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HoxA10 Represses Transcription of the Gene Encoding \( \text{p67}^{\text{phox}} \) in Phagocytic Cells

Stephan Lindsey,* Chunliu Zhu,* Yu Feng Lu,*† and Elizabeth A. Eklund1*†

\( \text{p67}^{\text{phox}} \) and \( \text{gp91}^{\text{phox}} \) are components of the phagocyte-specific respiratory burst oxidase that are encoded by the \( \text{NCF2} \) and \( \text{CYBB} \) genes, respectively. These genes are transcribed exclusively in myeloid cells that have differentiated beyond the promyelocyte stage. In mature phagocytes, \( \text{NCF2} \) and \( \text{CYBB} \) transcription continues until cell death and further increases in response to IFN-\( \gamma \) and other inflammatory mediators. Because \( \text{p67}^{\text{phox}} \) and \( \text{gp91}^{\text{phox}} \) expression profiles are similar, we hypothesize that common transcription factors interact with homologous \( \text{cis} \) elements in the \( \text{CYBB} \) and \( \text{NCF2} \) genes to coordinate transcription. Previously, we identified a negative \( \text{CYBB} \) promoter \( \text{cis} \) element that is repressed by the homeodomain transcription factor HoxA10. We found that transcriptional repression requires HoxA10-dependent recruitment of histone deacetylase activity to the \( \text{CYBB} \) \( \text{cis} \) element. In response to IFN-\( \gamma \), phosphorylation of two tyrosine residues in the HoxA10 homeodomain decreases binding to \( \text{CYBB} \) promoter, thereby abrogating HoxA10-mediated repression. In the current studies, we investigate the possibility that HoxA10 similarly represses \( \text{NCF2} \) transcription. We identify a sequence in the \( \text{NCF2} \) promoter that is homologous to the HoxA10-binding \( \text{CYBB} \) \( \text{cis} \) element. We find that this \( \text{NCF2} \) promoter sequence functions as a negative \( \text{cis} \) element that is repressed by HoxA10 in a tyrosine phosphorylation and histone deacetylation-dependent manner. Our results suggest that cytokine-stimulated pathways regulate HoxA10-mediated repression of the \( \text{CYBB} \) and \( \text{NCF2} \) genes in differentiating myeloid cells and in mature phagocytes during the inflammatory response. Because \( \text{p67}^{\text{phox}} \) and \( \text{gp91}^{\text{phox}} \) are rate-limiting components for respiratory burst activity, our studies may identify rational therapeutic targets to modulate free radical generation in pathological conditions. The Journal of Immunology, 2005, 175: 5269–5279.

Professional phagocytic cells, such as neutrophils, monocytes, and macrophages, contribute to the system of host defense by generating superoxide and other toxic free radicals via the respiratory burst. Signaling events initiated by inflammatory mediators such as IFN-\( \gamma \) modulate activity of the respiratory burst oxidase. Oxidase activation involves interaction of at least two cytosolic proteins (\( \text{p47}^{\text{phox}} \) and \( \text{p67}^{\text{phox}} \)) with a membrane-bound catalytic unit composed of \( \text{p22}^{\text{phox}} \), \( \text{p40}^{\text{phox}} \), and a flavoprotein (reviewed in Ref. 1). Inflammatory mediators induce transcription of genes encoding the rate-limiting components of the oxidase complex (2, 3): \( \text{p67}^{\text{phox}} \) and \( \text{gp91}^{\text{phox}} \), which is encoded by the \( \text{NCF2} \) gene (4); and \( \text{gp91}^{\text{phox}} \), which is encoded by the \( \text{CYBB} \) gene (5). Transcription of the \( \text{CYBB} \) and \( \text{NCF2} \) genes is restricted to myeloid cells that have differentiated beyond the promyelocyte stage (2, 3). Because transcription of these genes is concurrent during both myelopoiesis and the inflammatory response, we hypothesize that a common set of transcription factors regulates homologous \( \text{cis} \) elements in these two genes. Consistent with this hypothesis, we previously identified homologous \( \text{cis} \) elements in the proximal promoters of the \( \text{CYBB} \) and \( \text{NCF2} \) genes, which are necessary for IFN-\( \gamma \)-induced transcription (6–8). In both genes, the \( \text{cis} \) elements are activated by a multi-protein complex which includes the transcription factors PU.1, IFN-regulatory factor 1 (IRF1)\(^2\) and the IFN consensus sequence-binding protein (ICSBP) (6–8). Cooperation between these proteins recruits the CREB-binding protein to the \( \text{CYBB} \) and \( \text{NCF2} \) promoters. Assembly of this activation complex requires cytokine-induced, Jak2-dependent tyrosine phosphorylation of IRF1 and ICSBP (8, 9). Therefore, common events are necessary for activation of \( \text{CYBB} \) and \( \text{NCF2} \) transcription.

In previous investigations, we determined that the homeodomain transcription factor HoxA10 represses \( \text{CYBB} \) transcription. HoxA10 interacts with a proximal \( \text{CYBB} \) promoter element as part of a multiprotein complex which includes Pbx1a and the transcriptional corepressor protein histone deacetylase (HDAC) (2, 10). HoxA10-mediated repression of \( \text{CYBB} \) transcription requires HDAC2 activity but is Pbx1 independent (11). In myeloid cell lines, IFN-\( \gamma \) increases HoxA10 tyrosine phosphorylation in a Jak2-dependent manner (12). Tyrosine phosphorylation decreases HoxA10-binding affinity for the \( \text{CYBB} \) promoter, thereby abrogating repression (12). Decreased DNA-binding affinity requires interaction of two phosphorylated tyrosine residues in the HoxA10 homeodomain with an SH2-like domain that is N-terminal to the homeodomain (12). In the current studies, we investigated HoxA10 repression of \( \text{NCF2} \) transcription via a promoter sequence homologous to the HoxA10/Pbx-binding \( \text{CYBB} \) element. We also determine the impact of HoxA10 tyrosine phosphorylation on \( \text{NCF2} \) transcription. Previously, we found that histone deacetylase inhibition increases \( \text{p67}^{\text{phox}} \) mRNA abundance and \( \text{NCF2} \) transcription in myeloid cell lines (11). In the current studies, we investigated the roles of HoxA10 and Pbx1 in histone deacetylase-mediated \( \text{NCF2} \) repression.

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Abbreviations used in this paper: IRF1, IFN-regulatory factor 1; ICSBP, IFN consensus sequence-binding protein; HDAC, histone deacetylase; MSCV, murine stem cell retroviral vector; PTP, protein tyrosine phosphatase; TSA, trichostatin A; \( \beta \)-gal, \( \beta \)-galactosidase; CAT, chloramphenicol acetyltransferase.
Toxic free radicals generated via the respiratory burst contribute to tissue damage in conditions such as reperfusion injury, adult respiratory distress syndrome, and autoimmune disorders. The goal of our investigations is to identify mechanisms by which inflammatory mediators coordinate expression of gp91<sup>phox</sup> and p67<sup>phox</sup> during myelopoesis and the immune response. We hypothesize that inflammatory mediators increase CYBB and NCF2 transcription in phagocytic cells by inducing posttranslational modification of transcription factors that regulate homologous cis elements in these genes. Because the CYBB and NCF2 genes encode rate-limiting oxidase proteins, elucidation of the involved pathways may identify rational therapeutic targets to modulate the inflammatory response in pathological conditions.

Materials and Methods

Plasmids and PCR mutagenesis

**Reporter plasmids.** Constructs with various fragments of the NCF2 5’ flank in the pCATE reporter vector (Promega) have been previously described (7). For the current studies, a construct with the proximal 1000 bp of 5’ flank was used (+6 to −1000 bp relative to the ATG; referred to as −1000NCF2CATE) (7). This construct includes the positive cis element activated by PU.1, IRF1, and ICSBP, and the sequence homologous to both element. The sequences 5’ to −610 to −618 bp (also, artificially introduced promoter-reporter constructs were generated in the minimal promoter/reporter vector p-TATA CAT, as previously described (10, 13) (obtained from Dr. A. Kraft, Medical University of South Carolina, Charleston, SC). A construct was generated with four copies of the −600 to −637 bp sequence from the NCF2 promoter (in the forward direction, referred to as p-nC2TATACAT) (7).

**Plasmids with cDNAs.** The cDNA for human HoxA10 was obtained from C. Largman (University of California, San Francisco, CA) (14). This cDNA sequence represents the major transcript in mammalian hematopoietic cells, encoding a 393-aa, 55-kDa protein. Wild-type HoxA10 cDNA sequence was subcloned into the pDNAamp vector for in vitro translation, pSRα for expression in mammalian cells (15), and pMSCV (Stratagene) for expression in murine bone marrow cells. The Pbx1a cDNA was obtained from Dr. M. Cleary (Stanford University School of Medicine, Stanford, CA). This cDNA was subcloned into the pSRα vector for expression in mammalian cells.

HoxA10 cDNA with mutation of the Pbx1 interaction domain was generated by PCR-based site-directed mutagenesis, as described (11). Oligonucleotides were synthesized by the Core Facility of the Robert H. Lurie Comprehensive Cancer Center at Northwestern University as follows: dsA10 (derived consensus sequence for Pbx/HoxA10 binding): 5’-tcacctgattattcagacctgc-3'; dscybA10 (the Hox/Pbx-binding sequence from the CYBB promoter, −94 to −134 bp); 5’-tcacctgattattcagacctgctattcagacctgc-3'; dscybA10 (a homologous sequence from the NCF2 promoter, −610 to −618 bp); 5’-aagagtcatcctagctttctagcaagag-3' or dsmutnc2A10 (same sequence with mutation of the Hox/Pbx consensus sequence); 5’-aagagtcatcctagctttctagcaagag-3'. In these oligonucleotides, the HoxA10 core is bold, in the Pbx core is in italics, and ccaat boxes are underlined. Complementary single stranded oligonucleotides were annealed and used in EMSAs or subcloned to generate artificial promoter constructs, as described (10).

**Oligonucleotides.**

Oligonucleotides were synthesized by the Core Facility of the Robert H. Lurie Comprehensive Cancer Center at Northwestern University as follows: dsA10 (derived consensus sequence for Pbx/HoxA10 binding): 5’-tcacctgattattcagacctgc-3'; dscybA10 (the Hox/Pbx-binding sequence from the CYBB promoter, −94 to −134 bp); 5’-tcacctgattattcagacctgctattcagacctgc-3'; dscybA10 (a homologous sequence from the NCF2 promoter, −610 to −618 bp); 5’-aagagtcatcctagctttctagcaagag-3' or dsmutnc2A10 (same sequence with mutation of the Hox/Pbx consensus sequence); 5’-aagagtcatcctagctttctagcaagag-3'. In these oligonucleotides, the HoxA10 core is bold, in the Pbx core is in italics, and ccaat boxes are underlined. Complementary single stranded oligonucleotides were annealed and used in EMSAs or subcloned to generate artificial promoter constructs, as described (10).

**Myeloid cell culture.**

The human myelomonocytic cell line U937 (16) was obtained from Andrew Kraft (Medical University of South Carolina, Charleston, SC). Cells were maintained and differentiated as described (10–12). U937 cells were treated with 500 U/ml human rIFN-γ for 48 h, as indicated. Primary murine bone marrow myeloid progenitor cells were obtained and cultured as described (17). Briefly, bone marrow was harvested from the femurs of wild-type BALB/c mice according to a protocol approved by the Animal Care and Use Committee of Northwestern University and the Jesse Brown VA Medical Center. Bone marrow mononuclear or Scf<sup>−</sup> cells were separated and cultured in DME supplemented with 10% FBS, GM-CSF (10 ng/ml), and IL-3 (5 ng/ml) as described (17). After 48 h, cells were transduced with a retroviral vector to express HoxA10, Y326/343F HoxA10, or empty vector control. Retrovirus was generated using the murine stem cell retroviral vector (MSCV) as described (17). Cells were selected in puromycin for 96 h, treated with various cytokines, harvested, and RNA extracted.

**RNA extraction and Northern blots.**

Total cellular RNA was extracted from U937 cells or murine bone marrow cells by the single-step method, as described (18). RNA was separated on denaturing formaldehyde gels and transferred to nylon membranes according to standard techniques. Probes were generated by the random primer method, using cDNA sequences from genes of interest as a template.

**Nuclear extract and EMSAs.**

Nuclear extract proteins were prepared by the method of Dignam et al. (19) with protease inhibitors, as described (10). Oligonucleotides probes were prepared, and EMSA and Ab supershift assays were performed as described (10–12). Antibodies to HoxA10 (not cross-reactive with other Hox proteins) was obtained from Covance Research Products or from Santa Cruz Biotechnology.

**In vitro-translated proteins.**

In vitro-translated HoxA10 and Y326/343F HoxA10 mRNA were generated from linearized template DNA using the Riboprobe System, according to the manufacturer’s instructions (Promega). In vitro-translated proteins were generated in rabbit reticulocyte lysate also according to the manufacturer’s instructions (Promega). Control (unprogrammed) lysates were generated in similar reactions in the absence of input RNA. In vitro-translated proteins were protein dephosphorylated with Yop protein tyrosine phosphatase (Yop-PTP) (New England Biolabs). Proteins (5 μl of in vitro-translated protein) were incubated for 30 min at 30°C in a 20-μl reaction volume with 50 U of Yop-PTP and 1× reaction buffer, according to the manufacturer’s instructions. Control proteins were similarly incubated in 1× reaction buffer without Yop-PTP.

EMSA with in vitro-translated proteins was performed as described (12). The amount of mutant and wild-type HoxA10 proteins in DNA-binding reactions was equalized by SDS-PAGE of <sup>35</sup>S-methionine-labeled proteins. De-tyrosine phosphorylation and integrity of Yop-PTP-treated proteins were verified by anti-phosphoysotrine immuneprecipitation and autoradiography of SDS-PAGE, as described (12).

**Transfection and reporter gene assays.**

Cells were transfected by electroporation as described (10–12). U937 cells (2 × 10<sup>6</sup> per sample) were cotransfected with 50 μg of pCATE reporter vector control and 10000NCF2 or pSRα control plasmid (p-ncf2TATACAT or p-SRα-TATACAT; 30 μg of pSRα control, HoxA10/pSRα, N312A/W323T HoxA10/pSRα binding mutant), or Y326/343F HoxA10/pSRα (homeodomain tyrosine mutant) and 15 μg of p-CMV-β-gal (to normalize for transfection efficiency). Transfectants were incubated for 24 h at 37°C and 5% CO<sub>2</sub>, followed by 48 h with or without IFN-γ (500 U/ml). Preparation of cell extracts, β-galactosidase (β-gal) and chloramphenicol acetyltransferase (CAT) assays were performed as described (10–12).

In other experiments, cells were transfected with 70 μg of p-TATACAT or p-TATACAT; 30 μg of pSRα control, HoxA10/pSRα, N312A/W313T HoxA10/pSRα binding mutant, or Y326/343F HoxA10/pSRα (homeodomain tyrosine mutant) and 15 μg of p-CMV-β-gal (to normalize for transfection efficiency). Transfectants were transfected with a vector to co-overexpress Pbx1a or treated with 1000 ng/ml trichostatin A (TSA) for 24 h, as described (10–12).

**Immunoprecipitation and Western blots.**

Immunoprecipitation experiments were performed with cell lysates from cultured murine bone marrow myeloid cells. Cells were disrupted in denaturing lysis buffer, as described (12). Cellular proteins (100 μg) were diluted with radiolimmunoprecipitation assay buffer (with protease inhibitors), incubated with either HoxA10 antiserum (rabbit polyclonal; Covance) or irrelevant control Ab, and collected with Staphylococcus protein A-Sepharose as described (12, 17). Immunoprecipitates were separated by 10% SDS-PAGE, and proteins were detected by HoxA10 Ab (goat polyclonal; Santa Cruz) or anti-phosphoysotrine Ab. In other experiments, cultured, transduced murine bone marrow myeloid cells were lysed in 2× SDS sample buffer and equal amounts of protein (50 μg)
were separated by SDS-PAGE. Western blots were serially probed with Abs to gp91\textsubscript{phox}, p67\textsubscript{phox}, and HoxA10.

**Chromatin immunoprecipitation**

U937 cells were cultured with or without IFN-\(\gamma\) for 48 h, as described (10). Cells were incubated with formaldehyde before lysis, and lysates were sonicated to generate chromatin fragments with an average size of 2.0 kb, as described (20). Lysates were incubated with anti-HoxA10 Ab or pre-immune serum control, and immunoprecipitates were collected with Staphylococcus protein A beads. Coprecipitated chromatin was PCR amplified with primers flanking the Hox/Pbx consensus-like sequences in the CYBB and NCF2 genes. PCR products were analyzed by acrylamide gel electrophoresis and visualized by ethidium bromide staining. Total input chromatin (nonprecipitated) was a positive control, and preimmune serum-precipitated chromatin was a negative control. The identity of the PCR product was verified by dideoxy sequencing.

**Results**

**HoxA10 overexpression decreases p67\textsubscript{phox} expression in myeloid cells**

Our first goal in these investigations was to determine the impact of HoxA10 on p67\textsubscript{phox} expression. For initial studies, we used U937 myelomonocytic cells (16). Although constitutive expression of gp91\textsubscript{phox} and p67\textsubscript{phox} is low in this cell line, IFN-\(\gamma\) treatment increases CYBB and NCF2 gene transcription, resulting in respiratory burst competence and therefore functional differentiation (6, 7). In previous studies, we determined that HoxA10 overexpression in U937 cells decreases endogenous gp91\textsubscript{phox} mRNA abundance but that IFN-\(\gamma\)-treatment abrogates this effect (10). In those studies, we found that IFN-\(\gamma\)-induces HoxA10 tyrosine phosphorylation, which decreases binding affinity of HoxA10 for the CYBB promoter (12). Therefore, we investigated the impact of HoxA10-overexpression on p67\textsubscript{phox} mRNA in U937 cells, with and without IFN-\(\gamma\)-treatment. We used U937 cells stably overexpressing HoxA10 or transfected with empty control vector, as in our previous studies (10). To compensate for possible integration site effects, we used stable transfectant pools instead of clones. We find that HoxA10 overexpression decreases p67\textsubscript{phox} mRNA abundance in untreated U937 cells but not in IFN-\(\gamma\)-treated transfecants, consistent with gp91\textsubscript{phox} expression (Fig. 1A).

Because U937 cells are a leukemia-derived line, other leukemia-associated abnormalities may influence the impact of HoxA10 on myeloid gene transcription in these cells. Therefore, we also investigated the impact of HoxA10 overexpression on p67\textsubscript{phox} mRNA in primary murine bone marrow myeloid cells. For these experiments, murine bone marrow myeloid progenitors were isolated and cultured in GM-CSF and IL-3, and transduced with a retroviral vector to express HoxA10 or empty control vector (17). Cells were selected in puromycin, and HoxA10 overexpression was verified by Western blot (not shown). RNA from transfected cells was analyzed for p67\textsubscript{phox} and gp91\textsubscript{phox} expression by Northern blot (Fig. 1E). We find that HoxA10 overexpression decreases p67\textsubscript{phox} and gp91\textsubscript{phox} mRNA abundance in murine myeloid progenitors, consistent with our results in non-IFN-\(\gamma\)-treated U937 cells (6, 7). These studies provide support for use of U937 myeloid leukemia cells to investigate the impact of HoxA10 on NCF2 transcription.

**HoxA10 overexpression decreases NCF2 transcription in U937 cells**

We next determined whether HoxA10 overexpression influences p67\textsubscript{phox} expression by repressing NCF2 transcription. In previous studies, we analyzed the proximal 1000 bp of NCF2 5' flank in U937 transcription experiments (7). This 1000-bp sequence includes an IFN-\(\gamma\)-inducible cis element at −173 to −180 bp and transcription start sites −25, −42, and −58 bp from the ATG (7, 21). We noted that the −610- to −618-bp NCF2 promoter sequence is homologous to the derived HoxA10/Pbx-DNA-binding consensus sequence and the HoxA10/Pbx-binding cis element from the CYBB promoter (11, 12). Therefore, we investigated the impact of HoxA10 overexpression on NCF2 transcription using a reporter construct that includes this Hox/Pbx consensus-like sequence (+6 to −1000 bp; referred to as −1000NCF2pCATE).

U937 cells were cotransfected with a vector to overexpress HoxA10 or empty control vector and −1000NCF2pCATE or pCATE control. Reporter gene activity was determined with and without IFN-\(\gamma\)-treatment of the transfectants. We find that HoxA10 overexpression significantly decreases reporter expression from the −1000NCF2pCATE construct (difference in reporter activity with and without HoxA10; \(p = 0.005, n = 6\); Fig. 2A). IFN-\(\gamma\)-treatment increased reporter activity from this NCF2 promoter construct, consistent with our previous results (Fig. 2A) (7). However, HoxA10 overexpression in IFN-\(\gamma\)-treated transfectants does not significantly repress reporter expression from −1000NCF2pCATE (difference in reporter activity with and without HoxA10; \(p = 0.32, n = 6\); Fig. 2A). This is similar to the impact of IFN-\(\gamma\) on HoxA10-mediated CYBB repression (10, 12). These results suggest that HoxA10 impacts NCF2 transcription via the 5' flank. Therefore, we further investigated the Hox/Pbx consensus-like sequence in the NCF2 promoter.

**HoxA10 represses a Hox/Pbx consensus-like sequence in the NCF2 5' flank**

To determine the functional significance of the Hox/Pbx consensus-like sequence in the NCF2 5' flank, we generated an artificial promoter construct with four copies of the NCF2 −600- to −637-bp sequence linked to a minimal promoter and reporter gene (using the pTATACAT vector (13); referred to as ncf2A10TATACAT). U937...
cells were cotransfected with a HoxA10 expression vector or empty control vector and ncf2A10TATACAT or pTATACAT control. Transfectants were analyzed for reporter expression with and without IFN-γ treatment. We find that HoxA10 overexpression significantly represses ncf2A10TATACAT reporter expression in non-IFN-γ-treated U937 transfectants (difference in reporter activity with and without HoxA10; \( p < 0.001 \), \( n = 10 \); Fig. 2B). However, HoxA10 overexpression does not significantly repress the NCF2 sequence containing artificial promoter construct in IFN-γ-treated transfectants (difference in reporter activity with and without HoxA10; \( p = 0.92 \), \( n = 6 \) ) (Fig. 2B). HoxA10 overexpression did not significantly impact reporter expression from pTATACT control vector under any of these conditions.

These results suggest that HoxA10 represses transcription via the NCF2 −600- to −637-bp sequence in untreated U937 cells. To investigate HoxA10 binding to this sequence, EMSAs were performed with U937 nuclear proteins and a synthetic double-stranded oligonucleotide probe representing the NCF2 −600- to −637-bp sequence (referred to as dscybbA10). In EMSA with nuclear proteins from untreated U937 cells, a low mobility protein complex binds this probe (Fig. 3A). The mobility of this complex is similar to the HoxA10/Pbx complex that binds the homologous CYBB probe (dscybbA10). IFN-γ treatment of U937 cells decreases binding of this low mobility complex to the dscybbA10 probe (Fig. 3A), consistent with previous results with the dscybbA10 probe (10 –12).

We next investigated binding specificity of this complex. EMSA were performed with the dscybbA10 probe, nuclear proteins from U937 cells, and various unlabeled competitor oligonucleotides. We find that binding of the low mobility complex is specifically abolished by competitor oligonucleotides with the derived Hox/
FIGURE 2. HoxA10 overexpression decreases NCF2 transcription via a Hox/Pbx consensus-like cis element in the proximal promoter. A. HoxA10 overexpression decreases NCF2 promoter activity in untreated but not IFN-γ-treated U937 cells. U937 cells were co-transfected with a reporter vector with 1000 bp of NCF2 5′ flank (−1000NCF2CATE) or empty vector control (pCATE) and a vector to overexpress HoxA10 (HoxA10/pSRo) or empty expression vector control (pSRo). Reporter gene activity was assayed after 48 h of incubation, with or without IFN-γ. HoxA10 overexpression significantly represses reporter expression from −1000NCF2CATE in untreated U937 transfectants, but not in transfectants treated with IFN-γ. In contrast, there was no impact of HoxA10 overexpression on empty vector pCATE control. B. HoxA10 overexpression represses a Hox/Pbx consensus-like sequence in the NCF2 promoter in U937 transfectants. U937 cells were co-transfected with a minimal promoter-reporter construct with four copies of the Hox/Pbx consensus-like sequence from the NCF2 promoter (ncf2A10TATACAT) or empty vector control (pTATACAT); and a vector to overexpress HoxA10 (HoxA10/pSRo) or empty vector control (pSRo). Reporter gene expression was assayed after 48 h of incubation with and without IFN-γ. HoxA10-overexpression significantly repressed ncf2TATACAT reporter expression in untreated U937 transfectants. In contrast, HoxA10-overexpression did not repress ncf2TATACAT reporter expression in IFN-γ-treated transfectants. HoxA10 overexpression had no impact on reporter expression from control pTATACAT.

HoxA10 repression of NCF2 transcription is Pbx1 independent

Although these studies indicate that HoxA10 and Pbx1 interact with the NCF2 −600- to −637-bp sequence in undifferentiated cells, this does not indicate a role for Pbx1 in NCF2 transcriptional repression. Indeed, our previous studies of the homologous CYBB cis element indicated that Pbx1 interaction is dispensable for HoxA10 repression of CYBB transcription (11). However, because Pbx1 has been shown to interact with transcriptional corepressors and mediate repression of other genes, we determined whether Pbx1 is required for NCF2 repression. U937 cells were cotransfected with the artificial promoter construct with multiple copies of the NCF2 cis element described above (ncf2A10TATACAT) or vector control and vectors to express various combinations of HoxA10, Pbx1a, or empty control vector. Transfectants were analyzed for reporter gene activity. We find that overexpression of Pbx1a alone does not significantly alter reporter activity from the ncf2A10TATACAT construct (difference in reporter activity with and without Pbx1a; p = 0.78, n = 6; Fig. 4A). Additionally, Pbx1a does not increase HoxA10-mediated repression of the NCF2 the cis element (significance of difference in HoxA10 repression, with and without Pbx1a; p = 0.42, n = 6).

Although these results suggest that Pbx1a does not contribute to HoxA10 repression activity, it is possible that Pbx1 is not the rate-limiting protein in U937 cells. For example, overexpression of

Pbx-DNA-binding consensus sequence (dsA10 oligo) and the homologous CYBB cis element sequence, but not by a homologous oligonucleotide with mutation of the Hox/Pbx consensus-like sequence (dsmutncf2A10) (Fig. 3B). We also investigated whether the low mobility complex is cross-immunoreactive with Abs to HoxA10 or Pbx1 in EMSA with the dsncf2A10 probe and nuclear proteins from U937 cells. We find that the low mobility complex binding the NCF2 cis element probe is disrupted by Abs to either HoxA10 or Pbx1 (Fig. 3C), similar to previous investigations with the homologous CYBB cis element probe (10).

On the basis of these results, we investigated in vivo HoxA10-binding to the NCF2 promoter by chromatin immunoprecipitation. We also investigated the impact of IFN-γ treatment of U937 cells on in vivo HoxA10-NCF2 promoter interaction. For these studies, proteins were cross-linked to DNA in vivo in U937 cells, lysates were precipitated with an HoxA10 Ab, and coprecipitated DNA was PCR amplified with primers flanking the putative HoxA10-binding site in the NCF2 promoter. Total input (nonprecipitated) chromatin was a positive control and preimmune serum precipitating chromatin was a negative control in these studies. Consistent with EMSA and transfection results, we find that HoxA10 interacts in vivo with the NCF2 promoter in untreated, but not IFN-γ-treated, U937 cells (Fig. 3D). In vivo interaction of HoxA10 with the CYBB promoter under these conditions was a positive control in these experiments.
Pbx1a in U937 cells would not increase HoxA10-induced repression if Pbx1 is present in great excess. To test this possibility, we overexpressed a mutant form of HoxA10 with disruption of the Pbx interaction hexapeptide (referred to as N312A/W323T HoxA10). This mutation disrupts HoxA10 interaction with Pbx proteins, but not HoxA10 DNA binding or CYBB repression (see (11, 22, 23)). U937 cells were cotransfected with the artificial promoter construct with multiple copies of the NCF2 cis element (ncf2A10TATACAT) or empty vector control and wild-type or N312A/W323T mutant HoxA10. Transfectants were analyzed for reporter activity, as above. We find that repression of ncf2A10TATACAT reporter activity by N312A/W323T HoxA10 overexpression is not significantly different from repression by overexpressed wild-type HoxA10 (difference in reporter activity in transfectants with wild-type vs N312A/W323T HoxA10; p = 0.64, n = 7; Fig. 4B). These results suggest that HoxA10 repression of NCF2 transcription is Pbx1 independent.

Our previous studies indicated that HoxA10 repression of CYBB transcription requires histone deacetylase activity. Consistent with this, we demonstrated a direct interaction between HoxA10 and HDAC2 in vitro and in U937 cells (11). We also found that HDAC inhibition increases reporter expression from the −1000NCF2pCATE construct in U937 transfection experiments (11). Therefore, we investigated the role of HDAC activity in HoxA10 repression via the NCF2 cis element. U937 cells were cotransfected with either the...
ncf2A10TATACAT or pTATACAT control vector and a vector to express HoxA10 or vector control. Reporter gene assays were performed after 24 h of TSA treatment, as previously described (11). We find that TSA abolishes HoxA10-induced repression of the Hox/Pbx consensus-like NCF2 cis element (Fig. 4C). Indeed, ncf2A10TATACAT reporter expression is not significantly different in control transfectants in comparison with TSA-treated transfectants overexpressing HoxA10 (difference in reporter activity of control transfectants vs HoxA10-overexpressing and TSA-treated transfectants; $p = 0.22$, $n = 9$). This is consistent with our previous results...
indicating that TSA increases endogenous gp91phox and p67phox mRNA in treated U937 cells (11).

**HoxA10 binding to the NCF2 cis element is decreased by homeodomain tyrosine phosphorylation**

In previous studies, we found that IFN-γ treatment of U937 cells results in HoxA10 tyrosine phosphorylation without altering protein abundance (10). We also identified two highly conserved tyrosine residues in the HoxA10 DNA-binding homeodomain which, when phosphorylated, decrease HoxA10 affinity for the negative CYBB cis element (12). Therefore, we investigated whether HoxA10 tyrosine phosphorylation also decreases binding affinity for the negative NCF2 cis element. To determine this, we performed EMSA with the dsncf2A10 probe and in vitro-translated HoxA10, with and without in vitro dephosphorylation (10, 12).

**FIGURE 5.** Phosphorylation of two tyrosine residues in the HoxA10 homeodomain decreases HoxA10 binding to the Hox/Pbx consensus-like cis element in the NCF2 promoter. A, Tyrosine phosphatase treatment decreases binding of wild-type but not homeodomain tyrosine mutant HoxA10 (Y326/343F HoxA10) to the Hox/Pbx consensus-like NCF2 cis element in vitro. EMSA was performed with a probe representing the NCF2 Hox/Pbx consensus-like sequence and in vitro-translated wild-type or Y326/343F HoxA10 or control reticulocyte lysate, with and without Yop-PTP treatment, as indicated. Yop-PTP dephosphorylation increases wild-type HoxA10-binding to the NCF2 probe. In contrast, Y326/343F HoxA10 has increased affinity for the NCF2 probe which is not altered by Yop-PTP treatment. Upper arrowhead, HoxA10; lower arrowhead, a degradation product including the DNA-binding domain. B, Y326/343F HoxA10 overexpression represses NCF2 transcription in U937 cells via the Hox/Pbx consensus-like cis element. U937 cells were cotransfected with a minimal promoter-reporter construct with four copies of the Hox/Pbx consensus-like sequence from the NCF2 promoter (ncf2A10TATACAT) or empty vector control (pTATACAT); and a vector to overexpress wild-type HoxA10 (HoxA10/pSRα), HoxA10 with mutation of the two homeodomain tyrosine residues (Y326/343F HoxA10/pSRα), or empty vector control (pSRα). Reporter gene expression was assayed after 48 h of incubation. Repression of ncf2TATACAT reporter expression by wild-type and Y326/343F HoxA10 was not significantly different. In contrast, neither wild-type nor tyrosine mutant HoxA10 impacted control pTATACAT reporter expression. C, Y326/343F HoxA10 overexpression represses NCF2 transcription in IFN-γ-treated U937 cells via the Hox/Pbx consensus-like cis element. U937 cells were cotransfected with a minimal promoter-reporter construct with four copies of the Hox/Pbx consensus-like sequence from the NCF2 promoter (ncf2A10TATACAT) or empty vector control (pTATACAT); and a vector to overexpress wild-type HoxA10 (HoxA10/pSRα), HoxA10 with mutation of the two homeodomain tyrosine residues (Y326/343F HoxA10/pSRα), or empty vector control (pSRα). Reporter gene expression was assayed after 48 h of IFN-γ incubation. In contrast to overexpressed wild-type HoxA10, overexpressed Y326/343F HoxA10 significantly repressed ncf2TATACAT reporter expression in IFN-γ-treated transfectants. Neither wild-type nor tyrosine mutant HoxA10 impacted control pTATACAT reporter expression.
FIGURE 6. Homeodomain tyrosines mediate HoxA10 repression of p67phox and gp91phox in IFN-γ-treated, ex vivo-differentiated murine bone marrow myeloid progenitor cells. A. Overexpression of Y326/343F HoxA10, decreases p67phox and gp91phox mRNA expression in ex vivo M-CSF-differentiated murine bone marrow myeloid progenitor cells, with and without IFN-γ treatment. Murine bone marrow-derived myeloid progenitor cells were transduced with a retroviral vector to express HoxA10, Y326/343F HoxA10, or empty vector control. Transduced cells were selected in puromycin and differentiated with M-CSF, with or without IFN-γ, as indicated. RNA was extracted and analyzed by Northern blot for p67phox and gp91phox expression. HoxA10 overexpression did not decrease p67phox and gp91phox mRNA abundance in cells cultured in M-CSF or M-CSF plus IFN-γ. In contrast, overexpression of tyrosine mutant HoxA10 decreased both p67phox and gp91phox mRNA abundance under these culture conditions. B, IFN-γ treatment of M-CSF-differentiated murine bone marrow myeloid progenitor cells increases HoxA10 tyrosine phosphorylation. Bone marrow-derived myeloid progenitor cells were differentiated with M-CSF and cultured for 24 h with or without IFN-γ. Cell lysate proteins were immunoprecipitated with a HoxA10 Ab or preimmune serum, as indicated. Blots were serially probed with Abs to phosphoryrosine and HoxA10. HoxA10 tyrosine phosphorylation is increased by IFN-γ treatment of M-CSF-differentiated cells. In contrast, IFN-γ does not alter HoxA10 protein abundance under these conditions. C, Overexpression of Y326/343F HoxA10, but not wild-type HoxA10, decreases p67phox and gp91phox protein expression in ex vivo M-CSF-differentiated murine bone marrow myeloid progenitor cells. M-CSF differentiation of murine bone marrow-derived myeloid progenitor cells increases gp91phox and p67phox protein expression. Murine bone marrow-derived myeloid progenitor cells were transduced with a retroviral vector to express HoxA10, Y326/343F HoxA10, or empty vector control

HoxA10 repression of NCF2 transcription is regulated by homeodomain tyrosine phosphorylation

To determine the functional significance of these homeodomain tyrosines for HoxA10-induced NCF2 repression, transfection experiments were performed with wild-type and Y326/343F HoxA10. U937 cells were cotransfected with the NCF2 cis element containing artificial promoter construct or empty vector control and vectors to overexpress either wild-type or Y326/343F HoxA10. Reporter gene expression was determined with and without IFN-γ treatment, as described in Materials and Methods. We find that Y326/343F HoxA10 repression of ncf2A10/11005 reporter expression is not significantly different from wild-type HoxA10 repression in untreated U937 transfectants (difference in repression by wild-type vs Y326/343F HoxA10; p = 0.19, n = 6; Fig. 5B). However, in contrast to wild-type HoxA10, repression of the NCF2 cis element by Y326/343F HoxA10 is not abrogated by IFN-γ treatment of the transfectants (difference in reporter expression in Y326/343F HoxA10 vs wild-type HoxA10-expressing transfectants; p = 0.004, n = 6; Fig. 5C). Y326/343F HoxA10 has no impact on pTATACAT control reporter activity with or without IFN-γ treatment. These results suggest that phosphorylation of homeodomain tyrosines 326 and 343 decrease HoxA10 binding affinity and NCF2 repression in IFN-γ-treated U937 myeloid cells.

On the basis of these results, we also investigated the impact of tyrosine phosphorylation on HoxA10 repression of p67phox and gp91phox expression in differentiating murine bone marrow myeloid cells. In the experiments above, we determined that ex vivo monocyte differentiation of murine myeloid progenitor cells increases p67phox and gp91phox expression and HoxA10 tyrosine phosphorylation (Fig. 1, B and C). Therefore, we were interested in determining the role of HoxA10 homeodomain tyrosine phosphorylation on p67phox and gp91phox expression in these cells. Because IFN-γ treatment increases p67phox and gp91phox expression and HoxA10 tyrosine phosphorylation in U937 cells, we were also interested in determining the impact of IFN-γ on ex vivo M-CSF-differentiated murine monocytes. In initial studies, we performed Northern blots to determine the impact of IFN-γ treatment of M-CSF-differentiated cells on p67phox and gp91phox expression. We find that IFN-γ treatment increases p67phox and gp91phox expression and HoxA10 and tyrosine phosphorylation in ex vivo differentiated monocytes alters HoxA10 protein abundance or tyrosine phosphorylation. Similar to our results with U937 cells, IFN-γ increases

and selected in antibiotics, as above. Bone marrow myeloid progenitor cells were cultured in GM-CSF and IL-3, with or without M-CSF differentiation. Western blots of cell lysates were analyzed for expression of p67phox, gp91phox, and HoxA10, as indicated. HoxA10 overexpression decreased p67phox and gp91phox protein abundance in myeloid progenitor cells, but not in M-CSF-differentiated cells. In contrast, overexpression of tyrosine mutant HoxA10 decreased both gp91phox and p67phox protein abundance in myeloid progenitors and M-CSF-differentiated cells.

represor cis element but does not impair phosphorylation of other HoxA10 tyrosines (11, 12). We performed EMSA with the NCF2-negative cis element probe and in vitro-translated Y326/343F HoxA10 protein with and without Yop-PTP treatment. We find that Yop-PTP treatment does not increase binding of Y326/343F HoxA10 to the NCF2 cis element to the same extent as Yop-PTP treatment increases binding of an equivalent amount of wild-type HoxA10 to this probe (Fig. 5A). Indeed, the intensity of the shifted protein complex due to binding of Y326/343F HoxA10 to the dsncf2A10 probe is similar to the intensity of the shifted protein complex due to binding of wild-type, Yop-PTP-treated HoxA10 (Fig. 5A).
HoxA10 tyrosine phosphorylation in ex vivo monocyte-differentiated cells without altering total protein abundance (Fig. 6B).

These results suggest a correlation between HoxA10 tyrosine phosphorylation and increased p67phox and gp91phox expression in ex vivo-differentiating murine myeloid cells and in response to IFN-γ treatment of these cells. Therefore, we investigated the impact of phosphorylation of the conserved HoxA10 homeodomain tyrosines on p67phox and gp91phox expression in this nontransformed model. For these studies, bone marrow-derived myeloid progenitor cells were transduced with a retroviral vector to express HoxA10, Y326/343F HoxA10, or empty control vector and selected, as described above. Overexpression of the wild-type and tyrosine mutant HoxA10 was approximately equivalent by Western blot (not shown). Transduced cells were differentiated with M-CSF, with or without IFN-γ treatment for the last 48 h, and p67phox and gp91phox mRNA abundance was determined by Northern blot (Fig. 6A). We find that monocyte differentiation, with and without IFN-γ treatment, abolishes repression of gp91phox and p67phox expression by overexpressed wild-type HoxA10. In contrast, repression of gp91phox and p67phox expression by overexpressed Y326/343F HoxA10 is not altered by M-CSF differentiation or IFN-γ treatment. We also verified these results by examining the impact of wild-type and Y326/343F HoxA10 on expression of p67phox and gp91phox protein in ex vivo differentiating myeloid progenitors (Fig. 6C). Consistent with the mRNA expression results, we find that Y326/343F HoxA10 represses expression of these proteins with and without monocyte differentiation. In contrast, wild-type HoxA10 overexpression only impacts expression of these oxidase proteins in myeloid progenitor cells.

**Discussion**

Several lines of evidence suggest an important role for the homeodomain protein HoxA10 in regulating myelopoiesis. Specifically, HoxA10 overexpression induces myeloid leukemia in murine bone marrow transplantation experiments (24). Consistent with this, HoxA10 is overexpressed in human myelodysplastic syndromes and acute myeloid leukemia (25). However, the mechanisms by which HoxA10 influences myelopoiesis are not well understood. In previous investigations, we found that HoxA10 represses transcription of CYBB, the gene encoding the respiratory burst oxidase protein gp91phox (10–12). We determined that HoxA10 represses CYBB transcription by recruiting HDAC2 to a promoter cis element (11). This repression is abolished by HoxA10 tyrosine phosphorylation which decreases binding affinity for the CYBB promoter (12). In myeloid leukemia cell lines, we found that IFN-γ activates signaling events leading to HoxA10 tyrosine phosphorylation (9, 12). On the basis of these data, we hypothesized that HoxA10 influences myelopoiesis by repressing myeloid-specific gene transcription in undifferentiated cells. However, this hypothesis was based on regulation of only one myeloid gene. Additionally, this hypothesis did not address HoxA10 regulation of myeloid gene transcription in response to inflammatory mediators in mature phagocytic cells.

In the current investigations, we demonstrate that HoxA10 represses transcription of the NCF2 gene. This gene encodes another respiratory burst oxidase protein, p67phox. We demonstrate that NCF2 repression is HDAC dependent and Pbx independent and is negatively regulated by cytokine-induced HoxA10 tyrosine phosphorylation. Therefore, these studies provide the first demonstration that HoxA10 represses transcription of multiple myeloid genes characteristic of mature phagocytes. Additionally, these studies identify homeodomain tyrosine phosphorylation as the mechanism by which multiple cytokines regulate HoxA10 activity, thereby impacting gene expression. Because CYBB and NCF2 transcription occurs simultaneously in differentiating myeloid cells and in mature phagocytes in response to inflammatory mediators (2, 3), these studies also identify a mechanism that coordinates expression of the two genes.

Previously, we investigated the impact of HoxA10 on gene transcription in U937 myeloid leukemia cells. IFN-γ induces both monocytoid differentiation and functional activation of this transformed cell line (16). In the current studies, we extend our investigations to a nontransformed model; ex vivo differentiation of murine bone marrow-derived myeloid progenitor cells. We find that M-CSF differentiation of murine myeloid progenitors increases expression of gp91phox and p67phox in a HoxA10 homeodomain tyrosine phosphorylation-dependent manner. These results suggest that progression of myeloid differentiation requires cytokine-induced HoxA10 tyrosine phosphorylation. We were also interested in determining whether HoxA10 plays a role in inflammatory mediator-induced gp91phox and p67phox expression in mature phagocytes. Therefore, we investigated the impact of IFN-γ treatment of ex vivo-differentiated murine monocytes. We find that IFN-γ further increases expression of these oxidase components and leads to functionally significant hyper-tyrosine phosphorylation of HoxA10.

Therefore, these studies identify three important characteristics of HoxA10-mediated transcriptional repression. First, our studies indicate that HoxA10 regulates gene transcription in a tyrosine phosphorylation-dependent manner during both cytokine-induced differentiation and in response to inflammatory mediators. Second, our results suggest that the impact of HoxA10 on these target genes is not all or none, but a graded response to stimulation by different cytokines at various times during the life of the phagocyte. Third, because gp91phox and p67phox are the rate-limiting oxidase components, our results provide the first indication that HoxA10 regulates the function of mature phagocytes. These results expand the sphere of HoxA10 influence beyond myelopoiesis. In recent investigations, we determined that CYBB transcription is activated by HoxA9 in differentiating myeloid cells (26). These data are consistent with correlation of myeloid phenotype with HoxA9 expression in murine and human leukemia (27, 28). It will be of interest to determine whether HoxA9 also exerts an effect on phagocyte function that antagonizes HoxA10.

Despite these identified similarities in CYBB and NCF2 transcriptional regulation (6, 10), there are also differences between these two genes. For example, although the HoxA10-binding cis elements in the NCF2 and CYBB genes are homologous, the negative cis element in the CYBB promoter overlaps a positive cis element (29). Therefore, HoxA10 represses CYBB transcription by two mechanisms: recruitment of HDAC2 activity to the proximal promoter; and competition for binding site occupancy with transcriptional activators (10). However, in the current studies, we did not identify a positive cis element overlapping the NCF2 HoxA10-binding repressor element. Therefore, HoxA10 regulates NCF2 transcription by a single mechanism, recruitment of HDAC activity to the promoter. Another difference between regulation of CYBB and NCF2 transcription is the number and use of transcription start sites. CYBB transcription initiates from a single site adjacent to a proximal promoter TATA box (30, 31). In contrast, multiple p67phox transcripts are generated under various conditions from several start sites in the GC-rich NCF2 5′ flank (6, 21). Therefore, although there are common mechanisms of CYBB and NCF2 regulation, there are also important differences.

Free radicals generated by the phagocyte oxidase cause tissue damage in a number of disease states. Our investigations identify a mechanism that down-regulates expression of the two rate-limiting genes for respiratory burst activity. Because this mechanism
is relevant to induction of gene transcription by inflammatory mediators in mature cells, these studies may suggest novel therapeutic targets for pathological conditions related to free radical generation.

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Disclosures

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