HoxA10 Activates Transcription of the Gene Encoding Mitogen-activated Protein Kinase Phosphatase 2 (Mkp2) in Myeloid Cells*

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HoxA10 is a homeodomain transcription factor that is frequently overexpressed in human acute myeloid leukemia. In murine bone marrow transplantation studies, HoxA10 overexpression induces a myeloproliferative disorder with accumulation of mature phagocytes in the peripheral blood and tissues. Over time, differentiation block develops in these animals, resulting in acute myeloid leukemia. In immature myeloid cells, HoxA10 represses transcription of some genes that confer the mature phagocyte phenotype. Therefore, overexpressed HoxA10 blocks differentiation by repressing myeloid-specific gene transcription in differentiating myeloid cells. In contrast, target genes involved in myeloproliferation due to HoxA10 overexpression have not been identified. To identify such genes, we screened a CpG island microarray with HoxA10 co-immunoprecipitating chromatin. We identified the DUSP4 gene, which encodes mitogen-activated protein kinase phosphatase 2 (Mkp2), as a HoxA10 target gene. We analyzed the DUSP4 5′-flank and identified two proximal-promoter cis elements that are activated by HoxA10. We find that DUSP4 transcription and Mkp2 expression decrease during normal myelopoiesis. However, this down-regulation is impaired in myeloid cells overexpressing HoxA10. In hematopoietic cells, c-Jun N-terminal kinases (Jnk) are the preferred substrates for Mkp2. Therefore, Mkp2 inhibits apoptosis by dephosphorylating (inactivating) Jnk. Consistent with this, HoxA10 overexpression decreases apoptosis in differentiating myeloid cells. Therefore, our studies identify a mechanism by which overexpressed HoxA10 contributes to inappropriate cell survival during myelopoiesis.

The 39 human and murine HOX genes are arranged in four paralog groups on four different chromosomes. These genes encode homeodomain transcription factors that are highly conserved from Drosophila to humans. During embryogenesis, HOX gene transcription is activated 3′ to 5′ with the 3′-most genes regulating cephal development and the 5′-most genes regulating caudal development (1). Transcription of the HOX genes is also tightly regulated during definitive hematopoiesis.

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the possibility that HoxA10 is a multifunction transcription factor during myelopoiesis.

In contrast, relatively few target genes that encode proteins involved in regulating cell proliferation or survival have been identified for any Abd HoxA protein. In hematopoietic stem cells and myeloid progenitor cells, these functions are regulated by a variety of different mechanisms. To identify HoxA10 target genes involved in myeloproliferation in differentiating myeloid cells, we coupled chromatin co-immunoprecipitation with high throughput screening of a CpG island microarray (14). By this approach, we identified DUSP4 as a potential HoxA10 target gene in undifferentiated myeloid cells. This gene encodes Dusp4 (mitogen-activated protein kinase phosphatase 2).

Mkp1 and -2 are structurally related proteins that are expressed in hematopoietic cells but have different substrate specificities. In vivo, Mkp2 preferentially dephosphorylates (and therefore inactivates) c-Jun N-terminal kinases 1 and 2 (Jnk1 and -2) but not p38 or extracellular signal-regulated kinase mitogen-activated protein kinases (15). Jnk1 is activated (phosphorylated) in response to genotoxic stress or hematopoietic cytokines and mediates apoptosis in differentiating myeloid cells (16, 17). Therefore, if HoxA10 activates DUSP4 transcription, HoxA10 overexpression in myeloid malignancy might increase cell survival during cytokine-induced myelopoiesis. In this study, we investigate the role of HoxA10 in DUSP4 transcription and in regulation of apoptosis.

**MATERIALS AND METHODS**

**Plasmids and PCR Genomic Cloning**

**DUSP4 5’-Flank Reporter Constructs**—Genomic clones for the DUSP4 5’-flank were obtained by PCR using genomic DNA isolated from U937 cells. The 3’-primer for each of the reactions encompassed +30 to 0 relative to the ATG start codon. A series of 5’-primers were generated to amplify 5’-flank sequences, including −2933 to +30 bp, −1772 to +30 bp, −1722 to +30 bp, −1205 to +30 bp, and −1142 to +30 bp. These genomic sequences were subcloned into the pcATE reporter vector (Stratagene, Cedar Creek, TX). Clones were completely sequenced on both strands, and the sequence was compared with the NCBI Human Genome Data Base to ensure that no mutations had been introduced.

**Artificial Promoter Constructs**—Artificial promoter/reporter constructs were generated as previously described (10, 12) in the minimal promoter/reporter vector, p-TATACAT (18) (obtained from Dr. A. Kraft (Hollings Cancer Center at the Medical University of South Carolina, Charleston, SC)). Constructs were generated with four copies (in the forward direction) of the −1755 to −1717 bp sequence (referred to as “A”) or the −1174 to −1136 bp sequence (referred to as “B”) from the DUSP4 promoter (p-ADUSP4-TATACAT and BDUSP4-TATACAT, respectively).

**cDNA Sequences**—The cDNA for human HoxA10 was obtained from C. Largman (University of California, San Francisco, CA) (19). This cDNA sequence represents the major transcript in mammalian hematopoietic cells, encoding a 393-amino acid, 55-kDa protein (20). Wild type HoxA10 cDNA sequence was subcloned into the pSRα vector for expression in mammalian cells (per the manufacturer’s instructions (Stratagene)). The human Mkp2 cDNA was obtained from Clontech (Mountain View, CA) and subcloned into the pSRα vector for expression in mammalian cells.

**Plasmids for Short Hairpin RNA (shRNA) Expression**—Plasmids were generated to express shRNA to human Mkp2 using the plKO.1 vector (kindly provided by Dr. K. Rundell, Northwestern University, Chicago, IL). Oligonucleotide sequences for Mkp2-specific shRNA or scrambled control shRNA were designed with the assistance of the software on the Promega Web site (Promega, Madison, WI).

**Oligonucleotides**

Oligonucleotides were synthesized by the Core Facility of the Robert H. Lurie Comprehensive Cancer Center at Northwestern University. Double-stranded, synthetic oligonucleotides were generated representing the −1755 to −1717 bp “A” sequence from the DUSP4 promoter (5’-TAACTCCCTTGC-TCTGTGATTAAATTCTCATAACAGA-3’), the −1174 to −1136 bp “B” sequence from the DUSP4 promoter (5’-CTAT-TAAAACCTGATTTATGCTTTTAGATGAAAACTGCATC-3’), or the −94 to −134 bp sequence of the CYBB promoter (5’-ttcagttgcaacattgattaccccaattttctgataaaa-3’). In these oligonucleotides, the HoxA10 core is in boldface type, and the Pbx core is in italic type.

**Myeloid Cell Line Culture**

The human myelomonocytic cell line U937 (22) was obtained from Andrew Kraft. Cells were maintained and differentiated as described (10, 12, 23). U937 cells were treated with 500 units/ml human recombinant IFNγ for 24 or 48 h, as indicated (Hoffman-LaRoche).

**Chromatin Immunoprecipitation and CpG Island Screening**

U937 cells were cultured with or without IFNγ for 48 h, as described (10, 12, 23). Chromatin immunoprecipitation and CpG island microarray hybridization were performed as described (14). Briefly, cells were incubated with formaldehyde and lysed, and lysates were sonicated to generate chromatin fragments with an average size of 2.0 kb. Lysates were precipitated with HoxA10 antisera (Covance) or control preimmune serum. Chromatin was recovered by precipitation, proteins were stripped from the chromatin, and the chromatin was PCR-amplified. Several batches of immunoprecipitated, amplified chromatin were combined for each experiment.

Aliquots of HoxA10-specific and preimmune serum control-precipitated, amplified chromatin were labeled with Cy3 or Cy5 by the random primers method. Labeled DNA was used to probe a CpG island microarray, as described (14). CpG island microarrays were obtained from the Microarray Center, University Health Network (Ontario Cancer Institute, Ontario, Canada). “Spots” with 3-fold enhancement in HoxA10-specific versus control preimmune serum-precipitated chromatin in

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2 The abbreviations used are: shRNA, short hairpin RNA; IFNγ, interferon γ; EMSA, electrophoretic mobility shift assay(s); CAT, chloramphenicol acetyltransferase; Jnk, c-Jun N-terminal kinase.
three independent hybridization experiments were further considered. Dye swapping experiments were performed as controls for differences in efficiency in incorporation of Cy3 versus Cy5 into DNA. Arrays were scanned using an Agilent microarray scanner (G2565BA, Wilmington, DE), and feature intensity statistics were extracted using GenePix (Molecular Devices, Union City, CA). The GenBank™ accession number from the array was used to search the NCBI human genome data base for adjacent genes.

Specificity of chromatin immunoprecipitation was confirmed by independent chromatin immunoprecipitation with HoxA10-specific antiserum or preimmune serum control. Precipitated chromatin was PCR-amplified with overlapping primer sets that were designed to encompass the proximal 4 kb of 5′-flank sequences, based on location of the CpG island (−1734 to −2025 bp relative to the ATG start codon). Primer sets were designed to amplify −2025 to +30 bp or −2025 to −3613 bp.

Electrophoretic Mobility Shift Assays (EMSA)  
Nuclear extract proteins were prepared by the method of Dignam (24) with protease inhibitors (as described) (10). Oligonucleotides probes were prepared, and EMSA and antibody supershift assays were performed, as described (10, 12). For all experiments, at least three independent batches of nuclear proteins were tested in at least two independent experiments. Integrity of the nuclear proteins and equality of protein loading was determined in control EMSA with a classical CCAAT box (24) with protease inhibitors (as described) (10). Oligonucleotides probes were prepared, and EMSA and antibody supershift assays were performed, as described (10, 12). For all experiments, at least three independent batches of nuclear proteins were tested in at least two independent experiments. Integrity of the nuclear proteins and equality of protein loading was determined in control EMSA with a classical CCAAT box from the α-globin gene promoter. Antiserum to HoxA10 (not cross-reactive with other Hox proteins) was obtained from Covance Research Products (Richmond, CA) and from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to Pbx1 and Pbx2 (not cross-reactive with each other or other Pbx proteins) were obtained from Santa Cruz Biotechnology. All EMSA were performed several times with at least two different batches of nuclear proteins, and representative data are shown.

Transfection and Reporter Gene Assays  
Reporter Gene Assays—Cells were transfected by electroporation as described (10, 12, 23). U937 cells (32 × 10⁶ cells/sample) were transfected with 70 μg of reporter plasmid with various truncations of the DUSP4 5′-flank (or empty vector control), 50 μg of HoxA10/pSRα or control pSRα, and 15 μg of p-CMVβ-gal (to normalize for transfection efficiency). In other experiments, cells were transfected with p-TATACAT, ADUSP4-TATACAT, or BDUSP4-TATACAT minimal promoter reporter vectors, 50 μg of HoxA10/pSRα or control pSRα, and 15 μg of p-CMVβ-gal (to normalize for transfection efficiency). Transfectants were incubated for 48 h at 37 °C, 5% CO₂, with and without IFNγ (500 units/ml). Preparation of cell extracts, β-galactosidase, and chloramphenicol acetyltransferase (CAT) assays were performed as described (10, 12, 23).

Stable Transfectants—Stable U937 transfectants were generated with pSRα vectors to overexpress HoxA10, Mkp2, or empty vector control. Stable transfectants were selected in G418, as previously described (10). At least three transfectant pools were tested for each construct. In some experiments, U937 cells were transfected with PLKO.1 vectors to express shRNA for Mkp2 or scrambled shRNA control and selected with puromycin.

Western Blotting  
Total cell lysate proteins from U937 cells or U937 stable transfectants (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose. Western blots were serially probed with antibodies to various proteins. Antibodies to tubulin, HoxA10, Jnk1, and phospho-Jnk were obtained from Santa Cruz Biotechnology. Each experiment was performed several times, and representative blots are shown.

Quantitative Real Time PCR  
In some experiments, RNA was isolated from U937 cells using the Triazol reagent, according to manufacturer’s instructions (Invitrogen). RNA was tested by denaturing gel electrophoresis to determine the integrity of the 18 and 28 S ribosomal bands. Primers were designed with the software from Integrated DNA Technologies, and real time PCR was performed using SYBR green according to the “standard curve” method. Results were normalized to 18 S and actin to control for RNA abundance in various samples.

In other experiments, chromatin co-immunoprecipitating from U937 cells with antibody to HoxA10, anti-acetyl-histone 3, or control preimmune serum was analyzed by real time PCR. Primers were designed to amplify ~80 bp centered around the A and B HoxA10 DNA-binding consensus sequences using the Integrated DNA Technologies software, as above. Results were normalized to PCR product abundance in nonprecipitated, total chromatin samples. Experiments were performed in triplicate for each of three different immunoprecipitation experiments.

Apoptosis Assays  
Annexin V/projudin iodide (Beckman) double staining was used according to the manufacturer’s instructions. The cells were washed with ice-cold culture medium, adjusted to a concentration of 1 × 10⁶ cells/ml, and incubated with annexin V-fluorescein isothiocyanate solution (2.5 μg/ml) and propidium iodide (12.5 μg/ml) on ice for 15 min, and cell preparations were analyzed on a BD Biosciences FACScan flow cytometer.

Statistical Analysis  
Statistical significance of comparisons between two groups was determined by Student’s t test. Statistical significance of comparisons between larger groups was determined using analysis of variance. Statistical calculations were performed using the SigmaPlot and SigmaStat software.

RESULTS  
HoxA10 Interacts with the DUSP4 Promoter in Vitro and in Vivo—HoxA10 target genes were identified by chromatin co-immunoprecipitation from U937 myeloblast leukemia cells. This myeloid cell line undergoes differentiation in response to various cytokines, including IFNγ (22). U937 differentiation is characterized by acquisition of mature phagocyte characteristics, including respiratory burst activity and phagocytosis (10,
Differentiation is also characterized by cell cycle arrest within 24 h and programmed cell death over 72 h. Therefore, this cell line provides a reasonable model of the events of myelopoiesis.

For these studies, U937 cells were treated with formaldehyde to generate in vivo DNA-protein cross-links, and cell lysates were immunoprecipitated with antisera to HoxA10 or control preimmune serum. Prior to immunoprecipitation, the lysates were sonicated to generate 2.0-kb chromatin fragments (14). Precipitated chromatin was labeled with Cy3 or Cy5 and used to hybridize a human CpG island microarray, as described (14). Triplicate array experiments were performed (with independent co-precipitations), and only "spots" with at least 3-fold enhancement in all three were further considered. One of the identified CpG islands localized to chromosome 8 in the 5'-flank of the DUSP4 gene (Fig. 1A; location of the CpG island is −2025 to −1734 bp relative to the ATG start codon). This gene encodes dual specific phosphatase 4, also known as Mkp2 (mitogen-activated protein kinase phosphatase 2). This result was of considerable interest, since Mkp2 has been implicated in regulating apoptosis in myeloid cells via inactivation (dephosphorylation) of proapoptotic Jnk proteins (14).

Since co-precipitating DNA was sheared to generate 2.0-kb fragments, the location of the CpG island indicated that the HoxA10 binding site(s) should be within the proximal 4.0 kb of the DUSP4 5'-flank. Therefore, we performed initial experiments to verify interaction of HoxA10 with the DUSP4 5'-flank and to locate the binding site within either the proximal 2.0 kb of the 5'-flank or between 2.0 and 4.0 kb. To determine the impact of differentiation stage on HoxA10 binding, chromatin was co-immunoprecipitated from U937 cells with and without 48 h of IFNγ differentiation. Precipitating chromatin was amplified by PCR, separated by acrylamide gel electrophoresis, and visualized by ethidium bromide staining.
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Two primer sets were used to amplify fragments from −2025 bp to +30 bp (relative to the ATG) or from −2025 to −3613 bp. For these experiments, nonprecipitated chromatin was a positive control, and chromatin co-precipitating with preimmune serum was a negative control. We found specific amplification of HoxA10 co-precipitating chromatin with the proximal primer set (Fig. 1B). Although not quantitative, these studies also suggested that HoxA10 interaction with the DUSP4 5′-flank decreased during U937 differentiation. In contrast, HoxA10 antibody did not co-precipitate chromatin with the more distal 5′-flank sequence (not shown).

The results of these chromatin immunoprecipitation studies suggest that HoxA10 interacts with a binding site within the proximal 2.0 kb of DUSP4 5′-flank in vivo. Therefore, we analyzed this 2.0-kb sequence to determine if it contained any HoxA10 DNA-binding consensus sequences. This analysis resulted in identification of two sequences that conform to the derived consensus for DNA binding of HoxA10-Pbx heterodimers. These sequences were found at −1731 to −1738 bp (referred to as “A”) and −1158 to −1166 bp (referred to as “B”) relative to the ATG start codon in the DUSP4 5′-flank (Fig. 1C).

These results provided initial evidence in support of our hypothesis that DUSP4 is a HoxA10 target gene. Therefore, we performed additional experiments to determine the impact on Mkp2 protein abundance. For these studies, stable U937 transfectant pools were generated with a vector to overexpress HoxA10 or empty control vector. Transfectants were analyzed with and without IFNγ differentiation. Three independent transfectant pools were selected, and the results of representative experiments are presented. Western blots of total cell lysates were serially probed with antibodies to Mkp2, HoxA10 (to demonstrate overexpression), Jnk1, p-Jnk (to indicate activation), and tubulin (as a loading control). Mkp2 protein abundance decreases during IFNγ differentiation of control U937 cells. In comparison, Mkp2 protein abundance is relatively increased in HoxA10-overexpressing U937 transfectants after differentiation. Phospho-Jnk1, but not total Jnk1, increases during differentiation of control U937 transfectants. In contrast, Jnk1 phosphorylation does not similarly increase in HoxA10-overexpressing U937 stable transfectants in response to IFNγ differentiation. HoxA10 is overexpressed in U937 stable transfectants with the HoxA10 expression vector, consistent with expectations.

Mkp2 Expression Is Decreased by Differentiation and Increased by HoxA10 Overexpression in Myeloid Cells—Mkp2 is expressed in myeloid cells, but differentiation stage-specific regulation of Mkp2 expression has not been previously investigated (14). Differentiation of U937 cells is associated with increased Jnk activation (phosphorylation) and Jnk-induced apoptosis. Since Mkp2 dephosphorylates Jnk, one might anticipate a decrease in Mkp2 expression as differentiation proceeds. Therefore, we initially investigated the impact of IFNγ differentiation on Mkp2 mRNA abundance in U937 cells. For these studies, expression was analyzed by real time PCR using RNA isolated from U937 cells over a 72-h period of differentiation. We found that Mkp2 mRNA abundance in U937 cells decreased significantly after 24 h of IFNγ treatment but did not decrease further between 24 and 72 h post-IFNγ (F = 0.40, p = 0.68, n = 4) (Fig. 2A).

Based on these results, we investigated the impact of U937 differentiation and HoxA10 overexpression on Mkp2 protein abundance. For these studies, stable U937 transfectant pools were generated with a vector to overexpress HoxA10 or empty control vector. Transfectants were analyzed with and without IFNγ differentiation. Three independent transfectant pools were selected, and the results of representative experiments are presented. Western blots of total cell lysates were serially probed with antibodies to Mkp2, HoxA10 (to verify overexpression), Jnk and phospho-Jnk (as a downstream Mkp2 target), and tubulin (as a loading control).

In control transfectants, we found that IFNγ treatment decreased Mkp2 protein abundance and increased Jnk phosphorylation, consistent with our hypothesis (Fig. 2B). In contrast, Mkp2 protein expression was relatively preserved during differentiation of HoxA10-overexpressing U937 transfectants, and Jnk phosphorylation did not increase. These results suggest the possibility that HoxA10 overexpression decreases apoptosis in differentiating myeloid cells by impairing Jnk activation. Therefore, we performed additional studies to investigate the functional connection between HoxA10 overexpression, Mkp2 expression, and apoptosis in these cells.

HoxA10 Overexpression Decreases Mkp2-dependent Apoptosis in Differentiating Myeloid Cells—Based on the studies above, we hypothesized that HoxA10 overexpression in U937 cells would decrease apoptosis during IFNγ differentiation in a Mkp2-dependent manner. This would provide physiologic relevance to our identification of DUSP4 as a potential HoxA10 target gene. To investigate the impact of HoxA10-induced
Mkp2 expression on apoptosis, we first studied the effect of overexpressing these proteins in U937 cells. Stable transfectants pools of U937 cells were generated with vectors to overexpress either HoxA10 or Mkp2 or with empty control vector. Apoptosis of stable transfectants with and without 48 h of IFNγ differentiation was determined by annexin V staining.

In undifferentiated U937 transfectants, we found that overexpression of HoxA10 or Mkp2 did not significantly alter the percentage of apoptotic cells in comparison with control vector transfectants ($p > 0.7$, $n = 3$). Therefore, there were significantly more apoptotic cells in IFNγ-treated control transfectants compared to HoxA10- or Mkp2-overexpressing cells ($p = 0.002$, $F = 7.00$, $n = 3$). Overexpression of HoxA10 or Mkp2 in these stable transfectant pools was verified by Western blots of total cell lysates (representative blots are shown in Figs. 2B and 3B).

These results suggest that overexpression of either HoxA10 or Mkp2 protects differentiating myeloid cells from apoptosis. Therefore, we were interested in determining if the antiapoptotic effect of HoxA10 overexpression was dependent upon differentiation stage-inappropriate Mkp2 expression. To investigate this, U937 stable transfectant pools were generated with vectors to co-overexpress HoxA10 and an Mkp2-specific shRNA. Apoptosis in these cells was compared with control stable transfectants with empty expression vector plus vector to control Mkp2 expression. These results indicate that overexpression of HoxA10 or Mkp2 in differentiating myeloid cells protects against apoptosis.
express scrambled shRNA, a HoxA10 expression vector plus a vector to express scrambled shRNA, and an empty expression vector plus a vector to express Mkp2-specific shRNA. Apoptosis was determined as above (Fig. 3C).

In these studies, we found that the percentage of apoptotic cells significantly increased during IFNγ-induced differentiation of all of these stable transfectants with the exception of transfectants with HoxA10 expression vector plus control shRNA vector (Fig. 3C). Therefore, we found that expressing Mkp2-shRNA decreased the antiapoptotic effect of overexpressing HoxA10 in differentiating U937 cells. These results suggest that the antiapoptotic effect of HoxA10 overexpression in differentiating myeloid cells is at least partly abrogated by blocking Mkp2 expression. Efficient decrease in Mkp2 expression in stable transfectants with an Mkp2-specific shRNA vector was demonstrated by Western blot (Fig. 3D). These results suggest the functional significance of the impact of HoxA10-overexpression on Mkp2 expression. Therefore, we analyzed the DUSP4 5′-flank to identify cis element(s) activated by HoxA10.

**HoxA10 Overexpression Activates the DUSP4 Promoter in Myeloid Cells**—To investigate the functional significance of this interaction, we generated a series of reporter constructs with various sequences from the DUSP4 5′-flank. DUSP4 clones for these studies were obtained from U937 genomic DNA by PCR. To facilitate generating the potentially most informative set of constructs, the location of the A and B HoxA10/Pbx DNA-binding consensus sequences were considered in designing these experiments. Therefore, we generated CAT reporter constructs with 2.93, 1.77, 1.72, 1.20, and 1.14 kb of DUSP4 5′-flank (relative to the ATG start) (see Fig. 1C). Based on the location of identified consensus sequences, the 2.93- and 1.77-kb constructs include both potential HoxA10-binding sites. The 1.72- and 1.20-kb constructs include only the proximal (or B) HoxA10 binding site, and the 1.14-kb construct does not contain a HoxA10-binding consensus sequence.

**U937 Cells were co-transfected with these reporter constructs and a vector to overexpress HoxA10 or empty expression vector control.** Reporter gene assays were performed with and without IFNγ differentiation (Fig. 4A). In these studies, we found that IFNγ differentiation significantly decreased reporter expression from all of the constructs with DUSP4 5′-flank sequences except for the 1.14-kb construct. These results suggest that cis elements responsible for the differentiation stage-specific decrease in Mkp2 expression are found between 1.14 and 2.93 kb of the DUSP4 5′-flank. Additionally, we found that HoxA10 overexpression significantly increased reporter activity from all of these constructs, except for the 1.14-kb DUSP4 5′-flank construct, with and without IFNγ treatment. These results suggest that HoxA10-binding cis elements are located between 1.14 and 2.93 kb of the DUSP4 5′-flank, consistent with the location of the two HoxA10/Pbx DNA-binding consensus sequences. We found that the amount of HoxA10-induced reporter activity was not significantly different in transfectants with the 2.93- versus 1.77-kb construct, with and without IFNγ differentiation (p > 0.8, n = 4). Therefore, we compared HoxA10-inducible reporter activity from the 1.77-kb DUSP4 construct, which contains two potential Hox/Pbx binding sites (the A and B sequences), with the 1.72-kb DUSP4 construct, in which the distal consensus sequence has been eliminated (i.e. truncated to eliminate the A sequence). We found that HoxA10 overexpression induced significantly less reporter activity from the 1.72-kb construct in comparison with the 1.77-kb construct (p < 0.02, n = 4). This difference persisted with differentiation of the transfectants.

We also compared HoxA10-induced reporter activity from the 1.72- and 1.20-kb constructs, with and without differentiation. Both of these constructs include the proximal B consensus sequence but not the distal A sequence. We found that the amount of HoxA10-induced reporter activity of these constructs was not significantly different (p > 0.7, n = 4). Therefore, we compared HoxA10-induced reporter activity of the 1.20-kb DUSP4 5′-flank construct, which contains the proximal HoxA10-binding consensus (the B sequence), with the 1.14-kb construct, which does not include a HoxA10-binding consensus (i.e. truncated to eliminate the B sequence). We found that HoxA10 overexpression did not induce a significant increase in reporter expression of the 1.14-kb construct, with and without IFNγ differentiation.

These results suggest that HoxA10-inducible reporter activity decreases with deletion of the A sequence and is abolished by deletion of the B sequence. If the A and B sequences represent cis elements activated by HoxA10, HoxA10 overexpression would be expected to induce more reporter activity from constructs with both cis elements in comparison with constructs with only the more proximal B sequence. To test this hypothesis, we compared HoxA10-induced activation of constructs with both the A and B sequences (2.93- and 1.77-kb constructs) with activation of constructs with only the proximal B sequence (1.72- and 1.20-kb constructs). We found that overexpression of HoxA10 increased reporter expression from the 2.93- and 1.77-kb constructs by 79.5% ± 4.7%. In contrast, overexpressed HoxA10 increased reporter expression from the 1.72- and 1.20-kb constructs by 44.8% ± 3.1%. Therefore, overexpressed HoxA10 induced significantly more expression from constructs with both the A and B cis elements in comparison with constructs with only the B cis element (about 2-fold more, p < 0.02, n = 8). In contrast to these studies, neither HoxA10 overexpression nor IFNγ differentiation significantly altered expression from the empty, control CAT reporter vector.

**Overexpressed HoxA10 Activates Two Cis Elements in the DUSP4 Promoter**—These results suggest that HoxA10 activates two different cis elements in the DUSP4 5′-flank. Therefore, we investigated whether either the A or B DUSP4 sequence functions as a HoxA10-activated cis element. For these studies, we generated constructs with four copies of the DUSP4 A (−1755 to −1717 bp) or B (−1174 to −1136 bp) sequence linked to a minimal promoter and CAT reporter (referred to as ADUSP4-TATACAT and BDUSP4-TATACAT, respectively) (Fig. 1C). These constructs or empty control pTATACAT vector were co-transfected into U937 cells with a vector to overexpress HoxA10 or empty expression vector. Reporter gene studies were performed with and without IFNγ differentiation of the transfectants (Fig. 4B).

We found that the ADUSP4-TATACAT construct exhibited ∼3-fold more reporter activity in comparison with control
**FIGURE 4.** HoxA10 overexpression and myeloid differentiation influence activity of the DUSP4 promoter. 

**A**. HoxA10 overexpression increases activity of the DUSP4 promoter in U937 transfectants, with and without IFN-γ differentiation, and this activation depends on two consensus sequences for HoxA10-DNA binding. U937 cells were co-transfected with a reporter construct with −2.93, −1.77, −1.72, −1.20, or −1.14 kb of the DUSP4 5′-flank relative to the ATG start codon or empty reporter vector control and a vector to express HoxA10 or empty expression vector control. Reporter gene assays were performed with and without IFN-γ differentiation. Reporter activity of the 2.93- and 1.77-kb DUSP4 promoter constructs were not significantly different, were equivalently decreased by IFN-γ differentiation, and equivalently increased by HoxA10 overexpression. Activity of the 2.93- and 1.77-kb constructs (which contain both the A and B HoxA10-binding consensus sequences) was significantly greater than activity of the 1.72- and 1.2-kb constructs (which contain only the B HoxA10-binding consensus sequence), with and without IFN-γ differentiation. However, reporter activities of the 1.72- and 1.2-kb constructs were not significantly different, with and without IFN-γ, with and without HoxA10-overexpression. In contrast, HoxA10 overexpression does not influence reporter expression from the 1.14-kb DUSP4 construct (which does not contain a HoxA10 DNA-binding consensus sequence). Neither HoxA10 overexpression nor IFN-γ treatment significantly influences reporter expression from the empty control reporter vector.

**B**. HoxA10 activates an artificial promoter construct with multiple copies of the A or B sequence from the DUSP4 5′-flank linked to a minimal promoter in U937 transfectants with and without IFN-γ differentiation. U937 cells were co-transfected with a minimal promoter-reporter vector with four copies of the DUSP4 A or B sequence (ADUSP4-TATACAT or BDUSP4-TATACAT) or minimal promoter-reporter control vector (p-TATACAT) and a vector to express HoxA10 or empty expression vector control. Reporter activity was determined with and without 48 h of IFN-γ treatment of the transfectants. Both the DUSP4 A and B sequence-containing constructs have significantly more reporter expression in comparison with control p-TATACAT in U937 transfectants. Activity of both the ADUSP4-TATACAT and BDUSP4-TATACAT vectors was significantly decreased by IFN-γ treatment of the transfectants. HoxA10 overexpression significantly increased reporter activity of both the ADUSP4-TATACAT and BDUSP4-TATACAT constructs in U937 transfectants, with and without IFN-γ differentiation. In contrast, IFN-γ treatment and HoxA10 overexpression did not significantly influence reporter activity from the p-TATACAT control vector.

* and **, statistically significant differences in reporter expression of constructs with versus without truncation of the A HoxA10 binding consensus sequence in HoxA10-overexpressing transfectants. # and ##, statistically significant differences in reporter activity of constructs with versus without truncation of the B HoxA10-binding consensus sequence in HoxA10-overexpressing transfectants. ** and ###, statistically significant differences in reporter activity of constructs with versus without differentiation in HoxA10-overexpressing transfectants.
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pTATACAT vector in undifferentiated U937 transfectants, suggesting that this sequence functions as a cis element ($p = 0.002, n = 4$). However, we found that the A sequence was a significantly less efficient positive cis element in IFNγ-treated transfectants. We also found that HoxA10 overexpression significantly increased reporter expression from the A oligonucleotide-containing reporter construct in both untreated and IFNγ-differentiated U937 transfectants. Indeed, reporter expression from ADUSP4-TATACAT was not significantly different in IFNγ-treated transfectants overexpressing HoxA10 from that in undifferentiated transfectants without HoxA10 overexpression ($p = 0.63, n = 4$). In other words, HoxA10 overexpression prevented the normal IFNγ-induced decrease in activity of the DUSP4 A cis element.

We performed similar experiments to determine if the more proximal HoxA10-binding DUSP4 sequence was a functional cis element, activated by HoxA10. In these studies, we found that inclusion of the DUSP4 B sequence in the artificial promoter vector significantly also increased reporter activity, suggesting that this sequence also functions as a cis element ($p \leq 0.01, n = 4$). Similar to the DUSP4 A cis element, activity of the B cis element was significantly less in IFNγ-treated U937 transfectants in comparison with undifferentiated transfectants. We also determined the impact of overexpressing HoxA10 on the DUSP4 B cis element. In these studies, we found that HoxA10 overexpression significantly increased reporter activity from the DUSP4 B cis element-containing construct, with and without IFNγ differentiation. As with the DUSP4 A cis element, there was no significant difference in reporter activity from the BDUSP4-TATACAT construct in undifferentiated transfectants without HoxA10 overexpression in comparison with differentiated transfectants overexpressing HoxA10 ($p = 0.26, n = 4$). These results further suggest that HoxA10 overexpression can reverse the impact of myeloid differentiation on Mkp2 expression, consistent with the studies above.

We also determined whether there were differences between the DUSP4 A and B cis elements in terms of efficiency of activation by overexpressed HoxA10. In undifferentiated, HoxA10-overexpressing U937 transfectants, we found that reporter activity of the ADUSP4-TATACAT construct was significantly greater than the BDUSP4-TATACAT construct ($p = 0.02, n = 4$). However, we found that reporter activity of the ADUSP4-TATACAT and BDUSP4-TATACAT constructs was not significantly different in IFNγ-treated, HoxA10-overexpressing U937 transfectants ($p = 0.27, n = 4$). In control experiments, neither HoxA10 overexpression nor IFNγ differentiation significantly altered expression of pTATACAT vector.

HoxA10 Interacts with Two Cis Elements in the DUSP4 Promoter, in Vitro—These studies suggest that HoxA10 overexpression increases DUSP4 promoter activity in undifferentiated and differentiated U937 cells, and this effect depends on the A and B cis elements. We were interested in determining if either of these cis elements interact with HoxA10 in vitro and the impact of differentiation on this interaction.

Therefore, we performed EMSA to determine whether HoxA10 binds either of these DNA sequences in vitro. First, we investigated whether oligonucleotide probes representing either the A or B DUSP4 sequence interact with a specific protein complex in vitro. For these studies, EMSA were performed with radiolabeled, double-stranded oligonucleotide probes representing the A or B sequences and nuclear proteins isolated from U937 cells with or without IFNγ differentiation. At least three different batches of nuclear proteins were used for each of these experiments, and representative experiments are shown. For these studies, the same amount of nuclear protein was used in all of these binding assays, and the specific activities of these two probes were the same. Equality of protein loading was determined in control EMSA with probes representing the CCAAT box from the α-globin promoter, which binds the classical CCAAT factor CP1 (not shown) (10).

We found that these DUSP4 promoter sequence probes interact with a protein complex of similar mobility (Fig. 5A). We also found that binding of both of these complexes was decreased in assays with nuclear proteins from IFNγ-treated cells in comparison with untreated cells. Our results also suggest that probe A may have greater affinity for protein complex binding in comparison with probe B.

We further investigated these protein complexes for cross-reactive binding specificities. In these studies, EMSA was performed with the A or B probe; nuclear proteins from U937 cells; and excess double-stranded oligonucleotide competitors representing the A or B sequence, the CYBB HoxA10-binding cis element, or irrelevant control oligonucleotide (Fig. 5B). In these studies, we found that the low mobility complexes that bound in vitro to these two probes have cross-competitive binding specificities with each other and with the CYBB cis element. These studies also suggest that oligonucleotide B has a lower affinity for binding the protein complex in comparison with probe A.

As discussed above, the A and B cis elements contain sequences homologous to the derived consensus for HoxA10 binding to DNA as a heterodimer with Pbx, a frequent binding partner. Therefore, we next determined whether protein complexes interacting with the A and B DUSP4 oligonucleotide probes were cross-immunoreactive with HoxA10 or Pbx proteins. In these studies, EMSA was performed with the A or B oligonucleotide probe; U937 nuclear proteins; and antibodies to HoxA10, Pbx1, Pbx2, or irrelevant control antibody (Fig. 5C). We found that the low mobility protein complexes interacting with the A and B probes were cross-immunoreactive with HoxA10 and Pbx2 but not Pbx1 or irrelevant antibody. The B probe also bound a higher mobility complex, which was cross-immunoreactive with HoxA10 or Pbx2 antibody. This latter complex may represent HoxA10 or Pbx2 binding to the B sequence as a monomer.

HoxA10 Interacts with Two Cis Elements in the DUSP4 Promoter, in Vivo—Based on these results, we investigated in vivo HoxA10 binding to the A and B cis elements in the DUSP4 promoter and the effect of IFNγ differentiation on this binding. For these studies, chromatin co-immunoprecipitation was performed with a HoxA10 antibody or preimmune serum control using lysates of U937 cells with and without IFNγ differentiation (as in Fig. 1B). Chromatin was analyzed by quantitative real time PCR with primers flanking the A or B cis elements (Fig.
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In these studies, we identify DUSP4, the gene encoding Mkp2, as a HoxA10 target gene. We find that Mkp2 expression decreases during myeloid differentiation and that this decrease is at least partly due to decreased DUSP4 transcription. Also, we find that HoxA10 overexpression prevents down-regulation of Mkp2 expression and DUSP4 transcription during myelopoiesis. The downstream consequence of sustained Mkp2 expression is impaired apoptosis during differentiation of HoxA10-overexpressing myeloid cells. Therefore, these studies identify a novel HoxA10 target gene. Additionally, these studies provide a mechanism by which overexpressed HoxA10 induces resistance to apoptosis during myelopoiesis. These results are of potential significance to the phenotype of HoxA10-overexpressing malignant myeloid cells.

We demonstrate that HoxA10 activates DUSP4 transcription by interacting with at least two cis elements in the 5'-flank. These cis elements were identified by homology to a derived DNA-binding consensus sequences in the proximal DUSP4 5'-flank (A and B) interact in vitro with protein complexes of the same mobility. EMSA was performed with nuclear proteins from U937 cells (1 μg) and oligonucleotide probes representing either the A or B sequence from the DUSP4 promoter. An equivalent amount of probe of the same specific activity was used in each of these binding assays to permit comparison between the two different probes. Both the A and B probes bind a low mobility complex that decreases in intensity in EMSA with nuclear proteins from IFNγ-treated U937 cells. This complex appears to bind with greater affinity to the A probe than to the B probe. The low mobility complex is indicated by the upper arrow, and free probe is shown by the lower arrow. B, the A and B DUSP4 sequences bind protein complexes in vitro with cross-competitive binding specificities with each other and with other Hox/Pbx consensus oligonucleotides. EMSA were performed with U937 nuclear proteins (1 μg) and the A or B DUSP4 oligonucleotide probes. Binding reactions were incubated with a 200-fold molar excess of unlabeled, double-stranded oligonucleotide competitor. Homologous oligonucleotides compete for binding of the low mobility complex, but an unrelated oligonucleotide competitor does not. Also, there is cross-competitive binding specificity between the A and B probes and between these probes and the HoxA10/Pbx binding site from the CYBB promoter. Binding affinity of the B oligonucleotide is less than the A probe in these experiments. Because of the difference in intensity of the shifted bands generated by the A and B probes, different exposure durations were used for the two probes.

C, the protein complexes that bind to the A and B DUSP4 sequences in vitro are cross-immunoreactive with HoxA10 and Pbx2. EMSA were performed with U937 nuclear proteins (1 μg) and the A or B DUSP4 oligonucleotide probes. Binding reactions were preincubated with antibodies (Ab) to HoxA10, Pbx1, or Pbx2 or irrelevant control antibody (2 μg each), as indicated. Binding of the low mobility complex to either the A or B probe is specifically disrupted by antibody to HoxA10 or Pbx2. The upper arrow indicates the specific, low mobility complex, and the lower arrow indicates free probe.
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**Figure 6.** HoxA10 binds to the A and B DUSP4 sequences in vivo. A. HoxA10 binds to the A and B regions of the DUSP4 5′-flank in vivo. Chromatin immunoprecipitation experiments were performed with U937 cells, with and without 48 h of IFNγ-differentiation. Lysates were immunoprecipitated with an antibody to HoxA10 or control preimmune serum. Co-precipitating chromatin was analyzed by real time PCR using primers designed to amplify ~80-bp sequences centered around the A and B cis elements. Results were normalized to input (unprecipitated) chromatin to control for total DNA abundance in the samples. HoxA10 interaction with either the A or B sequence decreased significantly during IFNγ-differentiation of the cells. Also, significantly less HoxA10 interacted in vivo with the B cis element in comparison with the A cis element, with and without IFNγ. * and **, statistically significant difference in the amount of A versus B chromatin co-precipitating with HoxA10 antibody. Insignificant amounts of preimmune serum co-precipitating chromatin were amplified by either the A or B primer sets, as indicated. B. IFNγ-differentiation decreases interaction of acetylated histone 3 with the A and B regions of the DUSP4 5′-flank in vivo. Chromatin immunoprecipitation experiments were performed with U937 cells, with and without 48 h of IFNγ differentiation. Chromatin was immunoprecipitated with an antibody to acetyl-histone 3 (H3) or preimmune serum as a negative control. Co-precipitating chromatin was analyzed by real time PCR using primers designed to amplify ~80-bp sequences centered around the A and B cis elements. Results were normalized to input (unprecipitated) chromatin to control for total DNA abundance in the samples. IFNγ-differentiation significantly decreases interaction of acetylated histone H3 with the A and B regions of the DUSP4 promoter. Also, significantly more acetyl-H3 interacts with the A cis element in comparison with the B cis element, with and without IFNγ. * and **, statistically significant difference in A versus B chromatin co-precipitating with acetyl-H3 antibody. Insignificant amounts of preimmune serum co-precipitating chromatin were amplified by either the A or B primer sets, as indicated.

Consensus sequence for DNA binding of HoxA10/Pbx heterodimers. We found that the activity of these two cis elements is additive in the context of the intact promoter. We also found that these cis elements are more active in undifferentiated myeloid cells than after cytokine-induced differentiation. Consistent with this differentiation stage-specific activity, binding of HoxA10 and acetylated histones to these cis elements is greater in undifferentiated myeloid cells.

In contrast to the current studies, we previously found that HoxA10 represses transcription of the CYBB and NCF2 genes in immature myeloid cells (10, 12). Therefore, HoxA10 can either activate or repress transcription of various target genes in undifferentiated myeloid cells. In the current studies, we found that HoxA10 overexpression prevents the normal down-regulation of DUSP4 transcription during myelopoiesis. In contrast, in our previous studies, we found that HoxA10 overexpression did not induce differentiation stage-inappropriate repression of CYBB or NCF2 transcription (10, 12).

During cytokine-induced differentiation, HoxA10 binding affinity for the negative CYBB and NCF2 cis elements decreases. We found this decrease was due to phosphorylation of specific tyrosine residues in the HoxA10 homeodomain (11). In contrast, overexpressed-HoxA10 induces as much activity from the DUSP4 cis elements in differentiating myeloid cells as in undifferentiated cells. These results suggest that HoxA10 transcriptional activation and repression functions are not similarly regulated by cytokine-mediated post-translational modification of HoxA10.

However, although overexpressed HoxA10 induces the same percentage increase in activity of the two DUSP4 cis elements in undifferentiated transfectants as in differentiated transfectants, the total amount of cis element activity is greater in undifferentiated transfectants. One possible explanation for this might be that there is less endogenous HoxA10 in the IFNγ-treated transfectants in comparison with untreated transfectants. This could result in a lower base-line activity of the cis elements. However, our previous studies indicate that HoxA10 protein abundance is relatively constant during differentiation of U937 myeloid cells. This is in contrast to the decrease in HoxA10 expression during the CD34+ to CD34− transition in normal myelopoiesis and represents an aspect of the transformed phenotype of these cells.

An alternative explanation for the decreased activity of these DUSP4 cis elements after differentiation, even in HoxA10-overexpressing cells, is a decrease in activity of a crucial partner protein or co-activator. We find that HoxA10 binds the two DUSP4 cis elements as a heterodimer with Pbx2. This is in contrast to the HoxA10/Pbx1 heterodimer, which binds the CYBB and NCF2 cis elements. The two proteins in Hox/Pbx heterodimers are hypothesized to have different functions. For some cis elements, the Hox protein is thought to provide binding site specificity, and the Pbx protein is thought to increase binding affinity of the complex. It is possible that altered Pbx2 activity during differentiation influences HoxA10/Pbx2 complex binding to the two DUSP4 cis elements. This is an active area of investigation in the laboratory.
The two identified HoxA10-binding cis elements in the DUSP4 promoter are about 600 bp apart (−1157 to −1165 and −1132 to −1139 bp from the ATG start codon). Similarly, the CYBB promoter also includes several HoxA10-binding cis elements in the proximal promoter, each of which are several hundred bp apart (21). As in the current studies, these CYBB cis elements exhibit variable binding affinity for HoxA10. In the current studies, we found that the more proximal cis element had lower HoxA10 binding affinity, as determined by in vitro and in vivo binding studies. Consistent with this, the proximal cis element showed less activity in U937 transfection experiments, with and without IFNγ differentiation. However, in the context of the whole promoter, we found that the A and B cis elements exhibit approximately equivalent activity. This may be related to the relative proximity of the lower affinity, B cis element to the transcriptional start site in the DUSP4 gene.

It is possible that the variable affinity of HoxA10 for these two cis elements provides a mechanism for a graded response to HoxA10-induced activation at various points during the differentiation program. Specifically, a decrease in HoxA10 abundance, such as normally occurs during the transition from CD34+ to CD34− progenitors (3), would have a greater impact on the function of the proximal DUSP4 cis element. However, further events during terminal differentiation or phagocyte activation might influence HoxA10 binding to the distal cis element. This would decrease Mkp2 expression, increase Jnk1 activation, and lead to apoptosis. This process could be deranged at various points by the extent of differentiation stage-inappropriate HoxA10 expression or by other factors that influence binding affinity of the HoxA10/Pbx2 complex to the DUSP4 cis elements. Further clarification of the role of multiple HoxA10 binding cis elements in differentiation stage-specific regulation of various target genes will be of interest.

A number of pathways are involved in programmed cell death during myelopoiesis. One pathway involves Jnk, also referred to as Sapk (stress-activated kinases) (27). Jnk is activated in response to genotoxic stresses and also in response to the “stress” of cytokines, such as granulocyte-macrophage colony-stimulating factor, interleukin-3, and IFNγ (17). In human chronic myeloid leukemia, activation of Jnk1 can lead to apoptosis of the transformed leukocytes (28), suggesting a physiologic relevance to this pathway in malignant myeloid disease. Activation of Jnk is partly dependent upon activity of protein phosphatases of the Mkp family. In vivo, phospho-Jnk is the preferred substrate for Mkp2, and p38 mitogen-activated protein kinase is the preferred substrate for Mkp1 (15). These results suggest that HoxA10 induction of Mkp2 expression influences apoptosis via the Jnk phosphatase role of Mkp2.

We find that inhibition of differentiation-induced apoptosis by HoxA10 overexpression is at least partly dependent on increased Mkp2 expression in U937 cells. These results suggest that HoxA10 activation of DUSP4 transcription is physiologically significant to the phenotype of malignant myeloid cells overexpressing HoxA10. However, our studies indicate that not all of the apoptosis resistance of HoxA10-overexpressing cells was reversed by Mkp2 knockdown. It is possible that this reflects the efficiency of Mkp2 suppression by the specific shRNA. However, this result could also indicate that HoxA10 influences expression of additional target genes involved in programmed cell death in differentiating myeloid cells. Consistent with this latter possibility, not only did HoxA10 overexpression abolish differentiation-induced Jnk1 activation; phospho-Jnk1 is actually decreased in HoxA10-overexpressing U937 cells during IFNγ differentiation. By coupling chromatin co-immunoprecipitation with CpG island microarray screening, we identified a number of other potential HoxA10 target genes involved in apoptosis, proliferation, and cell cycle regulation. These additional genes will be the subject of future studies.

Regulation of apoptosis in differentiating myeloid cells is complex. The current studies represent the first identification of a role for HoxA10 in this process. HoxA10 abundance decreases during normal myelopoiesis. Our studies suggest this decreases HoxA10 activation of DUSP4 transcription, especially via the proximal, low affinity cis element. This has implications for the impact of sustained HoxA10 expression during differentiation in various forms of acute myeloid leukemia (3). Specifically, these results suggest that sustained HoxA10-expression in maturing progenitors would sustain Mkp2 expression, impairing Jnk activation and therefore apoptosis. This could be a mechanism that contributes to myeloid progenitor expansion and leukocytosis in HoxA10-overexpressing murine models. Therefore, these studies suggest a potential pathway for molecular therapeutic targeting in myeloid malignancies characterized by overexpression of Abd HoxA proteins.

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