Structural Determinants within Pbx1 That Mediate Cooperative DNA Binding with Pentapeptide-Containing Hox Proteins: Proposal for a Model of a Pbx1-Hox-DNA Complex

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Genetic studies have identified a family of divergent homeodomain proteins, including the human proto-oncoprotein Pbx1 and its *Drosophila* homolog extradenticle (Exd), which function as cofactors with a subset of Hox and HOM-C proteins, and are essential for specific target gene expression. Pbx1/Exd binds DNA elements cooperatively with a large subset of Hox/HOM-C proteins containing a conserved pentapeptide motif, usually YPWMR, located just N terminally to their homeodomains. The pentapeptide is essential for cooperative DNA binding with Pbx1. In this study, we identify structural determinants of Pbx1 that are required for cooperative DNA binding with the pentapeptide-containing Hox protein HoxA5. We demonstrate that the homeodomain of Pbx1 contains a surface that binds the pentapeptide motif and that the Pbx1 homeodomain is sufficient for cooperative DNA binding with a Hox protein. A sequence immediately C terminal to the Pbx1 homeodomain, which is highly conserved in Pbx2 and Pbx3 and predicted to form an α-helix, enhances monomeric DNA binding by Pbx1 and also contributes to maximal cooperativity with Hox proteins. Binding studies with chimeric HoxA5-Pbx1 fusion proteins suggest that the homeodomains of Pbx1 and HoxA5 are docked on the representative element, TTGATTGAT, in tandem, with Pbx1 recognizing the 5′ TTGAT core motif and the Hox protein recognizing the 3′ TGAT core. The proposed binding orientation permits Hox proteins to exhibit further binding specificity on the basis of the identity of the four residues 3′ to their core binding motif.

Homeobox genes are a large family of genes involved in the genetic control of cell fate and segmental patterning (30, 38, 50). Homeobox gene products share a conserved DNA-binding motif which is designated the homeodomain (HD [50]). The vertebrate Hox proteins and *Drosophila* HOM-C proteins represent a distinct group of homeotic selector proteins that specify the body plan (1, 30, 38). Mutations in these genes cause segmental transformations in which a particular segment develops morphological structures resembling those of another. Hox and HOM-C proteins share structural and functional features, including the primary sequence of their HDs, the chromosomal organization of their respective genes, and spatial and temporal expression patterns along the anterior-posterior axis (1, 30, 38). Functional conservation of homeotic selector genes is also evidenced by ectopic expression of mammalian Hox genes in the *Drosophila* embryo (36, 39).

The HDs of both Hox and HOM-C proteins bind DNA in vitro and contribute to function in vivo by recognizing specific DNA motifs in selected target genes (9, 13, 14, 19). HD proteins act with great biological specificity in development, and functional differences among HD proteins are largely mediated by their HDs (15, 18, 31, 37). Because many divergent HDs in Hox proteins bind similar DNA sequences containing a TAAT core (C/T-<u>TAAT</u>-G/T-G/A [9, 13, 14, 19, 32]), a central issue in development is to determine how specific HD proteins of the Hox/HOM-C family achieve their specificity. Mechanisms that account for differences in biological function of HD proteins in general include inherent differences as activators or repressors of transcription (3, 49), differences in sequence-specific DNA binding due either to the inclusion of

a second DNA-binding domain (12, 21, 54) or to differences in the sequence of the third helix of the HD itself (10), and a requirement for direct protein-protein interactions with other transcription factors (2, 5, 11, 63). In yeasts, interaction of the HD protein α2 with either a second HD protein, a1, or with MCM1 (a homolog of the serum response factor [SRF]) specifies different DNA recognition motifs, impacts transcription of different target genes, and specifies distinct yeast cell types (22). A short protein sequence of α2 immediately N terminal of the HD mediates α2-MCM1 interaction (56), while the C-terminal tail of α2 is involved in the α2-a1 interaction (51). Cooperative interactions involving HD proteins are also documented for Phox1 (17), POU HD proteins (21, 52, 53, 57, 62), and Paired class HD proteins (60).

Genetic studies have identified a new family of HD proteins that function as cofactors with a subset of Hox/HOM-C proteins (48). This family includes human proto-oncoprotein Pbx1 (26, 41), its homolog Pbx2 and Pbx3 (40), *Drosophila* Extradenticle (Exd [47]), and *Caenorhabditis elegans* ceh-20 (4). PBX1 was first identified because of its involvement in the t(1;19) chromosomal translocation in human pre-B-cell acute lymphoblastic leukemias (26, 41), in which a transcriptionally activated form of Pbx1 is formed by fusion with the E2A gene (producing E2A-PBX1) that functions as an oncoprotein (8, 24, 25, 27). In *Drosophila melanogaster*, mutations in EXD cause homeotic transformations in both embryos and adult flies (43, 58). Unlike other HOM-C genes, EXD does not regulate HOM-C gene expression, nor does its function depend on its regulation by HOM-C genes (43, 47), suggesting that EXD functions in parallel to HOM-C genes to specify segmental identity.

Pbx1/Exd interacts directly with a group of Hox/HOM-C proteins, exhibiting cooperative DNA binding (6, 7, 35, 45, 55) on the artificial elements TGATTGAT (35) and TGATTAAT (34). In the Hox-B1 promoter, two similar elements—TGATG

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GAT and AGATTGAT—are essential for stage-specific and tissue-specific expression of the Hox-B1 gene and serve as motifs for cooperative binding by Hox-B1 and Exd (45). In the somatostatin promoter, a heterodimer of Pbx and the pancreatic HD protein, STF-1, binds a TGATTAAT element (42) that is important for somatostatin expression in pancreatic islet cells (33). Transcription through this element is activated synergistically by STF-1 and E2A-Pbx1 (42). Each of these elements contains tandem repeats of TGAT, TAAT, or AGAT. On the TTGATTGAT site, DNase I footprint analysis indicates that the Hox protein binds 5′ to Pbx1 (34). These results suggest that target gene selection by certain Hox/HOM-C proteins during development could be achieved by cooperative interaction with Pbx/Exd proteins on closely spaced or partially overlapping half-sites.

More than 20 Hox/HOM-C proteins contain a highly conserved pentapeptide motif N terminal to the HD (usually YPW MR) that is essential for binding DNA as a heterodimer with Pbx1/Exd (7, 23, 29). In the present report, we identify structural determinants of Pbx1 required for cooperative binding with the pentapeptide-containing Hox protein HoxA5 on TTG ATTGAT. This element, which is designated the PRS (Pbx1-responsive element) is also transcriptionally activated by oncoprotein E2A-Pbx1 (35). We find that the Pbx1 HD and 17 amino acids immediately C terminal to the HD are both necessary and sufficient for maximal monomeric and cooperative DNA binding. The HD of Pbx1 harbors a surface for direct interaction with the pentapeptide motif and is sufficient for cooperative DNA binding, while the C-terminal conserved sequence enhances DNA binding of the Pbx1 HD and also contributes to maximal cooperativity. We present evidence that the HDs of both Pbx1 and HoxA5 are docked in tandem on the PRS and propose a model for cooperative DNA binding by Pbx1 and pentapeptide-containing Hox proteins on adjacent TGAT half-sites. The proposed binding orientation permits Hox proteins to exhibit further binding specificity on the basis of the identity of DNA residues 3′ to their core binding motif.

MATERIALS AND METHODS

Construction of recombinant plasmids. All deletion mutants of Pbx1 or HoxA5 and the Hox Pbx chimeras (HPC mutants) were constructed by introducing the designated sequence after the initiating methionine in pGEM 3zf⁻ (Promega) and were expressed by coupled transcription-translation with SP6 polymerase. Construction of Pbx1bN51S, HoxA5N51S, and hemagglutinin-tagged HoxA5 were as described previously (34, 35). Site-directed mutagenesis on Pbx residues 233 to 326 was performed with the Muta-Gene system (Bio-Rad) in accordance with the manufacturer's instructions. All mutants were verified by DNA sequence analysis.

Expression and purification of recombinant Pbx proteins. Deletions of Pbx1 were constructed as glutathione S-transferase (GST) fusion proteins in pGEX-2T (Pharmacia). For purification of recombinant Pbx proteins, 20 ml of Bl21 cells containing the GST-Pbx expression vectors was grown at 37°C to an A_{600} of 0.5. Expression of GST-Pbx proteins was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside for an additional 3 h. Cells were collected by centrifugation and lysed by three brief sonications (15 s each) in 1 ml of 25 mM Tris (pH 7.5)–1 mM EDTA–150 mM NaCl (buffer B). Insoluble material was removed by centrifugation in a 1.5-ml Eppendorf tube, and the soluble fraction was added to 100 μl of glutathione-agarose beads pre-equilibrated with buffer B at 4°C and mixed for 2 h at 4°C. The beads were then washed seven times with 1.0 ml of buffer B. Pbx1 proteins were cleaved from GST sequences by the addition to the beads of 200 μl of buffer B containing 10 mM CaCl₂ and 1 U of thrombin and by rocking the tube for 2 h at room temperature. Cleaved Pbx1 proteins were recovered, and protein concentrations were estimated by Coomassie blue staining. The proteins were stored at −70°C in buffer B containing 25% glycerol (0.5× buffer B, 25% glycerol).

Calculation of apparent K_ds. Apparent equilibrium dissociation constants (K_ds) were defined by the formula $K_d = [DNA][P]/[P\text{-}DNA]$, where [DNA] is the concentration of free DNA, [P] is the concentration of free protein, and [P-DNA] is the concentration of the protein-DNA complex. The concentration of ³²P-labeled DNA probes was 0.3 nM. The concentration of Pbx proteins was kept at a level 10-fold in excess over that of DNA, allowing the approximation [P] ≅
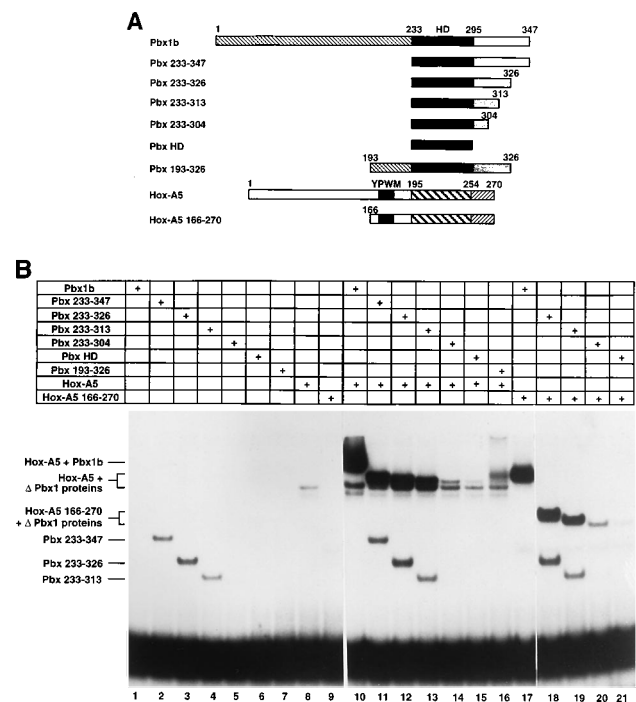

FIG. 1. Residues 233 to 313 constitute the minimal domain of Pbx1 required for strong monomeric and cooperative DNA binding. (A) Structures of Pbx1 and HoxA5 proteins used in this study. (B) Combinations of in vitro-translated full-length and truncation mutants of Pbx1 and HoxA5 proteins incubated with probe BS2 (CGAAA<u>TTGATTGAT</u>-GCGCCCCGCGCT); protein-DNA complexes were resolved by EMSA. Proteins added to each binding reaction are indicated at the top of the panel. A 2-μl volume of each translated HD protein was used, and a total of 4 μl of rabbit reticulocyte translation mix was added in all cases. Similar amounts of Pbx1 deletion mutants were added to each binding reaction mixture, as measured by inclusion of [³⁵S]methionine in parallel transcription-translation reactions and then by gel electrophoresis and autoradiography (not shown). When required, translation mix expressing the pGEM 3zf⁻ vector only was added to bring the total mix to 4 μl.

[P$_{total}$] to be used in calculating K_ds. A total of 100% of recombinant proteins were assumed to be active in binding. The value of [DNA]/[P-DNA] was determined by calculating the ratio of free counts per minute to bound counts per minute.

In vitro transcription-translation. In vitro transcription-translation was performed with the Promega TNT Coupled Reticulocyte Lysate system, in accordance with the manufacturer's protocol and by employing SP6 polymerase.

EMSA. Double-stranded oligonucleotides were labeled with [³²P]ATP by phosphorylating a short oligonucleotide that annealed to the 3′ portion of the binding-site oligonucleotide and then synthesizing the complementary strand by using deoxynucleoside triphosphates and Klenow. For electrophoretic mobility shift assays (EMSA), 15,000 cpm of probe was incubated with 2 to 4 μl of in vitro-translated proteins or appropriate amounts of recombinant proteins in the presence (for in vitro-translated proteins) or absence (for purified recombinant proteins) of 1 μg of poly(dI-dC) · (dI-dC) in a buffer containing 10 mM Tris (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 2.5 mM MgCl₂, 0.1% Nonidet P-40, and 5% glycerol for 20 min at room temperature. Bound and free probes were separated by electrophoresis in 5 to 8% acrylamide gels formed in 0.5× Tris-borate-EDTA and were run in the same buffer. After drying of the gel, the protein-DNA complexes were visualized by autoradiography.

RESULTS

Maximal monomeric and cooperative DNA-binding activity of Pbx1 requires the Pbx1 HD and the carboxyl-terminally conserved residues. Structural determinants of Pbx1 required for cooperative DNA binding with Hox proteins were examined by using HoxA5 as a heterodimer partner. In a manner similar to that with HoxB8, cooperative DNA binding of HoxA5 and Pbx1 was dependent on DNA binding by HoxA5, because an asparagine-to-serine point mutation at residue 51

of the HoxA5 HD abrogated both monomeric and cooperative DNA binding (data not shown). A series of Pbx1 deletion mutants were constructed (Fig. 1A), and their ability to bind the PRS as a monomer or to bind cooperatively with HoxA5 was examined (Fig. 1B). Unexpectedly, deletion of Pbx1 sequences N terminal to the HD (Pbx residues 233 to 347) strongly increased monomeric DNA binding (Fig. 1B, lane 2). Addition of only 30 residues N terminal to the HD (Pbx residues 193 to 326) was sufficient to suppress the inherent tight DNA binding of the HD and C terminus (lane 7). Beginning with Pbx residues 233 to 347, C-terminal deletions revealed that residues 296 to 313 were required for maximal DNA binding, since both Pbx residues 233 to 304 and Pbx HD failed to bind DNA detectably (lanes 5 and 6). The DNA-protein complexes observed in lanes 2 to 4 (Fig. 1B) represented monomeric binding of Pbx1 to the PRS, because mixing experiments using combinations of Pbx residues 233 to 347, 233 to 326, and 233 to 313 failed to yield complexes of intermediate mobility (data not shown). Efficient cooperative DNA binding with HoxA5 also required both the HD and C-terminal residues 296 to 313 (lanes 11 to 13), since both Pbx residues 233 to 304 (lane 14) and Pbx HD (lane 15) exhibited a significant loss of cooperative DNA binding with HoxA5. Thus, the Pbx1 HD plus residues 296 to 313 possess a strong DNA-binding activity that is not evidenced by full-length Pbx1, and this same sequence constitutes a minimal domain that is both necessary and sufficient for maximal cooperative DNA binding with Hox proteins.

Analysis of the minimal HoxA5 domain required for cooperative DNA binding with Pbx1 revealed that deletion of HoxA5 residues 1 to 165 reduced monomeric DNA binding by HoxA5 residues 166 to 270 (Fig. 1B, lane 8 versus 9) but retained strong cooperative DNA binding with Pbx1 (lane 17). HoxA5 residues 166 to 270 contain the YPWMR motif, the HD, and the short 16-amino-acid C terminus of HoxA5 (Fig. 1A). Like full-length HoxA5 (lane 10), HoxA5 residues 166 to 270 complexed efficiently with Pbx1 (lane 17), Pbx residues 233 to 326 (lane 18), and Pbx residues 233 to 313 (lane 19) but did not complex efficiently with Pbx residues 233 to 304 (lane 20) or Pbx HD (lane 21). Because HoxA5 residues 166 to 270 did not form a monomeric complex in EMSA that migrated near other Pbx1-HoxA5 complexes, HoxA5 residues 166 to 270 were used in subsequent experiments to simplify data analysis.

The HD of Pbx1 contains a surface for interaction with the YPWMR motif. Previous studies (7, 23, 29) demonstrated that cooperative DNA binding by Pbx1 and pentapeptide-containing Hox proteins was dependent on the Hox pentapeptide motif. A synthetic peptide from HoxA5 containing this motif (QPQIYPWMRKLH), which was designated PEP-A5[WT], also disrupts cooperative binding (29). Therefore, we used this synthetic peptide to examine whether the interaction between HoxA5 and the minimal cooperativity domain of Pbx1 was pentapeptide dependent. Such an approach has also provided evidence for direct coupling between the Stat transcription factor and the interleukin 4 (IL-4) receptor following addition of IL-4 (20). PEP-A5[WT] disrupted the Pbx residues 233 to 313-HoxA5 DNA-binding complex in a concentration-dependent manner (Fig. 2A), while a mutant peptide (QPQIYPFMRKLH), which was designated PEP-A5[MUT] and which contains a single W-to-F substitution within the YPWMR motif that also abolishes the ability of HoxA5 to bind DNA cooperatively with Pbx1, failed to inhibit complex formation (Fig. 2A, lanes 2 versus 4 and lanes 3 versus 5), suggesting that the pentapeptide contacts the surface of Pbx residues 233 to 313.

If the YPWMR motif binds Pbx residues 233 to 313, the resulting interaction could increase the affinity of DNA binding
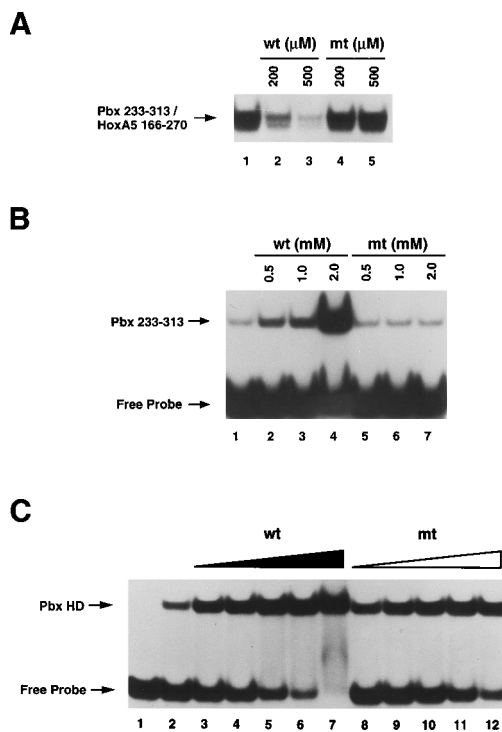

FIG. 2. A peptide from HoxA5 containing the pentapeptide disrupts Pbx1-Hox-DNA complexes and stimulates formation of Pbx1-DNA complexes. (A) A 2-μl volume of in vitro-translated Pbx residues 233 to 313 and 2 μl of HoxA5 residues 166 to 270 were incubated with probe BS2 in the absence (lane 1) or presence of PEP-A5[WT] (QPQIYPWMRKLH; lanes 2 and 3) or PEP-A5[MUT] (QPQIYPFMRKLH; lanes 4 and 5). DNA-binding complexes were resolved by EMSA. Free probe and monomeric DNA-binding complexes are not shown. (B) A 2-μl volume of in vitro-translated Pbx residues 233 were 313 was incubated in the absence of peptide (lane 1) or in the presence of PEP-A5[WT] (lanes 2 to 4,) or PEP-A5[MUT] (lanes 5 to 7). (C) A 1 nM concentration of purified recombinant Pbx HD was incubated in the absence of peptide (lane 2) or in the presence of PEP-A5[WT] (lanes 2 to 7) or PEP-A5[MUT] (lanes 8 to 12), with threefold increases in the concentrations of each peptide up to a final concentration of 3 mM in lanes 7 and 12. wt, wild type; mt, mutant.

by the HDs of either Pbx1, the Hox protein, or both (17). Therefore, we tested whether PEP-A5[WT] enhanced DNA binding by Pbx residues 233 to 313. Addition of PEP-A5[WT] increased the abundance of the Pbx1 residues 233 to 313-DNA complex in a concentration-dependent manner while PEP-A5[MUT] did not, suggesting that binding of PEP-A5[WT] increases the affinity of Pbx residues 233 to 313 for DNA (Fig. 2B, lanes 2 to 4 versus lanes 5 to 7). To distinguish whether C-terminal residues 296 to 313 were dispensable for enhancement of DNA binding by PEP-A5[WT], the ability of PEP-A5[WT] to stimulate DNA binding by the recombinant Pbx1 HD was examined. This analysis was performed in the absence of poly-(dI:dC), which is essential to observe DNA binding by the isolated Pbx1 HD. PEP-A5[WT] enhanced DNA binding of the HD alone (Fig. 2C, lanes 3 to 7 versus lane 2), and PEP-A5[MUT] also increased binding but was fourfold less effective (Fig. 2C, lanes 8 to 12 versus lanes 3 to 7). The ability of the mutant peptide to increase binding by Pbx HD but not by Pbx residues 233 to 313 may indicate that weak DNA binding by the HD can be increased by interaction with other residues in the pentapeptide but that these interactions do not significantly alter the tighter binding exhibited by Pbx residues 233 to 313 (see following paragraph). These experiments suggested that the Pbx1 HD binds the pentapeptide motif and that bind-

ing of the HoxA5 pentapeptide to Pbx1 stabilizes the Pbx1-HoxA5-DNA complex, possibly by increasing the affinity of Pbx1 for DNA.

Pbx1 sequences 296 to 313 increase the monomeric DNA-binding affinity of the Pbx1 HD. The fact that Pbx residues 233 to 313 bind the PRS probe in the presence of dI-dC (Fig. 1B, lane 4), while the Pbx HD does not (lane 5), suggested that the affinity of Pbx residues 233 to 313 for the PRS is greater than that of the isolated HD. To quantitate this effect, the apparent K_ds of DNA-protein complexes formed between the PRS and of individual recombinant Pbx proteins were measured. K_d values were as follows: Pbx1 HD, 5.2 ± 1.1 nM; Pbx1 residues 233 to 304, 5.5 ± 0.9 nM; Pbx1 residues 233 to 313, 1.1 ± 0.4 nM; and Pbx1 residues 233 to 326, 0.9 ± 0.3 nM (as determined by EMSA; measurements were taken at concentrations of 0.3 nM DNA probe and 3.0 nM each protein). These values demonstrated that addition of residues G-296 to Y-305 did not increase DNA binding, while further addition to residue A-313 increased DNA binding by approximately fivefold.

Cooperative DNA binding is mediated by the Pbx1 HD and is enhanced by inclusion of residues 296 to 313. The observation that PEP-A5[WT] can bind the Pbx1 HD directly (Fig. 2C) suggested that the Pbx1 HD contained either all or a significant portion of the structural determinants required for cooperative interactions with HoxA5 on DNA. If the HD contained all residues involved in cooperative interactions with Hox proteins, then the fact that addition of C-terminal residues 296 to 313 strongly increases cooperative DNA binding with HoxA5 (observed in Fig. 1B, lanes 14 and 15 versus lanes 11 to 13) could result exclusively from their enhancement of DNA binding (Fig. 1B, lanes 5 and 6 versus lanes 2 to 4) (see K_d values above). Alternatively, the Pbx1 HD might contain a subset of residues required for cooperativity, and amino acids 296 to 313 might contribute the remaining residues that either bind the Hox protein directly or induce a configuration upon the Pbx HD that permits it to bind the Hox protein with optimal affinity.

Two approaches were utilized to test the possibility that the Pbx1 HD itself mediates cooperative interactions with Hox proteins via their pentapeptide motif. First, a series of chimeric proteins were prepared by fusing the pentapeptide motif from HoxA5 to the N terminus of the Pbx1 HD (HPC [Fig. 3A]) and were tested for their monomeric and cooperative DNA-binding properties on the PRS element. One would predict that chimeric proteins containing Pbx1 sequences sufficient for cooperative DNA binding would be able to bind cooperatively as a homodimer on a favorable DNA site. Because the PRS was isolated by affinity to Pbx1 (35) and because it binds dimers of Pbx1 and Hox proteins, we reasoned that the PRS might also bind homodimers of a chimeric Pbx1 protein containing the Hox pentapeptide. Addition of the HoxA5 pentapeptide sequence to the N termini of a number of Pbx1 HD constructs, formerly shown to bind DNA as monomers, resulted in the formation of complexes larger than those observed for monomeric DNA binding (Fig. 3B, lane 1 versus lanes 2 to 5), implicating homodimer formation on the PRS. Mixing experiments confirmed that this complex was a dimer because addition of HPC313 plus HPC347 (lane 6), HPC304 plus HPC347 (lane 7), or HPC295 plus HPC347 (lane 8) each resulted in formation of a third complex of intermediate mobility. For each HPC protein in lanes 2 to 4, the homodimer bound DNA much more strongly than the monomer, whose binding to the probe was evident only for HPC313 (lanes 4 and 6). Similarly to their role in Hox-Pbx1 heterodimers, addition of residues 296 to 313 strongly enhanced the stability of HPC dimers bound to DNA (lane 2 versus lane 4). The fact that
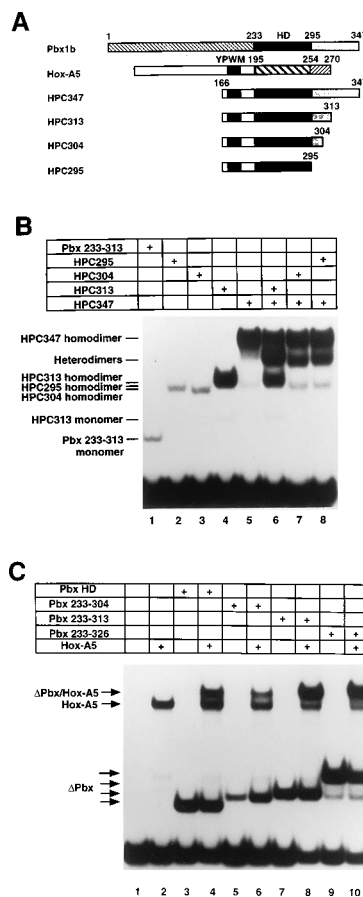

FIG. 3. Fusion of the Hox pentapeptide to Pbx1 induces homodimer formation on the PRS. (A) Schematic diagram of the HPC proteins. (B) Monomeric and cooperative binding by HPC proteins. A 2-μl (lanes 1, 4, and 5) or 4-μl (lanes 2 and 3) volume of each in vitro-translated protein was incubated with the BS2 probe. In lanes 2 and 3, 4 μl of translated protein was used to better visualize the homodimer complex. Mixtures of 2 μl of each translation product are analyzed in lanes 6 to 8. The identities of recombinant proteins added to binding reaction mixtures are indicated at the top of the panel. (C) Cooperative DNA binding by bacterially synthesized Pbx1 and HoxA5 polypeptides. A 1 nM concentration of Pbx HD or Pbx residues 233 to 304 or 0.25 nM Pbx residues 233 to 313 or 233 to 326 was incubated in the absence (lanes 3, 5, 7, and 9) or presence (lanes 4, 6, 8, and 10) of HoxA5 protein.

HPC295 bound the PRS cooperatively as a homodimer argues that the HD of Pbx1 contains most or all of the structural information required for cooperative interaction with Hox proteins via the pentapeptide motif. This assay also demonstrated that inclusion of Pbx1 residues 296 to 313 stabilizes the dimer but did not distinguish whether stabilization was due exclusively to protein-protein interactions between Pbx1 and HoxA5 in the trimeric complex, or whether DNA binding by either HoxA5 or Pbx1 might also be increased as a consequence of complex formation.

The second method used to test the ability of the Pbx1 HD to bind the PRS cooperatively with HoxA5 was direct analysis with recombinant full-length HoxA5 and the Pbx1 HD, synthesized in bacteria. EMSA were performed in the absence of poly(dI:dC). Concentrations of purified Pbx1 proteins were adjusted to yield approximately the same amounts of monomeric complex, and the abundance of the cooperative complex with HoxA5 was examined (Fig. 3C). None of the Pbx proteins bound the PRS as a dimer (lanes 3, 5, 7, and 9). All Pbx1 fragments, including the HD, formed a complex with HoxA5.
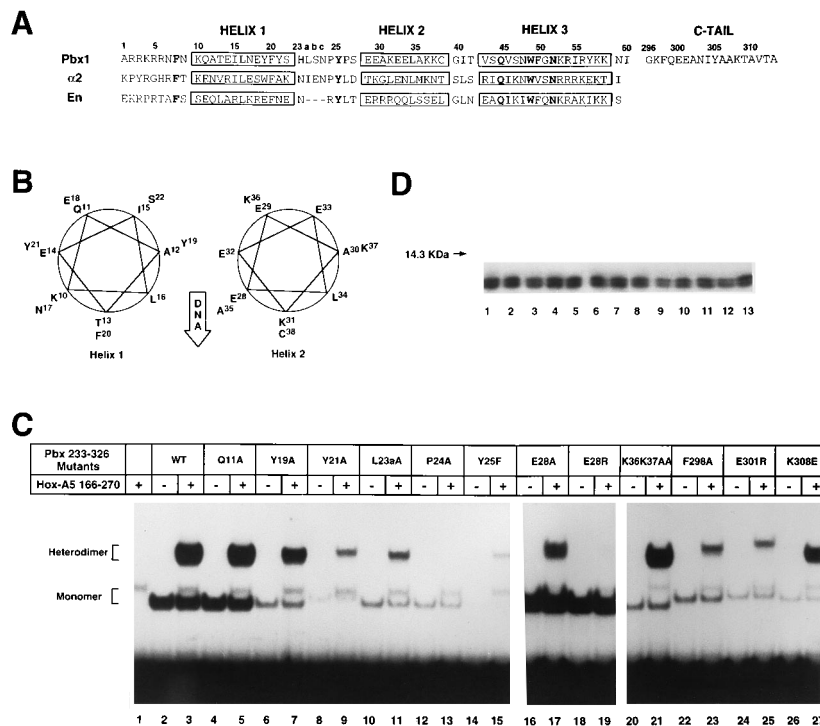
FIG. 4. Mutations in Pbx residues 233 to 326 differentially alter monomeric DNA binding and cooperative DNA binding with HoxA5. (A) Sequence alignment of the HDs of Pbx1, α2, and engrailed. The numbering of the Pbx1 HD was derived by comparison to that given by Wolberger et al. (61), and the numbering of the Pbx1 C-terminal sequence is from the numerical designations within Pbx1. (B) Helical wheel predictions of helices 1 and 2 of the Pbx1 HD. Residues predicted to face DNA are indicated. (C) A 2-μl volume of in vitro-translated mutants of Pbx residues 233 to 326 was incubated with probe BS2 in the presence or absence of 2 μl of in vitro-translated HoxA5 residues 166 to 270. Additions to binding reaction mixtures are indicated above the panel. WT, wild type. (D) Levels of expression of individual Pbx residue 233 to 326 proteins, analyzed on a sodium dodecyl sulfate-17.5% polyacrylamide gel. The position of the 14.3-kDa molecular mass marker is indicated at the left.

For Pbx HD or Pbx residues 233 to 304 (lanes 3 to 6), the abundance of the trimeric ΔPbx1-HoxA5-DNA was more than would be statistically expected on the basis of the abundance of the monomeric complexes, suggesting that weak protein-protein interactions were occurring between these ΔPbx and HoxA5 proteins. For instance, approximately 1/5 of the probe in lane 4 is bound to Pbx1 and 1/5 is bound to HoxA5. If no protein-protein interactions were operative, 1/25 of the probe should be represented in the trimeric complex, but instead, 1/5 is found in the trimeric complex. Addition of residues 304 to 313 or 304 to 326 produced significantly more of the heterodimer complex (lanes 4, 6, 8, and 10). Thus, while the HD of Pbx1 contains structural motifs that cooperate with HoxA5 to increase DNA binding, C-terminal residues 296 to 313 strongly enhance this effect.

DNA binding and cooperativity are separable functions in both the Pbx1 HD and in residues 296 to 313. To identify Pbx1 residues that contributed to either DNA binding or cooperative interactions with HoxA5, point mutations were introduced into Pbx1 residues 233 to 326, and their effects on DNA binding and cooperative binding with HoxA5 residues 166 to 270 were examined. A comparison of the amino acid sequence of the Pbx1 HD with that of yeast α2 and *Drosophila* engrailed (en) is depicted in Fig. 4A (numbering as in reference 61). On the basis of the conserved structural elements of the α2 and engrailed HDs bound to DNA (28, 61), Pbx1 HD side chains most likely to mediate protein-protein interaction were predicted to reside on the exposed faces of helices 1 and 2 (Fig. 4B) and residues contained in the loop between these helices (Fig. 4A). Therefore, these residues, as well as C-terminal

residues 296 to 313, were targeted for systematic site-directed mutagenesis. Comparable levels of mutant proteins were assayed (Fig. 4D). The results are summarized in Table 1, and representative mutants are analyzed by EMSA in Fig. 4C. The ratio of the abundance of the Pbx residues 233 to 326-HoxA5 complex to that containing Pbx residues 233 to 326 alone served as a measurement of the relative impact of a mutation on cooperativity. Monomeric DNA binding by the Pbx1 HD was reduced by many of the point mutations (Table 1; Fig. 4C). A comparison of mutants Y19A, L23aA, and P24A revealed that all substantially reduced DNA binding (Fig. 4C, lanes 6, 10, and 12); however, while P24A virtually eliminated cooperative DNA binding with HoxA5 (lane 13), L23aA retained significant cooperative DNA binding with HoxA5 (lane 11) and Y19A retained a majority of cooperative DNA binding with HoxA5 (lane 7). This suggests that DNA-binding mutations have different effects on the surface of the Pbx1 HD that mediates cooperative interactions with HoxA5. Mutant E28A suppressed cooperativity with HoxA5 by fivefold (lane 17), and mutant E28R abolished cooperativity without reducing monomeric DNA binding (lane 19). Double mutation of E32 and E33 to alanine (E32E33AA) also reduced cooperative DNA to binding by fivefold without altering monomeric DNA binding (Table 1). Thus, mutations along this face of helix 2 altered cooperative interactions with HoxA5 without impacting DNA binding substantially. This analysis suggested that the HD mediates two separable functions: DNA binding and cooperative interactions with HoxA5.

Three mutations in residues 296 to 313 reduced monomeric DNA binding (Table 1); however, while F298A (Fig. 4C, lane

TABLE 1. Analysis of mutations on Pbx1 residues 233 to 326[a]

Location	Mutant	Monomeric DNA binding	Cooperative DNA binding
Helix 1	Q11A	+ + + +	+ + + +
	E14A	+ + +	+ + +
	I15A	+ + + +	+ + + +
	E18A	+ + + +	+ + + +
	Y19A	+ +	+ + +
	Y21A	+	+
	S22A	+ + +	+ + + +
Loop 1-2	H23L23aAA	−	−
	H23A	+ +	+ + +
	L23aA	+ +	+ +
	S23bA	+ + + +	+ + + +
	N23cA	+ + + +	+ + +
	P24A	+	−
	Y25F	+/−	+
	P26A	+	+
	S27A	+ + +	+ + +
Helix 2	E28A	+ + + + +	+ + +
	E28R	+ + + + +	−
	E29A	+ + + +	+ + + +
	E32E33AA	+ + + +	+ + +
	E32K	−	−
	E33K	+ + +	+ + +
	K36K37AA	+ +	+ + + +
Turn 2-3	G39T41AA	+ + +	+ + +
	I40A	+/−	+
C-terminal tail	F298A	+ +	+ +
	Q299A	+ + + +	+ + + +
	E300R	+ + + +	+ + +
	E301R	+	+
	N303A	+ + + +	+ + + +
	I304S	+ + + +	+ + + +
	Y305F	+ + + +	+ + + +
	K308E	+	+ + +
	T309A	+ + + +	+ + + +
	V311A	+ + + +	+ + + +

[a] Summary of mutagenesis analysis of Pbx residues 233 to 326. + + + +, basal level of both monomeric and cooperative DNA binding by the wild-type Pbx residues 233 to 326; + + +, + +, or +, decrease in either DNA binding or cooperativity by approximately 4- to 6-fold, 8- to 10-fold, or greater than 10-fold, respectively. Amino acid residues on the HD were numbered according to the system used by Wolberger et al. (61). Those on the C-terminal tail were numbered according to their positions in Pbx1.

FIG. 5. Differential effects of PEP-A5[WT] on mutant Pbx residue 233 to 326 proteins. A 4-μl volume of each in vitro-translated Pbx residue 233 to 326 protein was incubated with probe BS2 in the absence (lanes 1, 3, 5, 7, 9, 11, and 13) or presence (lanes 2, 4, 6, 8, 10, 12, and 14) of 2 mM PEP-A5[WT]. The Pbx-DNA complex was resolved by EMSA. Free probe is located at the bottom of the autoradiogram, monomeric binding is indicated by the major gel shift complex in most lanes, and apparent dimeric binding is observed in lanes 2 and 14.

23) and E301A (lane 25) also reduced cooperative DNA binding dramatically, K308E (lane 27) did not. Thus, K308 is likely to contribute a critical function in enhancing DNA binding. The ability of this mutant to retain significant cooperativity with HoxA5 also suggests that residues 296 to 313 contribute an independent function to cooperative interactions with Hox proteins that is distinct from their ability to enhance DNA binding.

One interpretation of the Pbx1 HD mutational analysis is that the loop between helices 1 and 2, as well as the N-terminal portion of helix 2, provides an interaction surface with Hox proteins (Fig. 2C) (Table 1). If residues within this region interacted directly with the Hox pentapeptide, then mutations at these locations should reduce the enhancement of DNA binding by the mutant protein in response to PEP-A5[WT]. The effect of PEP-A5[WT] on monomeric DNA binding of all mutant Pbx1 residue 233 to 326 proteins was thus examined. Mutations that severely impaired stimulation by PEP-A5[WT] were located on the loop between helices 1 and 2, including L23a/A, P24/A, and Y25/F (Fig. 5 and data not shown). In fact, the relative abilities of Y19A, L23aA, and P24A to respond to PEP-A5[WT] (Fig. 5, lanes 2 to 8) paralleled their relative abilities to form a
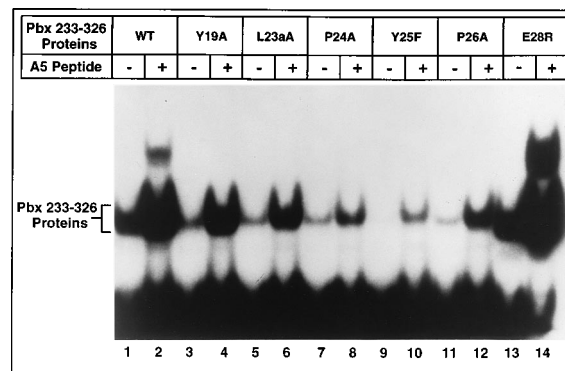
cooperative complex with HoxA5 (Fig. 4C, lanes 6, 7, 10 to 13), suggesting that these mutations directly alter the conformation of the surface that interacts with the pentapeptide. In contrast, DNA binding by the E28R mutation, which abolished cooperative binding without impairing monomeric DNA binding (Fig. 4C, lane 19), was efficiently stimulated by PEP-A5[WT] (Fig. 5, lanes 13 and 14). These results suggested that the loop between helices 1 and 2 may interact with the Hox pentapeptide, while surfaces of helix 2 may interact with other portions of the minimal Hox cooperativity domain, possibly only upon a conformational change accompanying complex formation. In support of this interpretation, direct interaction analysis with the yeast two-hybrid system demonstrated that mutations in the loop between helix 1 and helix 2 (L23aA, P24A, and Y25F) destroyed interaction with HoxA5, while mutation E28A in helix 2, which suppressed formation of the cooperative binding complex by fivefold, retained full interaction with HoxA5 (data not shown).

DISCUSSION

Cooperative DNA binding with Pbx1 provides a molecular mechanism that targets a subset of HD proteins to specific PRS-like DNA elements. To lay a foundation for understanding how cooperative interactions contribute to specific DNA binding by heterodimers of Pbx and pentapeptide-containing Hox proteins, we have examined the structural determinants of Pbx1 required for simple monomeric and cooperative DNA binding, using a canonical PRS element and a representative Hox protein, HoxA5. The conclusion that the HD of Pbx1 is sufficient for cooperative DNA binding is supported by two observations. First, engraftment of the pentapeptide-containing sequence of HoxA5 onto the HD of Pbx1 produces a chimeric protein that binds the PRS as a homodimer but fails to bind as a monomer (Fig. 3B, lane 2), indicating that the Pbx1 HD contains sufficient information for pentapeptide-mediated cooperative DNA binding. This results also indicates that in addition to the Hox HD, the pentapeptide motif is sufficient and may represent the sole determinant for mediating cooperative DNA binding with Pbx1. Second, a synthetic peptide containing the HoxA5 pentapeptide motif specifically enhances monomeric DNA binding by the Pbx1 HD (Fig. 2C), arguing strongly that the HD alone of Pbx1 contains a prom-

inent surface for direct protein-protein interaction with the pentapeptide motif.

Pbx1 residues 296 to 313 exhibit two functions: they increase the affinity of the HD for DNA (Fig. 1B; see Results), and they enhance maximal cooperative DNA binding with Hox proteins (Fig. 1B and 3C). The ability of residues 296 to 313 to increase the affinity of the monomeric Pbx1 HD for DNA was apparent in the presence of dI-dC (Fig. 1B) and was directly measured in the absence of dI-dC (see Results). Mutational analysis also suggested that residues 296 to 313 were directly responsible for enhancement of DNA binding, which was evidence by the fact that Pbx1 residue 233 to 326 mutants F298A, E301R, and K308E exhibited a substantial reduction in monomeric DNA binding at comparable protein levels (Fig. 4C). This sequence could enhance DNA binding through direct contact with DNA or by altering the conformation of the adjacent HD, causing it to adopt a conformation that binds DNA more tightly, or by both mechanisms. Residues 296 to 313 were concluded to enhance cooperativity on the basis of two lines of evidence. First, their addition to the recombinant Pbx1 HD fragment potentiated formation of the trimeric Pbx1-HoxA5-DNA complex in an experiment in which the concentrations of both the Pbx HD and Pbx residues 233 to 326 proteins were adjusted to yield equivalent monomeric DNA binding (Fig. 3C). Second, if residues 296 to 313 enhanced DNA binding exclusively without contributing to cooperativity, one would expect that loss of DNA binding would be paralleled by a commensurate reduction in the formation of the Pbx1-HoxA5-DNA complex. However, the facts that K308E and E301R are both severely compromised in monomeric DNA binding but that K308E retains a high level of cooperative DNA binding while E301R exhibits almost none argue that K308E alters the DNA-binding surface without altering the cooperative interaction surface, while E301R alters both surfaces. At this time, we cannot distinguish between the possibilities that residues 296 to 313 contribute directly to cooperativity by providing a protein-protein interaction surface or that they alter the conformation of the adjacent HD, maximizing its interaction with the Hox protein. In a somewhat similar case, fusion of the C-terminal tail of yeast α2 to a1 also enhanced the DNA binding by a1 HD, suggesting that in the a1-α2 complex, binding of the C-terminal tail of α2 to a1 enhances DNA binding by a1 (51). Unlike the C-terminal tail of α2, which mediates cooperative DNA binding between α2 and a1 (51), the C-terminal conserved sequence of Pbx1 is not absolutely essential for Pbx1/Hox heterodimerization. Residues 296 to 313 are highly conserved among Pbx1, Pbx2, and Pbx3 and are predicted to form an α-helix. After residue 313, the sequences of Pbx1, Pbx2, and Pbx3 diverge substantially. Thus, residues 296 to 313 would be proposed to exert similar functions in all Pbx proteins, as well as in the oncoprotein E2A-Pbx1.

Point mutational analysis of Pbx 233-326 also identified residues in the exposed surfaces of helices 1 and 2 and in the loop between these helices that may be involved in cooperative interactions with HoxA5 (Fig. 1B). The DNA-binding activity of the Pbx1 HD was influenced by mutations on multiple surfaces (Table 1), demonstrating that the proper folding of the HD is essential for optimal DNA binding. Surfaces of Pbx1 that contributed most strongly to cooperative interactions with HoxA5, as judged by comparing the degree of loss of monomeric DNA binding with the loss of cooperativity, included the loop between helices 1 and 2 of the HD, amino acid residues on helix 2 (E28 and E32E33), and residues on the C-terminal extension (F298 and E301). We propose that the Hox pentapeptide either is cradled between helices 1 and 2 of the Pbx1 HD or binds directly to the loop between helices 1 and 2
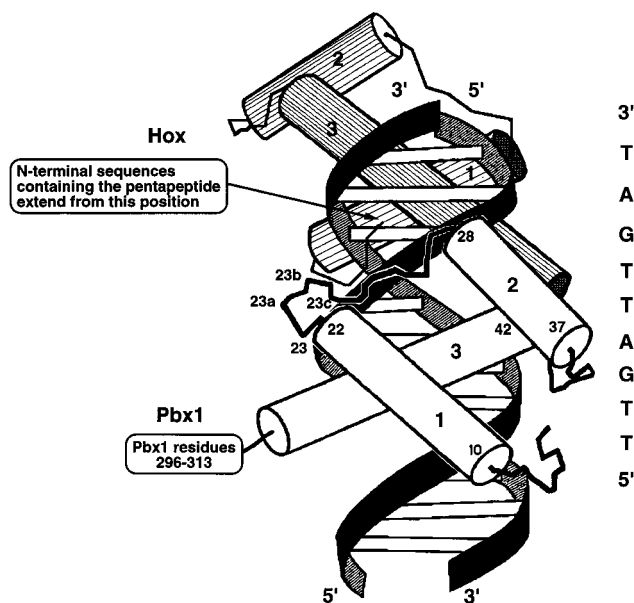

FIG. 6. A model for the binding of Pbx1 and Hox proteins on the PRS. The HD of Pbx1 is aligned on the PRS in tandem with that of the Hox protein, which is positioned 5′ to Pbx1 on the sequence 5′-TTGATTGAT-3′. The positioning of Pbx1 is by analogy with the crystal structure of yeast α2 (61).

because point mutants in this loop induced the largest reduction in both the ability of the Pbx1 HD to exhibit enhanced DNA binding by the synthetic pentapeptide and its ability to bind HoxA5 in a yeast two-hybrid interaction assay. Because mutations in this loop also strongly reduce monomeric DNA binding and thus could alter the positioning of helices 1 and 2, failure to bind cooperatively with HoxA5 could arise from failure of the pentapeptide either to bind the loop or to bind a constellation of residues in helices 1 and 2.

The mechanism by which E28 contributes to cooperativity is unclear. E28 is predicted to face the DNA. E28R exhibited a stronger DNA-binding activity than that of the wild-type protein, while cooperativity with HoxA5 was abolished, and DNA-binding by E28R was also stimulated by PEP-A5^{WT} to a degree similar to that of wild-type Pbx1 residues 233 to 313. This suggests that interaction of the Pbx1 HD with the pentapeptide motif is not appreciably altered by this mutation and thus that interaction with the pentapeptide alone may be insufficient for cooperative DNA binding. The importance of residue 28 of the HD of the *Drosophila* Paired protein was recently demonstrated in a study of the crystal structure of cooperative Paired homodimers (59). The crystal structure revealed that Ile-28 is located on the dimer interface and that replacement of Ile with Arg impairs the cooperativity. Although monomers constituting the Paired homodimer bind DNA in opposing orientations, it is possible that E28 of the Pbx1 HD is also positioned on the dimer interface and binds a residue(s) of the Hox HD (Fig. 6), contributing to cooperative interactions.

The crystal structure of Paired homodimers also revealed that a conformational change in the DNA occurred in the complex (59). A similar conformational change was also observed upon binding of a single engrailed HD to DNA (28). Therefore, it was suggested that the binding of one Paired HD may induce a conformational change in the DNA that facilitates the binding of the second HD (59). A similar scenario may also occur in cooperative DNA binding by Pbx1 and Hox proteins. Because Hox proteins bind the PRS more tightly than

does Pbx1, the initial binding of a Hox protein may induce a conformational change in DNA and/or the Hox HD that creates a favorable environment for binding of the Pbx1 HD to DNA, for the interaction of the Pbx1 HD with the Hox pentapeptide, and for interaction of E28 of Pbx1 with Hox residues or with DNA.

Finally, results from the analysis of homodimerization of HPC proteins on the PRS (Fig. 3B) suggest that the HDs of Pbx1 and Hox proteins are likely positioned on the PRS in tandem. The PRS (TTGATTGAT) consists of direct repeats of TTGAT or TGAT. Although the HD of Pbx1 exhibits the highest affinity for the PRS (5 nM), it also exhibits a strong affinity for ATCAA (14 nM, [35]), and the binding of the Pbx1 HD to CGAAAGGCATTGATGCGCC is also stimulated specifically by PEP-A5^{WT} (data not shown). The ability of pentapeptide-Pbx1 fusion proteins to effectively dimerize on the PRS element suggests that this element was first selected as a dimeric Pbx1 binding site rather than a single Pbx1 recognition sequence. The yeast a1 HD exhibits remarkable sequence identity in helices 2 and 3 with homologous positions in Pbx1 (17 of 23 residues [26]) and also binds a DNA sequence containing TGAT (16). These observation indicate that TGAT is the core Pbx1 recognition sequence and that HPC proteins bind in tandem to TTGATTGAT. An important observation raised by homodimer formation by HPC proteins on the PRS is that the Pbx1 HD must contain functional correlates of Hox HD residues that are normally involved in cooperative interactions with Pbx1 in the Hox-Pbx1 heterodimer complex. The interchangeability of the HDs of Pbx1 and HoxA5 in formation of the cooperative dimeric complex suggests that the Hox HD also binds in tandem with Pbx1 in the Hox-Pbx1-DNA complex.

On the basis of structural studies of the DNA-HD complex (28, 46, 61) and our results, we propose a model for the cooperative binding of Pbx1 and pentapeptide-containing Hox proteins on DNA (Fig. 6). The model suggests that Pbx1 and the Hox protein each bind a TGAT half-site in tandem, in a specific orientation, and with the Hox protein binding 3′ to Pbx1. The positioning of the Hox protein 3′ to Pbx1 is based on DNase I protection analysis (34). This orientation is also consistent with mutational analysis of the PRS, in which conversion of the 3′ G to A (TTGATT$\underline{A}$AT) did not alter the abundance of the Pbx1-HoxA5-DNA complex, while conversion of the 5′ G to A (TT$\underline{A}$ATTGAT) strongly suppressed cooperativity. The orientations of the HDs of HoxA5 and Pbx1 on their core sequences are based on analysis of the interaction of the yeast a1 and α2 HD proteins with DNA and on the crystal structures of both the engrailed-DNA complex (28) and the α2-DNA complex (61). In the complex of a1/α2 bound to the hsg operator, a1 is predicted to bind its DNA motif in the same orientation as that identified for α2 (61), which is known, on the basis of its crystal structure (16). Because the HDs of a1 and Pbx1 both contain the TGAT core, we predict that the Pbx1 HD aligns on DNA similar to that of a1, binding in a conformation that positions the loop between helices 1 and 2 toward the upstream Hox protein, and the N-terminal arm, which contacts the minor grove, toward downstream sequences. The Hox protein is predicted to bind the TGAT core sequence in the same manner in which engrailed binds its TAAT core sequence. This would position the turn between helices 1 and 2 in the 3′ direction and the N-terminal arm 5′ to the core sequence. This alignment produces the same tandem arrangement of the Hox and Pbx1 HDs predicted from HPC protein analysis. In this conformation, Asn-51 of the Hox HD would contact A in the TGAT sequence (61). Hox residues N terminal to the HD, including the pentapeptide, would have

the ability to interact with residues on the exposed surfaces of helices 1 and 2 of the Pbx1 HD, as well as with those constituting the loop between these helices. The recognition helix of the Hox HD lies beneath the surface of helix 2 of the Pbx1 HD, and both E28 and E32 project in this direction. C-terminal residues 296 to 313 of Pbx1 would be free to interact with the Pbx1 HD, with DNA, or with the adjacent Hox HD. Residues in the N-terminal arm of Hox helix 1 could interact with residues in helix 1 or helix 2 of Pbx1 or in the loop that joins these helices. The recognition helix of the Hox protein would be permitted to dictate further DNA sequence specificity in the 3′ direction, and specific nucleotides up to 4 bp from the TAAT core strongly effect binding affinity and differential DNA binding by pentapeptide-containing HD proteins (10). Interestingly, both of the sites in the mouse Hox B1 promoter that bind Hox B1-Exd heterodimers contain GG 3′ to the proposed Hox core binding site, and GG is a strongly favored sequence in Hox protein binding sites just 3′ to their TAAT core. In addition, the AT dinucleotide 5′ to the Hox core TAAT motif, which forms the predicted 3′ dinucleotide of the Pbx1 core motif (TGAT), permits strong overall DNA binding in HoxA5, -B4, -A7, -C8, and -B1 (44). The affinity for these nucleotides is dictated by contacts between the first 12 residues of the HD with the minor grove of DNA and varies among pentapeptide-containing members of the HOM-C (10). Thus, Pbx1 and Hox proteins may bind overlapping half-sites, and further specificity of discriminating among Hox partners may be dictated by the specificity of residues 3′ of the Hox core sequence as well as the affinity of the Hox protein for the AT sequence 5′ to its core sequence. A definitive identification of Pbx1 residues that bind Hox protein residues and therein mediate cooperative binding awaits the crystal structure of a Pbx1-Hox-DNA complex.

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