The homeodomain region controls the phenotype of HOX-induced murine leukemia

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HOX proteins are widely involved in hematopoietic development. These transcription factors combine a conserved DNA-binding homeobox with a divergent N-terminal that mediates interaction with variable cofactors. The resulting combinatorial diversity is thought to be responsible for mammalian HOX specificity. Contrasting this proposed mechanism for normal HOX function, here we demonstrate that, in the context of hematopoietic immortalization and leukemogenesis, individual HOX properties are governed almost exclusively by the homeodomain. Swap experiments between HOX1 and HOXA9, 2 members of nonrelated paralog groups, revealed that gene expression patterns of HOX transformed cells in vitro are determined by the nature of the homeodomain. Similar results were seen in vivo during HOX-mediated leukemogenesis. An exchange of the homeodomains was sufficient to convert the slow, low-penetrance phenotype of HOX1-induced leukemia to the aggressive fast-acting disease elicited by HOXA9 and vice versa. Mutation and deletion studies identified several subregions within the DNA binding domain responsible for paralog specificity. Previously defined binding sites for PBX cofactors within the exchangeable, nonhomeobox segment were dispensable for in vitro oncogenic HOX activity but affected in vivo disease development. The transcriptional activator domain shared by HOX1 and HOXA9 at the very N-terminal proved essential for all transformation. (Blood. 2012;120(19): 4018-4027)

Introduction

Next to their role in embryo patterning, HOX genes also play an eminent role during normal and malignant hematopoiesis.1-3 The mammalian HOX family is composed of 39 members that are clustered in 4 groups (A-D). Each contains between 9 and 11 different HOX paralogs classified by the homeodomain. Hematopoietic stem cells and early precursors mainly express HOX genes of groups A and B. During differentiation, these genes are gradually switched off in order of their genomic location. The “anterior” HOX genes corresponding to the antennapedia complex in Drosophila (HOX1-8 at the 3’ end of the cluster with respect to the transcriptional orientation) are extinguished before the “posterior” genes (HOX9-13 at the 5’ end of the cluster). Mature hematopoietic cells generally do not express HOX genes any more.

Increased HOX activity blocks hematopoietic differentiation and immortalizes highly proliferative progenitors. This preleukemic population can be converted to overt leukemia by additional mutations. Therefore, it is not surprising that HOX genes and in particular the “abdominal”-type members are involved in leukemogenesis at multiple instances. A general overexpression of all HOX genes is observed in many cases of acute myeloid leukemia. In clinical samples, HOXA9 has been shown to be a negative prognostic factor with survival inversely correlated to HOXA9 expression.4 The HOX-A cluster is also involved in acute T-cell leukemia where a chromosomal translocation brings part of this genomic locus under control of the strong enhancer normally driving the T-cell receptor.5,6 Furthermore, HOX genes participate in translocations that create highly leukemogenic HOX fusions with proteins of the nuclear pore complex.7 In addition, induction of HOX expression is the central mechanism of blood cell transformation caused by MLL fusion proteins. MLL, a trithorax protein, is an upstream regulator of HOX activity controlling HOX loci by chromatin modification. Oncogenic MLL fusions thwart this normal function and cause constitutive HOX expression.8-10 Similarly, other HOX upstream regulators, such as the CDX proteins, are found at higher than normal levels in acute leukemia.11-14 And finally, 2 very recent reports point out an important role for HOXA9 during blast crisis in chronic myeloid leukemia15 and in a subgroup of multiple myeloma.16

At the structural level, HOX proteins are transcription factors that display a bipartite composition consisting of a highly divergent N-terminal, including sequences with transcriptional activator function17 followed by a C-terminal DNA binding motif termed homeobox. This highly conserved domain is the common denominator of all HOX proteins and defines the phylogenetic relationship of different HOX paralog groups. Short AT-rich sequences were determined in site selection and other in vitro experiments as the preferred binding site for HOX proteins.18-21 As these motifs are very numerous in DNA, additional specificity is required. Therefore, significant advance was made when it was realized that, in analogy to Drosophila, also mammalian HOX proteins form cooperative DNA binding complexes with other homeodomain proteins of the PBX (extradenticle in fly) and MEIS (homothorax) families.22-27 Mammals possess 5 homothorax related genes (MEIS1-3, PKNOX1, PKNOX2) and 4 PBX genes that are each transcribed in various isoforms. Therefore, HOX/MEIS/PBX complexes can occur in a great combinatorial diversity. The importance of MEIS as HOX cofactor was corroborated by experiments in leukemia prone BXH2 mice where malignant cells frequently
carried retroviral insertions coactivating *Meis1* (hence, the name myeloid ectodort viral integration site) and either *Hoxa9* or *Hoxa7*.

In addition, it was shown several times that efficient leukemogenesis in experimental animals requires overexpression of a *Hox* gene together with *Meis*. The question of whether PBX proteins are necessary for HOX-mediated leukemogenesis is not completely settled yet. It was reported that coexpression of *Pbx1b* does not cooperate with *Hoxa9* in hematopoietic transformation and that deletion of a conserved Pbx interaction motif in either *Hoxa9* or a NUP98-HOXA9 fusion leaves transforming activity untouched.32,33 In contrast, other publications find the same motif essential for oncogenic activity and describe a reduction of transformation potential for MLL fusion proteins (that work through up-regulation of *Hox* genes) in cells with reduced *Pbx2* and *Pbx3*.34

Recently, we characterized the leukemogenic potential of each individual *HOXA* gene.34 In this study, we could demonstrate that, apart from the known oncogenes of the “posterior,” *abdominal B*-type genes also “anterior” *labial*-like HOX proteins have significant transforming capacity and cooperate with *Meis1*. However, the phenotype of the occurring leukemia was different. HOXA1 elicited a long-latency disease with reduced penetrance, and leukemic cells displayed myeloid differentiation markers. In contrast, HOXA9 caused a rapidly lethal, more stem-cell-like leukemia with 100% penetrance. To elucidate the basis for these observed differences, here we performed a structural study delineating the “specificity determinants” of HOXA1 and HOXA9. Unexpectedly, we found that the homeodomain alone is the major determinant of paralog specific features in all aspects tested. In contrast, the completely nonsynonymous N-termini of HOXA1 and HOXA9 were functionally equal. Targeting this common denominator might provide a possibility for a coordinated “anti-HOX” therapy instead of targeting HOX members individually.

### Methods

#### Plasmids, retroviral constructs, cell culture, antibodies

All HOX constructs and derivatives thereof were cloned by PCR using either human HOXA1 (NM_005522.4) or HOXA9 (NM_152739.3) as template. Amplicons were inserted into the pMSCV retroviral vector series (Clontech, TaKaRa) and supplemented with an N-terminal HA-tag to aid immunologic detection. For luciferase assays, GAL4 DNA-binding domain fusions were generated in the vector pSG424.35 All clones were sequenced to avoid inadvertent introduction of errors by Taq polymerase. A murine pMSCVneo-Meis1 plasmid was a laboratory stock. Retroviral packaging was done in the Phoenix-E packaging line.37 Antibodies for Western detection and FACS were from Sigma-Aldrich or BD Biosciences.

#### Replacing assays

Replacing or colony formation cell assays were done essentially as described.38,39 This assay allows assessment of the clonogenic capacity of hematopoietic precursor cells after repeated replacing in semisolid medium. An enhanced self-renewal capacity is detected as surrogate parameter for transformation activity. In short, hematopoietic precursor cells were isolated from the bone marrow of 8- to 12-week-old Balb/c mice by magnetic selection for CD117 (c-kit) according to the instructions of the manufacturer (Miltenyi Biotec). The cells were activated in the presence of recombinant murine IL-3, IL-6 (10 ng/mL), and SCF (100 ng/mL). After 2 rounds of retroviral infection by spinoculation (2.5 hours at 34°C and 2500g), cells were seeded in methocel media (M3234, StemCell Technologies) under appropriate selective conditions and with cytokines as in activation medium plus an addition of GM-CSF at 10 ng/mL.

### Colony counts were recorded after 2 rounds of replating. For the generation of permanent HOX-transformed lines, cells from the second replating were transferred to RPMI 1640 medium supplemented with the same cytokines.

#### Transplantation experiments

For induction of acute leukemia in syngenic recipients, 1 × 10⁶ transduced cells were transplanted together with 1 × 10⁶ normal bone marrow cells as radioprotectant into lethally irradiated (9 Gy) animals by intravenous injection. Animals were monitored on a daily basis. After first signs of overt disease were visible, the recipients were killed and a postmortem analysis was performed. Permanent cell lines were regrown in cytokine supplemented media from leukemic spleen. Engraftment of HOXA9ΔPBX(Meis cells was confirmed by PCR on genomic DNA isolated from peripheral blood of transplanted animals with primers ΔPBXf:agtcttggtctctcctcagg, and ΔPBXrev: ccagggtctggtgttttgtatagg. To avoid coamplification of the highly homologous mouse *Hoxa9* sequence, these primers were chosen to span intron 1 of endogenous *Hoxa9*. All animal procedures were performed according to the regulations of the local and institutional authorities (Federation for Laboratory Animal Science Associations [FELASA] recommendations, University of Erlangen Department of Animal welfare, regional licensing authority, license #TS-99/01, 621.2531.32-03/00, 54-2532.2-5/12).

#### Quantitative RT-PCR, microarray experiments, luciferase assays

Quantitative RT-PCR was performed by SYBR Green–based chemistry (Stratagene) on a MX3000P real-time cycler according to the instructions of the manufacturer. Primers were designed such that the resulting amplicons spanned introns. Primers used were: β-actin(forward): caacagggagacagctagag; β-actin(reverse): ctgcctagttgcacaggtcttg; Myb (forward): gacaagaagctggctggctgct; Myb (reverse): gcgcttaaagccgaggccgct; Mc141l(forward): ccggccagaccaacaatacgct; Mc141l(reverse): gcgtaaccgcacgacgaaggcc; Enpp1 (forward): cgggttgctgttg; Enpp1(reverse): ctcaccgcacctgaatttgttg. Expression normalized to actin was determined for each gene in 3 independent RNA samples. These values were scaled to relative expression across all samples, and principal components were built by addition of relative expression units. Luciferase assays were done by electroporation of REH cells according to standard protocols. A construct containing a minimal SV40 promoter preceded by a triplicate of the GAL4 binding site served as reporter (derived from pGL3-basic, Promega). Electroporations were done in triplicates with 0.1 µg reporter plasmid and 0.9 µg pSG424-based expression construct.

### Where appropriate, a Student’s *t* test was performed to probe for statistical significance which was assumed for *P* values < .05.

#### Immunoprecipitation

For immunoprecipitation of HOX, HOX derivatives, and PBX, epitope-tagged versions were coexpressed in 293T cells. Total cell extracts were prepared in lysis buffer (20mM HEPES, pH 7.5, 10mM KCl, 0.5mM EDTA, 0.1% Triton X-100, 10% glycerol, 300mM NaCl, supplemented with 1mM PMSF). Because efficient heterodimerization of HOX and PBX proteins requires DNA, 0.1 nmol of a double-stranded oligo corresponding to a HOX/PBX consensus (ccatcgatgagcagc) was added to total lysates. Precipitation was done with antitag agarose; and after thorough washing in lysis buffer, the precipitated material was analyzed by immunoblot.
solved. From these data, it can be inferred that this basic tetrad is acids (RKKR; Figure 1A). A crystal structure of HOXA9 has been preceded by an N-terminal extension that contains 4 basic amino components toward HOX specific function, we constructed swap to delineate the respective contribution of the 2 major HOX design leaves the vestigial arginine of the “basic tetrad” in HOXA1 intact and creates a small “duplication.” However, this feature of HOXA1 is moved upwards; and as suggested by the structure of the HOXA9 homeodomain, it is likely out of reach to make further contacts with DNA. No structural data are available for HOXA1. Therefore, a linear design was chosen for the reciprocal HOXA9/A1 swap, replacing all amino acids downstream of the conserved R210 in HOXA9 (R233 in HOXA1) with the respective residues of HOXA1. The chimeric HOX genes were cloned into a pMSCV retroviral backbone and tested in phoenix packaging cells for expression. Similar to the parental HOXA1 and HOXA9 constructs, all swaps carried an N-terminal HA-tag to aid detection by Western blot. All constructs were expressed, correctly indicating that swapping the homeodomain does not impair protein stability. To test the functionality of the modified HOX proteins, hematopoietic precursor cells were transduced with the respective swaps and parental constructs as controls (Figure 1C). Transduced cells were analyzed in replating assays. All constructs showed in vitro transforming activity enhancing self-renewal capability of primary hematopoietic cells. We consistently observed a small reduction in colony numbers for cells transduced by HOXA1 compared with HOXA9, probably reflecting the weaker transforming ability of HOXA1. Interestingly, this effect was correlated with the homeodomain. Grafting the HOXA9 homeodomain onto HOXA1 augmented the transforming capability of the HOXA1/9 chimera, whereas the reciprocal fusion blunted the response in the replating assay to a level reached with the parental HOXA1 construct. This phenomenon prompted us to investigate the underlying molecular principles.

**HOX-specific gene expression patterns are determined by the homeodomain**

Because differences in phenotype should have their reflection in a particular genotype, we analyzed paralog-specific gene expression (Figure 2). First, we concentrated on sentinel genes that are exclusively induced either by HOXA1, HOXA9, or by both proteins as previously identified in our laboratory (C.B. and R.K.S., unpublished data, 2011). The gene for the solute carrier protein Slc14a1 (NM_008813) encoding ectonucleotide pyrophosphatase/phosphodiesterase 1 shows the opposite behavior with expression levels — 30-fold higher in HOXA9-transformed cells. Enpp1 (NM_008813) encoding ectonucleotide pyrophosphatase/phosphodiesterase 1 shows the opposite behavior with expression levels — 30-fold higher in HOXA9-transformed cells. Myb (NM_010848) is a direct downstream target of both HOXA1 and HOXA9 and is expressed approximately at equal levels in cells transformed by these genes.34–41 Primary hematopoietic precursor cells were transduced with the constructs as indicated and replated twice in cytokine containing methocel media. Colony numbers are given as mean ± SD of 3 biologic replicates.

**Results**

**Design and functionality of swap constructs**

To delineate the respective contribution of the 2 major HOX components toward HOX specific function, we constructed swap clones exchanging the homeodomain of HOXA1 with that of HOXA9 and vice versa (Figure 1). The homebox of HOXA9 is preceded by an N-terminal extension that contains 4 basic amino acids (RKKR; Figure 1A). A crystal structure of HOXA9 has been solved.40 From these data, it can be inferred that this basic tetrad is positioned close to the minor groove opposite of the actual DNA recognition helix 3 (Figure 1B). Because this motif may increase general protein-DNA affinity, it was grafted together with some flanking amino acids and the actual core-homeodomain onto the N-terminus of HOXA1 to create a HOXA1/9 swap. This particular design leaves the vestigial arginine of the “basic tetrad” in HOXA1 intact and creates a small “duplication.” However, this feature of HOXA1 is moved upwards; and as suggested by the structure of the HOXA9 homeodomain, it is likely out of reach to make further contacts with DNA. No structural data are available for HOXA1. Therefore, a linear design was chosen for the reciprocal HOXA9/A1 swap, replacing all amino acids downstream of the conserved R210 in HOXA9 (R233 in HOXA1) with the respective residues of HOXA1. The chimeric HOX genes were cloned into a pMSCV retroviral backbone and tested in phoenix packaging cells for expression. Similar to the parental HOXA1 and HOXA9 constructs, all swaps carried an N-terminal HA-tag to aid detection by Western blot. All constructs were expressed, correctly indicating that swapping the homeodomain does not impair protein stability. To test the functionality of the modified HOX proteins, hematopoietic precursor cells were transduced with the respective swaps and parental constructs as controls (Figure 1C). Transduced cells were analyzed in replating assays. All constructs showed in vitro transforming activity enhancing self-renewal capability of primary hematopoietic cells. We consistently observed a small reduction in colony numbers for cells transduced by HOXA1 compared with HOXA9, probably reflecting the weaker transforming ability of HOXA1. Interestingly, this effect was correlated with the homeodomain. Grafting the HOXA9 homeodomain onto HOXA1 augmented the transforming capability of the HOXA1/9 chimera, whereas the reciprocal fusion blunted the response in the replating assay to a level reached with the parental HOXA1 construct. This phenomenon prompted us to investigate the underlying molecular principles.

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To get a more comprehensive picture of the gene expression associated with a particular homeodomain, global expression
profiling was done (Figure 2D-E). RNA was pooled from 2 biologic replicates according to the HOX gene used for transduction and hybridized to expression arrays. For evaluation, the HOXA1 and HOXA9/1 samples were compared with HOXA9 and HOXA1/9 cells. Approximately 4600 genes showed a 2-fold difference in expression levels between HOXA1 and HOXA9/1 versus HOXA9 and HOXA1/9.

For the top scoring 100 genes with the largest expression changes and lowest variation (\( P < 0.05 \)) a dendrogram and a heat map were developed (Figure 2D-E; supplemental Table 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Corroborating sentinel gene experiments, expression patterns in transformed cells clearly clustered with the respective homeodomain. To expand this analysis, we determined Pearson correlation coefficients for a larger dataset composing all 254 genes with a \( 10 \)-fold average difference in expression between HOXA1 and HOXA9 and a significant expression of \( > 2 \) log; transformed hybridization value (supplemental Figure 1; supplemental Table 2). As expected, a comparison of HOXA1 and HOXA9 scored low (\( r = -0.05 \)). In contrast, the RNA profile in the HOX9/1 swap cell lines was highly similar to that of HOXA1 cells (\( r = 0.81 \)). Correlation between cells transformed by HOXA1/9 and the original HOX9 constructs even reached a score of \( r = 0.90 \), indicating a nearly perfect match of the respective gene expression patterns.

The homeodomain is responsible for the leukemic phenotype

A good system to assess the paralog-specific properties of HOX genes is the induction of acute leukemia. As previously shown, wild-type HOXA1 causes leukemia with a long latency and reduced penetrance, whereas HOXA9 induces disease very rapidly and efficiently. Both proteins, however, need coexpression of Meis1 for efficient leukemogenesis. For that reason, hematopoietic precursor cells were cotransduced with Meis1 and the swap constructs or the parental HOX genes as control (Figure 3). Surface marker analysis of the double-transduced cells corroborated the homeodomain/phenotype correlation. HOXA9 as well as HOXA1/9 cells were arrested at an earlier stage of differentiation displaying higher c-Kit levels than the HOXA1 or HOXA9/1 populations (Figure 3A). For leukemia induction, these cells were transplanted into syngenic, lethally irradiated recipients together with nontransduced bone marrow as radioprotectant (Figure 3B). Animals were monitored on a daily basis; and on first signs of overt disease (cachexia, ruffled fur, altered behavior, abnormal posture), euthanasia and a thorough post mortem analysis were performed. Spleen weight was recorded, and permanent cell lines were generated from the spleen of the recipients as a definite proof for the presence of leukemia. Again, all aspects of in vivo leukemogenesis in these experiments...
were determined nearly exclusively by the nature of the homeodomain. Equipping the N-terminus of HOXA1 with a HOXA9 homeodomain decreased latency of the resulting leukemia dramatically. In contrast, a HOXA9 amino terminus with a HOXA1 homeodomain protracted the disease course. Another characteristic difference between HOXA1- and HOXA9-induced leukemia was the spleen weight of recipient animals (Figure 3C). ‘HOXA1 leukemia’ was generally associated with a more pronounced splenomegaly compared with HOXA9-initiated disease. This feature was also largely controlled by the homeodomain. However, similar to the gene expression experiments, HOXA1/9 was a better “mimic” of HOXA9 also in vivo, whereas the reciprocal fusion lead to a less complete conversion. This was also underlined by the phenotype of cell lines established directly from leukemic spleen (Figure 3D). An “A9-type” DNA binding domain led to the development of more stem cell-like features with a high level of c-Kit and no evidence for the presence of the differentiation marker Gr-1. The presence of a HOXA1-type homeobox area caused the opposite effect that was more pronounced in HOXA9/1 cells. Expression of HOX-sentinel genes in these cell lines followed the
same pattern as observed in vitro with the interesting exception that myb expression seemed to be proportional to disease latency (Figure 3E).

To test the correlation of the homeodomain and the phenotype of HOX-transformed cells in a different model, an additional HOXA9/13 swap was tested (supplemental Figure 2). As we have shown previously, HOXA13 directs differentiation of hematopoietic cells toward monocytes and macrophages, whereas HOXA9-transformed cells give rise to mainly mixed precursor/granulocyte populations. This particular phenotype of HOXA13-transduced cells manifests itself in the appearance of “mobile” cells that migrate through methylcellulose during replating assays. In addition, HOXA13 cells have the classic monocyte/macrophage morphology and display higher levels of Gr-1 and CD14 surface markers compared with HOXA9 transformed populations. Indeed, all of these features were completely recapitulated by the HOXA9/13 chimera corroborating the importance of the homeodomain as specificity factor.

Several features within the larger homeodomain region cooperate to determine HOX specificity

Because of the particular design, the HOXA9/1 fusion recreates the original HOXA9 basic tetrad upstream of the A1 core-homeobox. In normal HOXA1, this motif is reduced to a single arginine. A structure-function analysis (Figure 4) was performed to elucidate whether this vestigial feature of HOXA9 might be the molecular reason for the less complete conversion of the HOXA9/1 chimera compared with the reciprocal construct. For this purpose, 2 sets of HOXA1 mutants were created. First, additional HOXA1/9 swap derivatives were cloned with the junctions between HOXA1 and HOXA9 sequences moved incrementally toward the C-terminus (Figure 4A). In addition, 4 further mutants were tested that introduce single aspects of the HOXA9 homeodomain into the context of HOXA1: (1) the arginine preceding the core homeobox was changed to the more extended basic feature of HOXA9 (RKKR); (2) charged amino acids of recognition helix 3 in HOXA1 were converted to those present at the same position in HOXA9 (T271R/RE287IN); (3) the long C-terminal extension of HOXA1 following the homeobox that has no counterpart in HOXA9 was removed (ΔC); and (4) a combination of the 3 aforementioned features. After confirmation of correct expression (Figure 4B), hematopoietic precursor cells were transduced with the respective mutants. Each construct was capable of immortalizing hematopoietic progenitors in replating assays (Figure 4C), indicating that the altered homeodomain regions were still capable of inducing at least the minimal set of genes necessary for in vitro transformation. Three independent cell lines were generated for each mutant. The degree of “HOXA9-ness” was quantified by a modified principal component analysis. Expression of the 10 most discriminatory genes that distinguish cells transformed by HOXA1 from those expressing HOXA9 was determined by quantitative RT-PCR for each line. Relative expression values for HOXA1 and HOXA9 specific genes were calculated and added to yield a HOXA1 (PC1) and a HOXA9 (PC2) component (Figure 4D). All 3 major features within the homeodomain region (basic tetrad, helix 3, short C-terminal extension) were absolutely necessary to induce a full HOXA9-type expression pattern. Fusing only helix3 and/or the short HOXA9 C-terminus to HOXA1 (clones T237-A9; F250-A9; E270-A9) led to a loss of HOXA9-specific gene expression. The increased HOXA1 portion, though, did not yet bring back a complete HOXA1 specific pattern. This was only possible with complete HOXA1 where the reduced basic patch, helix3 and the C-terminal extension cooperated. The individual conversion of each of these features to HOXA9 identity (clones RKKR, T271R/RE287IN, ΔC) caused a loss of HOXA1 specific transcripts but did not rescue a “HOXA9-type” expression pattern. Even a combined conversion of these 3 determinants could not rescue the HOXA9 “genotype” indicating that the determinants interrogated by our mutants are necessary but not sufficient to determine the specificity of the DNA
binding domain. Therefore, the recognition of paralog-specific genes beyond a shared "transforming" target set is a consequence of a collaboration of multiple features within the extended homeodomain region. This gives a probable explanation why the reciprocal HOXA9/1 fusion did not completely mimic HOX A1.

**Essential features within the HOX N-termini**

Although there is no significant sequence homology between the N-termini of HOXA1 and HOXA9, our results show that the 2 domains are functionally interchangeable. As a consequence, they must share features that, in combination with the respective homeobox, allow establishment of a paralog specific gene expression pattern. The only common denominator of both proteins described in literature is an interaction with PBX cofactors. Pbx2/Hoxa9 complexes have been detected in myeloid cells and functional cooperation has been demonstrated on DNA templates. Association of Hoxa1 with Pbx1 has been inferred indirectly from EMSA experiments where Hoxa1 and Pbx1 bind cooperatively if tethered to DNA by adjacent recognition sites. In both cases, a tryptophan-containing motif was absolutely essential for interaction with PBX. Therefore, we asked whether PBX binding is a common biologic activity of the HOXA1 and HOXA9 N-termini by testing PBX binding mutants (Figure 5). Derivatives of HOXA1, HOXA9, and HOXA1/9 were created that deleted the crucial tryptophan, including several flanking amino acids (Figure 5A). This manipulation did not alter protein stability as all constructs were expressed at levels comparable to the wild-type versions (Figure 5B). In coimmunoprecipitation experiments, the deletion of the W-motif completely prevented DNA-dependent binding of HOXA9 to PBX2 (Figure 5C). Under identical conditions, HOXA1 did not coprecipitate with PBX1 or PBX2, indicating that the binding affinity was below our detection limit. Still, as deducible from the literature, similar deletions abolish cooperative DNA binding with PBX and mimic a knockout phenotype of HOXA1 during mouse development. Interference with PBX binding had no detectable effect on in vitro transformation as tested in replating assays (Figure 5D). Colony numbers for all PBX binding mutants did not differ significantly from those obtained with the parental constructs corroborating similar results previously reported for HOXA9. In contrast, the PBX binding motif was essential for efficient induction of leukemia in transplantation assays (Figure 5E). Whereas animals transplanted with HOXA9/Meis died of disease after 5-6 weeks, recipients receiving a HOXA9ΔPBX/Meis graft were still alive after 10 weeks. The HOXA9ΔPBX transgene was detectable in these animals by PCR in genomic DNA from peripheral blood indicating engraftment (Figure 5F). Yet, the recipients showed only slightly elevated WBC counts at that time point (Figure 5G). Eventually, leukemia also developed in HOXA9ΔPBX/Meis-transplanted animals after a protracted latency of 14-19 weeks. From the spleens of all mice, HOXA9ΔPBX/Meis cells could be rederived for in vitro culture and the presence of HOXA9 protein. As control, anti-flag and anti-HA blots of the input are shown alongside. (D) Colony numbers obtained in replating assays performed with primary bone marrow cells transduced either with HOX or a HOX construct as indicated. Data are mean ± SD of a biologic triplicate. (E) Kaplan-Meier survival plot of mice transplanted either with cells cotransduced with HOXA9/Meis (n = 3) or HOXA9ΔPBX/Meis (n = 5) as indicated. (F) Engraftment of HOXA9ΔPBX/Meis cells. Genomic DNA was isolated from a peripheral blood sample drawn at day 66 after transplantation from HOXA9ΔPBX/Meis recipients. The HOX transgene was detected by PCR with primers spanning the PBX deletion site. As controls, DNA from a HOXA9/Meis graft (lane HOXA9) and a plasmid containing HOXA9ΔPBX (lane plasmid) were used. Expected amplicon sizes are 190 bp for HOXA9 and 172 bp for HOXA9ΔPBX/Meis (n = 3) or HOXA9ΔPBX/Meis (n = 5) as indicated. (F) Engraftment of HOXA9ΔPBX/Meis cells. Genomic DNA was isolated from a peripheral blood sample drawn at day 66 after transplantation from HOXA9ΔPBX/Meis recipients. The HOX transgene was detected by PCR with primers spanning the PBX deletion site. As controls, DNA from a HOXA9/Meis graft (lane HOXA9) and a plasmid containing HOXA9ΔPBX (lane plasmid) were used. Expected amplicon sizes are 190 bp for HOXA9 and 172 bp for HOXA9ΔPBX. (G) White blood cell (WBC) count at day 66 after transplantation in peripheral blood of the HOXA9ΔPBX/Meis recipients and 2 nontransplanted animals (control). Spleen weight of normal animals and of HOXA9ΔPBX/Meis recipients at day of death is listed in the right column.
HOXA9 in a GAL4-based reporter assay. Indeed, in these experiments, both N-termini served as strong transcriptional activators. Transactivation and transformation were clearly correlated as deletion mutants that impaired colony formation also caused a significant loss in transactivation capacity.

Discussion

Here we report that HOX specific phenotypes in transformed hematopoietic cells are largely controlled by the identity of the HOX DNA binding module (ie, the core homeobox and adjacent sequences). In contrast, the very divergent N-termini of HOXA1 and HOXA9 could be exchanged without significant consequences. The problem of HOX target specificity is not completely understood. Most studies tackling this question have been done in Drosophila. Similar to our results early publications reported that replacing the homeodomain of fly Ultrabithorax (Ubx) with that of Antennapedia (Antp) converts Ubx to an Antp-like molecule. Gehring et al concluded that the homeodomain and neighboring sequences “almost entirely determine its functional specificity” in fly. In the meantime, however, the emerging picture is more complex, and it appears as if there are different classes of target genes. Some of those are recognized by the DNA binding region of the HOX protein alone, whereas others require the input of extradenticle/PBX or other cofactors. (For a comprehensive review of this problem see Mann et al.) The fruit-fly genome encodes only 1 PBX homolog (Extradenticle) and 1 MEIS-related protein (Homothorax) that interact with 8 Drosophila Hox proteins. This restricts combinatorial possibilities compared with the 39 HOX, 4 PBX, and 5 MEIS/PKNOX genes of mammals. Therefore, we expected that cofactor binding patterns would be distinct for different HOX proteins and thus contribute to paralog specific gene expression. Surprisingly, however, specificity was mostly determined by the homeodomain region. This predominance of the DNA binding motif for target gene recognition implies that the common denominator. Both HOXA1 and HOXA9 contain an evolutionary conserved tryptophan residue that has been shown in crystal structures to insert into a hydrophobic binding pocket of PBX. In addition, functional assays indicated that PBX increases DNA binding affinity of mammalian HOX proteins. The importance of PBX for progenitor immortalization in replating assays is not yet completely clear. Whereas Schnabel et al describe that deletion of the hexapeptide motif in HOXA9 impairs its transformation and transformation was clearly correlated as activator than HOXA9 despite its weaker oncogenic activity; however, this may be the result of the artificial environment of the GAL4-based reporter system. A previous report strongly corroborates the interdependence of transactivation and transformation. It has been shown that the VP16 transactivator domain can replace the N-terminus of HOXA9 without a deleterious effect on transforming efficiency. Transactivation does not seem to be a simple consequence of Meis1 binding as anterior HOX proteins HOXA1 to HOXA7 do not directly interact with MEIS. Still, HOXA1 collaborates with MEIS1 to induce leukemia. It may be possible that MEIS is recruited to HOXA1 through PBX/MEIS dimers that
have been reported. Alternatively, the cooperation of HOX and MEIS may occur at the genetic level. MEIS has its own set of targets that can be activated in the absence of HOX proteins. These include genes, such as cyclinD3, which are involved in control of self-renewal capacity and proliferation. In addition, MEIS alone without any additional HOX input can induce leukemia if the transactivation capacity of the protein is “boosted” by fusing it to a VP16 transactivation domain, and this phenomenon was accompanied by an up-regulation of endogenous HOX genes.

In conclusion, it appears as if transformation of hematopoietic cells by HOX proteins, a process much more complex than embryonic development, can be governed by a sort of “simplified” HOX code where intrinsic specificity conferred by the homeodomain is sufficient. This has consequences for strategies to develop therapies for a treatment of HOX-based leukemia. If all HOX proteins more or less depend on the same biologic mechanism to activate their targets, it may be possible to find inhibitors that aim at these oncogenes in general instead of having to deal with each HOX member individually.

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Authorship

Contribution: C.B., E.M., M.-P.G.-C., and R.K.S. performed and analyzed experiments; and R.K.S. designed research and wrote the paper.

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The homeodomain region controls the phenotype of HOX-induced murine leukemia

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