Molecular and Cellular Biology, Jan. 2001, p. 224–234 Vol. 21, No. 1
0270-7306/01/$04.00+0 DOI: 10.1128/MCB.21.1.224–234.2001
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Defining Roles for *HOX* and *MEIS1* Genes in Induction of Acute Myeloid Leukemia

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Received 21 July 2000/Returned for modification 13 September 2000/Accepted 12 October 2000

Complex genetic and biochemical interactions between HOX proteins and members of the TALE (i.e., PBX and MEIS) family have been identified in embryonic development, and some of these interactions also appear to be important for leukemic transformation. We have previously shown that *HOXA9* collaborates with *MEIS1* in the induction of acute myeloid leukemia (AML). In this report, we demonstrate that *HOXB3*, which is highly divergent from *HOXA9*, also genetically interacts with *MEIS1*, but not with *PBX1*, in generating AML. In addition, we show that the *HOXA9* and *HOXB3* genes play key roles in establishing all the main characteristics of the leukemias, while *MEIS1* functions only to accelerate the onset of the leukemic transformation. Contrasting the reported functional similarities between PREP1 and MEIS1, such as PBX nuclear retention, we also show that *PREP1* overexpression is incapable of accelerating the *HOXA9*-induced AML, suggesting that MEIS1 function in transformation must entail more than PBX nuclear localization. Collectively, these data demonstrate that *MEIS1* is a common leukemic collaborator with two structurally and functionally divergent *HOX* genes and that, in this collaboration, the *HOX* gene defines the identity of the leukemia.

The homeodomain-containing transcription factors of the *HOX* gene family, regulators of pattern formation and tissue identity during embryogenesis, have also been identified previously as regulators of hemopoietic cell proliferation and differentiation (40). In the hematopoietic system, *HOX* gene expression is largely confined to primitive cells (11, 32), and the enforced expression of *HOX* genes (i.e., *HOXB4*, *HOXB3*, and *HOXA10*) in mouse hemopoietic cells results in distinct phenotypes, affecting various hemopoietic lineages (33, 34, 39).

In agreement with their regulatory functions, aberrant expression of *HOX* genes is associated with leukemic transformation both in mice and in humans. In a subset of human myeloid leukemias, a recurrent translocation between the *HOXA9* and *NUP98* genes results in the expression of the fusion oncoprotein NUP98-HOXA9 (6, 24). Recently, the *HOXA9* gene was also shown to be the single most highly correlated gene (out of 6,817 genes tested) for poor prognosis in human acute myeloid leukemia (AML) (12), thus suggesting a potential key role for this gene in human leukemia, beyond that caused by the *HOXA9-NUP98* chromosomal translocation. By applying either retroviral insertional mutagenesis or retroviral overexpression, roles for the *HOXA7*, *HOXA9*, *HOXA10*, *HOXB3*, and *HOXB8* genes in leukemic transformation in mice have also been established previously (17, 25, 28, 34, 38, 39). The lineage-specific effects produced by the overexpression of different *HOX* genes in mouse bone marrow cells, which often precede acute leukemic transformation (34, 39), raise the possibility that *HOX* genes may influence the typical phenotypic variations seen between subsets of acute leukemia.

A number of studies have demonstrated that HOX proteins collaborate in the in vitro DNA binding with members of the TALE (three-amino-acid loop extension) subclass of homeodomain-containing proteins comprising the PBC (mammalian PBX and *Drosophila melanogaster* EXD proteins) and MEIS (mammalian MEIS and PREP1 and *Drosophila* HTH proteins) families (20). This interaction shows moderate specificity, with HOX proteins from paralog groups 1 to 10 interacting with PBX proteins, whereas interaction with MEIS proteins is limited to HOX paralogs 9 to 13 (36). The cooperative interaction between PBX (or EXD) and HOX proteins has been shown elsewhere to enhance the DNA binding affinity and specificity of HOX proteins (20) and is essential for at least some of the HOX-dependent developmental programs (2, 29). In contrast, a functional role for a dimeric HOX-MEIS complex has not been established so far (31). Members of the MEIS family can, however, form a stable heterocomplex with PBX (or EXD) in both DNA-dependent and -independent manners (5, 8, 30), and interaction with MEIS induces nuclear localization of PBX proteins by preventing their nuclear export (1, 3, 15, 27). Recently, indirect interaction between HOX and MEIS proteins (or HTH) was established by the identification of HOX-PBX-MEIS heterotrimeric complexes (4, 37). Studies both with *Drosophila* and with mice have, furthermore, shown that formation of such a trimeric complex is essential for the execution of at least some HOX-dependent developmental programs (9, 14, 31).

Members of the PBX and MEIS families are also involved in human and mouse leukemias. PBX1 is part of the fusion pro-

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tein E2A-PBX1 found in 10 to 20% of human pediatric pre-B acute lymphoblastic leukemia patients (16, 26). By applying retroviral co-overexpression, we have also previously demonstrated a strong collaboration between *HOXA9* and *E2A-PBX1* in the induction of AML (38). In addition, MEIS1 is frequently activated by retroviral integration in myeloid leukemias in BXH-2 mice and genetically interacts with *HOXA7* and *HOXA9* genes in AML (17, 22, 25). Thus, in leukemic transformation, as in the regulation of pattern formation and tissue identity during embryogenesis, an important genetic interaction has been established between *HOX* and *TALE* genes.

Based on the above results, we wanted to gain further insight into the nature of the collaboration between HOX proteins and members of the *MEIS* and *PBC* families in leukemic transformation. The results presented herein identify *MEIS1* as a common collaborator with two divergent *HOX* genes, i.e., *HOXA9* and *HOXB3*. The specificity of this collaboration was proven by the lack of genetic cooperativity between *HOX* and the two other *TALE* genes tested, i.e., *PBX1* and *PREP1*. Using overexpression studies in bone marrow cells, we also demonstrate that each *HOX* gene studied predisposes to leukemias that are phenotypically distinct and that *MEIS1* acts primarily to accelerate the occurrence of these leukemias without altering their phenotype.

TABLE 1. Absolute numbers of untransduced and transduced myeloid colony-forming cells[a] transplanted per mouse

Expt no. and mouse group (*n*)	No. of CFC injected/mouse[b]			
	Untransduced	G418[r]	Puro[r]	G418[r] and Puro[r]
Expt 1				
Neo (6)	2,200	2,100		
Puro (4)	4,200		2,300	
PBX1 (6)	3,300		1,400	
MEIS1 (6)	3,300		1,600	
HOXA9 (7)	950	2,200		
HOXB3 (6)	3,400	2,300		
HOXA9-MEIS1 (6)	2,100	1,800	1,000	250
HOXB3-PBX1 (6)	2,500	1,200	500	250
HOXB3-MEIS1 (6)	2,800	1,500	1,000	400
Expt 2				
HOXA9 (6)	1,200	700		
HOXA9-MEIS1 (6)	450	300	40	15
HOXA9-PREP1 (6)	1,600	650	500	300

[a] The number of transduced long-term repopulating cells (LTRC) injected per mouse can be estimated based on our previous results which determined the frequency of LTRC at 1 per 100 colony-forming cells (CFC) (33) and estimation of gene transfer to LTRC equal to that of CFC (33).

[b] The number of transduced CFC injected per mouse was determined as follows: (number of bone marrow cells injected per mouse) × (CFC frequency in the injected bone marrow inoculum) × (percentage of CFC resistant to puromycin and/or G418).

MATERIALS AND METHODS

Animals. All mice, both donors (C57BL/6Ly-Pep3b × C3H/HeJ)F_1 [(PepC3)F_1] and recipients (C57BL/6J × C3H/HeJ)F_1 [(B6C3)F_1], were bred and maintained as previously reported (38).

Generation of recombinant retroviruses. The retroviral vectors used in this study, i.e., *MSCV-HOXA9-pgk-neo* (no. 412), *MSCV-HOXB3-pgk-neo* (no. 245), *MSCV-PBX1b-pgk-puro* (no. 448), and *MSCV-MEIS1a-pgk-puro* (no. 515), have all been described before (17, 34). The *MSCV-PREP1-pgk-puro* (no. 682) retrovirus was generated by subcloning the human *PREP1* cDNA (5) into the *Hpa*I site of the *MSCV-pgk-puro* retrovirus. The *MSCV-pgk EGFP* vector (generous gift from K. Humphries, Terry Fox Laboratory, Vancouver, British Columbia, Canada) served as a backbone to generate the *MSCV-MEIS1a-pgk-EYFP* (no. 722) retroviral vector (enhanced yellow fluorescent protein [EYFP] cDNA from Clontech) used in part of these studies. High-titer helper-free retrovirus producer cells were generated from GP+E-86 and BOSC-23 viral packaging cells and tested as reported previously (17).

Retroviral infection and transplantation of primary murine bone marrow cells. Both double and single retroviral infections of primary murine bone marrow cells, followed by transplantation of infected cells, were done as previously described (38).

In vitro cultures and FACS analysis. For myeloid clonogenic progenitor assays, cells were cultured in methylcellulose cultures as described previously (38). Bone marrow cells harvested from the cocultivation with virus-producing cells or recovered from reconstituted leukemic mice were plated at a concentration of 2×10^3 to 8×10^3 cells/ml or 3×10^4 cells/ml, respectively. In an effort to derive cell lines from the leukemic mice, their bone marrow and/or spleen cells were grown 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 murine interleukin-3 (IL-3) per ml or 0.5 ng of granulocyte-macrophage colony-stimulating factor per ml. To analyze the effect of *MEIS1* or *HOXA9* on in vitro proliferation, $EYFP^+$ or enhanced green fluorescent protein-positive ($EGFP^+$) cells were purified, as previously described (39), from the bone marrow of the *EGFP*-control, *HOXA9-EGFP*, and *MEIS1-EYFP* mice and grown in Dulbecco's modified Eagle's medium containing 15% fetal calf serum, 10^{-5} M β-mercaptoethanol, 2 mM glutamine, 200 mg of transferrin per ml, 6 ng of murine IL-3 per ml, 10 ng of human IL-6 per ml, 50 ng of murine steel factor per ml, and 3 U of human urinary erythropoietin per ml. For fluorescence-activated cell sorting (FACS) analysis, cells from the bone marrow, spleen, and thymus of *EGFP* and *MEIS1-EYFP* mice were analyzed as previously described (17).

DNA, RNA, and protein analyses. The probes used for RNA and DNA analysis were a *Xho*I/*Sal*I fragment of pMC1*neo* (*neo*), a *Hin*dIII/*Cla*I fragment of *MSCV-pgk-puro*, or the full-length 1.4-kb *HOXA9*, 1.6-kb *HOXB3*, 1.5-kb *MEIS1*, 1.8-kb *PBX1*, and 1.8-kb *PREP1* cDNAs, labeled with ^{32}P by random primer extension. For Western blot analysis, total-cell lysates from HOXA9 or PREP1 viral producer cells (GP+E-86) were prepared as previously described (18). A polyclonal antibody to PREP1 was used as described previously (5).

RESULTS

Generation of bone marrow transplantation chimeras. To determine whether the leukemic transformation induced by the previously reported genetic interaction between *HOXA9* and *MEIS1* was specific for these two genes or whether similar interactions could be detected with other *HOX-TALE* pairs, transplantation chimeras were generated using bone marrow cells engineered to retrovirally overexpress *HOXB3* or *HOXA9* together with either *PBX1*, *MEIS1*, or *PREP1*. In addition, various control mice were also generated (all transplantation chimeras that were part of these studies are outlined in Table 1).

The decision to use *HOXB3* and *HOXA9* genes for these studies was based on the premises that the products of both of these genes have the capacity to induce AML when overexpressed (34, 38) and on sequence comparison studies which showed that the proteins encoded by these genes represent two of the most divergent (clustered) HOX proteins, which bear similarity only in their homeodomains (i.e., they are highly divergent in their N- and C-terminal regions [Fig. 1A]).

The choice of the *TALE* genes was based on previous studies which demonstrated their involvement in leukemic transformation (i.e., *PBX1* as part of *E2A-PBX1* or *MEIS1* as a genetic collaborator with *HOXA9*) (17, 21) or as a functional homolog to *MEIS1* (*PREP1*). *PREP1* was preferred over *MEIS2* or *MEIS3* because it is the most divergent member of the family (Fig. 1A), which still retains most of the functional capabilities of MEIS1, including its ability to regulate nuclear trafficking of

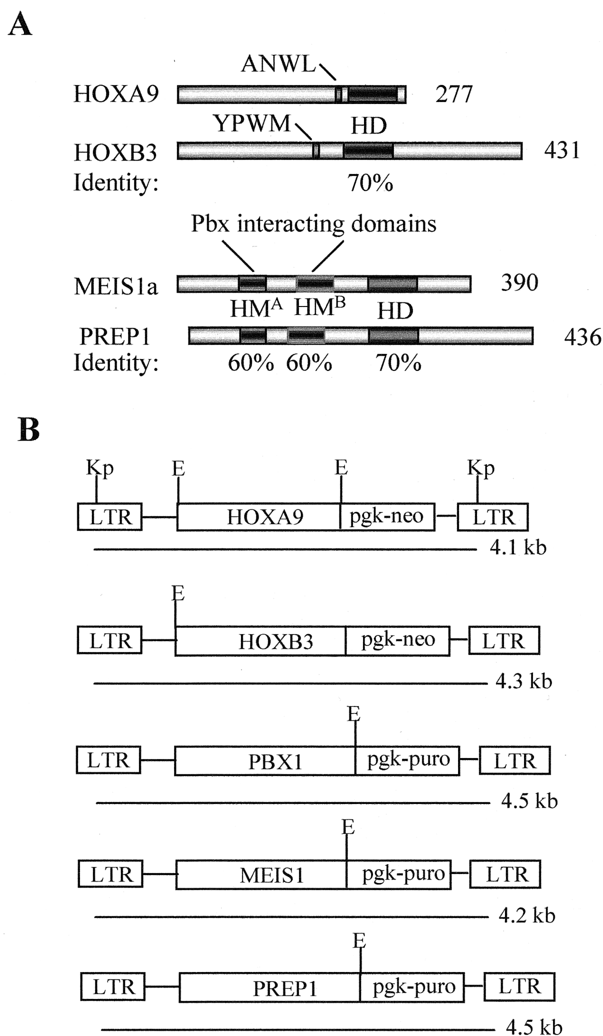


FIG. 1. Diagrammatic representation of the HOXA9, HOXB3, MEIS1a, and PREP1 proteins and the retroviral constructs used in this study. (A) Sequence comparison of the HOXA9 and HOXB3 proteins and the MEIS1a and the PREP1 proteins used in this study. Both HOXA9 and HOXB3 proteins have a motif (ANWL in HOXA9 and YPWM in HOXB3) N-terminal to the homeodomain that is essential for their interaction with PBX proteins. Apart from their homeodomains, which are 70% identical, these proteins do not display significant sequence similarity. The MEIS1a and the PREP1 proteins share sequence similarity only in their homeodomains (70%) and in the N-terminal HM^A (60%) and HM^B (60%) domains that mediate interactions with PBX proteins. (B) Diagrammatic representation of the integrated *MSCV-HOXA9*, *MSCV-HOXB3*, *MSCV-PBX1*, *MSCV-MEIS1*, and *MSCV-PREP1* proviruses. The expected sizes of the full-length long terminal repeat (LTR)-driven viral transcripts are shown. Restriction sites indicated are *Kpn*I (Kp) (shown only for the *HOXA9* virus but present in all constructs) and *Eco*RI (E). HD, homeodomain; HM, Homothorax-Meis domain.

PBX (1, 3, 15) and to bind identical DNA regulatory sequences (9, 14).

The bone marrow transplantation chimeras were generated by injecting bone marrow cells, immediately following their retroviral infection, into lethally irradiated mice. As the number of transduced cells transplanted per mouse can affect the time frame in which the leukemia develops (U. Thorsteinsdottir and G. Sauvageau, unpublished data), a proportion of the infected bone marrow cells was used to determine the number of transduced hemopoietic progenitors (resistant to G418 [Neo^r] and/or puromycin [$Puro^r$]) injected per mouse in each experimental group (Table 1). No preselection was performed prior to transplantation, thus rendering recipients of doubly infected cells (e.g., *MEIS1* plus *HOXB3*) chimeras consisting of a mixture of non-, single-, and double-transduced cells. The exact composition of each chimera at the time of bone marrow transplantation is detailed in Table 1.

HOXB3 **collaborates with** ***MEIS1*****, but not with** ***PBX1*****, to induce AML.** All recipients of *HOXB3*-transduced bone marrow cells (either alone or in combination with *PBX1* or *MEIS1*) eventually developed AML but with different latencies (Fig. 2A). *MEIS1*, but not *PBX1*, could significantly accelerate the occurrence of AML in the *HOXB3* chimeras, thus indicating a genetic collaboration between *HOXB3* and *MEIS1* in the induction of AML (Fig. 2A). The initial mixed nature of our chimeras (Table 1) can be exploited to further support these conclusions. Although doubly transduced cells represented only 21% of the *HOXB3*-transduced cells initially injected to generate the *HOXB3-MEIS1* chimeras (Table 1), the AML that developed in all of the *HOXB3-MEIS1* mice contained both intact *MEIS1* and *HOXB3* proviruses (Fig. 2B, right panel). In contrast, the presence of both the *PBX1* and *HOXB3* proviruses was detected in only two of the five *HOXB3-PBX1* chimeras analyzed (see mouse 4 and its secondary recipients 4.1 and 4.2 and mouse 5 spleen in Fig. 2B, left panel). This is consistent with the bone marrow transplantation inoculum in which approximately one-fifth of the *HOXB3*-transduced cells were also infected with the *PBX1* retrovirus, thus demonstrating the absence of oncogenic interaction between these two genes. This establishes that *HOXB3* collaborates with *MEIS1*, but not *PBX1*, in leukemic transformation.

Clonal analysis of proviral integration sites demonstrated that the *HOXB3*- and *MEIS1*-induced AMLs were mono- or biclonal (Fig. 2D). Furthermore, the numbers of clones detected with a probe (*neo*) that detects *HOXB3* proviral integration sites and with a probe (*puro*) that detects *MEIS1* proviral integrations were the same, strongly suggesting that all of the leukemic clones detected in the *HOXB3-MEIS1* mice contained both the *HOXB3* and *MEIS1* proviruses (Fig. 2D). Northern blot analysis of total RNA isolated from the leukemic cells confirmed that these clones expressed both retrovirally derived mRNAs (Fig. 2C).

The AML induced by co-overexpression of *HOXB3* and *MEIS1* was readily transplanted to secondary recipients that developed AML in 41 $\pm$ 6 days (data not shown). The leukemias in the secondary mice (labeled as a derivative of a number, e.g., 1.1 or 3.1, etc.) contained both the *HOXB3* and *MEIS1* proviruses with the same clonal composition as that detected in the primary mice (Fig. 2D). Growth factor-independent cell lines were as easily generated from the *HOXB3*- and *MEIS1*-induced leukemias (n = 6) as from the control *HOXA9-MEIS1*-induced leukemias (see below), and high expression of both the *HOXB3* and *MEIS1* retrovirally derived messages could be detected in these cell lines (CL in Fig. 2C). Collectively, these results, together with our previous demonstration of collaboration between *HOXA9* and *MEIS1* in AML induction (17), demonstrate that *MEIS1* can act as a common

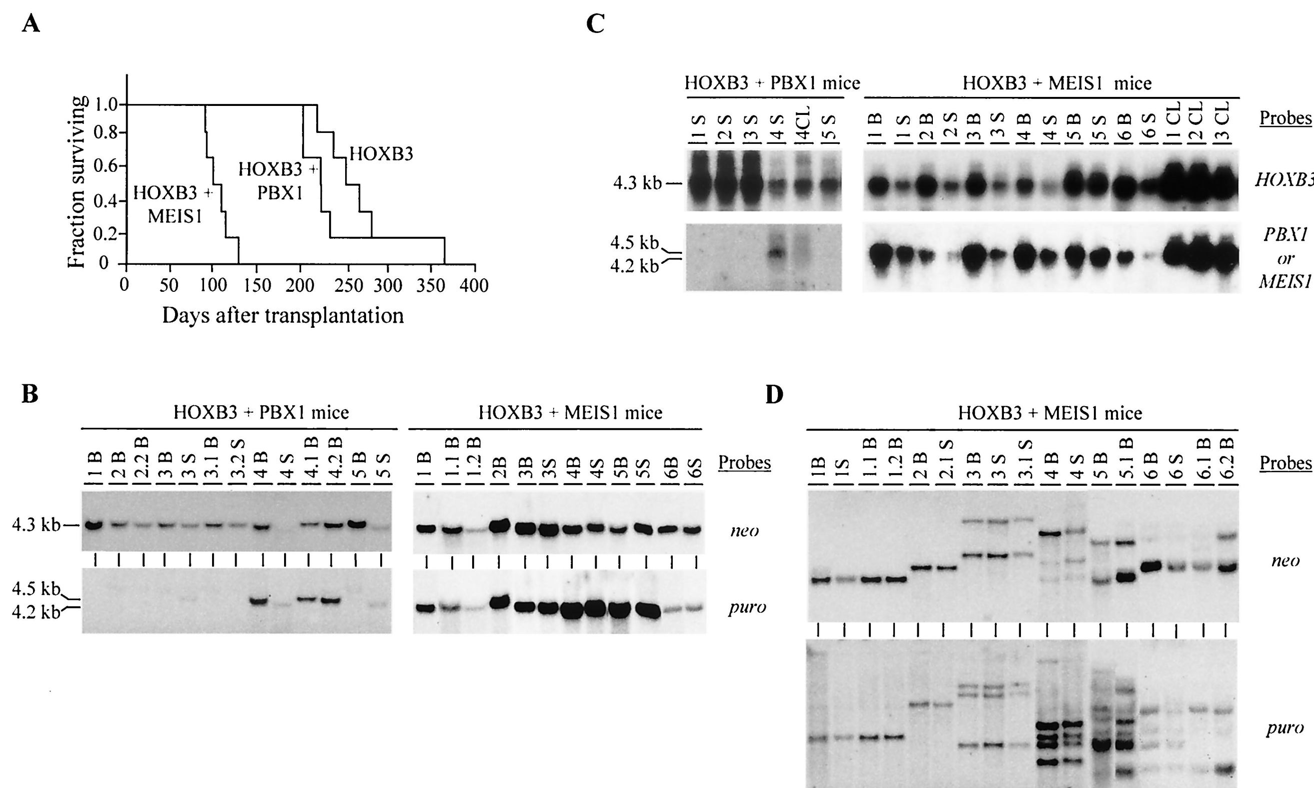
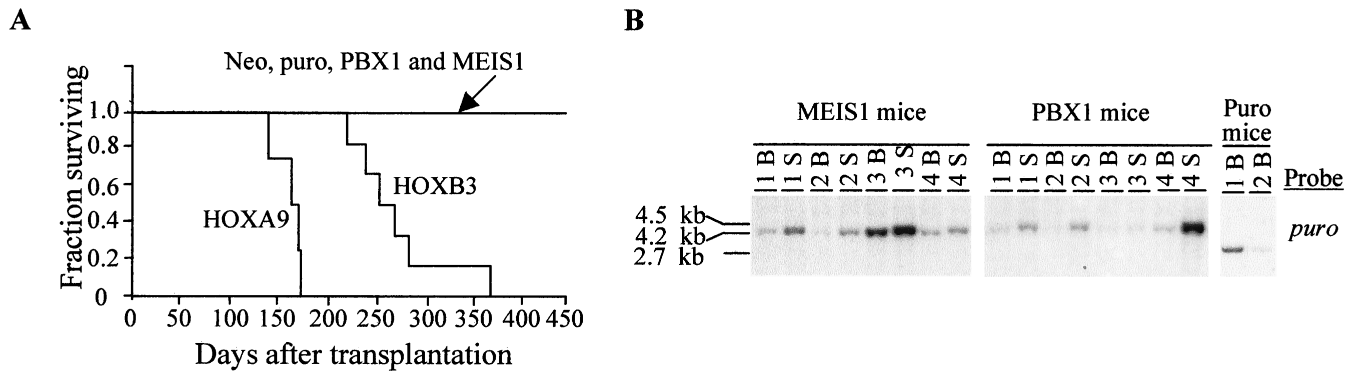


FIG. 2. Demonstration of collaboration between *HOXB3* and *MEIS1*, but not *HOXB3* and *PBX1*, in leukemogenesis. (A) Survival graph demonstrating the collaboration between *HOXB3* and *MEIS1*, but not *PBX1*, in the development of AML. The survival of the *HOXB3-MEIS1* mice was significantly shorter than that of the *HOXB3* mice ($P < 0.001$, two-tailed Student's t test) and the *HOXB3-PBX1* mice ($P < 0.007$). The survival of the *HOXB3-PBX1* mice was not significantly different from that of the *HOXB3* mice. (B) Southern blot analyses of genomic DNA isolated from the bone marrow and/or spleen of the *HOXB3-PBX1* and *HOXB3-MEIS1* chimeras. DNA was digested with *Kpn*I to release the integrated *HOXB3* (4.3-kb), *MEIS1* (4.2-kb), or *PBX1* (4.5-kb) proviral fragments. The membranes were hybridized with a *neo*-specific probe to detect the *HOXB3* provirus and a *puro*-specific probe to detect the *MEIS1* or *PBX1* provirus. (C) Northern blot analysis of total RNA (10 μg) isolated from bone marrow or spleen cells of the *HOXB3-PBX1* and *HOXB3-MEIS1* mice. The membranes were hybridized with full-length *HOXB3*, *MEIS1*, or *PBX1* cDNA probes. (D) Southern blot analysis of DNA isolated from bone marrow of primary and secondary *HOXB3-MEIS1* 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 hybridized first with a *neo*-specific probe for detection of the *HOXB3* proviral fragment(s) (top panel) and then subsequently with a *puro*-specific probe to detect the *MEIS1* proviral fragment(s) (bottom panel). In panels B, C, and D, each primary recipient is identified with a specific number and its secondary recipients or cell lines generated from each primary recipient, with a derivative thereof (e.g., 1.1 and 1.2 and CL1, CL2, etc.). B, bone marrow; S, spleen; CL, cell lines.

collaborator with highly structurally and functionally diverse *HOX* genes in leukemic transformation.

Overexpression of *MEIS1* alone does not predispose to leukemia. Although it has been previously demonstrated both for fibroblasts and for mouse bone marrow cells that *PBX1* lacks an inherent transformation ability (17, 18, 21), the oncogenic potential of *MEIS1* when activated alone has not been thoroughly evaluated. As outlined in Table 1, a number of control chimeras, overexpressing only a single *HOX* or *TALE* gene, were generated for the experiments described above. These chimeras were thus used to compare the leukemogenic potential of the *MEIS1* gene with that of *PBX1*, *HOXA9*, or *HOXB3*. The number of transduced cells transplanted per mouse was high for each of the four groups of chimeras, with *MEIS1* mice receiving numbers that were ~70% of those received by the *HOX* mice (Table 1).

At 13 months posttransplantation, all mice in both the *HOXA9* and *HOXB3* groups had developed AML (all leukemias were mono- or biclonal [data not shown]), whereas the *MEIS1* mice, like the *PBX1* mice, appeared to thrive normally for the observation period of 15 months (Fig. 3A). At that time, four *MEIS1* and *PBX1* mice were sacrificed for more detailed analysis. By FACS and morphological analyses together with in vitro progenitor assays, the only hematological abnormality detected in both groups of mice was a slight enlargement of their spleen (*MEIS1* mice, 0.23 ± 0.15 g, and *PBX1* mice, 0.24 ± 0.20 g, versus untransplanted control, 0.1 g), which, however, was also frequently detected in the *neo* and *puro* control mice (0.22 ± 0.20 g) analyzed at a similar time point. Southern blot analysis of DNA isolated from bone marrow and spleen demonstrated the presence of the intact *MEIS1* or *PBX1* provirus in these organs, indicating that these mice had indeed been repopulated by *MEIS1*- or *PBX1*-transduced hematopoietic stem cells capable of long-term repopulation (Fig. 3B). Furthermore, Northern blot analysis of total RNA isolated from these same tissues revealed the expected expression of the retrovirally derived *MEIS1* message ($n = 3$ mice [data not shown]). The low intensity of the proviral signal



C

EGFP control (30% transduced cells at BMT)

MEIS1-EYFP (69% transduced cells at BMT)

Bone marrow

89 | 11

24 | 6
65 | 5

43 | 4
46 | 7

71 | 29

17 | 1
54 | 28

23 | 22
52 | 3

B220

Mac1

EGFP

EYFP

Spleen

74 | 26

43 | 18
31 | 8

9 | 2
67 | 22

84 | 14

45 | 4
41 | 10

3 | 3
85 | 9

Thymus

80 | 20

74 | 19
5 | 2

69 | 17
11 | 3

53 | 47

49 | 46
4 | 1

47 | 42
7 | 4

CD4

CD8

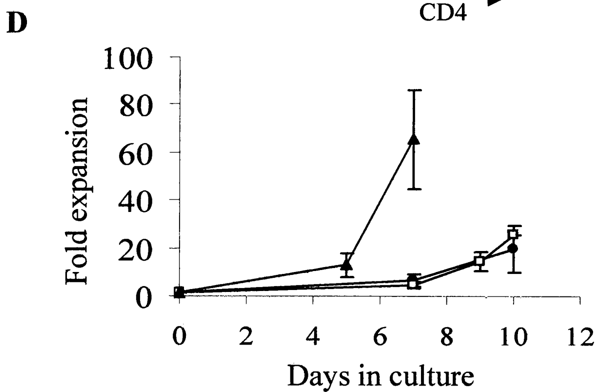
Gated on EGFP⁺ cells

4 | 77
5 | 14

Gated on EYFP⁺ cells

6 | 81
3 | 10

D



detected in hematopoietic tissues of most of the mice is an indicator of low-level repopulation by transduced cells, thus underscoring the fact that neither *MEIS1* or *PBX1* gave a proliferative advantage to hematopoietic cells.

In order to assess in greater detail the effect of overexpression of *MEIS1* on the regeneration of the various hemopoietic lineages, another set of transplantation chimeras were generated as described above, but this time bone marrow cells were engineered to overexpress *MEIS1* through the *MSCV-MEIS-pgk-EYFP* retroviral vector. These mice ($n = 4$ control mice, and $n = 4$ *MEIS1* mice) were then sacrificed at 60 days posttransplantation, and the contribution of transduced cells to the myeloid and T- and B-lymphoid lineages was analyzed by FACS (Fig. 3C). In the bone marrow and spleen of the *MEIS1* mice, the proportion and absolute numbers of myeloid cells (Mac1$^+$ [Fig. 3C and data not shown, respectively]) were within the normal range, whereas the B-lymphoid cells (B220$^+$) were slightly reduced. The contribution of transduced cells (EYFP$^+$) to the myeloid lineage was within the expected range considering the initial gene transfer (69%), thus suggesting that *MEIS1* had little effect on the proliferation or differentiation of myeloid cells in vivo. However, the contribution of *MEIS1*-transduced cells to the regeneration of B-lymphoid cells in both the bone marrow and the spleen was very low for all of the four mice analyzed (Fig. 3C), indicating that high levels of *MEIS1* are incompatible with B-cell development. In contrast, overexpression of *MEIS1* had no detectable effect on T-lymphoid development, as evidenced by a relatively high proportion of transduced cells in the thymus and their normal distribution in the thymic CD4 and CD8 subpopulations (Fig. 3C). As none of the *MEIS1* chimeras that have been generated in our laboratory have developed any hematological malignancies ($n = 20$, of which $n = 13$ were $\geq$14 months posttransplantation when analyzed), this effect of *MEIS1* on the B-lymphoid lineage does not appear to predispose such cells to leukemia (Fig. 3A and data not shown).

To determine the proliferative capacity of *MEIS1*-overexpressing cells, EYFP$^+$ bone marrow cells from the *MEIS1* chimeras were grown in vitro under conditions that stimulate the proliferation of primitive myeloid cells. In agreement with the finding of the effect of *MEIS1* in vivo, the proliferative capacity of *MEIS1*-overexpressing bone marrow cells in vitro was similar to that of control (EGFP$^+$) cells (Fig. 3D), thus supporting the conclusion that *MEIS1* does not confer a proliferative advantage to bone marrow cells. In contrast, *HOXA9*-transduced bone marrow cells derived from *HOXA9-EGFP* chimeras ($n = 4$ mice) showed ~10-fold-greater expansion than that of control bone marrow cells for a 7-day culture period (Fig. 3D).

Taken together, these data demonstrate that *MEIS1* displays a very low leukemogenic potential when overexpressed alone in hematopoietic cells, in contrast to its clear leukemogenic effect when co-overexpressed with *HOXA9* or *HOXB3* (Fig. 2A and 4A).

The phenotypes of the AMLs that developed in transplanted mice are *HOX* gene dependent. As discussed above, enforced expression of either *HOXB3* or *HOXA9* in mouse bone marrow cells induced mono- or biclonal transplantable AML in the recipients. However, despite transplantation of each *HOXB3* and *HOXA9* mouse with a similar dose of transduced cells (Table 1), the AMLs that developed in these two groups of primary recipients differed with respect to the latency (for *HOXB3* chimeras, two times longer than for *HOXA9* chimeras [Fig. 3A and 4A]), the differentiation status (much higher proportion of mature cells in the *HOXB3*-induced AML [Fig. 4]), and the tissue infiltration (much more pronounced in the *HOXB3*-induced AML [Fig. 4A]).

To determine the effects of *MEIS1* co-overexpression on the phenotype of these two different *HOX*-induced AMLs, the *HOXB3-MEIS1*-induced leukemias were compared to those that developed in chimeras transplanted with bone marrow cells overexpressing *HOXA9* or *HOXB3* alone or co-overexpressing *HOXA9* plus *MEIS1* (Table 1). Although *MEIS1* coexpression accelerated the occurrence of both the *HOXB3*- and *HOXA9*-induced AMLs by approximately threefold (Fig. 2A and 5A), it had no detectable effect on the phenotypic characteristics of their AML (Fig. 4 and data not shown). Thus, for example, the AML that developed in the *HOXB3-MEIS1* chimeras had all the main characteristics of the AML that developed in the *HOXB3* chimeras, such as the high proportion of mature myeloid cells and the massive greenish infiltration in nonhematopoietic tissue (Fig. 4). In contrast, the AMLs which occurred in the *HOXA9-MEIS1* chimeras, like those of the *HOXA9* chimeras, were characterized by only moderate infiltration into nonhematopoietic tissues and the presence of mostly immature (i.e., blast) cells in their hematopoietic organs (Fig. 4). Thus, although *MEIS1* accelerates the occurrence of the *HOXB3*- and *HOXA9*-induced leukemias, the *HOX* gene involved ultimately sets the limit for this acceleration and the phenotype of the leukemia. These data, together with the finding of the lack of leukemogenic effect by *MEIS1* when overexpressed alone in hematopoietic cells, thus strongly suggest that *HOX* genes determine the identity of the *HOX-MEIS1*-induced

FIG. 3. Overexpression of *MEIS1* is not permissive for B-lymphoid development but neither induces proliferation of bone marrow cells nor predisposes recipients to lymphoid or myeloid leukemias. (A) Survival graph of chimeras reconstituted with *HOXA9*-, *HOXB3*-, *MEIS1*-, or *PBX1*-transduced bone marrow cells, demonstrating, for the observation period of 450 days, that only the chimeras engineered to overexpress *HOXB3* or *HOXA9*, but not *MEIS1* or *PBX1*, developed leukemia. (B) Southern blot analyses of genomic DNA isolated from the bone marrow and spleen of *puro*-control, *PBX1*, and *MEIS1* mice. DNA was digested with *Kpn*I to release the integrated *puro* (2.7-kb), *MEIS1* (4.2-kb), or *PBX1* (4.5-kb) proviral fragments. The membranes were hybridized with a *puro*-specific probe to detect the control, *MEIS1*, and *PBX1* proviruses. (C) Flow cytometric analysis of hematopoietic cells from bone marrow, spleen, and thymus of *EGFP* control and *MEIS1-EYFP* mice transplanted 60 days earlier with *EGFP*- or *MEIS1-EYFP*-transduced bone marrow cells, respectively. Numbers in the inset quadrant represent the percentages of live cells in the corresponding quadrant. (D) In vitro proliferation of *HOXA9-EGFP* (▲)-, *EGFP*-control (□)-, and *MEIS1-EYFP* (●)-positive bone marrow cells isolated from corresponding mouse chimeras at 60 days after transplantation. B, bone marrow; S, spleen; BMT, bone marrow transplantation.

A

Characteristics of *HOXA9*- and *HOXB3*-induced AML.[a]

Mouse group(n)	Time to AML (days)	Spleen weight (g)	Greenish infilt. of non-hem. tissue	% immature cells[b]	
				PBL	BM
HOXB3(6)	276±64	1.1±0.3	6/6	55±10	65±9
HOXB3 + MEIS1(6)	105±14	0.7±0.2	6/6	46±10	54±19
HOXA9(6)	185±17	0.6±0.2	0/6	89±7	87±2
HOXA9 + MEIS1(6)	54±9	0.3±0.1	0/6	82±3	88±2

B

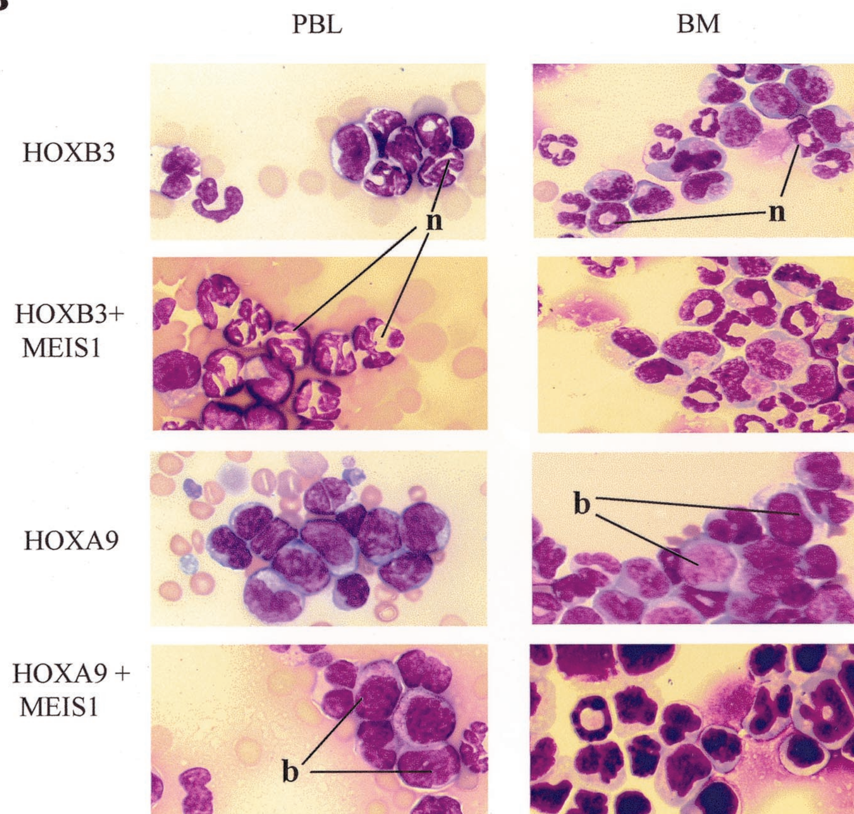


FIG. 4. Differences between *HOXB3*- and *HOXA9*-induced AMLs. (A) Main characteristics of the AMLs that developed in *HOXB3*, *HOXB3-MEIS1*, *HOXA9*, and *HOXA9-MEIS1* bone marrow chimeras. a, Results are expressed as the means ± standard deviations for the indicated number of mice. b, Determination of the proportion of immature and mature cells in hematopoietic tissue of the leukemic mice was based on morphological criteria, i.e., mature cells with segmented nuclei and immature cells, blast-like. For each tissue sample, $n = 200$ cells were counted from $n = 3$ representative mice in each group. infilt., infiltration; non-hem., nonhematopoietic. (B) Wright staining of peripheral blood smears (PBL) and bone marrow (BM) cytospins from representative leukemic *HOXB3*, *HOXB3-MEIS1*, *HOXA9*, and *HOXA9-MEIS1* mice. Magnification, ×100 for all. n, neutrophil; b, blast.

leukemias, with *MEIS1* acting mainly to heighten their leukemogenic potential.

***PREP1*, in contrast to *MEIS1*, does not accelerate the onset of *HOXA9*-induced AML.** To determine whether other members of the *MEIS* family could also accelerate the *HOX*-induced AML, *HOXA9* and *PREP1* were co-overexpressed in mouse bone marrow cells (see experiment 2, Table 1). PREP1 was selected for its reported functional similarities with MEIS1 (15) and its maximal divergence from MEIS1 in regions that exclude the conserved homeodomain and in the amino-terminal HM^A and HM^B motifs which mediate interaction with PBX proteins (5) (Fig. 1B).

Despite initial transplantation of the *HOXA9-PREP1* mice with an ~20-fold-higher number of doubly transduced cells

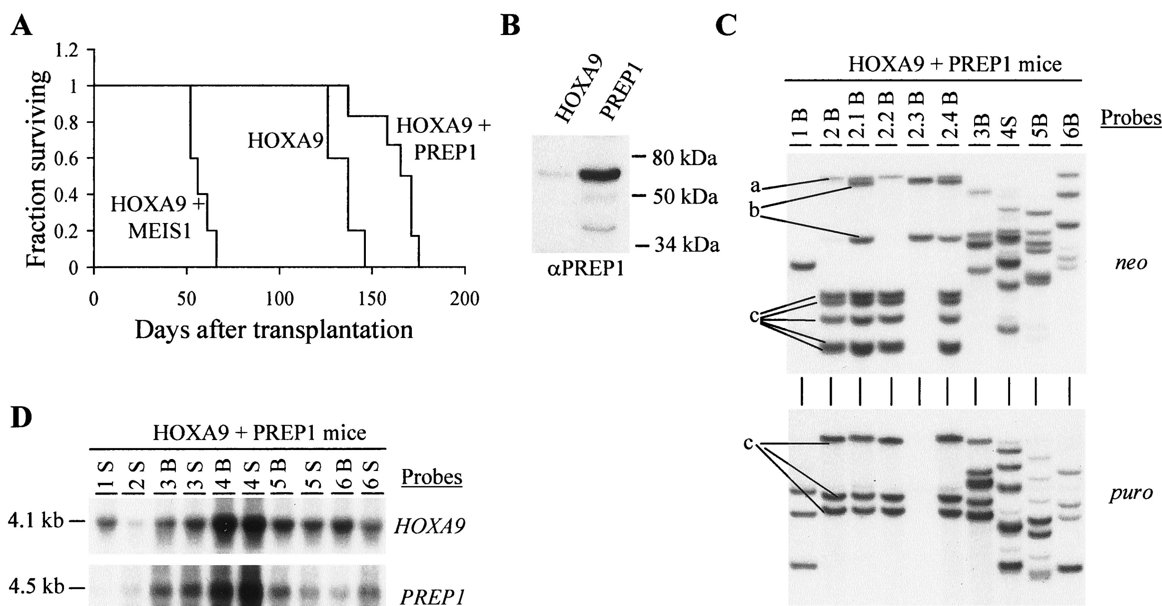


FIG. 5. Demonstration of lack of collaboration between *HOXA9* and *PREP1* in leukemogenesis. (A) Survival graph demonstrating that co-overexpression of *PREP1* with *HOXA9*, in contrast to that with *MEIS1*, does not accelerate the occurrence of the *HOXA9*-induced AML. The survival of the *HOXA9-MEIS1* mice was significantly shorter than that of the *HOXA9* mice ($P < 0.001$, two-tailed Student's t test) and the *HOXA9-PREP1* mice ($P < 0.001$). (B) Western blot analysis of total-cell lysates from the *HOXA9* and *PREP1* viral producer cells. The membrane was probed with rabbit anti-human PREP1 polyclonal antibody. The position of the full-length 64-kDa PREP1 protein is indicated. Two minor products, as previously described (5), are also detected (one generated by an internal ATG site). (C) Southern blot analysis of DNA isolated from bone marrow of primary and secondary *HOXA9-PREP1* 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 hybridized first with a *neo*-specific probe for the detection of the *HOXA9* proviral fragment(s) (top panel) and subsequently with a *puro*-specific probe to detect the *PREP1* proviral fragment(s) (bottom panel). For clarity, the three different clones detected in the primary and secondary recipients of mouse 2 are labeled a, b, and c. (D) Northern blot analysis of total RNA (10 μg) isolated from bone marrow and spleen cells of the *HOXA9-PREP1* mice. The membranes were hybridized with full-length *HOXA9* and *PREP1* cDNA probes. In panels C and D, each primary recipient is identified with a specific number, and its secondary recipients are identified with a derivative thereof (e.g., 1.1, 1.2, etc.). B, bone marrow; S, spleen.

than that for the *HOXA9-MEIS1* mice (Table 1, experiment 2), the *HOXA9-PREP1* mice developed AML with a latency similar to (or even longer than) that of the *HOXA9* mice (Fig. 5A). In contrast, the *HOXA9-MEIS1* mice, as previously reported (17), developed AML with an approximately three-times-shorter latency period (Fig. 5A). The leukemias that developed in the *HOXA9-PREP1* mice were all AML and were morphologically similar to those that developed with *HOXA9* (Fig. 4B). Thus, in contrast to other reported functional similarities with *MEIS1*, *PREP1* cannot accelerate the occurrence of *HOXA9*-induced leukemias.

To exclude the possibility that the lack of collaboration between *HOXA9* and *PREP1* was caused by a failure to generate PREP1 protein from the *PREP1* provirus, Western blot analysis was performed on total cellular lysates from the *PREP1* and *HOXA9* viral producer cells. As opposed to low levels of endogenous PREP1 present in the *HOXA9* viral producer cells, high levels of PREP1 protein were detected in the *PREP1* producer cells (Fig. 5B).

Interestingly, all leukemias that developed in the primary *HOXA9-PREP1* mice contained and expressed both the *HOXA9* and *PREP1* proviruses (Fig. 5C and 5D). This does not indicate genetic collaboration but rather reflects the very high double gene transfer for the *HOXA9* and *PREP1* retroviruses, as ~50% of *HOXA9*-transduced myeloid progenitors that were transplanted initially also contained the *PREP1* provirus (Table 1). Definitive proof for the absence of genetic interaction between *HOXA9* and *PREP1* was provided by the clonal analysis and transplantation of the leukemias that developed in these mice. For example, of three leukemic clones (i.e., Fig. 5C, a, b, and c) detected in primary recipient 2, only one clone (clone c) contained both the *HOXA9* and *PREP1* proviruses (*HOXA9* at five integration sites and *PREP1* at three), while the two other clones (a and b) contained only the *HOXA9* provirus (in clone a at one integration site and in clone b at two integration sites). When the leukemic cells from this primary mouse (mouse 2) were transplanted to secondary recipients, the *PREP1*-containing clone c could be outcompeted by clone b lacking *PREP1* (Fig. 5C, compare 2B with 2.3B). This demonstrates that *PREP1* was not essential for the maintenance of the *HOXA9*-induced leukemia.

These data demonstrate a lack of collaboration between *HOXA9* and *PREP1* in leukemic transformation, thus underscoring the specificity of the collaboration between *HOX* genes and *MEIS1*.

DISCUSSION

Previous studies favored the possibility that *HOX* and *TALE* genes would collaborate in specific pairs, with the pentapeptide-containing HOX proteins (e.g., HOXB3) collaborating with PBX and HOX proteins from paralogous groups 9 to 13

(e.g., HOXA9) collaborating with MEIS (17, 18). The studies reported in this paper clearly indicate that *MEIS1* is a common leukemogenic collaborator with the two highly divergent *HOX* genes *HOXB3* and *HOXA9*. These data would thus argue against the concept of specific collaborating pairs but would rather support a common mechanism in leukemias induced by *HOX* genes and *MEIS1*. The specificity of the *MEIS1-HOX* collaboration for leukemic transformation was evidenced by the inability of another *MEIS* family member, *PREP1*, to substitute for *MEIS1* in accelerating the *HOXA9*-induced AML. Evidence presented herein also establishes the lack of oncogenicity of *MEIS1* when overexpressed alone in primitive bone marrow cells and shows that the leukemogenic potential and phenotypes of the leukemias induced by the *HOX-MEIS1* pair are largely dependent on the *HOX* gene involved, with *MEIS1* acting mainly to accelerate the onset of these leukemias.

The nature of the collaboration between *MEIS1* and *HOX* genes in the induction of AML. Biochemical and genetic studies have demonstrated the importance of HOX-PBX (2, 29) and, most recently, HOX-PBX-MEIS heterocomplex formation for the execution of some HOX-dependent developmental programs (9, 14, 31). Previously, we showed that *HOXB3*- or *HOXB4*-induced transformation of Rat-1 fibroblasts is dependent on endogenous PBX1 levels and is enhanced by co-overexpression of *PBX1*, underscoring a role for a complex containing HOX and PBX in transformation (18). However, with respect to transformation of hemopoietic cells, no such collaboration can be detected between *HOXB3* and *PBX1* but can be detected rather between *HOXB3* and *MEIS1*. These findings were most surprising, considering that *HOXB3*-induced transformation of Rat-1 fibroblasts was not enhanced by the coexpression of *MEIS1* (J. Krosl and G. Sauvageau, unpublished observation). This emphasizes the importance of the cell type used to study *HOX*-induced transformation (i.e., primitive bone marrow cells for leukemias). In both *Drosophila* and mammalian development, the nuclear localization of EXD or PBX is dependent on the presence of HTH or MEIS, whereas a MEIS-independent mechanism appears to operate to maintain PBX nuclear localization in fibroblast cell lines (1, 3, 15, 27). Although it has not been determined for primitive hemopoietic cells, the inability of *PBX1* to accelerate the *HOX* (*-A9* or *-B3*)-induced leukemias when overexpressed could be explained by its cytoplasmic, rather than nuclear, localization in the absence of MEIS proteins. In support of a role for PBX proteins in *HOX*-induced leukemias, the tryptophan motif of HOXA9 (essential for HOXA9-PBX interaction) was recently demonstrated to be necessary for HOXA9-induced in vitro immortalization of myeloid progenitor cells (35), although another study suggests that it might be dispensable (7). The ability of MEIS1 to induce AML in collaboration with HOX proteins must, however, entail more than retaining endogenous PBX protein in the nucleus. This is evident by our demonstration here that the PREP1 protein, which is capable of inducing nuclear localization of EXD and PBX, in both *Drosophila* and mammalian cells (3, 15), lacks the ability to accelerate the *HOXA9*-induced leukemias. Together, these data indicate that in the HOX-induced leukemias the MEIS1 protein must have another role, in addition to one potentially involving PBX, which cannot be accomplished by PREP1.

It was recently demonstrated in two hematopoietic cell lines (i.e., U-937 and KG1) that the HOXA9 protein is part of a trimeric complex with both PBX2 and MEIS1 (37). This suggests that at least some *HOX* gene functions in hematopoietic cells could be dependent on such a trimeric complex formation. However, definitive proof of whether a similar trimeric complex is the foundation for the collaboration between HOX and MEIS1 proteins in leukemic transformation can be accomplished only with the use of appropriate HOX and TALE mutants, or by the identification of transforming targets which would require HOX-PBX and MEIS interactions for their full activation.

***HOX* genes determine the identity of the *HOX*- and *MEIS1*-induced AML.** Although co-overexpression of *MEIS1* accelerated the occurrence of both the *HOXB3*- and *HOXA9*-induced AML, their phenotypes remained *HOX* gene dependent. This observation is not restricted to *HOXB3* and *HOXA9*, as the occurrence of the AML induced by expression of the human fusion protein NUP98-HOXA9 is also accelerated by *MEIS1*, without affecting its phenotype (E. Kroon et al., unpublished data). The underlying mechanism responsible for the differences between the *HOXA9*- and *HOXB3*-induced leukemias is currently unknown. Previous and ongoing studies by our group have demonstrated that, when overexpressed in mouse bone marrow cells, the four *HOX* genes tested thus far generate distinct hematopoietic phenotypes (33, 34, 39; U. Thorsteinsdottir et al., unpublished data). This suggests that a subset of target genes, possibly responsible for cellular identity, is differentially regulated by each *HOX* gene product, thereby predisposing target cells to leukemias with different characteristics.

We show here that *MEIS1*, in contrast to most clustered and nonclustered *HOX* genes (e.g., *TCL-3* or *HOX11*), does not predispose target cells to leukemia when overexpressed in mouse bone marrow cells (13, 34, 39). This difference might be attributed to the inability of *MEIS1*, as shown here both in vivo and in vitro, to confer any proliferative advantage on primitive hematopoietic cells. In contrast, we and others have shown previously that overexpression of all of the *HOX* genes tested so far, as well as the nonclustered *HOX11* gene, enhances the proliferative potential of primitive hematopoietic cells (34, 38, 39). The ability of *MEIS1* to possess leukemogenic potential when co-overexpressed with *HOX* genes raises the possibility that it could engage in similar collaboration with other oncogenes that enhance cellular proliferation. This hypothesis is currently being evaluated in our laboratory.

Functional differences between the *MEIS* family members *MEIS1* and *PREP1*. Of the four mammalian MEIS family members, the MEIS1, MEIS2, and MEIS3 proteins share a high sequence similarity over the entire protein sequence (e.g., MEIS1 versus MEIS2, 77.2%, and MEIS1 versus MEIS3, 69.9%), which is highest in their homeodomain and their PBX interaction domain, HM (23). In contrast, apart from the homeodomain and the HM domain, the PREP1 protein does not share high sequence similarity with other members of the MEIS family. Despite this difference, PREP1 can substitute for MEIS1 or HTH in directing PBX or EXD nuclear localization (3, 15) and, like MEIS1, can form a heterotrimer with HOXB1 and PBX1 on the HOXB2 enhancer element (9, 14). In addition, recent studies using transgenic flies have shown functional conservation between HTH and PREP1 (15). The in-

ability of *PREP1* to accelerate the *HOXA9*-induced leukemias described here represents direct evidence for a functional difference between the PREP1 and MEIS1 proteins. On the basis of these studies, this difference is likely mediated through parts of the MEIS1 and PREP1 proteins other than the homeodomain or the HM domain and thus may involve functions other than DNA binding and interaction with PBC proteins.

The finding that PREP1 is incapable of accelerating the *HOX*-induced leukemias and the low overall sequence similarity between PREP1 and the three other MEIS family members also raise the possibility that, in vertebrates, PREP1 could have evolved to perform functions (perhaps antagonistic) distinct from those of other family members. This difference could thus allow an additional level of regulation within HOX- and TALE-dependent pathways. Interestingly, the PREP1 and MEIS1 protein levels are differentially regulated upon retinoic acid treatment of embryonic carcinoma P19 cells, with PREP1 protein levels dominating in untreated cells and MEIS1 dominating after retinoic acid treatment (9). Furthermore, in adult mouse tissues PREP1 is expressed ubiquitously (10), whereas MEIS1 expression appears to be more specific (10). These studies, together with the data presented here, are thus suggestive of dissimilar regulatory roles for MEIS1 and PREP1 proteins.

In summary, the results of the present study are highly suggestive that genetic interaction with *MEIS1* is part of a common mechanism in *HOX*-induced leukemias. These studies also establish that each of the two *HOX* genes tested has the capacity to determine the phenotype of the leukemias, independently of *MEIS1* co-overexpression. The inability of PREP1 to substitute for MEIS1 indicates that MEIS1 function in this collaboration must involve more than PBX nuclear retention. The existence of such a common mechanism, together with the growing evidence that *HOX* genes and their cofactors are causal oncogenes for human leukemia, reinforces the importance of defining the (common) molecular basis underlying HOX-induced transformation.

ACKNOWLEDGMENTS

We acknowledge Nadine Mayotte for expert technical assistance and Marie-Eve Leroux and Stephane Matte for their expertise and help regarding the maintenance and manipulation of the animals kept at the specific-pathogen-free facility. The support of Nathalie Tessier is also acknowledged for FACS analyses. Robert G. Hawley is acknowledged for his MSCV vectors.

This work was supported by a grant from the National Cancer Institute of Canada (NCI-C). U.T. is the recipient of a Leukemia Research Fund of Canada Fellowship, E.K. is the recipient of a Leukemia and Lymphoma Society of America Fellowship, L.J. is the recipient of a Medical Research Council (MRC) of Canada Fellowship, and Guy Sauvageau is an MRC Clinician-Scientist Scholar.

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