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Meis Proteins are Major In Vivo DNA Binding Partners for Wild-Type but Not Chimeric Pbx Proteins

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The *Pbx1* and *Meis1* proto-oncogenes code for divergent homeodomain proteins that are targets for oncogenic mutations in human and murine leukemias, respectively, and implicated by genetic analyses to functionally collaborate with Hox proteins during embryonic development and/or oncogenesis. Although Pbx proteins have been shown to dimerize with Hox proteins and modulate their DNA binding properties in vitro, the biochemical compositions of endogenous Pbx-containing complexes have not been determined. In the present study, we demonstrate that Pbx and Meis proteins form abundant complexes that comprise a major Pbx-containing DNA binding activity in nuclear extracts of cultured cells and mouse embryos. Pbx1 and Meis1 dimerize in solution and cooperatively bind bipartite DNA sequences consisting of directly adjacent Pbx and Meis half sites. Pbx1-Meis1 heterodimers display distinctive DNA binding specificities and cross-bind to a subset of Pbx-Hox sites, including those previously implicated as response elements for the execution of Pbx-dependent Hox programs in vivo. Chimeric oncoprotein E2a-Pbx1 is unable to bind DNA with Meis1, due to the deletion of amino-terminal Pbx1 sequences following fusion with E2a. We conclude that Meis proteins are preferred in vivo DNA binding partners for wild-type Pbx1, a relationship that is circumvented by its oncogenic counterpart E2a-Pbx1.

Hox proteins make critical contributions to cell fate and segmental patterning during embryonic development (30). As targets of oncogenic mutations in human and murine leukemias, they are also implicated in cancer pathogenesis (3, 4, 21, 34, 35), which likely reflects perturbations of their roles in normal hematopoietic cell differentiation (23). In these capacities, they are presumed to function as transcription factors whose DNA binding activities are mediated through a conserved motif known as the homeodomain, which is structurally related to the bacterial helix-turn-helix motif (48). However, at a molecular level, the contributions of Hox proteins to developmental processes and disease pathogenesis are inadequately explained, given their disappointingly poor in vitro DNA binding affinities and specificities as monomeric proteins. This has led to the proposal that additional factors are required to modulate the DNA binding and transcriptional properties of Hox proteins (13), which would be consistent with models for achievement of specificity by other classes of transcriptional proteins.

Genetic and biochemical studies support the argument for a role for members of the Pbx, exd, and ceh-20 subfamily (5) of divergent homeodomain proteins as potential Hox cofactors. In *Drosophila melanogaster*, exd is required for the execution of genetic programs that are also dependent on Hox proteins for appropriate segment-specific expression (40, 45, 46). A similar role for mammalian Pbx proteins is suggested by genetic analyses demonstrating that sequence elements with features of Pbx-Hox consensus sites are required for appropriate expression of murine *Hoxb-1* in the developing hindbrain (44). A number of studies have demonstrated that Pbx and exd proteins increase the in vitro DNA binding affinities of many Hox and several non-Hox homeodomain proteins (6–8, 16, 20, 28, 37, 39, 41, 43, 49, 50, 53). Pbx-Hox cooperative DNA binding occurs through adjacent DNA half sites and requires highly conserved motifs flanking the Hox and Pbx homeodomains (7, 20, 25, 37, 43, 50). Interactions with Pbx also result in enhanced DNA binding specificities by Hox proteins due to modulation of the Hox homeodomain N-terminal arm, which contacts nucleotides in the DNA minor groove (8, 26). However, despite compelling biochemical data that Pbx proteins serve as DNA binding cofactors for Hox proteins in vitro, the resultant binding activities of Pbx-Hox complexes can, at best, only partially account for the position-specific activities of Hox proteins in vivo, suggesting that current models of their biochemical interactions based on in vitro studies may be incomplete.

Recent studies implicate the Meis subfamily of homeodomain proteins as potential functional collaborators with Hox proteins in experimental tumor models. The proto-oncogene *Meis1* was originally identified at the sites of retroviral insertions in myeloid leukemias in the BXH-2 strain of mice (33, 35). *Meis1* codes for a homeodomain protein that has 44% amino acid identity with the homeodomains of Pbx proteins. Retroviral activation of *Meis1* is strongly correlated with viral activation of *Hoxa7* or *Hoxa9* in BXH-2 leukemias, implying that the latter cooperate with *Meis1* in leukemogenesis (35). Given the frequent coactivation of select *Hox* and *Meis* genes in BXH-2 leukemias, the current studies were undertaken to assess whether Meis proteins may serve as DNA binding partners for heterologous homeodomain proteins.

Our results demonstrate that Meis1 is capable of dimerizing with Pbx proteins on bipartite DNA sequences consisting of 5′ Pbx and 3′ Meis half sites. Pbx1-Meis1 heterodimers display distinctive DNA binding properties and cross-bind to a subset of Pbx-Hox sites, including those previously implicated as response elements in vivo for Pbx-Hox function. In nuclear extracts, Pbx1 and Meis1 (or proteins with similar DNA binding properties and electrophoretic mobilities) form abundant complexes that comprise a major cellular Pbx-containing DNA

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binding activity. Since Pbx1 and Meis1 heterodimerize in the absence of DNA, our findings suggest that Meis proteins are preferred partners for Pbx and provide new, testable models for formulating the molecular basis for their genetic interactions with Hox proteins.

MATERIALS AND METHODS

Plasmid constructions. In vitro expression clones for wild-type and mutant Pbx proteins under the control of the SP6 promoter have been described in previous studies (7, 8). A FLAG epitope-tagged form of Meis1 was constructed by oligonucleotide-directed mutagenesis to substitute the amino acids MDYKDDDKSS (FLAG tag) for the first 29 amino-terminal amino acids of Meis1 encoded by a full-length murine cDNA reported previously (35). In constructs $Meis_{60\text{-}390}$, $Meis_{112\text{-}390}$, and $Meis_{236\text{-}390}$, the first 59, 111, and 235 amino-terminal amino acids, respectively, of the Meis1 protein were deleted and replaced with the FLAG epitope tag. The maltose-binding protein (MBP)-Meis construct for expression of Meis1 in bacteria was generated by fusing the MBP in frame to amino acid 60 of Meis1. In the expression construct E2a-$Pbx1_{10\text{-}430}$, amino acids 10 to 430 of Pbx1 were fused with the E2a portion of the chimeric oncogene E2a-Pbx1a.

EMSA. Proteins for DNA binding and electrophoretic mobility shift assays (EMSA) were produced in vitro from SP6 expression plasmids by use of a coupled reticulocyte lysate system as described previously (7). DNA binding reactions were performed at 4°C for 30 min in a 15-μl reaction volume containing 2 μg of poly[d(I · C)], 75 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10 mM Tris-HCl (pH 7.5), 6% glycerol, and 2 μg of bovine serum albumin (BSA) as reported earlier (7) and subjected to EMSA (15) using 6% polyacrylamide gels (0.75-mm thickness) in 0.25× Tris-borate-EDTA (TBE) buffer. DNA probes (50,000 cpm/binding reaction) consisted of gel-purified, end-labeled, double-stranded oligonucleotides that had an identical backbone [5′-CTGCG$(X)_{11}$ CCGC-3′, where $(X)_{11}$ represents the binding site of interest]. The sequences of the relevant binding sites are indicated in the text and in the figure legends.

Nuclear extracts were prepared essentially as described previously (15), starting with 2×10^7 log-phase cultured cells that were washed in phosphate-buffered saline and then resuspended in 1 ml of hypotonic cell lysis buffer. For E14.5 dpc mouse embryos, cell suspensions were prepared in phosphate-buffered saline with a tissue grinder prior to hypotonic lysis. EMSA of nuclear proteins was performed as described elsewhere (15).

Site selections. A modification of the selective amplification and binding (SAAB) assay (2) was used to determine consensus binding sites for the monomeric Meis1 protein and Pbx-Meis heterodimers. A single-stranded oligonucleotide [5′-GAGGATCCAGTCAGCATG$(N)_{30}$CTCAGCCTCGAGATCTCG-3′, where $(N)_{30}$ represents 30 internal degenerate positions] was annealed to an oligonucleotide primer complementary to the 3′ arm and converted to double-stranded DNA with unlabeled nucleotides. The resultant double-stranded DNA was used in binding reactions with either in vitro-translated FLAG-tagged Meis1 protein or mixtures of in vitro-translated Pbx1 and Meis1 proteins. The binding reaction mixtures contained 10 μl of each translation product in a 100-μl total volume with 14 μg of poly[d(I · C)], 75 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM Tris-HCl (pH 7.5), 6% glycerol, 1% BSA, and 1% Nonidet P-40. Protein-DNA complexes were precipitated with an anti-FLAG monoclonal antibody (1 μg/100 μl for the monomeric Meis protein) or with an anti-Pbx1b monoclonal antibody (1 μg/100 μl for Pbx-Meis heterodimers) and protein G-Sepharose beads. The anti-Pbx1b antibody recognizes an epitope in the carboxy-terminal 14 amino acids of Pbx1b (14). Pellets were washed eight times with washing buffer (75 mM NaCl, 15 mM Tris-HCl [pH 7.5], 0.15% Triton X-100, and 1% BSA) and twice with washing buffer lacking BSA. The precipitated DNA was eluted in H_2O, boiled for 10 min, and amplified by 15 to 20 cycles of PCR with annealing at 52°C using primers complementary to the 5′ and 3′ arms. Approximately 10% of the amplified product was used for a subsequent round of selection. After six rounds of SAAB, the amplified product was digested with *Bam*HI and *Bgl*II and cloned into pBluescript (Stratagene, San Diego, Calif.). Nucleotide sequences of independent clones were determined and visually aligned.

Determination of high-affinity binding sites for endogenous Pbx1b-containing complexes employed Pbx1b immunopurified complexes from 100 μg of NIH 3T3 cell nuclear extract as starting material. Sepharose beads containing precipitated proteins were resuspended in binding buffer [10 mM Tris (pH 7.5), 75 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 10 ng of poly(dI-dC) per μl, 1% BSA, 6% glycerol] and incubated with 20 μg of degenerate oligonucleotide at 4°C for 1 h with gentle rocking. The Sepharose beads were sedimented and washed five times with 1-ml aliquots of binding buffer. The precipitated DNA-protein complexes were boiled in 50 μl of water, and a 10-μl aliquot was subjected to 25 cycles of PCR (denaturing, 1 min at 95°C; annealing, 1 min at 52°C; extension, 1 min at 72°C) in a 50-μl reaction mixture. After 10 rounds of selection, PCR products were cloned and sequenced.

Immunoprecipitations. In vitro-translated proteins in reticulocyte lysates (10 μl each of Pbx and Meis translates) were added to 100 μl of 1× binding buffer (10 mM Tris-HCl at pH 7.5, 75 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% BSA, 1% Nonidet P-40) in the presence or absence of oligonucleotide DNA (1 μg) and incubated at 4°C for 3 h with anti-Pbx1b monoclonal antibody (1 μg/100 μl) and protein G-Sepharose beads. Beads were precipitated and washed 10 times with 1× binding buffer. The precipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For precipitation of in vivo Pbx1-Meis1 complexes, 1 μg of anti-Pbx1b antibody was added to 300 μg of NIH 3T3 cell nuclear extract in 300 μl of immunoprecipitation buffer {20 mM HEPES [pH 7.9], 300 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate [CHAPS], 1 mM phenylmethylsulfonyl fluoride, leupeptin [1 μg/ml], pepstatin [1 μg/ml], aprotinin [1 μg/ml], and 10% glycerol)} and incubated on ice for 2 h. Protein G-Sepharose (20 μl) was added, and the reaction mixtures were incubated at 4°C for an additional 2 h with gentle mixing. Beads containing immune complexes were sedimented, washed five times with 1 ml of immunoprecipitation buffer, and then boiled in 20 μl of SDS-PAGE sample buffer for 10 min. Eluted proteins were electrophoresed in SDS–10% polyacrylamide gels, transferred to nitrocellulose membranes, and then subjected to Western blot analysis using an anti-Meis1 rabbit antiserum that was raised against an MBP-Meis1 fusion protein.

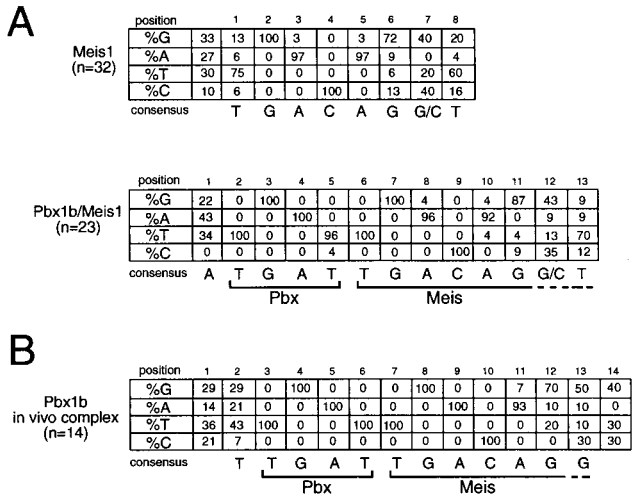

FIG. 1. High-affinity DNA binding sites for Meis1 and heterodimeric Pbx1-Meis1 complexes. The results of binding site selections for in vitro-produced monomeric Meis1 and Pbx1-Meis1 heterodimers (A) or Pbx1b complexes immunopurified from NIH 3T3 cells (B) are displayed as the percent frequency of each nucleotide at each position. The consensus binding sites are listed below the percentages. Brackets denote Pbx and Meis half sites inferred from mutational analyses. The number (n) of individual sequences on which the consensus is based appears to the left.

RESULTS

The Meis1 homeodomain protein recognizes a DNA binding site containing an atypical TGACAG core sequence. To investigate potential interactions between Meis1 and heterologous homeodomain proteins, we employed a modified SAAB procedure to determine high-affinity DNA binding sites for Meis1 alone or in the presence of Hox or Pbx proteins. The experimental approach involved incubation of in vitro-translated Meis1 or a mixture of Meis and heterologous proteins with an oligonucleotide containing 30 degenerate nucleotides flanked by PCR "handles." DNA-protein complexes were affinity purified with antibodies directed against Pbx (for Pbx-Meis heterodimers) or an epitope tag at the N terminus of Meis1 (for Meis monomeric DNA binding). DNA in the purified complex was amplified by PCR and used for subsequent rounds of selection and amplification. After six complete rounds to enrich for high-affinity sites, the DNA products were cloned and sequenced.

For monomeric Meis1, the consensus DNA binding site consisted of an 8-nucleotide sequence, TGACAG(G/C)T, in which nucleotides 1 to 6 were highly conserved with little variation (Fig. 1A). The TGACAG hexameric core is unusual for homeodomain proteins, which typically bind a tetrameric TAAT

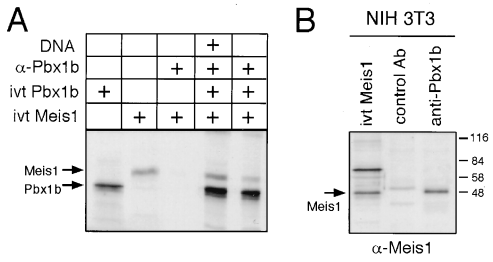

FIG. 2. Coprecipitation analyses demonstrate in vitro interactions of Pbx and Meis proteins in the absence of DNA and identify Meis proteins as components of endogenous Pbx complexes in nuclear extracts. (A) In vitro-synthesized, radiolabeled proteins were mixed and allowed to associate in the presence (+) or absence (−) of oligonucleotide DNA containing the consensus Pbx-Meis site as indicated above the gel lanes. Protein complexes were precipitated with a monoclonal anti-Pbx1b antibody (α-Pbx1b) and then analyzed by SDS-PAGE and autoradiography. ivt, in vitro. (B) Nuclear proteins prepared from NIH 3T3 cells were subjected to immunoprecipitation analysis using an anti-Pbx1b or control antibody (Ab). Immune precipitates were fractionated by SDS-PAGE and subjected to Western blot analysis using an anti-Meis1 antiserum (α-Meis1). Coprecipitation of an immunoreactive protein that comigrated with in vitro-produced Meis1 (ivt Meis1) was observed in the anti-Pbx1b but not the control antibody lane. The anti-Meis1 serum cross-reacts with an unknown 70-kDa protein in the reticulocyte lysate. Molecular masses (in kilodaltons) are shown to the right.

core (22). However, a similar core sequence preference was recently reported for TGIF, a homeodomain protein originally isolated based on its ability to bind an RXR response element (1). The TGIF homeodomain is highly similar to that of Meis1 (50% identity), suggesting that these proteins constitute a structurally distinct subclass of homeodomains that bind sites with an atypical TGACAG core consensus.

Meis1 and Pbx1 dimerize on the composite DNA sequence TGATTGACAG containing 5′ Pbx and 3′ Meis half sites. No consensus sites were obtained for Meis1-Hox-a7 complexes (data not shown). However, high-affinity DNA-binding sites were obtained for Pbx1-Meis1 complexes and revealed a 12-nucleotide consensus sequence [TGATTGACAG(G/C)T] in which the first 10 nucleotides formed an almost invariant core sequence (Fig. 1A). This core (TGATTGACAG) appeared to consist of two closely linked half sites, in which the 5′ TGAT site is identical to that determined previously for Pbx proteins (8, 24, 28, 52) and the 3′ TGACAG site is identical to the monomeric Meis1 DNA-binding sequence determined above. This suggested that the Pbx component of the Pbx-Meis heterodimeric complex contacts a 5′ half site and Meis1 occupies a 3′ half site. As a monomer or heterodimer, Meis1 displayed a modest preference for 3′ nucleotides flanking the core. Pbx1 did not appear to alter the DNA binding specificity of Meis1, since the sequence selected by Meis1, including the core and flanking region, remained constant whether or not Pbx was present. This contrasts with Pbx-Hox heterodimers in which Pbx modulates the DNA binding specificities of Hox partners (8) by interacting with conserved motifs that flank the Hox homeodomains.

Pbx and Meis proteins coimmunoprecipitate in the absence of cognate DNA binding sites and form heterodimeric complexes in nuclear extracts. To further demonstrate the Pbx1-Meis1 interaction, we performed immunoprecipitations of Pbx1 and Meis1 in the presence or absence of Pbx-Meis binding site DNA. Using an anti-Pbx1b monoclonal antibody to immunoprecipitate a mixture of ^{35}S-labeled, in vitro-translated proteins, Meis1 coprecipitated with Pbx1 regardless of whether Pbx-Meis DNA was present (Fig. 2A). The Pbx-Meis site only marginally enhanced coprecipitation.

Based on the observed ability of Pbx1 and Meis1 to dimerize in solution in the absence of specific DNA, we investigated whether comparable interactions may occur under more physiologic conditions. Endogenous Pbx1 was immunoprecipitated from nuclear extracts of NIH 3T3 cells, and the immune complexes were then subjected to Western blot analysis using a polyclonal antiserum specific for Meis1. An immunoreactive protein whose migration coincided with that of in vitro-produced Meis1 was present in the anti-Pbx1 immunoprecipitate but not in precipitations using a control antibody of the same isotype (Fig. 2B). These data conclusively demonstrated that at least a fraction of Pbx1 is complexed in vivo with Meis1.

Pbx-Meis heterodimeric complexes constitute a major Pbx-containing DNA binding activity in nuclear extracts. To determine high-affinity DNA binding sites for Pbx-containing complexes in nuclear extracts of NIH 3T3 cells, complexes were immunopurified and used for binding site selections. The consensus DNA site obtained after 10 rounds of selection (TGATTGACAG) matched the core sequence obtained for in vitro-formed Pbx1-Meis1 complexes (Fig. 1B). Notably, no sequences were obtained that corresponded to Pbx-Hox sites, indicating that the predominant DNA binding form of Pbx1 that was precipitated from nuclear extracts was complexed with Meis1 or proteins with similar DNA binding specificities.

EMSA were employed to further study the interactions of Meis and Pbx proteins. In vitro-produced Pbx1 and Meis1 displayed robust cooperative DNA binding on a synthetic oligonucleotide containing the Pbx-Meis consensus site, whereas monomeric Pbx1 or Meis1 displayed poor or no binding (Fig. 3A, lanes 1 to 5). Two isoforms of Pbx1 (Pbx1a and Pbx1b), resulting from differential splicing at its carboxy terminus (31), formed complexes with slightly different mobilities with Meis1. Inclusion of specific antibodies in binding reaction mixtures demonstrated that both Pbx1 and Meis1 were present in the protein-DNA complexes (data not shown).

When the Pbx-Meis consensus DNA site was used for EMSA of proteins prepared from NIH 3T3 cell nuclear extracts, two complexes (L and S) that specifically bound the probe were observed (Fig. 3B, lane 1). Complex S, whose migration matched that of in vitro-formed Pbx1b-Meis1, was almost completely shifted to a lower mobility by the presence of an anti-Pbx1b antibody in the DNA binding reaction mixture (Fig. 3B, lane 2). Complex L, which was not affected by anti-Pbx1b antibodies, comigrated with in vitro-formed Pbx1a-Meis complexes and was shifted to a lower mobility by an antibody (anti-PbxL) (14) reactive with Pbx1a as well as Pbx family members Pbx2 and Pbx3a (31) (Fig. 3B, lanes 5 and 6). Both complexes (L and S) were either partially disrupted or supershifted by the presence of anti-Meis1 antiserum in the binding reaction mixtures (Fig. 3B, lane 4). Partial disruption of the complexes by anti-Meis1 indicated the presence of Meis1 in each but also that a subset of complexes was likely to contain Meis1-related proteins, possibly Meis2 or Meis3 (36) or the more distantly related Pknox1 (10), that display low or no cross-reactivities with the serum raised against Meis1 (data not shown). Similar results were obtained with nuclear extracts prepared from E14.5 dpc mouse embryos (Fig. 3B, lanes 7 to 10), which express abundant amounts of Pbx1 proteins (14). Furthermore, DNA binding complexes with mobilities identical to those of L and S were present in nuclear extracts of human cells from various lineages, including hematopoietic, epithelial, and glial (Fig. 3C) lineages, although complex S was much less abundant in K562 cells, resulting in a fainter supershift band. Complex L contained a mixture of Pbx proteins (data not shown) and was partially supershifted by antibodies

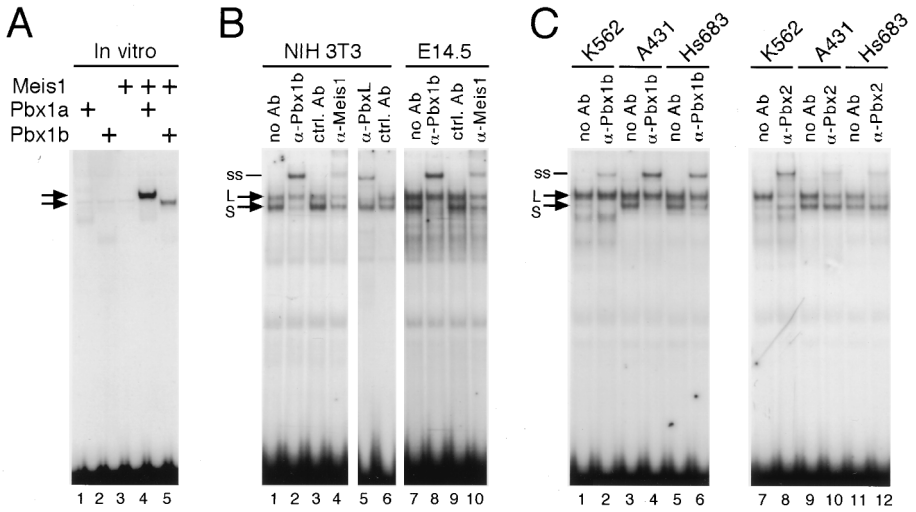

FIG. 3. DNA binding complexes containing Pbx1 and Meis1 are present in nuclear extracts from a variety of cell types. (A) In vitro-synthesized Pbx1 and Meis1 proteins cooperatively bound the consensus Pbx-Meis site (oligonucleotide M) under conditions (7) under which neither protein bound alone (lanes 1 to 5). (B) EMSA was performed on nuclear proteins extracted from NIH 3T3 cells or E14.5 dpc whole mouse embryos. The DNA probe consisted of a radiolabeled oligonucleotide containing the Pbx-Meis consensus site. Antibodies were added to selected binding reaction mixtures as indicated above the gel lanes. ctrl. Ab, control antibody; ss, antibody complex resulting from supershift analyses using anti-Pbx1b or anti-Meis1 antibodies (α-Pbx1b or α-Meis1, respectively); L and S, Pbx-Meis complexes with different mobilities containing long and short isoforms of Pbx, respectively. (C) EMSA was performed on nuclear proteins extracted from cell lines of human hematopoietic (K562), epithelial (A431), or glial (Hs683) origin. The DNA probe consisted of a radiolabeled oligonucleotide containing the Pbx-Meis consensus site. Anti-Pbx antibodies were added to binding reactions as indicated above the gel lanes. The higher-mobility bands in the K562 extract resulted from nonspecific interactions of nuclear proteins with the probe.

specific for human Pbx2 in the different cell lines (Fig. 3C, lanes 7 to 12).

Pbx-Meis complexes display distinctive DNA binding specificities and recognize a subset of Pbx-Hox sites. The half-site configurations of the Pbx-Meis consensus recognition sequence are similar to Pbx-Hox consensus sites shown in earlier studies to accommodate DNA binding by heterodimers of Pbx and Hox proteins. When complexed with Pbx, Hox proteins displayed an enhanced ability to distinguish a single nucleotide in the core of the Hox half site (designated nucleotide 7) (8). Pbx-Meis heterodimers were therefore assessed in EMSAs for their ability to bind a panel of Pbx-Hox sites that differed from each other only at nucleotide 7 (Table 1). In vitro-formed Pbx1a-Meis1 heterodimers bound only the site containing G_7 in the core of the Hox half site (Fig. 4A, lanes 2 to 5). Therefore, similar to Pbx-Hox complexes, this nucleotide plays a critical role for DNA recognition by Pbx1-Meis1 complexes. Conversely, none of the Pbx-Hox heterodimers tested bound under steady-state conditions to the Pbx-Meis site (Fig. 4A, lanes 8 to 13). The G_7 and/or C_9 nucleotides in the core of the Meis half site appeared to be critical for excluding binding by various Hox partners (data not shown). Since Pbx-Hox sites differ from the Pbx-Meis consensus only in the region of the Meis half site (Table 1), the present studies revealed that Meis DNA recognition is essential for cooperativity with Pbx. Mutation of the Pbx half site from TGAT to TGCG demonstrated that it is also essential for Pbx-Meis cooperative binding (Fig. 4A, lane 6). These data indicate that both proteins contact DNA and that the Pbx1-Meis1 heterodimer forms a DNA binding surface that recognizes adjacent half sites. Furthermore, although the overall binding specificity of Pbx1-Meis1 was distinctly different from that of Pbx-Hox complexes, there was limited overlap in the spectrum of sites recognized by the two types of complexes.

Pbx-Meis complexes in nuclear extracts of NIH 3T3 cells were also assessed for their abilities to bind Pbx-Hox sites. Similar to in vitro-formed Pbx1-Meis1 complexes, the endogenous binding species recognized a G_7-containing Pbx-Hox site but not comparable sites that contained A, T, or C at position 7 of the consensus (Fig. 4B, lanes 2 to 5). Complexes with mobilities different from Pbx-Meis complexes L and S formed on the Pbx-Hox sites, but these appeared to result from nonspecific interactions with the probe and did not contain Pbx proteins as demonstrated by supershift analyses (data not

TABLE 1. Summary of DNA binding results obtained with oligonucleotide probes employed for EMSA of nuclear proteins

Site	Oligonucleotide sequence[a]	Binding[b]
Pbx-Meis	CTGCGA TGATTGACAG CCGC	+
Pbx-Hox G_7	....TG.	+
Pbx-Hox A_7	....A.TG.	−
Pbx-Hox T_7	....T.TG.	−
Pbx-Hox C_7	....C.TG.	−
Mutated Pbx half site	..... .GCG.....	−
CRS1	T ....G..... TGAGCAAGC	+
Hoxb-1 R1	GCT.TC A...G..... G.T.	+
Hoxb-1 R2	TCAGAG .....AGT GTCT	+
Hoxb-1 R3	GG.G.G ....G..TG. G.G.	+
Consensus[c]	$\begin{matrix}A\\T\end{matrix}GAT\begin{matrix}T\\G\end{matrix}GAT\begin{matrix}C\\A\end{matrix}\begin{matrix}A\\G\end{matrix}\begin{matrix}G\\T\end{matrix}$	
Pbx-Hox nucleotide position[d]	1 3 5 7 9 11	

[a] Nucleotide sequences are shown, and the core binding sites are separated from their flanking nucleotides by spaces. Residues identical to those in the Pbx-Meis oligonucleotide are indicated by periods.

[b] +, positive reaction; −, negative reaction.

[c] Consensus for sites bound by Pbx1-Meis1 complexes.

[d] Nucleotide position numbering is based on consensus Pbx-Hox sites reported previously (8).

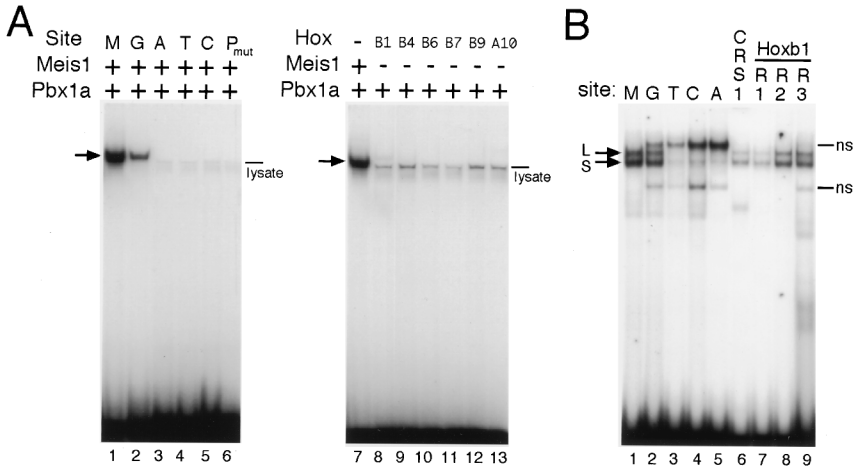

FIG. 4. Pbx-Meis complexes display distinctive DNA-binding specificities and recognize a subset of Pbx-Hox sites. (A) EMSA was performed with in vitro-translated proteins whose identities are indicated above the gel lanes. When oligonucleotides containing Pbx-Hox consensus binding sites were employed as probes (lanes 2 to 5, site identities indicated above the gel lanes), Pbx1-Meis1 heterodimers formed only on oligonucleotide G. Pbx1-Meis1 complexes did not form on a Pbx-Meis site in which the Pbx half site was mutated (lane 6). The consensus Pbx-Meis site did not support steady-state binding by various Pbx-Hox heterodimeric complexes (lanes 8 to 13). Arrows indicate Pbx-Meis heterodimeric complexes. Dashes indicate bands resulting from nonspecific interactions of endogenous proteins in the reticulocyte lysate with the DNA probes. Single letters refer to the oligonucleotides shown in Table 1: M, Pbx-Meis; G, Pbx-Hox G_7; A, Pbx-Hox A_7; T, Pbx-Hox T_7; C, Pbx-Hox C_7. (B) Nuclear proteins extracted from NIH 3T3 cells were subjected to EMSA using different DNA probes, whose identities are indicated above the gel lanes. Probe sequences are provided in Table 1. CRS1 refers to the Pbx consensus site in the *CYP17* gene (17). R1, R2, and R3 refer to conserved repeat elements in the *Hoxb-1* r4 enhancer (44). L and S, Pbx-Meis complexes with different mobilities containing long and short isoforms, respectively, of Pbx; ns, bands resulting from nonspecific interactions of endogenous proteins.

shown). Pbx-Meis complexes also formed on sites previously proposed to accomodate Pbx-Hox binding in vivo (Fig. 4B and Table 1). These included a cyclic AMP (cAMP) regulatory sequence (CRS1) in the *CYP17* gene that contains a Pbx consensus site (17) and three conserved repeat elements in the *Hoxb-1* rhombomere 4 (r4) enhancer (44).

The Meis interaction site on Pbx1 is separate from its Hox cooperativity motif and is deleted in E2a-Pbx1 chimeric proteins. Cooperative interactions between Hox and Pbx proteins require highly conserved motifs flanking the homeodomains of each protein, i.e., the Hox hexapeptide or tryptophan motifs and the Pbx Hox cooperativity motif (HCM), respectively (7, 9, 20, 37, 43). The apparent lack of hexapeptide or tryptophan motifs in Meis1, and its noncanonical DNA binding site suggested that Meis1 may interact with Pbx through a distinctly different mechanism than that employed by Hox proteins. Therefore, structure-function studies were performed to evaluate which portions of Pbx1 are required for its dimerization with Meis1.

A Pbx1 deletion mutant lacking the HCM and all amino acids carboxy terminal to the homeodomain retained its ability to cooperatively bind DNA with Meis1 in contrast to complete abrogation of binding with HoxB7 (Fig. 5A, lanes 3 and 4). These data indicated that the Pbx HCM, which is essential for Pbx-Hox cooperativity, is not necessary for Pbx1-Meis1 interactions. In contrast, deletion of the first 88 Pbx amino acids substantially abrogated cooperative binding with Meis1 but left Pbx-HoxB7 DNA binding unaffected (Fig. 5A, lanes 5 and 6). Consistent with these findings, a glutathione *S*-transferase–Pbx1 fusion protein, containing the minimal Pbx sequences (homeodomain plus HCM) necessary for Pbx-Hox DNA binding, cooperatively bound DNA with HoxB7 but not Meis1 (lanes 9 and 10). Not unexpectedly, the homeodomain of Pbx1 was required for DNA binding with HoxB7 and Meis1 (lanes 7 and 8). Therefore, two distinctly different, nonoverlapping regions of Pbx1 are required for cooperative DNA binding with Hox or Meis partners, respectively (Fig. 5C). The first 88 Pbx amino acids are referred to hereafter in this work as the Meis interaction motif (MIM) since they are required for optimal Pbx-Meis interactions.

In acute lymphoblastic leukemias with t(1;19) translocations, Pbx1 undergoes fusion with the heterologous E2a protein to form chimeric E2a-Pbx1 oncoproteins that lack the first 88 amino acids of Pbx1 (18, 38). Although E2a-Pbx1 was fully competent to bind Pbx-Hox sites with many Hox partners, including HoxB7 (7, 8, 28), it was unable to bind a Pbx-Meis site with Meis1 (Fig. 5A, lanes 11 and 12). The different capacities of heterologous partners to cooperatively bind DNA with E2a-Pbx1 reflect the fact that the HCM but not the MIM is preserved in the chimeric protein. This was further investigated by reinserting Pbx1 amino acids 10 to 88 into E2a-Pbx1, making a new fusion protein containing both the MIM and HCM (E2a-$Pbx1_{10\text{-}430}$). This construct bound DNA cooperatively with Meis1 without compromising its ability to cooperate with HoxB7 (Fig. 5A, lanes 13 and 14). Therefore, the MIM is a modular motif whose consistent deletion in E2a-Pbx1 results in a loss of function (i.e., Meis interaction).

Cooperative DNA binding with Pbx1 is not a general feature of homeodomain proteins that recognize the Meis half site. Structure-function analyses with Meis1 deletion mutants demonstrated that Meis1 amino acids 30 to 60 constitute the minimal interaction requirements, since all constructs lacking them were unable to cooperatively bind DNA with Pbx1 (Fig. 5B). Since the homeodomain protein TGIF binds to a noncanonical TGACA site (1), EMSA was employed to assess whether TGIF interacts with Pbx1 on a Pbx-Meis consensus site. Under these conditions, TGIF was unable to bind DNA cooperatively with Pbx (Fig. 5B, lane 5). This, together with the fact that the similarity between TGIF and Meis1 is limited to the homeodomain, further supports the hypothesis that regions of Meis1 outside of the homeodomain are required for dimerization and cooperative DNA binding with Pbx1.

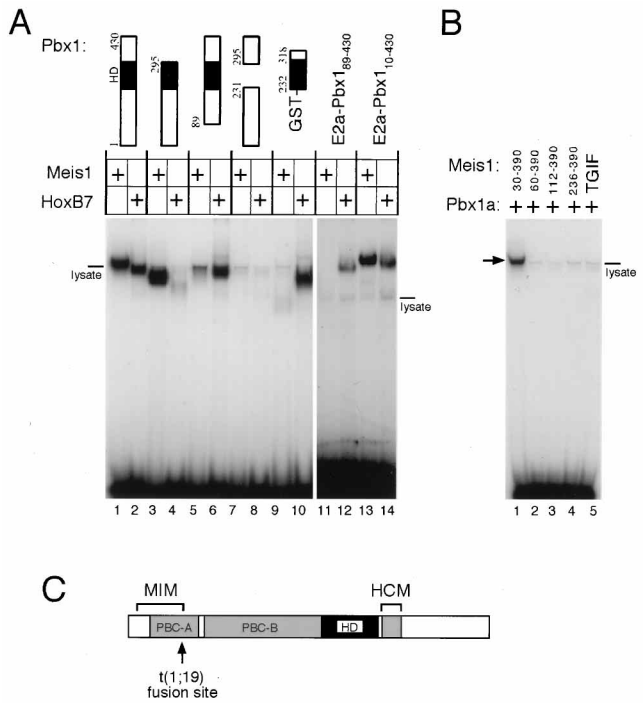

FIG. 5. The Meis interaction site on Pbx1 is different from its HCM and is deleted in E2a-Pbx1 chimeric proteins. (A) In vitro-translated proteins were incubated in DNA binding reactions in the presence of radiolabeled probe and then subjected to EMSA. The specifically programmed translation products (2 μl of Pbx protein plus 2 μl of HoxB7 or Meis1) added to individual binding reaction mixtures are indicated above the gel lanes. Pbx1 mutants are shown schematically, with a solid box indicating the homeodomain. DNA probes consisted of oligonucleotides encoding Pbx-Meis or Pbx-HoxB7 consensus sites for reactions containing Meis1 or HoxB7, respectively. Numbers correspond to amino acid positions (31) at deletion endpoints. (B) Highly conserved amino-terminal residues of Meis1 are required for interaction with Pbx1. In vitro-translated proteins were incubated in DNA binding reactions in the presence of a radiolabeled Pbx-Meis consensus DNA probe and then subjected to EMSA. The Meis1 or TGIF constructs contained in the binding reaction mixtures are indicated above the gel lanes. Meis1 nomenclature refers to residues contained in each construct according to an amino acid numbering system reported previously (35). (C) Schematic illustration of Pbx1, showing separate motifs required for Meis (MIM) and Hox (HCM) interactions. PBC-A and PBC-B, conserved sequences shared by members of the Pbx, exd, and ceh-20 homeodomain subfamily (5); HD, Pbx homeodomain.

DISCUSSION

The studies reported here identify products of the *Meis1* proto-oncogene as in vivo DNA binding partners for wild-type Pbx proteins. Pbx1-Meis1 heterodimers, whether formed in vitro or purified from nuclear extracts, displayed distinctive DNA binding preferences for bipartite DNA sequences that adhere to a consensus consisting of 5′ Pbx (TGAT) and 3′ Meis (TGACAG) half sites. The requirement for contiguous half sites is similar to the requirements for DNA binding by heterodimeric Pbx-Hox complexes; however, the half site preference exhibited by Meis1 is distinctly different from those of Hox and most homeodomain proteins. Pbx1 increases the DNA binding affinity of Meis1 comparable to its effects on a subset of Hox and non-Hox homeodomain proteins that contain conserved hexapeptide or tryptophan motifs. Unexpectedly, Meis1 does not contain one of these highly conserved dimerization motifs but rather requires, at a minimum, sequences near its amino terminus for cooperative interactions with Pbx. Perhaps reflecting this difference, Pbx1 utilizes alternative portions of itself to interact with Meis versus Hox proteins. The HCM, which is required for optimal cooperative DNA binding by Pbx-Hox complexes and for transformation by E2a-Pbx1 (9), is entirely dispensable for interactions with Meis1. Significantly, Pbx1 does not appear to modulate the DNA binding specificity of Meis1, unlike its effects on Hox proteins which, in the presence of Pbx, display enhanced discrimination for nucleotides contacted by the homeodomain N-terminal arm through the DNA minor groove (8, 26). Thus, there are important differences between Pbx-Meis and Pbx-Hox interactions, and these have potential implications for how Pbx, Meis, and Hox proteins may contribute to development and oncogenesis.

Several observations indicate that Meis proteins are highly preferred endogenous DNA-binding partners for Pbx1. Coprecipitation analyses demonstrated that Pbx1 and Meis1 physically coexist in preformed complexes in nuclear extracts of NIH 3T3 cells. Furthermore, when employed for site selections, immunopurified Pbx1-containing complexes selected high-affinity binding sites that were virtually identical to those selected by in vitro-derived Pbx1-Meis1 heterodimers. Although these studies do not rule out the possibility of additional in vivo partners for Pbx1, they indicate that Meis1 or related proteins are the predominant partners, perhaps due to their ability to dimerize with Pbx in solution. Notably, DNA sites characteristic of those bound in vitro by Pbx-Hox heterodimers were not selected by Pbx1-containing complexes in NIH 3T3 cells, in spite of the fact that the latter express several Hox proteins (11, 12, 19, 47). Although this may reflect a reduced ability of Hox proteins to dimerize in solution and coprecipitate with Pbx proteins, Pbx-Hox DNA-binding complexes were also not observed in EMSA of nuclear extracts. Taken together, these findings suggest that simple, heterodimeric Pbx-Hox complexes either do not form in vivo or are considerably less abundant than Pbx-Meis complexes. The predominant Pbx-containing DNA-binding activities observed in nuclear extracts of cultured cells and embryos were Pbx-Meis complexes, which formed not only on synthetic Pbx-Meis sites but also on a subset of Pbx-Hox sites. The identities of these endogenous complexes were convincingly demonstrated by their reactivities with anti-Pbx1 or anti-Meis antibodies and their DNA-binding specificities and migrations in EMSA, which were identical to those of in vitro-formed Pbx-Meis complexes. Therefore, Meis family members serve as endogenous dimerization partners for Pbx1, and the available biochemical data suggest that they are highly preferred partners for Pbx1 in vivo.

The complexes we have identified as Pbx-Meis heterodimers also recognize sites previously implicated as response elements through which Pbx may effect subordinate gene regulation. The cAMP-regulatory sequence (CRS1) in the *CYP17* gene (17) contains a sequence motif that displays identity (9 out of 10 nucleotides) with the core Pbx-Meis consensus. In our EMSA, CRS1 supported the binding of two complexes whose migrations matched those of Pbx-Meis heterodimers. Using CRS1 as an affinity probe, Kagawa et al. (17) purified four proteins from mouse adrenal cortical Y1 cells. Two of four CRS1-binding proteins were identified as Pbx1a and Pbx1b, respectively, by sequence analyses. The remaining two proteins were not identified, but their reported mobilities are highly similar to those for Meis1 isoforms (predicted molecular masses, 53 and 63 kDa) (33, 35). Taken together, the data strongly implicate Pbx1-Meis heterodimers as potential effectors in the cAMP-mediated regulation of *CYP17*, which codes for cytochrome P-450c17, a required enzyme for glucocorticoid and sex hormone biosynthesis.

Pbx proteins are also implicated as participants in a con-

served autoregulatory loop that controls the segmental expression of *Hoxb-1* in the mouse hindbrain. Genetic analyses have defined three DNA sequence motifs, R1, R2, and R3, within the r4 enhancer of *Hoxb-1* that direct its expression in r4 (44). They adhere to the consensus site established for Pbx-Hox heterodimeric binding sites and support in vitro binding by Hoxb-1 in the presence of exd. Furthermore, r4 enhancer-mediated expression in the fly was demonstrated to be dependent on labial and exd, the *Drosophila* homologs of Hoxb-1 and Pbx, respectively, providing additional genetic support for the argument concerning their cooperative in vivo interactions. Our present studies show that r4 enhancer elements are also bound in vitro by Pbx1-Meis1 heterodimers, and these proteins, or highly similar family members, constitute the only Pbx-containing complexes that formed on r4 enhancer elements in nuclear extracts prepared from mouse embryos. These data raise new possibilities concerning the composition of protein assemblies that could positively or negatively regulate *Hoxb-1* expression through the r4 enhancer elements. Given their potential to accommodate binding by either Pbx-Hox or Pbx-Meis complexes, it will be of interest to assess the differential effects of Pbx and Meis null mutations on *Hoxb-1* autoregulation.

Previous studies suggested that Meis1 may be a functional homolog of Pbx proteins. This was based on (i) homology between the Meis and Pbx homodomains and (ii) genetic interactions between Meis1 and Hoxa-7 and/or Hoxa-9 in leukemogenesis (35). Our studies are consistent with previous genetic data supporting the argument for a Pbx-like role for Meis1 but suggest that this may not occur as simple heterodimers with Hox proteins. We have not been able to demonstrate direct physical interactions between Meis and Hoxa-7 proteins analogous to those that occur in vitro for Pbx with a subset of Hox proteins. Site selections failed to yield consensus DNA binding sites for Meis1-Hoxa-7 (data not shown), and no cooperativity was observed in DNA binding assays employing consensus sites that support Pbx-Hox or Pbx-Meis DNA binding. We cannot exclude the possibility that Meis1 may physically interact with a subset of Hox proteins on DNA sites that were not determined in our SAAB procedures (a possibility currently under investigation).

At least two potential models for integrating Pbx, Meis, and Hox function within the context of available biochemical and genetic data are suggested by our observations. In a so-called competitive model, Pbx proteins may serve as heterodimeric partners for either Hox or Meis proteins. As we observed in vitro, heterodimers could recognize three categories of binding sites consisting of exclusive Pbx-Hox sites, exclusive Pbx-Meis sites, or sites that accommodate either type of heterodimeric complex. Under this scenario, Meis and Hox proteins would compete (along with other non-Hox homeodomain proteins) for Pbx proteins as binding partners. Pbx-Meis complexes, given their formation in the absence of DNA, would likely constitute the predominant form of Pbx against which Hox proteins must compete for binding partners. The observations that Meis and Hox proteins bind 3′ of Pbx on their respective consensus sites would seem to favor this model. In an alternative, so-called cooperative model, Hox proteins would cooperatively interact with preformed Pbx-Meis heterodimers. Although Pbx-Meis-Hox complexes have not been observed in our experiments, the requirements for different regions of Pbx1 to dimerize with Meis versus Hox partners raise the possibility that Pbx could simultaneously interact with Meis and Hox proteins to form higher-order complexes. This model would account for the observed genetic interactions in different settings between *Hox* and *Pbx*, *exd*, or *Meis* genes, the apparent prevalence of Pbx-Meis heterodimers in various cell types, and the inability to detect simple Pbx-Hox heterodimers in vivo. Clearly, additional studies are needed to address the molecular mechanisms by which Hox proteins achieve their in vivo specificities within the context of Pbx and Meis cofactors, and our results suggest possible models for further testing.

Representatives of each of the Meis, Pbx, and Hox homeodomain subfamilies are implicated in oncogenesis. *Meis1* is ectopically expressed as a result of adjacent retroviral insertions in a subset of BXH-2 myeloid leukemias, whereas *Pbx1* undergoes fusion with the heterologous *E2A* gene following t(1;19) chromosomal translocations in pre-B-cell acute lymphoblastic leukemia (18, 33, 35, 38). A subset of *Hox* genes is oncogenically activated by similar mechanisms in murine and human myeloid leukemias (3, 4, 21, 34, 35). It is unclear how ectopic Meis1 expression contributes to leukemogenesis, particularly since deregulated expression of Hox proteins alone is sufficient for induction of transformed phenotypes in experimental models (29, 42, 51). The oncogenic properties of Meis1 remain uncharacterized, and it hasn't been determined whether ectopic expression of Meis1 alone results in oncogenic changes or whether it facilitates transformation only within the context of coexpressed Hox proteins. As a group, the three known *Meis* genes are widely expressed in many cell types (36); however, myeloid cells typically do not express Meis1, whose expression is redirected to this cellular compartment by retroviral insertions in BXH-2 leukemias. This suggests either that the quantitative levels of Meis proteins are critical or that various members of the Meis homeodomain subfamily may be functionally distinct, perhaps in their abilities to bind response elements and/or interact with heterologous partners. Although our studies did not reveal differences in the dimerization and DNA binding properties of Meis1 with Pbx family members (unpublished observations), further studies are required to address this in greater detail.

Our studies clearly demonstrate fundamental differences in the abilities of wild-type and oncogenic Pbx proteins to utilize Meis1 as a dimerization partner. In contrast to Pbx1, chimeric E2a-Pbx1 oncoproteins were unable to cooperatively bind DNA with Meis1. The amino-terminal 88 Pbx1 residues (the MIM), which are deleted following fusion with E2a, are critical for Pbx1-Meis1 cooperative DNA binding. Therefore, fusion with E2a results in both a loss of function, i.e., an inability to dimerize with Meis proteins, and a gain of function previously shown to result from acquisition of E2a transactivation motifs (24, 27, 52). The latter is essential for oncogenic activity of E2a-Pbx1 in experimental models of oncogenesis (32). By comparison, wild-type and chimeric Pbx1 proteins display identical abilities to dimerize and bind DNA with Hox proteins. E2a-Pbx1 retains the HCM, a highly conserved helical motif flanking the Pbx1 homeodomain, which is required for optimal cooperative interactions with Hox partners and also essential for E2a-Pbx1-mediated oncogenesis (9). An absolute requirement for the HCM in oncogenesis suggests that Hox or Hox-like proteins are involved in the oncogenic effects of E2a-Pbx1. It is unclear whether loss of ability to dimerize with Meis proteins is necessary for leukemogenesis or an incidental consequence of protein fusion, although it appears to be insufficient, since mutant Pbx1 proteins lacking the MIM were incapable of transforming NIH 3T3 cells (32). Although the significance remains to be determined, our current findings indicate that in its oncogenic form, E2a-Pbx1 circumvents interactions with Meis proteins, the apparent predominant DNA binding partners for wild-type Pbx proteins.

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