HOXA9 Activates Transcription of the Gene Encoding gp91\(^{\text{Phox}}\) during Myeloid Differentiation*

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The CYBB gene encodes gp91\(^{\text{Phox}}\), a component of the phagocyte respiratory burst oxidase. CYBB transcription is restricted to myeloid cells differentiated beyond the promyelocyte stage. In undifferentiated myeloid cells, the homeodomain (HD) transcription factor HoxA10 represses CYBB transcription via a cis element in the proximal promoter. During myelopoiesis, phosphorylation of conserved tyrosine residues in the HD decreases HoxA10 binding to this CYBB cis element. In the current studies, we found HoxA9 activates CYBB transcription in differentiated myeloid cells via the same cis element. We find HoxA9-mediated CYBB-transcription requires Pbx1 but is inhibited by Meis1. Additionally, phosphorylation of the conserved HD tyrosines increases HoxA9 binding to the CYBB promoter. The HOXA9 gene is involved in leukemia-associated translocations with the gene encoding Nup98, a nucleoporin protein. We find expression of a Nup98-hoxA9 fusion protein blocks HoxA9-induced CYBB transcription in differentiating myeloid cells. In comparison to HoxA9, Nup98-hoxA9 has greater binding affinity for the CYBB cis element, but binding is not altered by HD tyrosine phosphorylation. Therefore, these studies identify CYBB as a common target gene repressed by HoxA10 and activated by HoxA9. These studies also suggest overexpression of Meis1 or Nup98-hoxA9 represses myeloid-specific gene transcription, thereby contributing to differentiation block in leukemogenesis.

Hox proteins are homeodomain transcription factors that are highly conserved from Drosophila to human (1). Human HOX genes are arranged in four paralog groups (HOX-A–D) on four chromosomes. During definitive hematopoiesis, HOX gene transcription proceeds 3’ to 5’ through each paralog group (1). Therefore, HOX1–4 genes are maximally transcribed in hematopoietic stem cells and HOX7–13 in committed progenitors (2). Recent investigations indicate “Abd” HoxA proteins (HOX7–11) are of particular interest to myelopoiesis and myeloid leukemogenesis. One HoxA protein that has received particular attention is HoxA9.

Although few genuine HoxA9 target genes have been identified, much is known about the impact of this protein on myelopoiesis and leukemogenesis. For example, HoxA9 overexpression immortalizes cultured murine bone marrow myeloid cells, but does not block ex vivo differentiation (4). Similarly, HoxA9 overexpression expands the progenitor pool but does not block differentiation in murine bone marrow transplantation experiments (5). In such studies, HoxA9 overexpression induces leukemia only inconsistently, after a long latency (5, 6). Of interest to the current studies, HoxA9 overexpression in murine myeloid cells does not impair granulocyte-colony stimulating factor- or macrophage-colony stimulating factor-induced expression of gp91\(^{\text{Phox}}\), a phagocyte respiratory burst oxidase protein (4). Gp91\(^{\text{Phox}}\) is encoded by the CYBB gene, and transcription of this gene is restricted to myeloid cells differentiated beyond the promyelocyte stage (7, 8).

Other investigations associate HoxA9 with myeloid leukemia. For example, HoxA9 overexpression is highly correlated with myeloid phenotype in pediatric leukemia (9). Additionally, expression of leukemia-associated MLL (mixed lineage leukemia) fusion proteins increases expression of HOX7–13 in committed progenitors (10). Human leukemia and murine models (10, 11). However, MLL fusion proteins also induce leukemia in HoxA9-deficient mice, although the phenotype is “less myeloid” (12). The HOXA9 gene is itself involved in leukemia-associated translocations. For example, translocations involving HOXA9 and the gene encoding the nucleoporin protein Nup98 occur in some myeloid leukemias (13). These leukemias express a fusion protein with N-terminal Nup98 domains and the HOX9 HD. Nup98-HoxA9 overexpression rapidly induces leukemia in murine bone marrow transplantation experiments (14). In microarray experiments, differences in gene expression were identified in myeloid leukemia cell lines overexpressing HoxA9 versus Nup98-A9 (3). These results suggest HoxA9 and Nup98-hoxA9 either interact with different target genes, exhibit differential impact on myelopoiesis (and therefore secondarily on gene expression), or both.

HoxA9 binds DNA as a multiprotein complex with Pbx and/or Meis HD proteins. A DNA-binding site consensus sequence has been derived for HoxA9/Pbx, but few genuine target genes have been identified (15). Function of DNA-bound HoxA9/Pbx may vary during differentiation, because Pbx proteins interact with either co-repressors or co-activators in a signal-dependant manner (16). HoxA9 also partners with Meis1 to bind DNA, with and without Pbx1 (17). Overexpression of HoxA9 plus Meis1 (but not HoxA9 plus Pbx1) rapidly induces myeloid leukemia in murine bone marrow transplantation experiments (6). Overexpression of HoxA9 plus Meis1 also blocks ex vivo cytokine-induced myeloid differentiation (18). Of interest to the current studies, this includes blocked induction of gp91\(^{\text{Phox}}\) expression in differentiating cells (18).

HoxA9 and -10 are similarly expressed during myeloid dif-

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1 The abbreviations used are: HD, homeodomain; HA, hemagglutinin; IFN, interferon; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus; CAT, chloramphenicol acetyl transferase.

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fication and in myeloid leukemia (2). Additionally, both interact with Pbx proteins and recognize similar DNA-binding consensus sequences (15). We previously found HoxA10 represses transcription of the CYBB gene via a cis element homologous to the Hox/Pbx-DNA binding consensus (19). HoxA10 repression of the CYBB gene is Phx1-independent and occurs in undifferentiated myeloid cells (20). During differentiation, phosphorylation of conserved tyrosine residues in the HoxA10 HD decreases HoxA10 interaction with the CYBB cis element, decreasing repression (21).

Although conserved through the HD and Pbx interaction domains, HoxA9 and -10 proteins are otherwise divergent. Perhaps consistent with this, overexpressed HoxA10 induces myeloid leukemia in a Meis1-independent manner in murine bone marrow transplantation experiments (22). One mechanism contributing to leukemogenesis is the HoxA10 differentiation block due to repression of myeloid specific genes (i.e. the CYBB gene). However, repression activity is abrogated by cytokine-induced HoxA10 tyrosine phosphorylation. This suggests that differentiation block by overexpressed HoxA10 would require additional mutations in cytokine signaling pathways. Consistent with this, leukemia induction due to HoxA10 overexpression has a latency of several months in murine bone marrow transplantation experiments (22). Although this latency is longer than in experiments with Nup98-A9 or Meis1 plus HoxA9 overexpression, it is rapid and reproducible in comparison to HoxA9-induced leukemia (6).

Based on these results, the current studies investigate whether CYBB is a HoxA9 target gene. The first goal of these investigations is to determine whether HoxA9 and HoxA10 have redundant function with respect to regulation of myeloid-specific gene transcription during differentiation. Because of specific structural similarities and differences between HoxA9 and HoxA10, the second goal of these studies is to determine whether HD tyrosine phosphorylation similarly regulates DNA-binding affinity of the two proteins. Also, any model of HoxA9 function must address differences between HoxA9 and Nup98-A9, and the role of Meis1 in modulating HoxA9 activity. Our third goal is to address these issues in the context of CYBB gene regulation.

**MATERIALS AND METHODS**

**Plasmide and PCR Mutagenesis**—An HA-tagged HoxA9 cDNA was obtained from Dr. Corey Largman (California Veterans Affairs Medical Center, San Francisco, CA), and subcloned into the pcDNAamp vector (HA-HoxA9/pcDNAamp). HoxA9 (not tagged) was obtained by PCR, and also subcloned into the pcDNAamp vector (HoxA9/pcDNAamp). The cDNA for Nup98 and Nup98A-A9 were obtained from Nabel Yaseen (Northwestern University, Chicago, IL). These cDNAs were subcloned into the pcDNAamp vector with and without HA epitope tags. The cDNA for Pbx1a was obtained from M. Cleary (University of California, San Francisco, CA) and subcloned into the pcSRα vector, as previously described (19). The cDNA for Meis1b was obtained from Dr. Jeffrey Lawrence (University of California, San Francisco, CA) and subcloned into the pcSRα vector.

A HoxA9 cDNA sequence with point mutation of two residues in the Pbx interaction domain (residues 198 and 199 mutated to alanine, referred to as 198/199 HoxA9) or the two tyrosine residues in the homeodomain (tyrosines 212 and 225 to phenylalanine, referred to as Y212F/Y225F HoxA9) were generated by site-directed mutagenesis using the Clontech QuikChange protocol. These mutant cDNAs were subcloned into the pcDNAamp vector for in vitro translation and transfection experiments. Mutant cDNAs were sequenced to verify that no unintended mutations had been introduced.

**Reporter gene assays** were performed with a promoter/reporter construct with 450 bp of the promoter from the gene encoding gp91<sup>+</sup> (the CYBB gene) in the pcATE vector (Promega). This construct has differentiation-inducible reporter activity, as previously described (20, 21). For other assays, an artificial promoter construct was generated with four copies of a CYBB cis element homologous to the Hox/Pbx binding consensus linked to a minimal promoter and a CAT reporter was used (the pTATACAT vector) (23). This construct is referred to as cybbTATACAT and has been previously described (19, 20, 24).

**Oligonucleotides**—Oligonucleotides were synthesized by the Core Facility of the University of Alabama, Birmingham Comprehensive Cancer Center, or the Core Facility of the Robert H. Lurie Comprehensive Cancer Center at Northwestern University. Oligonucleotides: derived consensus sequence for HoxA10/Pbx binding (da10); 5’- tcgattatgattagcg-3’, the Hox/Pbx-binding sequence from the CYBB promoter (106 to 125 bp) (dyccylb); 5’- caatgattatgattagc-3’.

In these oligonucleotides, the Hox core is in boldface, the Pbx core is in italics, and coaat boxes are underlined. Oligonucleotide nucleotides used in PCR for chromatin immunoprecipitation experiments were designed to flank the Hox/Pbx binding CYBB cis element: 5’- PCR oligonucleotide (133 to 106) - 5’- tcaatgctaatagc-3’, 3’ PCR oligonucleotide (6 to 32) - 5’- csgatggattataaatgac-3’.

**Myeloid Cell Line Culture**—The human myelomonocytic cell line U937 (25) was obtained from Andrew Krance (Medical University of South Carolina, Charleston, SC). Cells were maintained and differentiated as described previously (19). U937 cells were differentiated for 48 h with 500 units/ml human recombinant IFNγ (Roche Applied Science).

**Electrophoretic Mobility Shift Assays**—Nuclear extract proteins were prepared by the method of Dignam (27) with protease inhibitors (as described) (28). Oligonucleotides probes were prepared, and EMSA and antibody supershift assays were performed as described (19). An antibody specific to HoxA9, Pbx1a, Meis1, and HA epitope tag were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The HoxA9 antibody recognizes a peptide present in Nup98-hoxA9 but is not cross-reactive with other Hox proteins.

**In Vivo Translated Proteins**—In vitro transcribed HoxA9, Y212F/Y225F HoxA9, and Nup98-A9 mRNA were generated from linearized templates using the Biotools Transcription System, according to manufacturer’s instructions (Promega, Madison, WI). In vitro translated proteins were generated in rabbit reticulocyte lysate, according to manufacturer’s instructions (Promega). Control lysates were generated in similar reactions in the absence of input RNA. In vitro translated proteins were tyrosine de-phosphorylated with Yop protein tyrosine phosphatase (New England Biolabs). Proteins (10 µl of in vitro translated protein) were incubated 30 min at 30 °C in a 20-µl reaction volume with 50 units of Yop and 1 × reaction buffer. Control proteins were incubated similarly in 1 × reaction buffer without Yop. De-phosphorylation of [35S]methionine-labeled, in vitro translated proteins was verified by SDS-PAGE and autoradiography of anti-phospho tyrosine immunoprecipitated proteins, as previously described (21). EMSA with in vitro translated proteins was performed as described (21). The amounts of in vitro translated proteins in DNA binding reactions were equalized by SDS-PAGE of [35S]methionine-labeled proteins.

**Transfection and Reporter Gene Assays**—Cells were transfected by electroporation as described (21). U937 cells (32 × 10⁶ per sample) were transfected with 50 µg of 450pCATE or pCATE (see above); 60 µg of pcDNAmp, wild type or mutant HoxA9/pcDNAmp, or Nup98-A9/pcDNAmp, 50 µg of pSRα, Pbx1a/pSRα, or Meis1b/pSRα; and 15 µg of p-CMVβ-gal (to normalize for transfection efficiency). Transfectants were incubated for 24 h at 37 °C, 5% CO2 followed by 48 h with or without IFNγ (500 units/ml). Preparation of cell extracts, β-galactosidase and chloramphenicol acetyl transferase (CATs) assays were performed as described (29, 30).

To generate lines stably expressing HoxA9 or Nup98-hoxA9, U937 cells were transfected with the mammalian expression vector pcDNAamp with HoxA9 or Nup98-hoxA9 with and without an HA-epitope tag or empty vector control plus the pSRα vector (for antibiotic selection). Cells were transfected and selected in G418 as previously described (28). Stable transflectants were analyzed for HoxA9 or Nup98-hoxA9 overexpression by Western blot. Each experiment was repeated with two or three different transflectant pools and representative results are shown.

**Immunoprecipitation and Western Blotting**—Immunoprecipitation was performed with 300 µg of nuclear proteins from U937 cells. Proteins were immunoprecipitated with anti-HoxA9 antibody (Santa Cruz Biotechnology) or irrelevant rabbit anti-mouse antibody. Proteins were immunoprecipitated for 4 h at 4 °C with 2 µl of HoxA9 antibody or 2 µl of control rabbit antibody followed by 1-h incubation with 30 µl of 50% Staphylococcus protein A-Sepharose bead slurry, as described (21). Immunoprecipitated proteins were washed with radiomime precipi—
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RESULTS

HoxA9 Overexpression Increases CYBB Transcription in Differentiating U937 Myeloid Cells—Previously, we found HoxA10 represses CYBB transcription in undifferentiated U937 myeloid cells (19). This repression requires HoxA10 interaction with a CYBB cis element homologous to the derived Hox/Pbx DNA-binding consensus sequence (referred to as the Hox/Pbx consensus-like CYBB cis element). U937 myeloid leukemia cells represent a pro- monocytic stage of differentiation and express very low levels of gp91^Phox. IFNγ differentiation increases CYBB transcription and gp91^Phox expression in these cells (19, 24). Also, HoxA10 becomes tyrosine-phosphorylated during U937 differentiation (19). Therefore, because tyrosine phosphorylation decreases HoxA10 binding affinity for the CYBB gene, input chromatin was a positive control and chromatin precipitated with glutathione S-transferase antibody was a negative control. PCR products were analyzed by acrylamide gel electrophoresis. The identity of the PCR product was verified by subcloning into a plasmid vector followed by dideoxy sequencing.

For these studies, U937 cells were stably transfected with a vector to express HoxA9 or empty vector control. Transfectant pools were selected (instead of clones) to compensate for potential integration site effects and three independent pools were studied. In initial experiments, we analyzed HoxA9 protein expression in these transfectants. Consistent with previous reports (32), we found little HoxA9 expression in control U937 transfectants. In contrast, HoxA9 protein abundance was increased in stable transfectants with HoxA9 expression vector (Fig. 1A). Therefore, we investigated the impact of HoxA9 overexpression on gp91^Phox mRNA abundance in U937 cells, with and without IFNγ differentiation. Total cellular RNA was isolated and analyzed by Northern blot for gp91^Phox message (Fig. 1B). We found HoxA9 overexpression does not alter gp91^Phox expression in undifferentiated U937 cell in comparison to control transfectants. In contrast, overexpression of HoxA9 in IFNγ-treated cells increases gp91^Phox expression in comparison to similarly treated control U937 transfectants.

To determine whether this increase in gp91^Phox mRNA represents increased CYBB transcription, the impact of HoxA9 on CYBB promoter activity was determined. U937 cells were transfected with a CAT reporter construct with 450 bp of the proximal CYBB promoter (previously described) (20, 21) or reporter vector control. This 450 bp has IFNγ-inducible promoter activity and includes the Hox/Pbx consensus-like cis element, discussed above. U937 cells were co-transfected with vectors to overexpress various combinations of HoxA9 and Pbx1a. Transfectants were analyzed for reporter expression with and without IFNγ differentiation. In undifferentiated U937 transfectants, overexpression of HoxA9 did not alter CYBB promoter activity, with or without Pbx1a co-overexpression (Fig. 1C). Consistent with our previous results (24), IFNγ increased CYBB promoter activity in U937 cells (Fig. 1C). In addition, HoxA9 overexpression induced a significant increase in CYBB promoter activity in differentiated transfectants (n = 7, p < 0.001). This HoxA9-transcriptional effect was significantly augmented by co-overexpression of Pbx1a in IFNγ-treated transfectants (n = 7, p < 0.001).

Nup98-HoxA9 Blocks Induction of CYBB Transcription during U937 Differentiation—Overexpression of the Nup98-hoxA9 leukemia-associated fusion protein induces acute myeloid leukemia in murine bone marrow transplantation experiments (19). Any model of HoxA9 function must address differences between overexpressed HoxA9 and this leukemia-associated protein. Since Nup98-hoxA9 includes the HoxA9 DNA-binding HD, we investigated the impact of Nup98-hoxA9 expression on endogenous gp91^Phox message abundance and CYBB promoter activity. For these studies, stable U937-transfectant pools were generated expressing the larger of the two previously described Nup98-hoxA9 isoforms (28). Expression of a protein of the appropriate size was verified by Western blot (59 kDa) (Fig. 1A). Stable Nup98-hoxA9 transfectants consistently express a HoxA9 cross-immunoreactive protein of lower molecular weight than endogenous HoxA9. Based on the size of this protein and immunoreactivity with various HoxA9 antibodies, this band represents a cleavage product containing the portion of HoxA9 protein present in the Nup98-hoxA9 fusion. Northern blots were performed with RNA isolated from Nup98-hoxA9 expressing cells. As with overexpressed HoxA9, we found little impact of Nup98-hoxA9 on gp91^Phox mRNA in undifferentiated U937 cells (Fig. 1B). However, in contrast to HoxA9, we found Nup98-hoxA9 expression blocked IFNγ induction of gp91^Phox expression in U937 cells (Fig. 1B).

Based on these results, we investigated the impact of Nup98-hoxA9 expression on CYBB promoter activity in U937 transfectants, as above. Because Nup98-hoxA9 includes the Pbx interaction domain from HoxA9, we also determined the effect of Pbx1a co-overexpression. Similarly to HoxA9, we found little impact of Nup98-hoxA9 on CYBB promoter activity in undifferentiated U937 transfectants, with and without Pbx1a co-overexpression (Fig. 1C). However, we found expression of Nup98-hoxA9 blocked IFNγ induction of CYBB promoter activity in U937 transfectants. This effect was not impacted by co-overexpression of Pbx1a.

HoxA9 Activates the Hox/Pbx Consensus-like CYBB cis Element in Differentiated U937 Cells—We next investigated whether HoxA9 activates transcription via the Hox/Pbx consensus-like CYBB cis element. In our previous investigations, we determined HoxA10 represses CYBB transcription via this cis element in undifferentiated U937 myeloid cells. For those studies, we used an artificial promoter construct with multiple copies of the Hox/Pbx consensus-like CYBB cis element linked to a minimal promoter and a CAT reporter (cybbTATACAT) (7, 8). Although this cis element is repressed by overexpressed HoxA10 in undifferentiated U937 transfectants (19), this CYBB sequence functions as a positive cis element in IFNγ-differentiated cells (19, 20). Transcriptional activation is associated with binding of an unidentified, specific protein complex in vitro. Therefore, we investigated the impact of HoxA9 overexpression on this artificial promoter construct, with and without Pbx1a co-overexpression. Transfectants were assayed with and without IFNγ differentiation and reporter activity compared with control transfectants with minimal promoter control vector (TATACAT), as described (19).

In undifferentiated U937 cells, we found HoxA9 overexpres-
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Fig. 1. Induction of gp91 \textsuperscript{Phox} expression during U937 differentiation is increased by HoxA9 overexpression and inhibited by Nup98-hoxA9 expression. A, HoxA9 and Nup98-hoxA9 expression in U937 stable transfectants. U937 cells were transfected with vector to express HoxA9 or Nup98-hoxA9 or empty expression vector. Protein expression was determined by Western blot of cell lysate proteins, as indicated. Increased HoxA9 protein abundance is demonstrated in transfectants with the HoxA9 expression vector. A protein of the appropriate size is expressed in cells transfected with Nup98-hoxA9 expression vector. The asterisk represents a degradation product of Nup98-hoxA9 consistently seen in these studies. B, expression of gp91 \textsuperscript{Phox} in IFN-\gamma-differentiated U937 cells is increased by HoxA9 overexpression and decreased by expression of Nup98-hoxA9. Total RNA was isolated from U937 stable transfectants with HoxA9 or Nup98-hoxA9 expression vector or empty control vector, with and without IFN-\gamma differentiation. Expression of gp91 \textsuperscript{Phox} and \gamma-actin control was determined by Northern blot. HoxA9 increases and Nup98-hoxA9 decreases gp91 \textsuperscript{Phox} mRNA abundance in IFN-\gamma-differentiated cells. C, CYBB promoter activity is increased by HoxA9 overexpression and decreased by Nup98-hoxA9 expression during IFN-\gamma-differentiated U937 cells. U937 cells were transfected with a reporter vector with 450 bp of the CYBB promoter (450cybbpCATE) or empty vector control and vectors to overexpress various combinations of HoxA9, Nup98-hoxA9, Pbx1a, or control expression vector. Reporter gene assays were performed with and without 48 h of IFN-\gamma differentiation. Reporter activity from control pCATE transfectants was subtracted from 450cybbpCATE transfectant reporter activity. Overexpression of HoxA9 increases reporter expression from the CYBB promoter in IFN-\gamma-differentiated transfectants, with and without co-overexpression of Pbx1a. In contrast, Nup98-hoxA9 expression blocks IFN-\gamma-induced CYBB promoter activity, with and without Pbx1a overexpression. None of these proteins impact CYBB promoter activity in undifferentiated transfectants. None of these proteins impacted background reporter activity from the pCATE empty vector.

PCR products were analyzed by acrylamide gel electrophoresis. Lysates from undifferentiated U937 cells were compared with lysates from IFN-\gamma-differentiated cells. We found association of endogenous HoxA9 with the CYBB promoter in IFN-\gamma-differentiated, but not undifferentiated, U937 cells (Fig. 2B).

We also investigated interaction of overexpressed HoxA9 with the CYBB promoter in vivo by chromatin immunoprecipitation. For these experiments, U937 stable transfectants overexpressing HA-epitope-tagged HoxA9 were generated and compared with control vector transfectants. Chromatin was co-precipitated with an anti-HA tag antibody from lysates of stable transfectants and precipitated chromatin analyzed as described above (Fig. 2C). Consistent with the endogenous protein, overexpressed HoxA9 interacted with the CYBB Hox/Pbx consensus-like cis element only in differentiated myeloid cells.

Nup98-hoxA9 Interacts with Hox/Pbx Consensus-like CYBB Cis Element in Differentiated and Undifferentiated U937 Cells—We next investigated the impact of Nup98-hoxA9 on this CYBB cis element during U937 differentiation. Although Nup98-hoxA9 expression blocked IFN-\gamma induction of gp91 \textsuperscript{Phox} mRNA and CYBB transcription in U937 cells, these investiga-
tions did not indicate whether Nup98-hoxA9 impacts function of the Hox/Pbx consensus-like cis element. Therefore, we co-transfected U937 cells with the CYBB cis element-contain-
ing artificial promoter construct (cybbTATACAT) or TATACAT control vector (TATACAT) and vectors to overexpress various combinations of HoxA9, Nup98-hoxA9, Pbx1a, or empty control vector. Reporter gene expression was determined after 48 h, with (black bars) and without (white bars) IFNγ treatment. In untreated U937 transfectants, although cybbTATACAT has increased reporter activity relative to empty vector control none of the overexpressed proteins significantly influence reporter expression. IFNγ-increases reporter expression from cybbTATACAT, as previously described. Additionally, reporter expression from this construct is significantly increased by overexpression of HoxA9 with or without co-overexpression of Pbx1a, but inhibited by Nup98-hoxA9 with and without Pbx1a. B, endogenous HoxA9 interacts with the Hox/Pbx consensus-like CYBB cis element in IFNγ-differentiated U937 cells. Lysates of untreated and IFNγ-differentiated U937 cells were analyzed by chromatin immunoprecipitation. After in vivo cross-linking, chromatin was co-precipitated from cell lysates by an anti-HoxA9 antibody. Co-precipitated chromatin was PCR-amplified with primers flanking the Hox/Pbx consensus-like CYBB cis element and separated by acrylamide gel electrophoresis. Irrelevant antibody was a negative control and total input chromatin a positive control. HoxA9 specifically co-precipitates this CYBB cis element only from lysates of IFNγ-differentiated U937 cells. C, overexpressed HoxA9 and Nup98-hoxA9 interact in vivo with the Hox/Pbx consensus-like CYBB cis element promoter in U937 cells. Lysates of untreated and IFNγ-treated U937 cells stably transfected with vectors to overexpress epitope-tagged HoxA9 or Nup98-hoxA9 or empty vector were analyzed by chromatin immunoprecipitation. Chromatin was co-precipitated with an anti-tag antibody, precipitated chromatin PCR-amplified with primers flanking the Hox/Pbx consensus-like CYBB cis element and separated by acrylamide gel electrophoresis. Irrelevant antibody was a negative control and total input chromatin a positive control. Overexpressed HoxA9 interacts with the CYBB promoter in differentiated U937 cells only, similar to the endogenous protein. However, Nup98-hoxA9 interacts with this CYBB cis element in co-precipitation experiments with both differentiated and undifferentiated U937 cells.

**Fig. 2.** HoxA9 and Nup98-hoxA9 interact with the Hox/Pbx consensus-like cis element in the CYBB promoter. A, HoxA9 and Nup98-hoxA9 have no significant functional impact on the Hox/Pbx consensus-like CYBB cis element in undifferentiated U937 cells, but HoxA9 increases and Nup98-hoxA9 decreases IFNγ-induced transcription via this cis element. U937 cells were co-transfected with an artificial promoter construct with multiple copies of a Hox/Pbx consensus-like CYBB cis element linked to a minimal promoter and a reporter (cybbTATACAT) or control vector (TATACAT) and vectors to overexpress various combinations of HoxA9, Nup98-hoxA9, Pbx1a, or empty control vector. Reporter gene expression was determined after 48 h, with (black bars) and without (white bars) IFNγ treatment. In untreated U937 transfectants, although cybbTATACAT has increased reporter activity relative to empty vector control none of the overexpressed proteins significantly influence reporter expression. IFNγ-increases reporter expression from cybbTATACAT, as previously described. Additionally, reporter expression from this construct is significantly increased by overexpression of HoxA9 with or without co-overexpression of Pbx1a, but inhibited by Nup98-hoxA9 with and without Pbx1a. B, endogenous HoxA9 interacts with the Hox/Pbx consensus-like CYBB cis element in IFNγ-differentiated U937 cells. Lysates of untreated and IFNγ-differentiated U937 cells were analyzed by chromatin immunoprecipitation. After in vivo cross-linking, chromatin was co-precipitated from cell lysates by an anti-HoxA9 antibody. Co-precipitated chromatin was PCR-amplified with primers flanking the Hox/Pbx consensus-like CYBB cis element and separated by acrylamide gel electrophoresis. Irrelevant antibody was a negative control and total input chromatin a positive control. HoxA9 specifically co-precipitates this CYBB cis element only from lysates of IFNγ-differentiated U937 cells. C, overexpressed HoxA9 and Nup98-hoxA9 interact in vivo with the Hox/Pbx consensus-like CYBB cis element promoter in U937 cells. Lysates of untreated and IFNγ-treated U937 cells stably transfected with vectors to overexpress epitope-tagged HoxA9 or Nup98-hoxA9 or empty vector were analyzed by chromatin immunoprecipitation. Chromatin was co-precipitated with an anti-tag antibody, precipitated chromatin PCR-amplified with primers flanking the Hox/Pbx consensus-like CYBB cis element and separated by acrylamide gel electrophoresis. Irrelevant antibody was a negative control and total input chromatin a positive control. Overexpressed HoxA9 interacts with the CYBB promoter in differentiated U937 cells only, similar to the endogenous protein. However, Nup98-hoxA9 interacts with this CYBB cis element in co-precipitation experiments with both differentiated and undifferentiated U937 cells.
transfectant lysates with anti-HA-tag antibody and interaction with the CYBB cis element determined by PCR, as above (Fig. 2C). In contrast to our results with HoxA9, Nup98-hoxA9 interacted with the Hox/Pbx consensus-like CYBB cis element in U937 cells, with and without IFNγ differentiation. The same results were obtained when chromatin was co-precipitated with an anti-HoxA9-antibody that recognizes a peptide present in Nup98-hoxA9 (not shown). These results suggest important differences in HoxA9 and Nup98-hoxA9 binding to the CYBB promoter.

HoxA9 Activation of the Hox/Pbx Consensus-like CYBB cis Element Requires Pbx1—Results of the transfection experiments above indicate co-overexpression of Pbx1a augments HoxA9-induced transcriptional activation of the CYBB cis element. However, HoxA9 alone has some trans-activation effect in these studies. This implies either HoxA9 activates transcription via Pbx1 requiring and independent mechanisms, or that overexpressed HoxA9 interacts with endogenous Pbx1 in U937 transfectants. To distinguish between these possibilities, a mutant form of HoxA9 with disruption of the Pbx interaction domain was generated by site-directed mutagenesis. This mutant form (mutation of arginine 198 and tryptophan 199 to alanine, referred to as 198/199 HoxA9) has been previously described and characterized by other investigators (4, 15). This mutation abolishes interaction with Pbx proteins, but not with DNA-binding consensus sequences (4, 15).

U937 cells were co-transfected with the CYBB cis element-containing artificial promoter construct (cybbTATACAT) or TATACAT control vector and vectors to overexpress various combinations of HoxA9 or Pbx1a, or empty vector. Reporter gene expression was determined after 48 h with (black bars) or without (white bars) IFNγ differentiation. IFNγ increases reporter expression from cybbTATACAT, as previously described. Unlike wild type HoxA9, 198/199 HoxA9 has no impact on reporter expression from cybbTATACAT in IFNγ-treated transfectants with or without co-overexpression of Pbx1a. Overexpressed Nup98-hoxA9 binds the Hox/Pbx consensus-like CYBB cis element with Pbx1 in EMSA with nuclear proteins from undifferentiated U937 cells, but HoxA9 does not. EMSA were performed with a probe representing the CYBB Hox/Pbx consensus-like sequence and nuclear proteins from U937 transfectants with vectors to express HoxA9 or Nup98-hoxA9 or empty vector. Binding reactions were incubated with an anti-HoxA9 antibody (recognizing HoxA9 domains present in Nup98-hoxA9), anti-Pbx1 antibody, or control antibody, as indicated. The previously described low mobility complex is cross-immunoreactive with antibodies to both HoxA9 and Pbx1.

These complexes are cross-immunoreactive with antibodies to both HoxA9 and Pbx1.

In EMSA with nuclear proteins from undifferentiated U937 cells, but HoxA9 does not. EMSA were performed with a probe representing the CYBB Hox/Pbx consensus-like sequence and nuclear proteins from U937 transfectants with vectors to express HoxA9 or Nup98-hoxA9 or empty vector. Binding reactions were incubated with an anti-HoxA9 antibody (recognizing HoxA9 domains present in Nup98-hoxA9), anti-Pbx1 antibody, or control antibody, as indicated. The previously described low mobility complex is cross-immunoreactive with antibodies to both HoxA9 and Pbx1.

These complexes are cross-immunoreactive with antibodies to both HoxA9 and Pbx1.

In comparison, this complex is more abundant in EMSA with nuclear proteins from IFNγ-treated transfectants overexpressing either HoxA9 or Nup98-hoxA9. These complexes are cross-immunoreactive with antibodies to both HoxA9 and Pbx1.

**FIG. 3. Pbx1 interaction is necessary for HoxA9-mediated CYBB transcription in IFNγ-differentiated U937 cells.** A. HoxA9 with mutation of the Pbx interaction domain has no significant functional impact on the Hox/Pbx consensus-like CYBB cis element in U937 cells with or without IFNγ differentiation. U937 cells were co-transfected with an artificial promoter construct with multiple copies of the Hox/Pbx consensus-like CYBB cis element linked to a minimal promoter and a reporter (cybbTATACAT) or control vector (TATACAT) and vectors to overexpress various combinations of HoxA9 with mutation in the Pbx interaction domain (198/199 HoxA9), Pbx1a, or empty control vector. Reporter gene expression was determined after 48 h with (black bars) or without (white bars) IFNγ differentiation. IFNγ increases reporter expression from cybbTATACAT, as previously described. Unlike wild type HoxA9, 198/199 HoxA9 has no impact on reporter expression from cybbTATACAT in IFNγ-treated transfectants with or without co-overexpression of Pbx1a.

B. Overexpressed Nup98-hoxA9 binds the Hox/Pbx consensus-like CYBB cis element with Pbx1 in EMSA with nuclear proteins from undifferentiated U937 cells, but HoxA9 does not. EMSA were performed with a probe representing the CYBB Hox/Pbx consensus-like sequence and nuclear proteins from U937 transfectants with vectors to express HoxA9 or Nup98-hoxA9 or empty vector. Binding reactions were incubated with an anti-HoxA9 antibody (recognizing HoxA9 domains present in Nup98-hoxA9), anti-Pbx1 antibody, or control antibody, as indicated. The previously described low mobility complex is cross-immunoreactive with Pbx1, consistent with our previous results. In contrast, the only complex generated by nuclear proteins from Nup98-hoxA9 transfectants is disrupted by HoxA9 antibody. C. HoxA9 and Nup98-hoxA9 bind the Hox/Pbx consensus-like CYBB cis element with Pbx1 in EMSA with nuclear proteins from IFNγ-differentiated U937 cells. EMSA were performed with a probe representing the CYBB Hox/Pbx consensus-like sequence and nuclear proteins from U937 transfectants with vectors to express HoxA9 or Nup98-hoxA9, or empty control vector. Binding reactions were incubated with anti-HoxA9 antibody, anti-Pbx1 antibody, or irrelevant control antibody, as indicated. In EMSA with nuclear proteins from control vector transfectants, IFNγ treatment decreases the abundance of the low mobility complex, consistent with previous results. In comparison, this complex is more abundant in EMSA with nuclear proteins from IFNγ-treated transfectants overexpressing either HoxA9 or Nup98-hoxA9. These complexes are cross-immunoreactive with antibodies to both HoxA9 and Pbx1.

We also investigated in vitro interaction of HoxA9, Nup98
hoxA9, and Pbx1 with the Hox/Pbx consensus-like CYBB cis element by EMSA. For these experiments, nuclear proteins isolated from U937 cells were preincubated with antibody to HoxA9, Pbx1, or irrelevant control antibody. Our previous investigations demonstrated binding of a low mobility complex containing HoxA10, Pbx1, and HDAC2 in EMSA with nuclear proteins from undifferentiated U937 cells (20). In EMSA with nuclear proteins from IFN-γ-treated U937 cells, this low mobility complex decreases in abundance and is no longer cross-immunoreactive with HoxA10 (19). These results suggest that differentiation alters composition of the protein complex that interacts with the Hox/Pbx consensus-like CYBB cis element.

Therefore, we tested whether endogenous HoxA9 is present in the low mobility complex binding the Hox/Pbx consensus-like CYBB cis element in EMSA with nuclear proteins from U937 cells. Additionally, we investigated in vitro binding of overexpressed HoxA9 or Nup98-hoxA9 to this cis element. For these experiments we used a HoxA9 antibody that recognizes a peptide present in the Nup98-hoxA9 fusion protein.

Similar to our previous results, we found Pbx1 (Fig. 3B) and HoxA10 (not shown) present in the low mobility complex binding the Hox/Pbx consensus-like CYBB cis element probe in EMSA with nuclear proteins from undifferentiated U937 cells. Consistent with results of chromatin immunoprecipitation, neither endogenous nor overexpressed HoxA9 binds this CYBB cis element in EMSA with nuclear proteins from undifferentiated U937 cells. However, in EMSA with nuclear proteins from undifferentiated Nup98-hoxA9-expressing U937 cells, HoxA9 antibody partly disrupts the low mobility complex binding this probe.

IFN-γ treatment of these U937 stable transfectants decreases in vitro binding of the low mobility complex to the Hox/Pbx consensus-like CYBB cis element probe, consistent with previous results. However, the low mobility complex is relatively more abundant in EMSA with nuclear proteins from differentiated U937 cells. Consistent with results of chromatin immunoprecipitation, neither endogenous nor overexpressed HoxA9 binds this CYBB cis element in EMSA with nuclear proteins from undifferentiated U937 cells. However, in EMSA with nuclear proteins from undifferentiated Nup98-hoxA9-expressing U937 cells, HoxA9 antibody partly disrupts the low mobility complex binding this probe.

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Because Nup98-hoxA9 contains the DNA-binding homeodomain, we also investigated the effect of tyrosine phosphorylation on binding of this oncprotein to the CYBB cis element. As above, we performed EMSA with the Hox/Pbx consensus-like CYBB cis element probe and in vitro translated Nup98-hoxA9 with or without Yop treatment. The same controls were performed as described for HoxA9. Equal amounts of HoxA9 and Nup98-hoxA9 fusion protein were used in these experiments as determined by autoradiography of 35S-labeled proteins. We found Nup98-hoxA9 has greater binding affinity for the CYBB cis element than HoxA9 (Fig. 4B). However, in contrast to both HoxA9 and HoxA10, Yop treatment does not influence Nup98-hoxA9 DNA binding to the CYBB cis element probe.

These results suggest wild type HoxA9 tyrosine phosphorylation increases DNA-binding affinity. Therefore, we performed specific experiments to determine the role of the conserved HD tyrosine residues. By site-directed mutagenesis, a mutant HoxA9 was generated with both HD tyrosine residues changed to phenylalanine (Y212F/Y225F HoxA9). This HoxA9 mutant protein was in vitro translated and used in EMSA with the Hox/Pbx consensus-like CYBB cis element probe, as above. As in our previous studies of HoxA10, mutation of these two residues did not prevent phosphorylation of other HoxA9 residues during in vitro translation (not shown) (21). We found binding affinity of Y212F/Y225F HoxA9 to the CYBB cis element probe is decreased in comparison to wild type HoxA9 (Fig. 4D). We also found DNA-binding affinity of this mutant HoxA9 protein is not altered by Yop treatment. Loading of wild type and mutant HoxA9 was equalized by autoradiography of 35S-labeled proteins.

These results suggest tyrosine phosphorylation during differentiation increases HoxA9-binding affinity for the CYBB cis element. Therefore, we hypothesized HD tyrosine mutant HoxA9 would not activate transcription via this cis element in differentiating U937 cells. To test this hypothesis, we co-transfected U937 cells with the artificial promoter construct with multiple copies of the Hox/Pbx consensus-like CYBB cis element (cybbTATACAT, as above) or control TATACAT vector and various combinations of vectors to overexpress Y212F/Y225F HoxA9 and Pbx1a. Transfectants were analyzed for reporter gene activity with and without IFN-γ differentiation. Similar to experiments with wild type HoxA9, Y212F/Y225F HoxA9 has no significant effect on cybbTATACAT reporter expression in undifferentiated U937 transfectants, with and without co-overexpression of Pbx1a (Fig. 4E). However, in con-
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Fig. 4. HoxA9 interaction with the Hox/Pbx consensus-like CYBB cis element is increased by phosphorylation of tyrosine residues in the homeodomain, but tyrosine phosphorylation does not alter Nup98-hoxA9 interaction with this cis element. A, HoxA9 is tyrosine phosphorylated during IFN-γ differentiation of U937 cells. Nuclear proteins from untreated and IFN-γ-differentiated U937 cells were immunoprecipitated under denaturing conditions with an antibody to HoxA9 or irrelevant antibody. Immunoprecipitated proteins were separated by SDS-PAGE and Western blots were probed with antibodies to HoxA9 and phospho-tyrosine. IFN-γ increases tyrosine-phosphorylated but not total HoxA9 protein. B, tyrosine phosphorylation increases HoxA9 binding to the Hox/Pbx-binding CYBB cis element, but does not alter Nup98-hoxA9 binding to this probe. EMSA were performed with a probe representing the Hox/Pbx consensus-like CYBB cis element and in vitro translated HoxA9, Nup98-hoxA9, or control reticulocyte lysate. In vitro translated proteins were de-phosphorylated by incubation with Yop tyrosine phosphatase, as indicated. Yop treatment decreases HoxA9 binding to this probe, but does not alter binding of Nup98-hoxA9. The lower, thin arrow indicates HoxA9, the thick black arrow indicates Nup98-hoxA9, and the asterisk indicates a degradation product of Nup98-hoxA9, as has been seen in previous investigations (14). C, tyrosine phosphorylation increases HoxA9 plus Pbx1a binding to the Hox/Pbx-binding CYBB cis element. EMSA were performed with a probe representing the Hox/Pbx consensus-like CYBB cis element and in vitro translated HoxA9, Pbx1a, or both. In vitro translated HoxA9 was de-phosphorylated with Yop tyrosine phosphatase, as indicated. Yop treatment decreases HoxA9 binding to this probe, with and without Pbx1a. The upper arrow indicates the HoxA9/Pbx1a complex and the lower arrow HoxA9. D, mutation of conserved HD tyrosine residues decreases HoxA9 binding affinity for the Hox/Pbx consensus-like CYBB cis element. EMSA were performed with a probe representing the Hox/Pbx consensus-like CYBB cis element and in vitro translated HoxA9, HoxA9 with mutation of the conserved tyrosine residues in the HD (Y212F/Y225F HoxA9), or control reticulocyte lysate. In vitro translated proteins were de-phosphorylated by incubation with Yop tyrosine phosphatase, as indicated. The abundance of DNA-bound HoxA9 was decreased by Yop treatment, as above. Binding of Y212F/Y225F HoxA9 was not altered by Yop treatment and was equivalent to Yop-treated wild type HoxA9. E, Y212F/Y225F HoxA9 has no functional impact on the Hox/Pbx consensus-like CYBB cis element in U937 cells with or without IFN-γ differentiation. U937 cells were co-transfected with an artificial promoter construct with multiple copies of a Hox/Pbx consensus-like CYBB cis element linked to a minimal promoter and a reporter (cybbTATACAT) or control vector (TATACAT) and vectors to overexpress various combinations of Y212F/Y225F HoxA9, Pbx1a, or empty control. Reporter gene expression was determined after 48 h, with (black bars) and without (white bars) IFN-γ. In contrast to the increase in cybbTATACAT reporter activity seen with overexpression of wild type HoxA9 with and without Pbx1a, reporter expression was not increased in transfectants with Y212F/Y225F HoxA9 with and without Pbx1a.

Contrast to wild type HoxA9, Y212F/Y225F HoxA9 has no significant effect on transcriptional activation of the CYBB cis element in IFN-γ-treated U937 cells (p = 0.51, n = 9) (Fig. 4E, compare with Fig. 2A, noting difference in scale). This is not altered by co-overexpression of Pbx1a (p = 0.55, n = 9).

Meis1b Blocks HoxA9 Induction of CYBB Transcription in
cis element interaction, or secondary impact on gp91 overexpress various combinations of HoxA9, Pbx1a, and neuronal (cybbTATACAT or control TATACAT) and vectors to differentiated U937 transfectants (Fig. 5). U937 cells were co-transfected with the artificial promoter construct with multiple copies of the cis element. U937 cells were co-transfected with a reporter vector with CYBB promoter activity (difference in reporter vector versus HoxA9 plus Pbx1a expression vectors p = 0.65, n = 5). Co-overexpression of Meis1b also blocked induction of reporter expression by HoxA9 plus Pbx1a in IFNγ-treated transfectants (Fig. 5B) (p = 0.47, n = 5 for difference between transfectants with control expression vector versus HoxA9 plus Pbx1a plus Meis1b).

We next investigated whether this Meis1b effect on CYBB promoter activity was mediated via the Hox/Pbx consensus-like cis element. U937 cells were co-transfected with the artificial promoter construct with multiple copies of the CYBB cis element (cybbTATACAT or control TATACAT) and vectors to overexpress various combinations of HoxA9, Pbx1a, and Meis1b. Reporter activity was assayed with and without IFNγ treatment. In undifferentiated U937 cells, CYBB promoter activity was not significantly altered by overexpression of Meis1b alone, or in any combination with HoxA9 or Pbx1a (Fig. 5B). However, in IFNγ-differentiated transfectants, co-overexpression of Meis1b blocked HoxA9-induced CYBB promoter activity (difference in reporter activity between 450pCATE transfectants with control expression vector versus HoxA9 plus Meis1b expression vectors p = 0.65, n = 5). Co-overexpression of Meis1b also blocked induction of reporter expression by HoxA9 plus Pbx1a in IFNγ-treated transfectants (Fig. 5B) (p = 0.47, n = 5 for difference between transfectants with control expression vector versus HoxA9 plus Pbx1a plus Meis1b).

Differentiated U937 Cells—Previous studies indicate co-overexpression of HoxA9 and Meis1 rapidly induces leukemia in murine bone marrow transplantation experiments. Additionally, Meis1 overexpression blocks ex vivo cytokine-induced differentiation in murine bone marrow myeloid cells overexpressing HoxA9 (18). This differentiation block specifically includes impaired cytokine-induced gp91Phox expression (18). Possible mechanisms include direct interaction of Meis1 with HoxA9 at a CYBB cis element, interference by Meis1 with HoxA9 CYBB cis element interaction, or secondary impact on gp91Phox expression by Meis1. Therefore, we investigated the impact of Meis1 on HoxA9-induced CYBB transcription during myeloid differentiation. In initial experiments, Meis1b overexpression in U937 transfectants was verified by Western blot (Fig. 5A). Consistent with previous reports, little Meis1 is detected in nuclear proteins from U937 cells. However, Meis1 is easily detected in nuclear proteins from U937 cells transfected with Meis1b expression vector. Expression of endogenous and overexpressed Meis1 is not altered by IFNγ differentiation (not shown).

Therefore, we determined the functional impact of overexpressed Meis1b on CYBB promoter activity in U937 transfectants. U937 cells were transfected with a reporter vector with the proximal 450 bp of the CYBB promoter (or empty vector control, as above) and various combinations of vectors to overexpress HoxA9, Pbx1a, and Meis1b. Transfectants were assayed with and without IFNγ treatment. In undifferentiated U937 cells, CYBB promoter activity was not significantly altered by overexpression of Meis1b alone, or in any combination with HoxA9 or Pbx1a (Fig. 5B). However, in IFNγ-differentiated transfectants, co-overexpression of Meis1b blocked HoxA9-induced CYBB promoter activity (difference in reporter activity between 450pCATE transfectants with control expression vector versus HoxA9 plus Meis1b expression vectors p = 0.65, n = 5). Co-overexpression of Meis1b also blocked induction of reporter expression by HoxA9 plus Pbx1a in IFNγ-treated transfectants (Fig. 5B) (p = 0.47, n = 5 for difference between transfectants with control expression vector versus HoxA9 plus Pbx1a plus Meis1b).

We found induction of Meis1 with the Hox/Pbx consensus-like CYBB cis element in IFNγ-treated transfectants overexpressing Meis1b but not undifferentiated transfectants. Endogenous Meis1 interaction with the CYBB promoter was not detected under these assay conditions. We also investigated whether Meis1b overexpression inhibits HoxA9 interaction with the Hox/Pbx consensus-like CYBB cis element. For these studies, chromatin was co-precipitated with an anti-HoxA9 antibody from lysates of U937 cells overexpressing Meis1b or transfected with empty vector. Lysates from IFNγ-differentiated and undifferentiated transfectants were analyzed, as above (Fig. 5D). In these studies, HoxA9 interacted with the CYBB cis element in IFNγ-differentiated cells and Meis1b overexpression did not block this interaction. These results suggest overexpressed Meis1b participates in a complex with HoxA9 binding the CYBB promoter in differentiated myeloid cells.

**DISCUSSION**

Studies of human leukemia and animal models implicate HoxA9 and HoxA10 in myeloid progenitor expansion and regulation of myelopoiesis. However, characterization of the biochemical mechanisms involved in these effects has been impaired by the paucity of identified target genes. In these studies, we identify the gene encoding gp91Phox (the CYBB gene) as a HoxA9 target gene. These studies focus on CYBB transcription, because we previously found HoxA10 represses this gene in undifferentiated myeloid cells (19). In contrast, our current studies indicate HoxA9 activates CYBB transcription in differentiated myeloid cells. These results represent the first example of HoxA9 and HoxA10 regulating a common target gene, but exhibiting opposite effects on transcription of the gene. Previously, we found HoxA10 tyrosine phosphorylation during myeloid differentiation decreases binding to a Hox/Pbx consensus-like CYBB cis element (20). Our current studies indicate HoxA9 tyrosine phosphorylation increases binding to the same CYBB cis element in differentiated myeloid cells. These results provide the first mechanistic explanation for differences in the functional impact of HoxA9 and HoxA10 on myeloid gene regulation during myelopoiesis.

Our previous studies indicate HoxA10 overexpression blocks differentiation and contributes to leukemogenesis by repressing genes expressed in mature phagocytes, such as the CYBB gene. Conversely, our current studies indicate HoxA9 activates transcription of such genes. HoxA9 overexpression is associated with myeloid progenitor expansion and myeloid phenotype in leukemia (9, 22). Therefore, our results suggest a mechanism by which leukemia might be phenotypically “less myeloid” in the absence of HoxA9. However, our current investigations do not indicate a mechanism for expansion of the progenitor pool in HoxA9 overexpressing cells. This will likely be clarified by identification of additional target genes. Based on studies of the homologous *Drosophila* proteins (32), one would expect HoxA9 to recognize different target genes at various stages of myelopoiesis. For example, although HoxA9 tyrosine phosphorylation increases CYBB-promoter interaction, it may decrease interaction with unidentified genes in undifferentiated cells. Also, the *Drosophila* proteins activate or repress transcription at various development stages (32). HoxA9 function is likely to be similarly complex.
FIG. 5. Meis1 interacts with the Hox/Pbx consensus-like CYBB cis element and inhibits HoxA9-induced transcriptional activation in IFNγ-differentiated U937 cells. A, overexpression of Meis1b in U937 transfectants. U937 cells were transfected with a vector to express Meis1b or empty expression vector. Meis1 overexpression was verified by Western blot of cell lysate proteins, as indicated. Meis1b is detected only in cells transfected with Meis1b expression vector. B, Meis1b overexpression inhibits HoxA9-induced CYBB promoter activity in IFNγ-differentiated U937 cells. U937 cells were transfected with a reporter vector with 450 bp of the CYBB promoter (450cybbpCATE) or empty vector control and vectors to overexpress various combinations of HoxA9, Pbx1a, Meis1b, or control expression vector. Reporter gene assays were performed with and without 48 h of IFNγ differentiation. Reporter activity from control pCATE transfectants is subtracted from 450cybbpCATE transfectant reporter activity. Overexpression of Meis1b significantly inhibits HoxA9-induced activity of the CYBB promoter in IFNγ-differentiated transfectants, with and without co-overexpression of Pbx1a. In contrast, overexpressed Meis1b alone has no impact on IFNγ-induced CYBB promoter activity. None of these proteins impact either CYBB promoter activity in undifferentiated transfectants, or background reporter activity from the empty pCATE vector. C, Meis1b has no functional impact on the Hox/Pbx consensus-like CYBB cis element in undifferentiated U937 cells, but
CYBB was not identified as a HoxA9 target gene in previous gene-expression profiling studies because of the differentiation-stage specificity of CYBB transcription. These previous studies used RNA from undifferentiated K562 or U937 cells (3, 33), neither of which expresses gp91Phox. These data are consistent with our results indicating HoxA9 overexpression increases gp91Phox expression only in differentiated U937 cells. HoxA9 overexpression also did not alter gp91Phox expression in gene-expression profiling studies with CD34+ cells (33). Combined with these results, our studies suggest cytokine-induced tyrosine phosphorylation increases HoxA9-DNA binding, influencing differentiation-stage-specific CYBB transcription.

We find HoxA9 and HoxA10 DNA binding is modulated by phosphorylation of conserved HD tyrosines. However, phosphorylation increases HoxA9 DNA binding and decreases HoxA10 binding. Previously, we found decreased HoxA10 DNA binding requires interaction of phosphorylated HD tyrosines with a HoxA10 domain not conserved in HoxA9 (23). This suggests divergence outside of the HD is important in differentially regulating HoxA9 and HoxA10 activities. Significantly, we found phosphorylation does not alter binding of the isolated HD (19) or Nup98-hoxA9 to this CYBB cis element. Therefore, regulation of HoxA9 DNA binding requires HD tyrosines and domains not conserved in HoxA10 or present in Nup98-hoxA9. Previous structural studies do not address this issue. The crystal structure of DNA-bound HoxA9 was solved using a truncated protein with the HD and Pbx interaction domain (34). In those studies, the truncated protein was expressed in Escherichia coli, and phosphorylation was not addressed. However, those studies found HD tyrosine 212 distorts DNA binding, which might be consistent with modification of this residue increasing DNA-binding affinity.

In our studies, HoxA9 activation of CYBB transcription requires an intact Pbx interaction domain. This is consistent with activation by Pbx-dependant recruitment of transcriptional co-activator proteins, as previously described for Hox/Pbx transcription of other genes (16). In contrast, HoxA10 repression involves direct recruitment of co-repressors by a HoxA10 domain not conserved in HoxA9 (20). Taken together, these results suggest a mechanism for Pbx-independent differentiation block (and leukemogenesis) by HoxA10, but not HoxA9. These results also suggest why co-overexpression of HoxA9 plus Pbx does not induce leukemia in murine bone marrow transplantation experiments. In contrast, these proteins would be anticipated to increase functional competence of mature phagocytes.

CYBB regulation provides a good model for understanding functional differences between HoxA9 and Nup98-hoxA9. Although Nup98-hoxA9 includes the HoxA9 Pbx interaction domain, it did not activate CYBB transcription in our studies. Indeed, we found Nup98-hoxA9 expression blocks differentiation-induced CYBB transcription. In EMSA with the Hox/Pbx consensus-like CYBB cis element, we found either overexpressed HoxA9 or Nup98-hoxA9 formed a DNA-bound complex with Pbx1. However, because Nup98-hoxA9 is substantially larger than HoxA9, it is possible steric hindrance quantitatively decreased Pbx1 interaction with this cis element in vivo. Alternatively, Nup98 domains may sterically hinder Pbx1 recruitment of co-activators to the CYBB promoter. In either case, HoxA9 domains are required for inhibition of differentiation-induced CYBB transcription by Nup98-hoxA9, because overexpression of the Nup98 portion of the fusion protein had no effect. In contrast to both HoxA9 and HoxA10, Nup98-hoxA9 DNA binding was not influenced by tyrosine phosphorylation. Therefore, these studies suggest an explanation for rapid differentiation block and leukemogenesis by Nup98-hoxA9 in comparison to HoxA10. Overexpression of either HoxA10 or Nup98-hoxA9 blocked differentiation by preventing myeloid-specific gene transcription. However, differentiation block by overexpressed HoxA10 required inactivating mutations in pathways that induced HD tyrosine phosphorylation during differentiation. In contrast, Nup98-hoxA9 interacted with myeloid gene promoters (such as the CYBB gene) and blocked differentiation with these pathways was intact.

Our studies of CYBB regulation also provide a mechanism for differentiation blocking by overexpression of HoxA9 plus Meis1. We found that Meis1 overexpression blocked HoxA9-induced CYBB transcription in differentiated myeloid cells. Previous investigations indicate overexpression of HoxA9 plus Meis1 rapidly induces leukemia in murine transplantation experiments. This is consistent with the fact that no additional mutations were required for differentiation blocking in cells overexpressing HoxA9 plus Meis1. We found that Meis1 overexpression did not interfere with HoxA9 interaction with the CYBB cis element in vivo. HoxA9 has previously been found to form hetero-trimeric DNA-bound complexes with Pbx1 and Meis1 (17). Therefore, Meis1 overexpression may inhibit transcription by sterically hindering recruitment of co-activator proteins to myeloid gene promoters by Pbx1.

These studies of CYBB transcription indicate a mechanism by which HoxA9 contributes to the myeloid phenotype. Additionally, these studies identify mechanisms for functional differences between HoxA9 and Nup98-hoxA9 and for differentiation block by overexpressed HoxA9 plus Meis1. Similar to our previous studies of HoxA10, the current investigations provide further indication of the importance of understanding Hox protein functional regulation in understanding regulation of normal myelopoiesis and myeloid leukemogenesis.

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