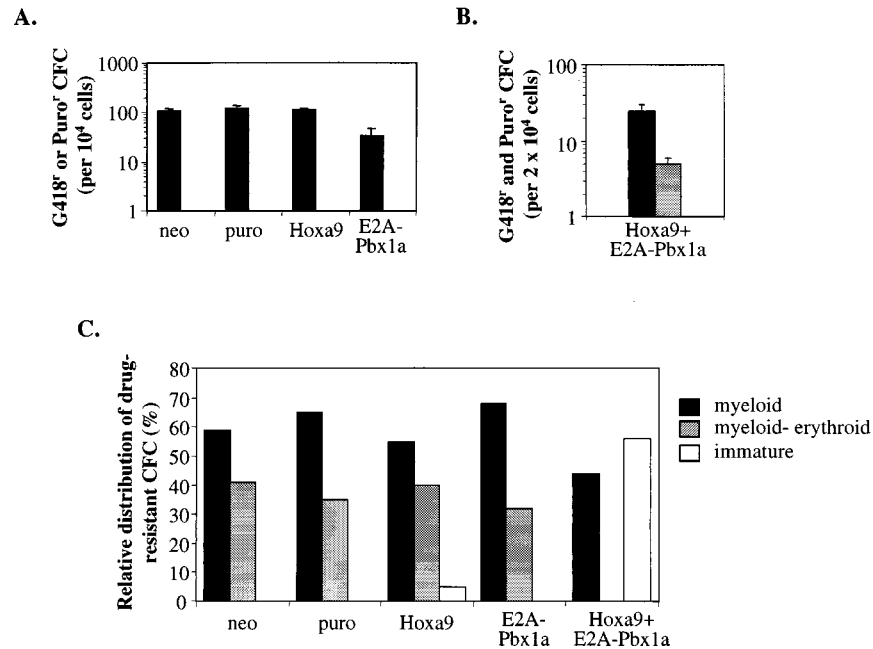
A.

B.

C.

FIG. 2. Absence of differentiation and cytokine-independent growth in vitro characterizes myeloid CFC overexpressing both *Hoxa9* and *E2A-Pbx1a*. (A) Total number of G418- or puromycin-resistant colonies generated per 10^4 bone marrow cells immediately following infection with *neo* control, *puro* control, *E2A-Pbx1a*, and *Hoxa9* retroviruses, in the presence of added growth factors. No colonies grew in the absence of added growth factors. (B) Total number of G418- and puromycin-resistant colonies generated from 2×10^4 bone marrow cells immediately following infection with *Hoxa9* and *E2A-Pbx1a* retroviruses, in the presence (black bars) and absence (gray bars) of added growth factors. Results shown in panels A and B represent the mean ± standard deviation of the number of G418 (*neo* and *Hoxa9*)- and/or puromycin (*puro* and *E2A-Pbx1a*)-resistant colonies detected on four different plates. (C) Well-isolated G418-, puromycin- or G418- and puromycin-resistant colonies were randomly picked (n = 16 to 20 for each infection type, from two different plates) on days 10 to 12 of incubation and examined by Wright staining. Colony types detected were classified as myeloid (mainly containing granulocytes and/or macrophages), myeloid-erythroid (containing granulocytes, macrophages, megakaryocytes, and erythrocytes), and immature (containing undifferentiated blast-like cells).

bone marrow cells by inducing autonomous, cytokine-independent proliferation and simultaneously suppressing differentiation of primitive CFCs.

Cooverexpression of *Hoxa9* with *E2A-Pbx1a* is acutely transforming. The capacity of *Hoxa9*- and *E2A-Pbx1a*-transduced bone marrow cells to induce acute leukemia in vivo was directly tested by transplanting *Hoxa9*- and *E2A-Pbx1a*-transduced bone marrow cells into lethally irradiated syngenic mice. In addition to the *neo* and *puro* controls, mice were also transplanted with bone marrow cells engineered to overexpress *Hoxa9* or *E2A-Pbx1a* alone. To facilitate comparison of the dose of transduced cells transplanted in each group, the number of transduced myeloid progenitor cells (G418 and/or puro-

mycin resistant) and the estimated number of long-term repopulating cells (LTRC) that were injected per mouse are shown in Table 1.

All recipients of *Hoxa9*- and *E2A-Pbx1a*-transduced bone marrow cells (*Hoxa9* + *E2A-Pbx1a* mice) developed acute leukemia in 39 ± 2 days (Table 2). In contrast, when overexpressed alone, neither *Hoxa9* nor *E2A-Pbx1a* was acutely transforming. In fact, it took between 80 and 167 days for leukemia to developed in these recipients (Table 2; see also below).

At the time of sacrifice, the *Hoxa9* + *E2A-Pbx1a* mice were runted, were short of breath, and had very high (>50,000/μl) peripheral leukocyte counts (Fig. 3). In these mice, bone marrow, spleen, and lymph nodes as well as liver, lungs, and brain

TABLE 2. Characteristics of diseases that developed in primary recipients[a]

Mouse group (n)	Time of death or sacrifice (days post-Tx)[b]	No. with		No. of NC (10^6)/femur (% BM blast)[b]	Spleen wt (g)[b]	Clonality	Disease
		HL paralyses	PBL blastosis				
neo control (4)	56[c]	0	0	19.0 ± 4 (<1)	0.1 ± 0.01	Oligo/poly	NOD
Hoxa9 nonleukemic (3)	56[c]	0	0	19.0 ± 5 (<1)	0.13 ± 0.01	Oligo/poly	NOD
Hoxa9 leukemic (4)	167 ± 32[d]	0	4	22.5 ± 5 (70 ± 20)	0.6 ± 0.1	Mono/bi	AML
E2A-Pbx1a (6)	51 ± 3[e]	6	0	7.0 ± 2 (25 ± 8)	0.7 ± 0.3	Poly	MPS
Hoxa9 + *E2A-Pbx1a* (6)	39 ± 2	0	6	17.0 ± 8 (90 ± 5)	0.8 ± 0.3	Oligo	AML

[a] Abbreviations: Tx, transplantation; HL, hind leg; PBL, peripheral blood; NC, nucleated cells; NOD, no observed disease; AML, acute myeloid leukemia; MPS, myeloproliferative disorder.

[b] Results are expressed as mean values ± SD.

[c] The *neo* control and *Hoxa9* nonleukemic mice were healthy at the time of sacrifice. Additional data for the three nonleukemic *Hoxa9* mice (mice 1 to 3) sacrificed 56 days after transplantation are presented in Fig. 3, 6A and B, and 7B.

[d] Sacrificed when presenting with acute leukemia (mice 4 to 7 in Fig. 4A and B and 7B).

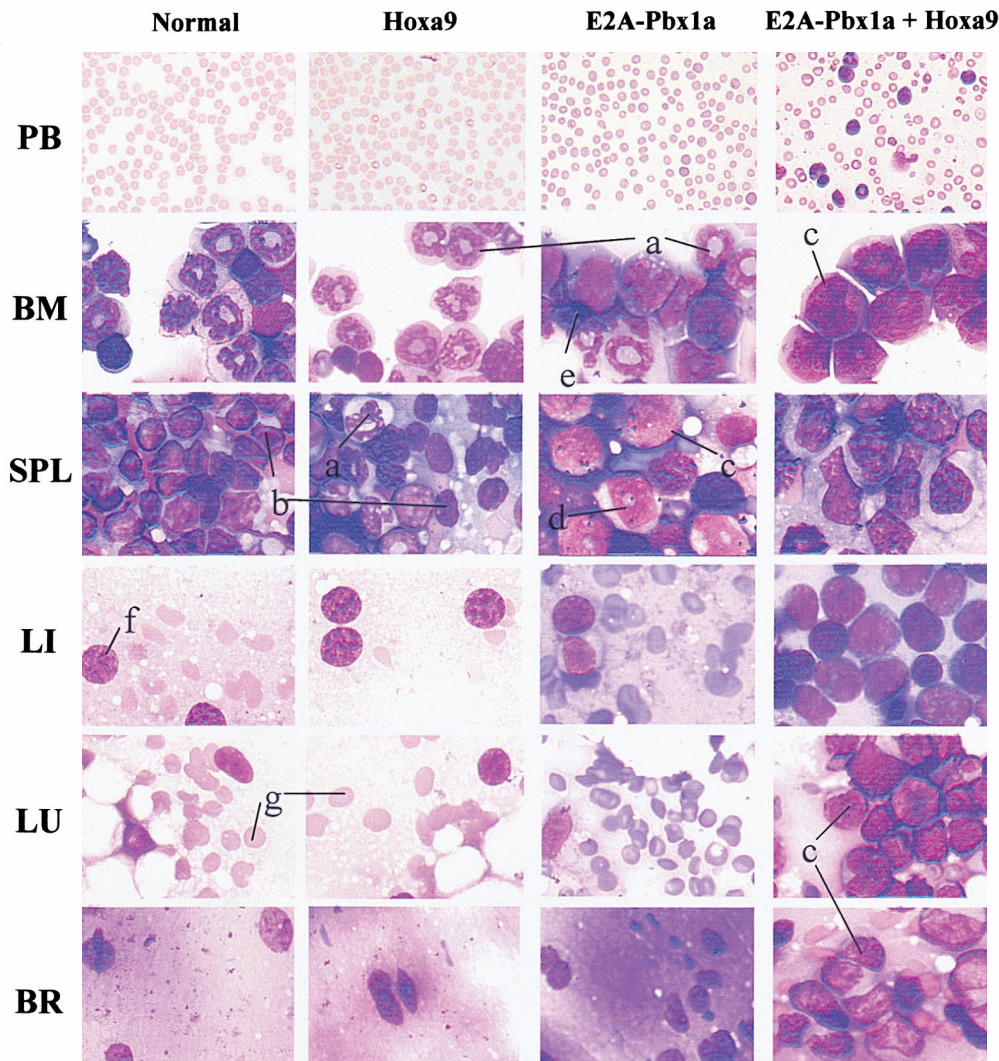[e] Sacrificed when presenting with hind leg paralyses.

FIG. 3. Cytopathological examination of hematopoietic and nonhematopoietic tissues from *neo*, *Hoxa9*, *E2A-Pbx1a*, and *Hoxa9 + E2A-Pbx1a* mice after Wright staining of peripheral blood smears (PB), bone marrow (BM) cytospins, and touch preparations of spleen (SPL), lung (LU), liver (LI), and brain (BR) tissues from representative *neo* control, *E2A-Pbx1a*, nonleukemic *Hoxa9*, and *Hoxa9 + E2A-Pbx1a* mice described in Table 2. Note the infiltration by immature blast cells in all six tissues of the *Hoxa9 + E2A-Pbx1a* mouse. In contrast, the spleen is the only tissue in the *E2A-Pbx1a* mouse that is infiltrated by such a cell type in addition to immature granulocytic cells. An increase in immature granulocytic cells is also detected in the spleen of the *Hoxa9* mouse. Magnification, ×100 for all except the peripheral blood (×20). a, granulocyte; b, lymphocyte; c, blast; d, immature granulocyte; e, erythroblast; f, hepatocyte; g, erythrocyte.

were highly infiltrated with leukemic blasts (Fig. 3 and data not shown). Morphological (Fig. 3), histochemical, and fluorescence-activated cell sorting (FACS) analyses showed that 30 to 40% of the leukemic blasts were of myeloid origin, with moderate Sudan black staining (myeloid), and coexpressed low levels of Mac-1 and Gr-1 (data not shown). None (<1%) of these leukemic cells expressed B- or T-cell markers such as B220, CD4, or CD8, and these cells were also negative for periodic acid-Schiff reaction characteristic of lymphoblastic leukemia (data not shown). Thus, each of the leukemias in *Hoxa9 + E2A-Pbx1a* mice had the capacity for partial myeloid differentiation, with a significant proportion of the blasts (60 to 70%) remaining uncommitted to either the myeloid or lymphoid lineage.

Southern blot analysis of DNA isolated from leukemic cells present in the bone marrow or spleen of the *Hoxa9 + E2A-Pbx1a* mice verified the presence of the *Hoxa9* (4.1-kb) and *E2A-Pbx1a* (5.8-kb) proviruses in these cells (Fig. 4A, top pan-

el). Furthermore, Northern blot analysis of total RNA from these same tissues detected high levels of the retrovirally derived *Hoxa9* and *E2A-Pbx1a* messages in all of these mice (Fig. 4B, top panel).

Analysis of secondary recipients of leukemic bone marrow cells derived from *Hoxa9 + E2A-Pbx1a* mice. The acute leukemia induced by cooverexpression of *Hoxa9* and *E2A-Pbx1a* was readily transplantable to secondary recipients, and clonal analysis of proviral integration sites demonstrated that the leukemias were derived from leukemic clone(s) present in the original donor leukemia (Fig. 5A). To determine the frequency of the biologically relevant cell in *Hoxa9 + E2A-Pbx1a* mice that can generate leukemia in secondary recipients (the leukemia repopulating cell [LRC]), bone marrow cells from *Hoxa9 + E2A-Pbx1a* mice 4 and 5 were injected at limiting dilution into lethally irradiated secondary recipients, along with a radioprotective dose of normal bone marrow cells. The frequency of the LRC was estimated at 1 in 124 and 1 in 60
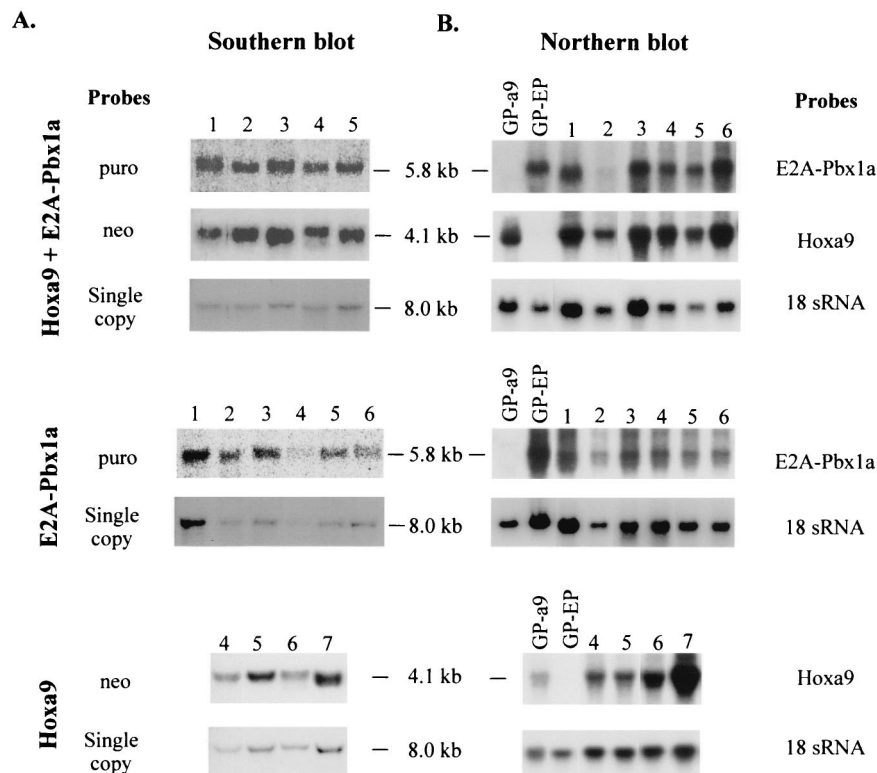
FIG. 4. Demonstration of the presence and expression of the integrated *Hoxa9* and *E2A-Pbx1a* proviruses in primary mice. (A) Southern blot analyses of genomic DNA isolated from the bone marrow or spleen of the primary *Hoxa9* + *E2A-Pbx1a* (top), *E2A-Pbx1a* (middle), and *Hoxa9* (bottom) mice described in Table 2. DNA was digested with *Kpn*I to release the integrated *E2A-Pbx1a* (5.8-kb) or *Hoxa9* (4.1-kb) proviral fragment. The membranes were hybridized with a *neo*-specific probe to detect the *Hoxa9* provirus and a *puro*-specific probe to detect the *E2A-Pbx1a* provirus. To provide a single-copy control of loading, the membranes were subsequently probed with full-length *Hoxa9* cDNA probe. Note that the signal shown for the 8.0-kb single copy is that of endogenous *Hoxa9*. (B) Northern blot analysis of total RNA (10 μg) isolated from bone marrow or spleen cells of the primary *Hoxa9* + *E2A-Pbx1a* (top), *E2A-Pbx1a* (middle), and *Hoxa9* (bottom) mice described in Table 2. The membranes were hybridized with full-length *E2A-Pbx1a* or *Hoxa9* cDNA probe. Each number assigned to a lane in panels A and B identifies a specific primary *Hoxa9*, *E2A-Pbx1a*, or *Hoxa9* + *E2A-Pbx1a* mouse that is also identified with this same number in Fig. 5 or 7. GP-a9 or -EP, GP+E-86 Hoxa9 or E2A-Pbx1a viral producer cells.

bone marrow cells for *Hoxa9* + *E2A-Pbx1a* mice 4 and 5, respectively (Fig. 5B). Interestingly, these studies also demonstrated a linear correlation between the number of LRC transplanted and the time required for the development of acute leukemia in the secondary recipients (Fig. 5C).

Southern blot analysis of genomic DNA isolated from the leukemic cells of the secondary recipients of *Hoxa9* + *E2A-Pbx1a* mice 4 and 5 confirmed the presence of *Hoxa9* (detected with the *neo* probe)- and *E2A-Pbx1a* (detected with the *puro* probe)-transduced clones in all mice that succumbed to acute leukemia, including those receiving only 60 bone marrow cells, estimated to contain only ~1 LRC (Fig. 5A, mice 4.9, 5.5, and 5.6). In each secondary recipient, the numbers of leukemic clones detected either with the *neo* or the *puro* probe were identical, as shown for clones a, b, and c, which are detected as dominant clones in seven of nine secondary recipients of bone marrow cells from *Hoxa9* + *E2A-Pbx1a* mouse 4 (Fig. 5A). These three clones were, however, not detected in mice 4.8 and 4.9, which were repopulated either by clone d (mouse 4.8), which contains *Hoxa9* provirus at four integration sites and *E2A-Pbx1a* at two, or by clone e (mouse 4.9), which contains *Hoxa9* provirus at two integration sites and *E2A-Pbx1a* at one (Fig. 5A). In addition, other clones could be detected with both probes, e.g., clone f in mouse 4.4, which contains *Hoxa9* provirus at three integration sites and *E2A-Pbx1a* at one. Expression of the transduced genes in leukemic cells of all of these secondary recipients was confirmed by Northern blot analysis

that detected high levels of expression of both the *Hoxa9* and *E2A-Pbx1a* retrovirally derived mRNAs (data not shown).

Together, these data confirm that the acute leukemia that developed in the primary *Hoxa9* + *E2A-Pbx1a* mice was caused by leukemic cells overexpressing both *Hoxa9* and *E2A-Pbx1a*. These cells thus outcompeted those overexpressing either *Hoxa9* or *E2A-Pbx1a* alone, which were in ~6-fold (*Hoxa9*) and ~3-fold (*E2A-Pbx1a*) excess in the inoculum originally transplanted in the primary recipients (Table 1). Furthermore, these studies also demonstrated that between one (mouse 2) and seven (mouse 4) transplantable leukemic clones were present in the primary *Hoxa9* + *E2A-Pbx1a* mice (Fig. 5A).

Leukemic progenitor cells overexpressing *Hoxa9* and *E2A-Pbx1a* do not require hematopoietic growth factors to proliferate in vitro. The leukemic cells in the bone marrow and spleen of *Hoxa9* + *E2A-Pbx1a* mice formed colonies in methylcellulose cultures supplemented with growth factors with plating efficiencies of 2.4% ± 1.2% and 1.7% ± 0.2% for bone marrow and spleen cells, respectively, or with 8- and 2,800-fold increases compared to *neo* control mice (Fig. 6A and B). For the five mice tested, nearly 100% of their leukemic progenitor cells were resistant to both G418 and puromycin, demonstrating that these cells were all transduced with both *Hoxa9* and *E2A-Pbx1a*. Interestingly, and in sharp contrast to bone marrow and spleen cells derived from nonleukemic *Hoxa9* or *E2A-Pbx1a* mice sacrificed 56 and 51 ± 3 days after transplantation, the leukemic cells from *Hoxa9* + *E2A-Pbx1a* mice had a similar
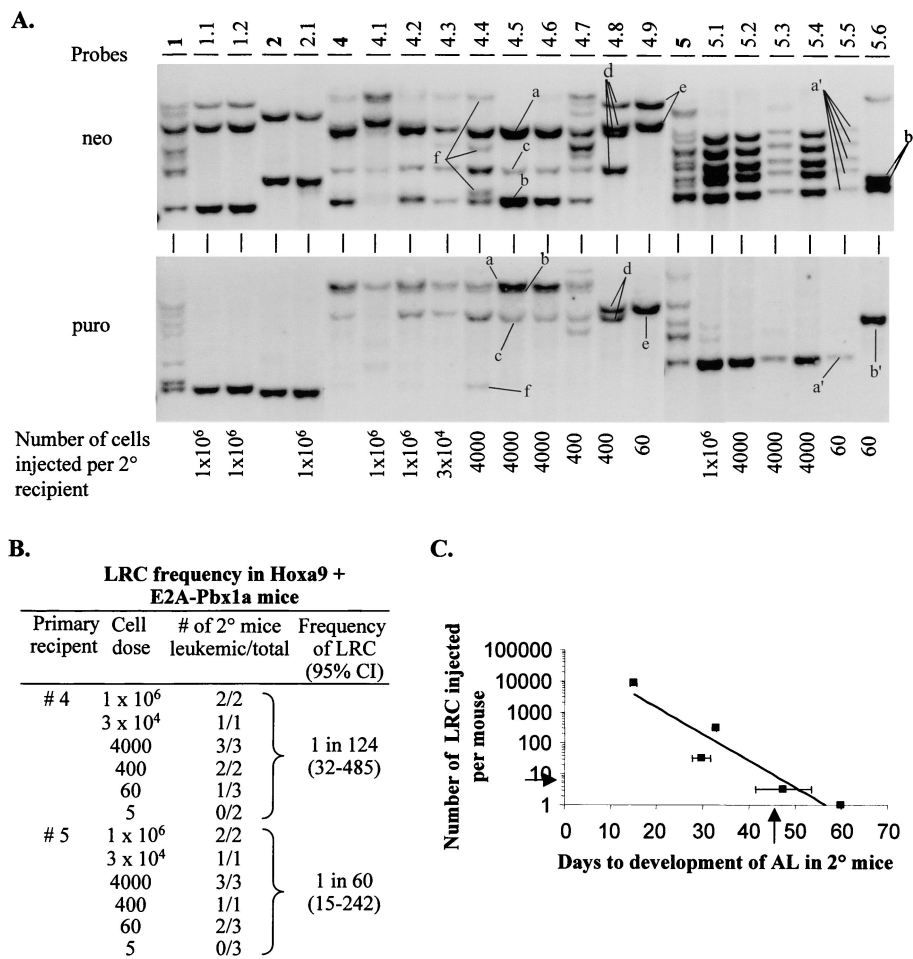
FIG. 5. Clonal analysis of the acute leukemia that developed in primary and secondary *Hoxa9 + E2A-Pbx1a* recipients. (A) Southern blot analysis of DNA isolated from bone marrow of primary and secondary *Hoxa9 + E2A-Pbx1a* mice. The DNA was digested with *Eco*RI, which cuts the integrated provirus once, thus generating a unique fragment for each proviral integration site. The membranes were first hybridized with a *neo*-specific probe to detect *Hoxa9* proviral fragments (top panel) and subsequently with a *puro*-specific probe to detect *E2A-Pbx1a* proviral fragments (bottom panel). Each primary recipient of *Hoxa9*- and *E2A-Pbx1a*-transduced cells is identified with a specific number shown in bold, and its secondary recipients are identified with derivatives thereof (1.1, 1.2, etc.). The number of leukemic cells transplanted per secondary recipients is indicated below each lane. For clarity, different leukemic *Hoxa9*- and *E2A-Pbx1a*-transduced clones detected in secondary recipients of mouse 4 are labeled from a to f; in secondary recipients of mouse 5, they are labeled a′ and b′. (B) Evaluation by limiting dilution analysis of the frequency of the LRC in *Hoxa9 + E2A-Pbx1a* mice 4 and 5. Clonality of the majority of the secondary recipients used in this assay is shown in panel A. (C) Graphic display of the correlation between the number of LRC transplanted per secondary recipient of *Hoxa9 + E2A-Pbx1a* mouse 4 and the time needed for the development of leukemia in those recipients. The arrow on the *x* axis indicate the predicted time required for seven LRC (arrow on *y* axis) to give rise to overt leukemia in secondary mice (see Discussion). AL, acute leukemia; 2°, secondary; Cl, confidence interval.

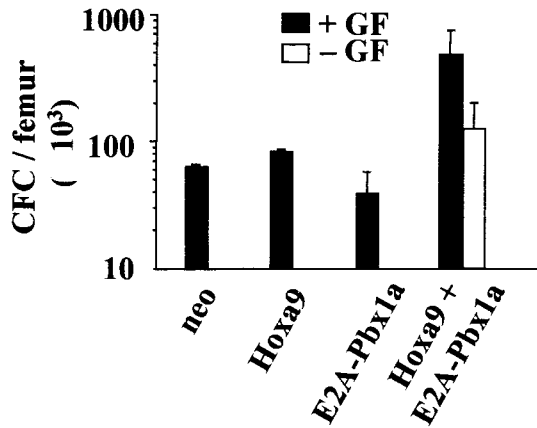capacity for colony formation in the presence or absence of added hematopoietic growth factors (Fig. 6A to C).

To test whether overexpression of *Hoxa9* and *E2A-Pbx1a* in these leukemic cells was inducing their production of hemopoietic growth factors, supernatants harvested from liquid cultures initiated with spleen cells from six *Hoxa9 + E2A-Pbx1a* mice were tested for the presence of IL-3, G-CSF, and Steel. Five of six leukemic samples tested produced measurable levels (0.1 to 1 ng/ml) of IL-3 and/or G-CSF (Fig. 6E; one sample produced both growth factors), and in the remaining sample secretion of Steel, but not IL-3 or G-CSF, was detected (data not presented). No production of G-CSF or Steel could be detected in the supernatants from the five cultures that were initiated with spleen cells from *E2A-Pbx1a* primary mice (not leukemic; see below), and only one of these contained measurable amounts of IL-3 (Fig. 6E).

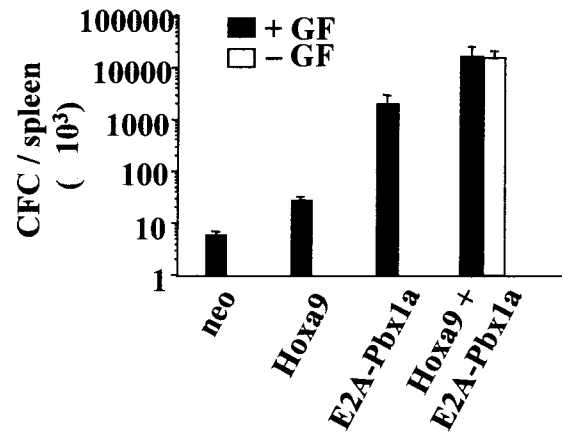Consistent with their production of growth factors, the leukemic cells from the *Hoxa9 + E2A-Pbx1a* mice were capable of proliferation in liquid cultures with or without added growth factors for a prolonged period of time. These cells grew in macroscopic clumps (Fig. 6D) containing mostly viable cells (*n* = 3 of three different leukemic cells tested) that were morphologically identical to the original donor leukemia, i.e., immature blast cells with rare differentiated elements (Fig. 6D). Clonal analysis performed on DNA isolated from these cells demonstrated that each cell line was derived from the same clone(s) identified in the spleen of a corresponding leukemic mouse (data not shown). In addition, it was possible to demonstrate by Western blot analysis that these cell lines expressed high levels of E2A-Pbx1a and Hoxa9 (Fig. 6F).

In contrast to the *Hoxa9 + E2A-Pbx1a* leukemic cells, the spleen cells from the *E2A-Pbx1a* mice did not survive (80 to 90% mortality at day 5) or proliferate in the absence of added growth factors. However, in the presence of IL-3 or GM-CSF, these cells proliferated initially, but while forming a feeder layer, the cultures became exhausted in 4 to 5 weeks.
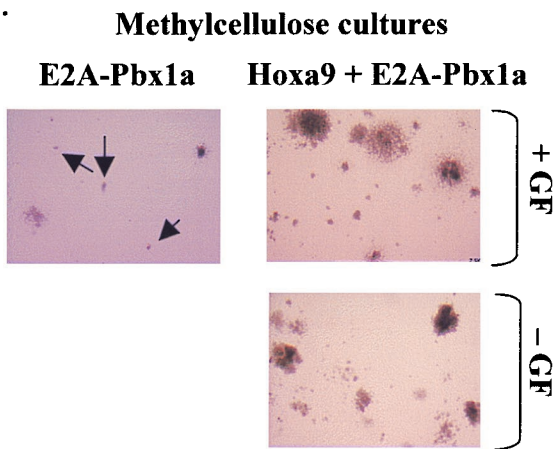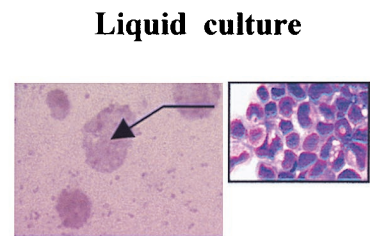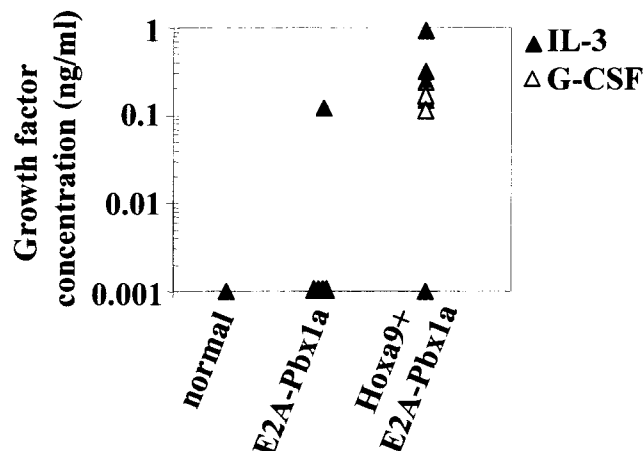
A.

B.

C. Methylcellulose cultures

E2A-Pbx1a   Hoxa9 + E2A-Pbx1a

+ GF

− GF

D. Liquid culture

E.

▲ IL-3
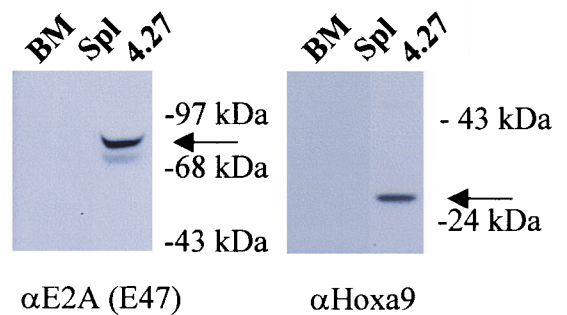△ G-CSF

F.

αE2A (E47)   αHoxa9

FIG. 6. Growth factor-independent proliferation of leukemic blasts isolated from primary recipients of bone marrow cells overexpressing *Hoxa9* and *E2A-Pbx1a*. The number of colonies generated from bone marrow (A) and spleen (B) cells of *neo* control, *Hoxa9*, *E2A-Pbx1a*, and *Hoxa9 + E2A-Pbx1a* mice, in the presence or absence of added growth factors, are shown. The results shown represent the mean ± standard deviation of the number of myeloid CFC in one femur and in the spleen of *neo* control ($n = 2$) and *Hoxa9* ($n = 3$) mice at 56 days following transplantation and that of *E2A-Pbx1a* ($n = 6$) and *Hoxa9 + E2A-Pbx1a* ($n = 6$) mice when sacrificed as outlined in Table 2. (C) Microscopic view of colonies generated in methylcellulose cultures of spleen cells from *E2A-Pbx1a* and *Hoxa9 + E2A-Pbx1a* mice 13 days

Overexpression of *E2A-Pbx1a* leads to the development of a highly polyclonal myeloproliferative syndrome (MPS). The recipients of *Hoxa9*-transduced bone marrow cells thrived normally for an extended period of time. At 56 days posttransplantation, three of these mice were sacrificed to allow comparison with the leukemic *Hoxa9* + *E2A-Pbx1a* mice. At this time, hemopoietic regeneration by *Hoxa9*-transduced cells in these mice was polyclonal, similar to that detected in the *neo* control mice (Table 2 and Fig. 7A and B). The only hematological abnormality detected in these mice was in their spleens, which were slightly enlarged (Table 2), with some increase in cells with immature granulocytic morphology (Fig. 3) and myeloid CFC (Fig. 6A and B). As we previously reported (13), the remaining four *Hoxa9* mice eventually developed mono- or biclonal AML with latency of 167 ± 32 days (Table 2 and Fig. 7B).

The recipients of *E2A-Pbx1a*-transduced bone marrow, however, developed progressive hind limb paralysis and for that reason were sacrificed at 51 ± 3 days following transplantation (Table 2). At the time of their sacrifice, these mice had normal blood cell counts and the cellular constituents of their bone marrow were not overtly altered except for higher than normal proportions of mature neutrophils and monocytes, with some (<30%) immature forms (Fig. 3). Their bone marrow cellularity was, however, decreased threefold compared to the control *neo* mice (Table 2), and all animals had large spleens (Table 2), characterized by an accumulation of immature granulocytic cells together with few blast-like cells (Fig. 3). These immature cells did, however, not infiltrate their lymph nodes and thymus or nonhematopoietic tissue such as liver, lung, or brain (Fig. 3). The in vitro clonogenic progenitor assays revealed that the numbers of bone marrow-derived CFC in recipients of *E2A-Pbx1a*-transduced bone marrow cells were not significantly altered (Fig. 6A), whereas the numbers of their spleen-derived CFC were ~300-fold higher than those determined for the *neo* control at 56 days following transplantation (Fig. 6B). Of the bone marrow and the spleen CFC, 64% ± 30% and 85% ± 30%, respectively, were puromycin resistant, demonstrating the in vivo expansion of *E2A-Pbx1a*-transduced myeloid CFC. Although *E2A-Pbx1a*-transduced CFC were capable of generating all types of colonies normally present in these cultures, the majority (up to 60%) generated small macrophage colonies of 40 to 50 cells (Fig. 6C). Together, these observations suggest that *E2A-Pbx1a* induced the expansion of a pool of relatively mature CFC, which preferentially homed to and/or proliferated in the spleen and, although not verified, in the lumbosacral region of the spinal cord, leading to hind limb paralysis.

Southern blot analyses of DNA isolated from the bone marrow and spleens of the affected mice verified the presence of the intact *E2A-Pbx1a* provirus (Fig. 4A, middle panel), and high levels of the *E2A-Pbx1* retrovirus-derived mRNA were detected in these tissues by Northern blot analysis (Fig. 4B, middle panel). Interestingly, Southern blot analysis of proviral integration sites indicated that the *E2A-Pbx1a* mice were repopulated by numerous *E2A-Pbx1a*-transduced clones (Fig.

7C). This finding, together with the expansion of myeloid CFC with low proliferative potential in these mice, suggests that they suffered from MPS. In support of that view, with the exception of mouse 1, this polyclonal disease that developed in *E2A-Pbx1a* mice could not be transplanted to secondary recipients, which succumbed to monoclonal AML with a latency of 75 ± 23 days (Fig. 7C). These secondary mice were, in contrast to the primary *E2A-Pbx1a* recipients, characterized by high leukocyte counts and infiltration by myeloid blasts of the bone marrow, spleen, and lymph nodes as well as liver and lungs (data not shown). The low efficiency in transplanting the polyclonal MPS, as well as the long latency and the monoclonal origin of the leukemic disease in secondary recipients, demonstrates that the development of *E2A-Pbx1a*-associated leukemia depends on accumulation of additional genetic events.

DISCUSSION

Several studies have suggested that cellular transformation induced by *E2A-Pbx1* may result from collaborative interactions with products of the *Hox* gene complex. The studies described herein provided the first direct evidence demonstrating that E2A-Pbx1 collaborates with a Hox gene product (i.e., Hoxa9) and that this collaboration is sufficient to acutely transform primary bone marrow cells. Furthermore, bone marrow cells which coexpressed these two genes were able to proliferate in the absence of exogenous hematopoietic growth factors that, at least in part, can be explained by their production of IL-3 and/or G-CSF.

Cooverexpression of *Hoxa9* and *E2A-Pbx1a* is sufficient to directly transform primitive hematopoietic cells. Three main observations in this study strongly suggest that cooverexpression of *Hoxa9* and *E2A-Pbx1a* is sufficient to directly transform primitive bone marrow cells. These are (i) the ability of cooverexpression of *Hoxa9* and *E2A-Pbx1a* to block differentiation and induce cytokine-independent growth of freshly infected primitive CFC (Fig. 2), (ii) the relatively short time required for the development of acute leukemia in the primary *Hoxa9* + *E2A-Pbx1a* mice (39 ± 2 days), and (iii) the evidence that secondary recipients which receive a fixed number of *Hoxa9*- and *E2A-Pbx1a*-transduced LRC developed leukemia within a similar time frame (or even longer) as the primary recipients repopulated with comparable numbers of *Hoxa9*- and *E2A-Pbx1a*-transduced leukemic clones. For example, primary mouse 4, which had seven detectable leukemic clones (labeled a to f in Fig. 5A), exhibited full-blown leukemia in 40 days, whereas its secondary recipients of seven LRC would develop leukemia in 45 days (Fig. 5C). This finding suggests that the seven leukemic clones active in primary recipient 4 were already transformed when they were originally transplanted into this recipient.

Since the cooverexpression of *Hoxa9* and *E2A-Pbx1a* appears to be sufficient to transform a given subpopulation of bone marrow cells, then the frequency of that target cell population can be estimated by comparing the number of transduced cells in the transplanted bone marrow inoculum to the

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after initiation of the cultures. The arrows indicate the presence of small macrophage colonies that dominated the cultures from the *E2A-Pbx1a* mice. GF, growth factor. (D) Liquid culture of *Hoxa9*- and *E2A-Pbx1a*-overexpressing cells after 2 months of in vitro growth, demonstrating the growth of these cells in macroscopic clumps (magnification, ×10). The callous shows a Wright-stained cytospin preparation of the cells in these clumps, showing the immature morphology of these cells (magnification, ×100). (E) The presence of bioactive IL-3 and G-CSF in conditioned medium obtained from 4-day-old cultures of cells obtained from the spleens of a normal mouse, or from *E2A-Pbx1a* (n = 5) and *Hoxa9* + *E2A-Pbx1a* (n = 6) mice and seeded at 10^6 cells/ml, was tested as outlined in Materials and Methods. (F) Western blot analysis of cell lysates from bone marrow (BM) and spleen (Spl) cells of normal mice and from one culture of *Hoxa9* + *E2A-Pbx1a* leukemic cells. The membrane was probed with anti-E2A monoclonal antibody and then with polyclonal antisera directed against Hoxa9. The position of the 85-kDa E2A-Pbx1a protein and the 36-kDa Hoxa9 protein is indicated (arrows).
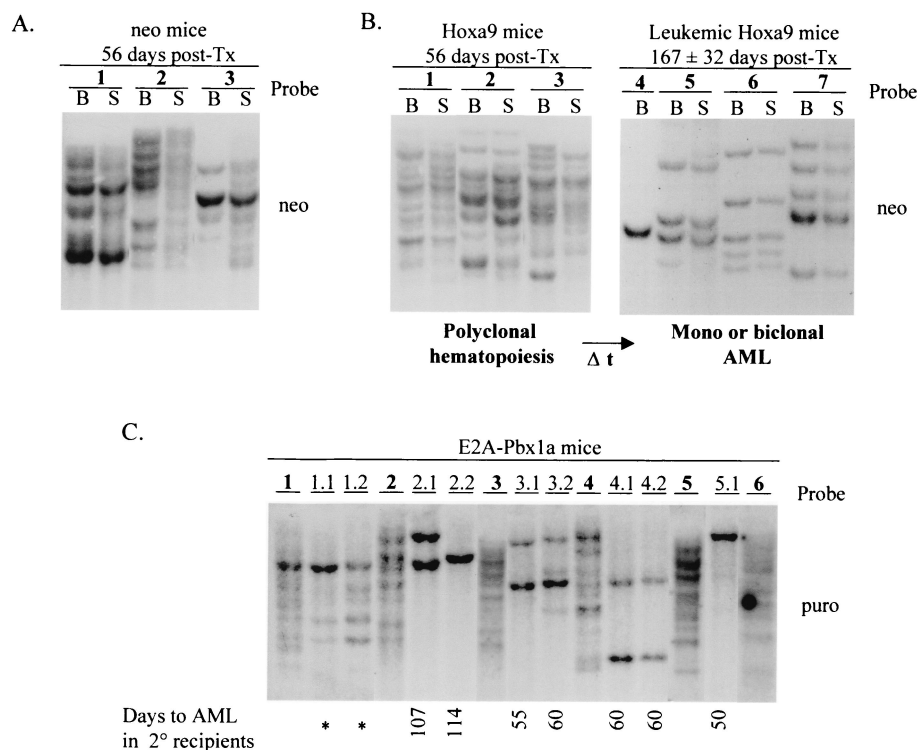
FIG. 7. Southern blot analysis of DNA isolated from bone marrow of primary *neo* (A) and *Hoxa9* (B) mice and primary and secondary *E2A-Pbx1a* mice (C). The DNA was digested with *Eco*RI, which cuts the integrated provirus once, thus generating unique fragments specific for the proviral integration site(s). The membranes were hybridized with a *neo*-specific probe to detect the *neo* control and *Hoxa9* proviral fragments and a *puro*-specific probe to detect those of the *E2A-Pbx1a* provirus. Each primary mouse is identified with specific number in bold, and its secondary recipients are identified with derivatives thereof. All secondary recipients were transplanted with 1×10^6 to 2×10^6 bone marrow or spleen cells from the donor mouse. Where applicable, the time (in days posttransplantation [post-Tx]) for the development of acute leukemia in the secondary *E2A-Pbx1a* mice is shown. Asterisks denote secondary (2°) mice that developed MPS.

number of leukemic clones detected in each primary recipient. Table 1 shows that on average, 252 and 3 *Hoxa9* and *E2A-Pbx1a*-transduced CFC and LTRC, respectively, were transplanted per primary recipient. Between one and seven leukemic clones were recovered per primary recipient, which suggests that the target for leukemic transformation is a primitive bone marrow cell which is found in a similar frequency as LTRC.

E2A-Pbx1a enhances proliferation of committed progenitors in vivo. Recipients of *E2A-Pbx1a*-transduced bone marrow cells developed a polyclonal MPS characterized by large spleens and hind leg paralysis. With the exception of one mouse, this disease could not be transplanted to secondary recipients, suggesting that the target cell was not a stem cell but rather a more differentiated (and more frequent) short-lived progenitor cell. In support of this conclusion, the number of clones containing the *E2A-Pbx1a* provirus was much higher than the number of transduced stem cells injected per mouse (Table 1 and Fig. 7B). In addition, this MPS evolved to clonal AML in secondary recipients following a latency period which exceeded that required for disease to occur in primary mice, proving that *E2A-Pbx1a* alone was incapable of full leukemic transformation of stem cells.

Previous work by Kamps and Baltimore (10) using retroviral expression of *E2A-Pbx1a* in murine bone marrow cells demonstrated that *E2A-Pbx1a* causes monoclonal AML with long latencies (155 ± 45 days), indicating that *E2A-Pbx1a* alone was insufficient to transform hemopoietic cells and that additional somatic mutations were essential for the development of AML. However, none of the primary recipients of *E2A-Pbx1a*-trans-

duced bone marrow cells described by Kamps and Baltimore developed the MPS observed in our studies (10). Several experimental differences between these two studies may explain this difference. First, different retroviral vectors were used to transduce and express the *E2A-Pbx1a* gene in mouse bone marrow cells (the pGD retroviral vector versus the MSCV vector used here), suggesting that different expression levels or promoter specificity could contribute to the differences observed. In support of that view, we were unable to induce the expansion of GM-CSF-responsive progenitor cells as described in their study (10). Second, the hemopoietic growth factors and conditions used to infect bone marrow cells differed significantly between the two studies.

One common feature of the disease that developed in the primary recipients of *E2A-Pbx1a*-transduced cells in these two studies was the high frequency of hind limb paralysis. This, combined with the unusually low number of cells in the bone marrow (three- to fourfold reduction) of the *E2A-Pbx1a* mice described here, suggests the presence of a unique set of adhesion molecules on the surface of *E2A-Pbx1a*-transduced bone marrow progenitors allowing them to home preferentially to the spleen and potentially also to nervous tissues (possibly the lumbosacral plexus).

Growth factor independence and production by *Hoxa9*- and *E2A-Pbx1a*-transduced bone marrow cells. *Hoxa9*- and *E2A-Pbx1a*-transduced bone marrow cells had the capacity to grow in the absence of growth factors when they were analyzed both immediately following retroviral gene transfer (Fig. 2B) and after being harvested from the bone marrow or spleens of leukemic mice (Fig. 6A to C). Except for bone marrow cells

engineered to express either a signal-transducing kinase such as Tel-Jak2 (33) or an activated growth factor receptor (or a growth factor such as IL-3), we believe that these studies are the first to demonstrate the capacity of transcription factors to directly induce growth factor-independent proliferation of primary bone marrow cells (i.e., immediately following gene transfer [Fig. 2B]).

Furthermore, it appears that at least three different growth factors (i.e., IL-3, G-CSF, and Steel) are being produced by the cells obtained from the spleen of *Hoxa9 + E2A-Pbx1a* mice. Curiously, the majority of leukemic blasts produced detectable levels of only one growth factor (e.g., IL-3 or G-CSF), suggesting that *Hoxa9* and *E2A-Pbx1a* may regulate a master program which controls the production of several hemopoietic growth factors. Alternatively, it cannot be excluded that *Hoxa9* and *E2A-Pbx1a* regulate the production of a growth factor that was not tested here (GM-CSF, IL-6, etc.). Furthermore, the variations in the production of these factors may also reflect the heterogeneity in the population of hemopoietic progenitors that were transformed even if the cells were phenotypically undifferentiated by several criteria (morphology, FACS, etc.).

Together, the studies presented herein prove the existence of a collaboration in leukemic transformation between the fusion oncoprotein E2A-Pbx1a and one of its presumed DNA binding partners, Hoxa9. Future goals will include the determination of *Hox* gene expression patterns in *E2A-Pbx1*-expressing human leukemia samples and determining whether other Hox gene products can also collaborate with the E2A-Pbx1 oncoprotein to transform primary hemopoietic cells. Also, it will be important to determine whether this oncogenic collaboration involves cooperative DNA binding between these two proteins.

REFERENCES

1. **Azpiazu, N., and G. Morata.** 1998. Functional and regulatory interactions between *Hox* and *extradenticle* genes. Genes Dev. **12:**261–273.
2. **Borrow, J., A. M. Shearman, V. P. Stanton, R. Becher, T. Collins, A. J. Williams, I. D. Dubé, 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 leukemia fuses the genes for nucleoporin NUP98 and class I homeoprotein HOXA9. Nat. Genet. **12:**159–167.
3. **Carroll, A. J., W. M. Crist, R. T. Parmley, M. Roper, M. D. Cooper, and W. M. Finley.** 1984. Pre-B cell leukemia associated with chromosome translocation 1;19. Blood **63:**721–724.
4. **Chan, S. K., L. Jaffe, M. Capovilla, J. Botas, and R. S. Mann.** 1994. The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with extradenticle, another homeoprotein. Cell **78:**603–615.
5. **Chang, C. P., I. de Vivo, and M. L. Cleary.** 1997. The Hox cooperativity motif of the chimeric oncoprotein E2a-Pbx1 is necessary and sufficient for oncogenesis. Mol. Cell. Biol. **17:**81–88.
6. **Chang, C. P., W. F. Shen, S. Rozenfeld, H. J. Lawrence, C. Largman, and M. L. Cleary.** 1995. Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. Genes Dev. **9:**663–674.
7. **Davis, L., M. Kuehl, and J. Battey.** 1994. Preparation and analysis of RNA from eukaryotic cells. Appleton and Lange, Norwalk, Conn.
8. **Dedera, D. A., E. K. Waller, D. P. LeBrun, A. Sen-Majumdar, M. E. Stevens, G. S. Barsh, and M. L. Cleary.** 1993. Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. Cell **74:**833–843. (Erratum, **75:**826.)
9. **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. USA **76:**3629–3633.
10. **Kamps, M. P., and D. Baltimore.** 1993. E2A-Pbx1, the t(1;19) translocation protein of human pre-B-cell acute lymphocytic leukemia, causes acute myeloid leukemia in mice. Mol. Cell. Biol. **13:**351–357.
11. **Kamps, M. P., C. Murre, X. H. Sun, and D. Baltimore.** 1990. A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in Pre-B ALL. Cell **60:**547–555.
12. **Kamps, M. P., D. D. Wright, and Q. Lu.** 1996. DNA-binding by oncoprotein E2A-Pbx1 is important for blocking differentiation but dispensable for fibroblast transformation. Oncogene **12:**19–30.
13. **Kroon, E., J. Krosl, U. Thorsteinsdottir, S. Baban, A. M. Buchberg, and G. Sauvageau.** 1998. Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1 but not PBX1. EMBO J. **17:**3714–3725.
14. **Krosl, J., S. Baban, G. Krosl, S. Rozenfeld, C. Largman, and G. Sauvageau.** 1998. Proliferation and transformation by HOXB4 and HOXB3 genes involves cooperation with PBX. Oncogene **16:**3403–3412.
15. **Krumlauf, R.** 1994. Hox genes in vertebrate development. Cell **78:**191–201.
16. **Lawrence, H. J., G. Sauvageau, A. Ahmadi, T. Lau, A. R. Lopez, M. M. Le Beau, M. Link, and C. Largman.** 1995. Stage and lineage-specific expression of the HOXA10 homeobox gene in normal and leukemic hematopoietic cells. Exp. Hematol. **23:**1160–1166.
17. **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:**281–290.
18. **Lu, Q., P. S. Knoepfler, J. Scheele, D. D. Wright, and M. P. Kamps.** 1995. Both Pbx1 and E2A-Pbx1 bind the DNA motif ATCAATCAA cooperatively with the products of multiple murine Hox genes, some of which are themselves oncogenes. Mol. Cell. Biol. **15:**3786–3795.
19. **Maconochie, M. K., S. Nonchev, M. Studr, S. K. Chan, H. Poepperl, M. H. Sham, R. S. Mann, and R. Krumlauf.** 1997. Cross-regulation in the mouse HoxB complex: the expression of Hoxb2 in rhobdomere 4 is regulated by Hoxb1. Genes Dev. **11:**1885–1895.
20. **Monica, K., D. P. LeBrun, D. A. Dedera, R. Brown, and M. L. Cleary.** 1994. Transformation properties of the E2a-Pbx1 chimeric oncoprotein: fusion with E2a is essential, but the Pbx1 homeodomain is dispensable. Mol. Cell. Biol. **14:**8304–8314.
21. **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.** 1996. Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15; p15) in human myeloid leukaemia. Nat. Genet. **12:**154–158.
22. **Nakamura, T., D. A. Largaespada, J. D. Shaughnessy, N. A. Jenkins, and N. G. Copeland.** 1996. Cooperative activation of Hoxa and Pbx1-related genes in murine myeloid leukaemias. Nat. Genet. **12:**149–153.
23. **Nourse, J., J. D. Mellentin, N. Galili, J. Wilkinson, E. Stanbridge, S. D. Smith, and M. C. Cleary.** 1990. Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell **60:**535–546.
24. **Peltenburg, L. T., and C. Murre.** 1996. Engrailed and Hox homeodomain proteins contain a related Pbx interaction motif that recognizes a common structure present in Pbx. EMBO J. **15:**3385–3393.
25. **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.
26. **Phelan, M. L., and M. S. Featherstone.** 1997. Distinct HOX N-terminal arm residues are responsible for specificity of DNA recognition by HOX monomers and HOX-PBX heterodimers. J. Biol. Chem. **272:**8635–8643.
27. **Phelan, M. L., I. Rambaldi, and M. S. Featherstone.** 1995. Cooperative interaction between HOX and PBX proteins mediated by a conserved peptide motif. Mol. Cell. Biol. **15:**3989–3997.
28. **Piper, D. E., A. H. Batchelor, C. P. Chang, M. L. Cleary, and C. Wolberger.** 1999. Structure of a HoxB1-Pbx1 heterodimer bound to DNA: role of the hexapeptide and a fourth homeodomain helix in complex formation. Cell **96:**587–597.
29. **Pöpperl, H., M. Bienz, M. Studer, S. K. Chan, S. Aparicio, S. Brenner, R. S. Mann, and R. Krumlauf.** Segmental expansion of Hoxb-1 is controlled by a highly conserved autoregulatory loop dependent upon exd/pbx. Cell **81:**1031–1042.
30. **Rocco, G. D., F. Mavilio, and V. Zappavigna.** 1997. Functional dissection of a transcriptionally active, target-specific Hox-Pbx complex. EMBO J. **16:**3644–3654.
31. **Sauvageau, G., U. Thorsteinsdottir, C. J. Eaves, H. J. Lawrence, C. Largman, P. M. Lansdrop, 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:**1753–1765.
32. **Sauvageau, G., U. Thorsteinsdottir, M. R. Hough, P. Hugo, H. J. Lawrence, C. Largman, and R. K. Humphries.** 1997. Over-expression of HOXB3 in

hematopoietic cells causes defective lymphoid development and progressive myeloproliferation. Immunity **6:**13–22.

33. **Schwaller, J., J. Frantsve, J. Aster, I. R. Williams, M. H. Tomasson, T. S. Ross, P. Peeters, L. Van Rompaey, R. A. Van Etten, R. Ilaria, Jr., P. Marynen, and D. G. Gilliland.** 1998. Transformation of hematopoietic cell lines to growth-factor independence and induction of fatal myelo- and lymphoproliferative disease in mice by retrovirally transduced TEL/JAK2 fusion genes. EMBO J. **17:**5321–5333.

34. **Shen, C. P. Chang, S. Rozenfeld, G. Sauvageau, R. K. Humphries, M. Lu, H. J. Lawrence, M. J. Cleary, and C. Largman.** 1996. Hox homeodomain proteins exhibit selective complex stabilities with Pbx and DNA. Nucleic Acids Res. **24:**898–906.

35. **Shen, W. F., J. C. Montgomery, S. Rozenfeld, J. J. Moskow, H. J. Lawrence, A. M. Buchberg, and C. Largman.** 1997. AbdB-like proteins stabilize DNA binding by the Meis1 homeodomain proteins. Mol. Cell. Biol. **17:**6448–6458.

36. **Shen, W. F., S. Rozenfeld, H. J. Lawrence, and C. Largman.** 1997. The Abd-B-like Hox homeodomain proteins can be subdivided by the ability to form complexes with Pbx1a on a novel DNA target. J. Biol. Chem. **272:**8198–8206.

37. **Szilvassy, S. J., R. K. Humphries, P. M. Lansdorp. A. C. Eaves, and C. J. Eaves.** 1990. Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulations strategy. Proc. Natl. Acad. Sci. USA **87:**8736–8740.

38. **Thomas, K. R., and M. R. Capecchi.** 1987. Site-directed mutagenesis by gene targetted in mouse embryo-derived stem cell. Cell **51:**503–512.

39. **Thorsteinsdottir, U., G. Sauvageau, M. R. Hough, H. J. Lawrence, C. Largman, and R. K. Humphries.** 1997. Overexpression of HOXA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia. Mol. Cell. Biol. **17:**495–505.

40. **Thorsteinsdottir, U., G. Sauvageau, and K. Humphries.** Enhanced polyclonal regeneration of hematopoietic stem cells overexpressing *HOXB4* following bone marrow transplantation. Blood, in press.