Tyrosine Phosphorylation of HoxA10 Decreases DNA Binding and Transcriptional Repression during Interferon \(\gamma\)-induced Differentiation of Myeloid Leukemia Cell Lines*

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The DNA binding affinity of HoxA10 is increased by partnering with Pbx proteins. A consensus sequence for Pbx1-HoxA10 DNA binding has been derived, but genuine target genes have not been identified. We noted that the derived Pbx-HoxA10 DNA-binding consensus is similar to a repressor element in the CYBB promoter. The CYBB gene, which encodes the respiratory burst oxidase component gp91\(^{phox}\), is expressed only in myeloid cells that have differentiated beyond the promyelocyte stage. In these studies, we demonstrate that interferon \(\gamma\) (IFN-\(\gamma\))-induced differentiation of myeloid cell lines abolishes in vitro Pbx-HoxA10 binding to either the derived consensus or the similar CYBB sequence. We also demonstrate that HoxA10, overexpressed in myeloid cell lines, represses reporter gene expression from artificial promoter constructs with Pbx-HoxA10 binding sites. We determine that HoxA10 has endogenous repression domains that are not functionally altered by IFN-\(\gamma\) treatment. However, IFN-\(\gamma\)-induced differentiation of myeloid cell lines leads to HoxA10 tyrosine phosphorylation, which decreases in vitro DNA binding to Pbx-HoxA10 binding sites. Therefore, these investigations identify the CYBB gene as a potential target for HoxA10 and define repression of genes expressed in mature myeloid cells as a novel role for HoxA10 during myeloid differentiation.

The 39 human and murine \(HOX\) genes encode homeodomain transcription factors necessary for embryogenesis (1, 2) and definitive hematopoiesis (3). Genes of the \(HOX\) A and B paralog groups are preferentially expressed in CD34\(^+\) bone marrow progenitor cells and are activated 3' to 5' during hematopoiesis (3). Expression of 3' \(HOX\) A and B genes increases in early CD34\(^+\) cells and decreases in CD34\(^+\) committed progenitors. In contrast, transcription of the 5' genes (i.e. \(HOX9\)-13) is invariant in CD34\(^+\) cells, and decreases in mature phagocytes (3).

In comparison with normal, mature myeloid cells, expression of HOXA10 is increased in acute myeloid leukemia, chronic myeloid leukemia, or myelodysplasia (4). Consistent with this, overexpression of HoxA10 in murine bone marrow induces a myeloproliferative disorder, which evolves to acute leukemia (5). These results suggest that HoxA10 is involved in progression of myeloid differentiation. Although the most abundant HoxA10 transcript in human myeloid cells encodes a 406-amino acid protein (predicted molecular mass of 50 kDa) (6), alternatively spliced transcripts have been described at various stages of murine embryogenesis (7) and in transformed cell lines (4). In myeloid leukemia cell lines, a HoxA10 transcript is present encoding a protein that initiates 20 amino acids N-terminal to the homeodomain (6). It is hypothesized that the 80-amino acid (15-kDa) "short A10" may contribute to immortalization of myeloid cell lines, although the role of short A10 in normal myelopoiesis is unknown.

Similar to the other Abd-like Hox proteins (Hox9–13), DNA binding affinity of HoxA10 is increased by partnering with Pbx proteins (8). Although consensus sequences for Pbx-HoxA10 binding have been derived (8–10), genuine Pbx-HoxA10 target genes have not been identified. It has been hypothesized that HoxA10 regulates myeloid differentiation by activating transcription of genes that are necessary for progression of myelopoiesis. Conversely, HoxA10 might repress transcription of genes characteristic of differentiated myeloid cells, or HoxA10 might activate transcription at one stage of myelopoiesis and repress transcription at another, as has been described for homologous Drosophila proteins, during embryogenesis (11).

We have been studying regulation of genes encoding the respiratory burst oxidase proteins, gp91\(^{phox}\) (the CYBB gene) (12) and p67\(^{phox}\) (the NCF2 gene) (13). These genes are transcribed in cells differentiated beyond the promyelocyte stage and therefore provide a model for gene regulation during late myelopoiesis. Both the CYBB and NCF2 genes contain sequences similar to the Pbx-HoxA10 binding consensus sequence. One of these CYBB sequences is within a previously described repressor element (14), which binds the CCAAT displacement protein (CDP)\(^3\) in electrophoretic mobility shift assays (EMSA) with HeLa or K562 nuclear proteins (14, 15). In NIH 3T3 cells, overexpression of CDP represses an artificial promoter construct containing the CYBB element (16).

In contrast, our previous investigations demonstrated that CDP is not a component of the complex binding to the CYBB repressor element in EMSA with nuclear proteins from the myeloid lines PLB985 and U937 (17). Since HoxA10 mRNA is present in these myeloid cell lines, but not in HeLa or K562 cells (4), our current studies investigate the hypothesis that, in committed myeloid progenitors, HoxA10 interacts with repressor elements and suppresses transcription of some myeloid-specific genes until later stages of myelopoiesis. Previously, we demonstrated that

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1 The abbreviations used are: CDP, CCAAT displacement protein; IFN-\(\gamma\), interferon \(\gamma\); bp, base pair; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis.
IFN-γ-induced myeloid differentiation decreases in vitro protein binding to the CYBB repressor element, coincident with increased CYBB transcription (17). Therefore, we also investigate the effect of IFN-γ-induced differentiation on HoxA10 DNA binding and functional activity in myeloid cells.

MATERIALS AND METHODS

Plasmids and Site-directed Mutagenesis: Reporter Constructs and Assays for Protein Expression—Artificial promoter/reporter constructs were generated as described previously (18), in the minimal promoter/reporter vector, p-TATACAT (19) (obtained from Dr. A. Kraft, University of Colorado, Denver). Constructs were generated with three copies (in the forward orientation) of the consensus sequence for HoxA10-Pbx binding (p-A10TATACAT) (8) or four copies (in the forward direction) the −94 to −134 bp sequence from the CYBB promoter (p-cybba10TATACAT). This CYBB promoter sequence has previously been demonstrated to function as a repressor element in myeloid cell lines (16). An artificial promoter construct with five copies of the Gal4 DNA binding site and a minimal promoter from the thymidine kinase gene linked to a chloramphenicol acetyltransferase (CAT) reporter (p-gal4TKCAT) was obtained from T. Gabig (Indiana University, Indianapolis).

The cDNAs for human HoxA10 and “short A10” were obtained from C. Largman (University of California, San Francisco) and subcloned into the vector p-gal4TKCAT (obtained from T. Gabig, Indiana University, Indianapolis). This promoter/reporter vector, p-TATACAT (19) (obtained from Dr. A. Kraft, University of Colorado, Denver). Constructs were generated with three copies (in the forward orientation) of the consensus sequence for HoxA10-Pbx binding (p-A10TATACAT) (8) or four copies (in the forward direction) the −94 to −134 bp sequence from the CYBB promoter (p-cybba10TATACAT). This CYBB promoter sequence has previously been demonstrated to function as a repressor element in myeloid cell lines (16). An artificial promoter construct with five copies of the Gal4 DNA binding site and a minimal promoter from the thymidine kinase gene linked to a chloramphenicol acetyltransferase (CAT) reporter (p-gal4TKCAT) was obtained from T. Gabig (Indiana University, Indianapolis).

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In vitro Translated Proteins and Tyrosine Dephosphorylation—In vitro translated HoxA10, short A10, and Pbx1 mRNA were generated from linearized template DNA, using the Riboprobe System, according to the manufacturer’s instructions (Promega, Madison, WI). In vitro translated proteins were generated in rabbit reticulocyte lysate, 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 and nuclear proteins were tyrosine-dephosphorylated with Yop protein-tyrosine phosphatase (New England Biolabs, Beverly, MA). Proteins (either 10 μl of in vitro translated protein or 2 μg of nuclear proteins) were incubated 30 min at 30 °C, in a 20-μl reaction volume with 50 units of Yop and 1× reaction buffer, according to the manufacturer’s instructions. Control proteins were incubated, similarly, in 1× reaction buffer without Yop.

EMSA with the in vitro translated proteins was performed as described (18). Binding assays with in vitro translated proteins and the dsyba10 oligonucleotide were performed in the presence of a 200-fold molar excess of the urc oat oligonucleotide. The urc oat oligonucleotide competes for binding of CP1, found in reticulocyte lysate, to the dsyba10 probe.

RESULTS

HoxA10 DNA Binding Decreases during IFN-γ-induced Myeloid Differentiation—The consensus sequence for HoxA10 binding to DNA as a heterodimer with Pbx1 (8) consists of a Pbx1 site adjacent to a HoxA10 site: 5′-ATGATTATGTA-3′.
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(HoxA10 site in boldface type, the Pbx site in italics) (9). Inspection of the promoter regions of the genes encoding gp91phox (the CYBB gene) and p67phox (the NCF2 gene) identified sequences similar to the Phox-HoxA10 consensus (Fig. 1A). The CYBB sequence includes the core consensus preferred in HoxA10 binding site selection experiments (TAAT) and the Pbx core (7). However, unlike sequences identified by binding site selection, there was overlap of the Pbx and Hox binding sites. The NCF2 sequence includes an alternative HoxA10 consensus (TAAT) and a Pbx core, identified by binding site selection (TAAT) (7), and a Pbx core, altered at position 3.

The Pbx-HoxA10-like sequence in the CYBB promoter is within a 30-bp region previously demonstrated to function as a repressor element in undifferentiated cells (16). In EMSA with nuclear proteins from the promyelocytic leukemia cell line PLB985, an unidentified, specific protein complex interacts with this CYBB repressor element (referred to as complex A) (14, 15). We previously demonstrated that IFN-γ-induced differentiation of PLB985 cells abolishes in vitro binding of complex A to the repressor element, coincident with an increase in CYBB transcription (17).

We hypothesized that HoxA10 is a component of complex A binding the CYBB repressor element and that myeloid differentiation decreases HoxA10 DNA binding. To pursue this hypothesis, we investigated whether in vitro protein binding to the derived Pbx-HoxA10 consensus sequence decreases during IFN-γ-induced myeloid differentiation. In these experiments, we used U937 cells, a monocyte-committed cell line that expresses HoxA10 mRNA (4). IFN-γ treatment of U937 cells results in monocyte differentiation and increased CYBB and NCF2 transcription (17). EMSAs were performed, using nuclear proteins from U937 cells and a radiolabeled probe with the derived consensus sequence for Pbx-HoxA10 binding (referred to as dsA10 oligonucleotide) (Fig. 1B). A complex binds to dsA10 that is of similar mobility to complex A generated by U937 nuclear proteins and the homologous CYBB sequence.

A. Sequence Homology with Derived Pbx/HoxA10 Consensus

| Consensus             | Sequence Homology |
|-----------------------|-------------------|
| Derived Pbx/HoxA10    | $5'$-ATGATTTATGA-3' |
| CYBB sequence         | $5'$-ATGA TTATTA-3' |
| NCF2 sequence         | $5'$-ATATAAATGG-3' |

B. EMSA

Lane 1, no competitor; lane 2, homologous dsA10 oligonucleotide; lane 3, dsncybbA10 oligonucleotide; lane 4, dsncf2A10 oligonucleotide; lane 5, urccaat unrelated oligonucleotide; lane 6, no competitor. The arrowhead indicates the A1 complex. C. Binding of a specific protein complex (complex A) to the dsncybbA10 probe is decreased during IFN-γ-induced U937 differentiation. EMSA was performed with the dsncybbA10 probe and nuclear proteins from U937 cells (2 μg) without (lane 1) and with (lane 6) 48-h IFN-γ differentiation. The arrowhead represents binding of specific complex A (17), and the asterisk shows binding of protein complex, previously demonstrated to represent the classical CCAAT binding complex, CP1 (14). D. Binding of complex A to the dsncybbA10 probe is competed for by the Pbx-HoxA10 binding consensus and other similar sequences. EMSA was performed with the dsncybbA10 probe and nuclear proteins from U937 cells (2 μg) in the presence of unlabeled, synthetic oligonucleotide competitor (200-fold molar excess). Lane 1, no competitor; lane 2, homologous dsncybbA10 oligonucleotide; lane 3, mutant dsncybbA10mut oligonucleotide; lane 4, unrelated dsncf2irf oligonucleotide; lane 5, similar dsncf2A10 oligonucleotide; lane 6, mutant dsncf2A10mut oligonucleotide; lane 7, consensus dsA10 oligonucleotide; lane 8, unrelated urccaat oligonucleotide. Complex A is indicated by an arrowhead.

Fig. 1. Sequences in the CYBB and NCF2 genes, similar to the derived Pbx-HoxA10 consensus, have cross-competitive binding specificities. A. Sequence analysis identifies sequences from the CYBB and NCF2 promoters similar to the derived consensus for Pbx1-HoxA10 binding. The HoxA10 binding core is indicated in boldface type, and the Pbx core is shown in italics. Note that, in the CYBB promoter sequence, there is a 1-bp overlap between the HoxA10 and Pbx core sequences.

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Fig. 2. HoxA10 from U937 nuclear proteins interacts in vitro with DNA probes containing the derived Pbx-HoxA10 DNA-binding consensus or the similar CYBB promoter sequence. A, a U937 nuclear protein, cross-immunoreactive with HoxA10, binds in vitro to the dsA10 probe. EMSA was performed with the dsA10 probe and nuclear proteins from U937 cells (2 μg), preincubated with the following. Lane 1, rabbit preimmune serum (2 μl); lane 2, HoxA10 specific rabbit serum (2 μl). The arrowhead indicates complex A1, and the double arrows indicate higher mobility complexes, also cross-immunoreactive with HoxA10. B, a U937 nuclear protein, cross-immunoreactive with HoxA10, binds in vitro to the dscybbA10 probe. EMSA was performed with the dscybbA10 probe and nuclear proteins from U937 cells (2 μg), preincubated with the following. Lane 1, rabbit preimmune serum (2 μl); lane 2, HoxA10-specific rabbit serum (2 μl). The arrowhead indicates complex A; the asterisk indicates CP1 binding to a CCAAT box in the probe. C, a U937 nuclear protein, cross-immunoreactive with Pbx1, binds in vitro to the dsA10 probe. EMSA was performed with the dsA10 probe and nuclear proteins from U937 cells (2 μg), preincubated with the following. Lane 1, Pbx antibody (2 μg) and blocking peptide (1 μl); lane 2, Pbx antibody alone (2 μg). The arrowhead indicates complex A; the asterisk indicates CP1 binding to a CCAAT box in the probe.

To determine if HoxA10 is a component of either complex A1 binding to the dsA10 probe or complex A binding to the dscybbA10 probe, we used an antibody raised to the HoxA10 C terminus that does not recognize other Hox proteins. In preliminary experiments, we determined that this antibody does not recognize recombinant CDP (data not shown). In EMSA with the dsA10 probe and nuclear proteins from U937 cells, binding of complex A1 is disrupted by HoxA10 antibody but not preimmune serum (Fig. 2A). Antibody to HoxA10 also disrupts binding of complex A to the dscybbA10 probe (Fig. 2B). Identical results were obtained with nuclear proteins from PLB985 cells (not shown). In addition, HoxA10 antibody disrupts two high mobility complexes binding the dsA10 probe, suggesting that they contain the HoxA10 C terminus, consistent with previously described, alternatively spliced messages (6, 7). Interestingly, the dscybbA10 probe does not bind high mobility species, cross-immunoreactive with HoxA10.

EMSAs were also performed to determine if Pbx1 is a component of either complex A1 binding to the dsA10 probe or complex A binding to the dscybbA10 probe. EMSAs were performed with U937 nuclear proteins and Pbx antibodies, with or without blocking peptides. In EMSA with an antibody to a peptide in the N terminus of Pbx1, inconsistent disruption of both complexes A and A1 is demonstrated (not shown). However, in EMSA with an antibody to a peptide in the Pbx C terminus, binding of complex A1 to the dsA10 probe and of complex A to the dscybbA10 probe is disrupted (Fig. 1, C and D). However, these results do not exclude the possibility that additional, uniden-
tified proteins bind to dsA10 or dscybbA10 and participate in these complexes.

Consistent with our previously reported EMSA with PLB985 nuclear proteins and the dscybbA10 probe (17), neither complex A binding to the dscybbA10 probe nor complex A1 binding to the dsA10 probe is disrupted by either of two antibodies to CDP (not shown).

**HoxA10 Represses Transcription of Artificial Promoter Constructs with Pbx-HoxA10 Binding Sites**—To determine if HoxA10 represses transcription in myeloid cells, U937 cells were transfected with artificial promoter constructs containing multiple copies of the derived Pbx-HoxA10 binding site (p-a10TATACAT) or the repressor element from the CYBB gene (p-cybba10TATACAT). Transfectants with these constructs demonstrated increased promoter activity that is statistically significant in comparison with control, empty vector (p-TATA-CAT) transfectants ($p < 0.05$ for both) (Fig. 3A). Reporter gene expression from U937 transfectants with the p-a10TATACAT construct is significantly repressed by overexpression of HoxA10 ($p = 0.012, n = 6$). Overexpression of HoxA10 also represses the p-cybba10TATACAT construct in U937 transfectants ($p = 0.001, n = 5$). However, HoxA10 has no significant effect on p-TATACAT control vector expression ($p > 0.5, n = 6$) (Fig. 3A).
Since the “short A10” form of HoxA10, present in myeloid cell lines, contains the DNA-binding homeodomain, we investigated whether overexpressed short A10 represses transcription. U937 cells were co-transfected with either p-a10TATACAT, p-cybbTATACAT, or control p-TATACAT and a vector to overexpress short A10. Short A10 also significantly represses reporter gene expression from the p-a10TATACAT and p-cybba10TATACAT constructs (p < 0.05) but less than full-length HoxA10 (Fig. 3A).

In contrast, overexpression of Pbx1, in U937 cells co-transfected with either p-a10TATACAT, p-cybb10TATACAT, or control p-TATACAT, did not significantly alter reporter gene expression (Fig. 3A). However, in U937 cells co-transfected with either p-a10TATACAT or p-cybb10TATACAT and vectors to overexpress both HoxA10 and Pbx1, repression of reporter gene expression is significantly greater than with HoxA10 alone (Fig. 3A; difference in reporter gene activity with HoxA10 versus HoxA10 plus Pbx1, p = 0.015 for p-a10TATACAT and p = 0.026 for p-cybb10TATACAT). Short A10 does not include the HoxA10 Pbx1 interaction domain (6). Consistent with this, repression of the two HoxA10-Pbx-containing constructs in U937 transfectants overexpressing short A10 and Pbx1 is not significantly different from the repression with short A10 alone (data not shown).

Since IFN-γ treatment of U937 cells decreases Pbx-HoxA10 binding to both the derived Pbx-HoxA10 consensus sequence and the CYBB repressor element, we investigated whether overexpressed HoxA10 represses transcription in IFN-γ-treated U937 cells. U937 cells were co-transfected with p-a10TATACAT, p-cybb10TATACAT, or empty vector control and a vector to overexpress HoxA10, short A10, Pbx1, or empty vector control. U937 transfectants incubated for 48 h with IFN-γ (200 units/ml) were compared with transfectants incubated for the same time without IFN-γ. Treatment with IFN-γ significantly increases expression from the p-a10TATACAT and p-cybb10TATACAT constructs (p < 0.05) but not p-TATACAT control transfectants (Fig. 3B).

In contrast to undifferentiated U937 transfectants, overexpression of HoxA10 did not significantly repress reporter gene expression from these constructs in IFN-γ-treated cells, with or without Pbx1 (p > 0.40 for all combinations in comparison with empty expression vector control). However, overexpressed short A10 represses reporter gene expression from p-a10TATACAT and p-cybb10TATACAT constructs in IFN-γ-treated U937 transfectants (Fig. 3B). Reporter gene activity in short A10 overexpressing U937 cells co-transfected with p-a10TATACAT is decreased 78.3% without versus 64.1% with IFN-γ treatment. In U937 transfectants with p-cybbTATACAT, overexpression of short A10 decreases reporter activity 49.3% without versus 88.9% with IFN-γ treatment. Overexpression of short A10 does not repress p-TATACAT reporter expression in U937 cells with IFN-γ treatment.

HoxA10 Contains Transcriptional Repression Domains That Are Not Functionally Altered by IFN-γ—To determine if HoxA10 protein possesses endogenous repression domains, the full-length protein and short A10 were expressed as fusion proteins with the DNA binding domain of GAL4 (A10gal4DB and SA10gal4DB, respectively). U937 cells were co-transfected with these fusion protein constructs (or empty gal4DB vector control) and an artificial promoter construct with multiple copies of a GAL4 DNA-binding site, linked to a minimal promoter and a CAT reporter (p-gal4TKCAT) (20 μg). Reporter gene expression is significantly repressed by overexpression of A10gal4DB (p = 0.00029, n = 11, Fig. 3C). This repression is not significantly altered by 48 h of IFN-γ treatment (p = 0.000017, n = 8; p value for reporter activity with or without IFN-γ was 0.949). However, overexpression of SA10gal4DB does not significantly repress reporter gene activity in U937 transfectants, with or without 48 h of IFN-γ (without IFN-γ, p = 0.50, n = 7; with IFN-γ, p = 0.77, n = 11; p value for reporter activity with or without IFN-γ was 0.65, Fig. 3C). U937 transfections were also performed with one-tenth the amount of reporter plasmid and the same amounts of A10gal4DB, SA10gal4DB, and control gal4DB plasmids. In these experiments, A10gal4DB repressed reporter expression to a greater extent than in the previous transfections with 10-fold more input reporter plasmid DNA (Fig. 3D).

Overexpressed HoxA10 Repressed Endogenous gp91phox and p67phox Expression in U937 Cells—RNA isolated from U937 transfectants was analyzed by Northern blot to determine if overexpression of HoxA10 was associated with decreased abundance of gp91phox or p67phox mRNA. U937 cells were transfected with either control pSRa or control pSRb (30 μg) and harvested 48 h later for extraction of total cellular RNA. RNA (20 μg) was analyzed by Northern blot, probed for gp91phox, p67phox, and γ-actin mRNA as indicated. U937 cells transfected with HoxA10/pSRa demonstrated decreased gp91phox and p67phox mRNA abundance in comparison with control transfectants. Blots were probed for γ-actin mRNA to control for loading. Overexpressed HoxA10 is detected by Western blot in U937 transfectants. U937 cells were transfected with FLAG epitope-tagged HoxA10/pSRa or control pSRa (30 μg) and harvested 48 h later for extraction of nuclear proteins. Nuclear proteins (30 μg) were separated by 12% SDS-PAGE and Western blots performed with anti-FLAG antibody.
epitope-tagged protein by Western blot (Fig. 4B). No immunoreactive protein species were detected when the blot was probed with irrelevant antibody (mouse anti-rabbit IgG) (not shown).

HoxA10 Is Tyrosine-phosphorylated during IFN-γ-induced U937 Differentiation—Several mechanisms might decrease HoxA10 DNA binding, and therefore transcriptional repression, during myeloid differentiation. Decreased HoxA10 abundance might result in successful competition for an adjacent or overlapping element by transcriptional activators. Conversely, increased activator abundance, during differentiation, might result in successful competition for the DNA binding site, or post-translational modification of HoxA10, such as phosphorylation, might decrease HoxA10 affinity for the DNA-binding site.

To investigate the effect of myeloid differentiation on HoxA10 abundance, U937 cells were treated with for 48 h with IFN-γ. By Western blot, nuclear proteins from treated and untreated U937 cells demonstrate three HoxA10 cross-immunoreactive species: a 50-kDa species, the predicted size of the protein encoded by the major transcript in myeloid cells (4); a 42-kDa species, the predicted size of a protein encoded by an alternatively spliced HoxA10 mRNA described in murine tissues (7); and a 15-kDa species, the predicted size of short A10 (6). IFN-γ treatment of U937 cells does not alter the abundance of any of these HoxA10 species (Fig. 5A), although binding to Pbx-HoxA10 consensus sequences is decreased by IFN-γ treatment in EMSA with the same nuclear proteins (Fig. 1, B and C). No protein species were detected when the blot was probed with rabbit pre-immune serum (not shown).

To determine if IFN-γ-induced differentiation results in HoxA10 phosphorylation, both IFN-γ-treated and undifferentiated U937 cells were 32P-labeled, and lysate proteins were analyzed. IFN-γ treatment of U937 cells increases the phosphorylation of anti-HoxA10 immunoprecipitable 50- and 42-kDa HoxA10 species, but not of short A10 (Fig. 5B). Since HoxA10 includes tyrosine, threonine, and serine residues (4), we next investigated whether HoxA10 was tyrosine-phosphorylated during myeloid differentiation. U937 cells were lysed, after 48 h of incubation with or without IFN-γ, and lysate proteins were immunoprecipitated with an anti-phosphotyrosine antibody or irrelevant control antibody. In Western blots of immunoprecipitated proteins, IFN-γ treatment of U937 cells increased immunoprecipitable, tyrosine-phosphorylated 50- and 42-kDa HoxA10 (Fig. 5C). In contrast, no immunoreactive species were detected when the blot was probed with rabbit preimmune serum (not shown).

HoxA10 Tyrosine Phosphorylation Decreases DNA Binding Affinity—To determine if HoxA10 tyrosine phosphorylation influences DNA binding affinity, in vitro translated protein was dephosphorylated and used in EMSA with Pbx-HoxA10 binding probes. In preliminary experiments, in vitro translated HoxA10 was immunoprecipitated with anti-phosphotyrosine antibody (Fig. 6A). To generate tyrosine-dephosphorylated HoxA10, in vitro translated protein was treated with the specific tyrosine phosphatase, Yop. Yop-treated in vitro translated HoxA10, was not immunoprecipitated by anti-phosphotyrosine antibody, although Yop treated protein was intact (Fig. 6A).

Tyrosine-dephosphorylated HoxA10 and control proteins were used in EMSA with the dsA10 and dsycbbA10 probes. As controls, in vitro translated HoxA10 was incubated in Yop reaction buffer without enzyme, and control reticulocyte lysate was incubated with and without Yop. Tyrosine-dephosphorylated HoxA10 demonstrates increased binding to the Pbx-HoxA10 consensus probe (Fig. 6B). Similar results were obtained with the dsycbbA10 probe (Fig. 6C). Binding of in vitro translated HoxA10 to the dsycbbA10 probe was competed for by unlabeled homologous oligonucleotide, by the dsA10 and dscf2A10 oligonucleotides, and by an oligonucleotide representing another CYBB promoter sequence (dsycbb5′A10), similar to the derived Pbx-HoxA10 consensus (Fig. 6D).

To determine if HoxA10 tyrosine dephosphorylation influences interaction with Pbx, in vitro translated HoxA10, with and without Yop treatment, was incubated in binding reactions with in vitro translated Pbx1. Both tyrosine-dephosphorylated HoxA10 and control HoxA10 interact with Pbx1 to form a low mobility complex with the Pbx-HoxA10 consensus probe. However, binding affinity of the Pbx-HoxA10 complex is reproducibly increased with Pbx1-tyrosine-dephosphorylated HoxA10, in comparison with Pbx1-control HoxA10 (Fig. 6B). Identical
Tyrosine phosphorylation decreases DNA binding affinity of HoxA10 for Pbx-HoxA10 binding sites. A, in vitro translated HoxA10 is tyrosine-phosphorylated. In vitro translated HoxA10 (10 μl), either with or without Yop tyrosine phosphatase treatment, was immunoprecipitated with anti-phosphotyrosine antibody or irrelevant control antibody. Unprogrammed reticulocyte lysate was included as a control. Immunoprecipitated proteins, separated by 12% SDS-PAGE, were detected by autoradiography as indicated. B, tyrosine phosphatase treatment of in vitro translated HoxA10 increases DNA binding to the derived Pbx-HoxA10 consensus sequence, with and without Pbx1. EMSA was performed with the Pbx-HoxA10 consensus sequence probe (dsA10) and in vitro translated HoxA10 or control rabbit reticulocyte lysate, with and without in vitro translated Pbx1. Lane 1, control reticulocyte lysate (1.5 μl); lane 2, Yop buffer-incubated control reticulocyte lysate (1.5 μl); lane 3, Yop-treated control reticulocyte lysate (1.5 μl); lane 4, HoxA10 (1.0 μl) plus control reticulocyte lysate (0.5 μl); lane 5, Yop buffer-incubated HoxA10 (1.0 μl) plus control reticulocyte lysate (0.5 μl); lane 6, Yop-treated HoxA10 (1.0 μl) plus control reticulocyte lysate (0.5 μl); lane 7, Pbx1 (0.5 μl) and control reticulocyte lysate (1.0 μl); lane 8, Pbx1 (0.5 μl) and Yop buffer-incubated control reticulocyte lysate (1.0 μl); lane 9, Pbx1 (0.5 μl) and Yop-treated control reticulocyte lysate (1.0 μl); lane 10, Pbx1 (0.5 μl) and HoxA10 (1.0 μl); lane 11, Pbx1 (0.5 μl) and Yop buffer-incubated HoxA10 (1.0 μl); lane 12, Pbx1 (0.5 μl) and Yop-treated HoxA10 (1.0 μl). The upper arrowhead indicates a complex formed by HoxA10 with Pbx1, and the lower arrowhead represents binding of HoxA10. Control reticulocyte lysate generates a complex with the dsA10 probe, consistent with observations of other investigators (8). C, tyrosine phosphatase treatment of in vitro translated HoxA10 increases DNA binding to the similar CYBB promoter sequence, with and without Pbx1. EMSA was performed with the CYBB promoter sequence probe (dscybbA10) and in vitro translated HoxA10, or control rabbit reticulocyte lysate, with and without in vitro translated Pbx1. Comparison of DNA binding affinity with and without tyrosine treatment was made. Lane 1, Yop buffer-incubated control reticulocyte lysate (2.5 μl); lane 2, Yop buffer incubated HoxA10 (2.0 μl) and control reticulocyte lysate (0.5 μl); lane 3, Yop-treated HoxA10 (2.0 μl) and control reticulocyte lysate (0.5 μl); lane 4, Pbx1 (0.5 μl) and Yop-treated control reticulocyte lysate (2.0 μl); lane 5, Pbx1 (0.5 μl) and Yop buffer-incubated control reticulocyte lysate (2.0 μl); lane 6, Pbx1 (0.5 μl) and Yop-treated HoxA10 (2.0 μl). The upper arrowhead indicates a complex formed by HoxA10 with Pbx1, and the lower arrowhead represents binding of HoxA10. Control reticulocyte lysate generates a complex with the dscybbA10 probe, similar to the dsA10 probe. D, in vitro translated HoxA10 binds specifically to the CYBB promoter sequence, similar to the Pbx-HoxA10 binding consensus. EMSA was performed with the CYBB promoter sequence probe (dscybb10) and in vitro translated, Yop-treated HoxA10 in the presence of competitor oligonucleotides (200-fold molar excess). Lane 1, dsA10 oligonucleotide; lane 2, homologous dscybb10 oligonucleotide; lane 3, dsncf2A10 oligonucleotide; lane 4, dscybb5 A10 oligonucleotide (another CYBB promoter sequence similar to the Pbx-HoxA10 consensus); lane 5, dscybb10mut oligonucleotide (mutant homologous sequence); lane 6, no competitor. The specific HoxA10 protein complex is indicated by the arrowhead. E, in vitro translated HoxA10, binding to the dscybbA10 probe, is recognized by HoxA10 antibody. EMSA was performed with the CYBB promoter sequence probe (dscybbA10), in vitro translated, Yop-treated HoxA10 and in vitro translated Pbx. Binding reactions were incubated in the presence of HoxA10 antibody (αHoxA10), rabbit preimmune serum, or no antibody, as indicated. HoxA10 antibody disrupted both complexes generated by HoxA10 with or without Pbx1, binding to the dscybb10 probe. The arrowheads indicate HoxA10 cross-immunoreactive complexes. F, tyrosine phosphatase treatment of “short A10” does not increase DNA binding affinity. EMSAs were performed with the CYBB promoter sequence probe (dscybb10) and in vitro translated
results were obtained with the dscybbA10 probe (Fig. 6C). In addition, the complexes generated by binding of in vitro translated HoxA10 and HoxA10 plus Pbx1 to the dscybbA10 probe were disrupted by antibody to HoxA10, but not by preimmune serum (Fig. 6E). Identical results were also obtained with the dsA10 probe (data not shown).

We also generated in vitro translated short A10 protein, in reticulocyte lysate, and performed similar experiments. In contrast to our results with HoxA10, binding of short A10 to the dscybbA10 probe is not increased by Yop treatment (Fig. 6F). Identical results were obtained with the dsA10 probe (not shown).

Since HoxA10 is phosphorylated during IFN-γ-induced U937 differentiation, we hypothesized that tyrosine dephosphorylation of endogenous HoxA10, from IFN-γ-treated U937 cells, would restore binding to the dsA10 and dscybbA10 probes. U937 nuclear proteins from undifferentiated U937 cells and from U937 cells treated for 48 h with IFN-γ were incubated with Yop. Control extracts were incubated under the same conditions in the absence of the enzyme. Consistent with our hypothesis, Yop treatment of nuclear proteins from IFN-γ-treated U937 cells increases binding of the HoxA10-containing protein complex to the dsA10 and dscybbA10 probes (Fig. 7, A and B). This complex was verified to contain immunoreactive HoxA10 in EMSA with the HoxA10-specific antibody (not shown). Yop treatment of nuclear proteins from undifferentiated U937 cells also increases abundance of the HoxA10-containing protein complex binding these probes.

DISCUSSION

Previous investigations suggest that HoxA10 increases proliferation and blocks differentiation during early myelopoiesis. HoxA10 function may be difficult to determine if there is variation in protein-protein interactions, protein-DNA interactions, or activity of functional domains during myelopoiesis. Our investigations determined that tyrosine phosphorylation of HoxA10 occurs during IFN-γ-induced myeloid differentiation and that tyrosine-phosphorylated HoxA10 has decreased DNA binding affinity. Additionally, we demonstrate, in U937 myeloid cells, that HoxA10 represses expression from artificial promoter constructs with Pbx-HoxA10 binding sites. We determine that HoxA10 has endogenous repression domains, not affected by IFN-γ-induced myeloid differentiation. Perhaps most interestingly, we identify the CYBB gene as a potential target for Pbx-HoxA10 repression, in undifferentiated myeloid cells.

We found that IFN-γ-induced myeloid differentiation decreases in vitro HoxA10 DNA binding to the derived Pbx-HoxA10 consensus, and to a similar CYBB promoter sequence. The CYBB sequence includes Pbx (5′-ataat-3′) and HoxA10 (5′-tat-3′) cores, previously identified by binding site selection (7, 8). However, unlike the derived consensus, there was a 1-bp overlap between the two sites. Despite this difference, the Pbx-HoxA10 consensus and CYBB sequence have cross-competitive binding specificities, although the derived consensus binds the complex with lower affinity. Also, the complex shifted by the CYBB probe migrates as a broader band than the complex shifted by the derived Pbx-HoxA10 consensus, suggesting that the CYBB sequence recruits additional proteins to the binding site. Other investigators found that HoxA9 interacts simultaneously with Pbx1 and Meis1 at DNA-binding sites (29, 30). It is similarly possible that a Meis protein participates in Pbx-HoxA10 binding.

We identified a similar sequence in the NCF2 gene (31), with cross competitive binding specificity to the derived Pbx-HoxA10 consensus. The NCF2 sequence has a bp change in position 3 in the Pbx core (5′-ataat-3′) and an alternative HoxA10 core (5′-tat-3′) (7). The NCF2 gene encodes the respiratory burst oxidase protein p67phox and is transcriptionally activated at the same point in myelopoiesis as the CYBB gene, suggesting that HoxA10 interacts with multiple genes activated during late myeloid differentiation. Previous investigations identified two other sequences in the CYBB promoter with cross-competitive binding with the CYBB sequence investigated in the current studies (17, 32). Therefore, HoxA10 may exert an effect on transcription by interacting with multiple promoter sites. We are currently investigating the significance of these other CYBB promoter sequences, and several similar NCF2 sequences (31).

In our investigations, overexpression of HoxA10 represses reporter gene expression from constructs with the derived Pbx-HoxA10 binding consensus. A, tyrosine phosphatase treatment of nuclear proteins from U937 cells increases HoxA10 DNA binding to the derived Pbx-HoxA10 consensus. EMSAs were performed with the dsA10 probe and nuclear proteins (3 μg) isolated from U937 cells that were either treated for 48 h with IFN-γ (lanes 1 and 2), or untreated (lanes 3 and 4). Nuclear proteins were either Yop-treated (lanes 1 and 3) or sham-incubated in Yop buffer (lanes 2 and 4). The arrowhead indicates binding of the A complex. B, tyrosine phosphatase treatment of nuclear proteins from U937 cells increases HoxA10 DNA binding to the CYBB promoter sequence, similar to the derived Pbx-HoxA10 consensus. EMSAs were performed with the dscybbA10 probe and nuclear proteins (3 μg) isolated from U937 cells that were either untreated for 48 h with IFN-γ (lanes 1 and 2), or untreated (lanes 3 and 4). Nuclear proteins were either Yop-treated (lanes 1 and 3) or sham-incubated in Yop buffer (lanes 2 and 4). The arrowhead indicates binding of the A complex, and the asterisk indicates CP1 binding to the CCAAT box in the probe.
HoxA10 consensus or the similar CYBB promoter sequence in U937 cells. Although Pbx1 overexpression augments HoxA10 repression, Pbx1 alone did not repress reporter gene expression. Since U937 cells have endogenous HoxA10, this suggests that Pbx1 is not rate-limiting. Our results differ from U937 transfection experiments by other investigators with HoxA9 and an artificial promoter construct with the Phx-Meis-HoxA9 consensus (30). In those studies, HoxA9 overexpression did not alter reporter expression. Differences in experimental design may explain the discrepancy, including shorter post-transfection incubation (6 versus 48 h), less transfected DNA (0.5 µg/10⁶ cells versus 3.0 µg/10⁶ cells), and differences in the minimal promoter. Or there may be differences in HoxA9 and HoxA10 function, despite similarity in protein-protein interactions and DNA binding specificity. In these investigations, we determined that overexpression of HoxA10 decreases endogenous gp91phox and p67phox mRNA abundance. Although this result is reassuringly consistent with our reporter gene assays, we cannot exclude the possibility that HoxA10 influences abundance of these transcripts indirectly by altering transcription of other genes or disrupting differentiation.

We also investigated the significance of the "short A10" transcript (6). We identified a HoxA10 cross-immunoreactive protein the predicted size of short A10 but were unable to demonstrate IFN-γ-induced phosphorylation in U937 cells. Also, DNA-binding of in vitro translated short A10 is not increased by tyrosine phosphate treatment. Consistent with this, short A10-induced repression of reporter expression from constructs with Phx-HoxA10 binding sites is not decreased by IFN-γ treatment. Since short A10 has insignificant endogenous repression activity, these results suggest that repression is due to successful competition with transcriptional activators for the same (or adjacent) DNA sequences.

Therefore, the mechanism of HoxA10 transcriptional repression is 2-fold: repression due to endogenous HoxA10 domains and binding site competition with transcriptional activators. Our studies also imply a function for short A10 in immortalized cell lines. Since short A10 represses transcription but is not modulated by differentiation-induced phosphorylation, it might contribute to transformation by repressing transcription of genes that are necessary for differentiation progression, or characteristic of the differentiated phenotype.

Previous studies of the CYBB promoter indicated that CDP represses transcription by binding within a 30-bp sequence that includes the Phx-HoxA10-like site (15, 16). CDP binds to this sequence in EMSA with nuclear proteins from the epithelial cell line HeLa and the erythroblukemia cell line K562. Additional studies determined that CDP DNA binding is modulated by another homeodomain protein, SATB1 (33). These factors are nuclear matrix-associated, and relative abundance regulates DNA interactions (33). These studies, in combination with our current observations, suggest a mechanism for transcriptional repression by homeodomain proteins in cells of various lineages. In nonmyeloid cells, transcription of CYBB may be repressed by CDP. In committed myeloid progenitors, SATB1 increases, decreasing CDP association with the repressor element, coincident with nuclear matrix disassociation. Transcriptional repression in myeloid progenitors would be maintained by Phx-HoxA10 binding until later in differentiation. This interaction could be rapidly (and reversibly) modulated by HoxA10 tyrosine phosphorylation, during differentiation or the inflammatory response (both increasing CYBB and NCF2 transcription).

Our investigations suggest that one role of HoxA10 during myeloid differentiation is repression of transcription of genes characteristic of mature myeloid cells, such as components of the phagocyte respiratory burst oxidase. It will be of interest to investigate other genes also transcriptionally activated during late myeloid differentiation (or actively transcribed during the immune response) to determine the significance of this HoxA10 function.

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Tyrosine Phosphorylation of HoxA10 Decreases DNA Binding and Transcriptional Repression during Interferon-γ-induced Differentiation of Myeloid Leukemia Cell Lines

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