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Pbx Modulation of Hox Homeodomain Amino-Terminal Arms Establishes Different DNA-Binding Specificities across the *Hox* Locus

CHING-PIN CHANG,[1] LUCIANO BROCCHIERI,[2] WEI-FANG SHEN,[3] COREY LARGMAN,[3] AND MICHAEL L. CLEARY[1]*

Laboratory of Experimental Oncology, Department of Pathology,[1] and Department of Mathematics,[2] Stanford University, Stanford, California 94305, and Department of Medicine, San Francisco VA Medical Center and University of California, San Francisco, California 94121[3]

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Pbx cofactors are implicated to play important roles in modulating the DNA-binding properties of heterologous homeodomain proteins, including class I Hox proteins. To assess how Pbx proteins influence Hox DNA-binding specificity, we used a binding-site selection approach to determine high-affinity target sites recognized by various Pbx-Hox homeoprotein complexes. Pbx-Hox heterodimers preferred to bind a bipartite sequence 5′-ATGATTNATNN-3′ consisting of two adjacent half sites in which the Pbx component of the heterodimer contacted the 5′ half (ATGAT) and the Hox component contacted the more variable 3′ half (TNATNN). Binding sites matching the consensus were also obtained for Pbx1 complexed with HoxA10, which lacks a hexapeptide but requires a conserved tryptophan-containing motif for cooperativity with Pbx. Interactions with Pbx were found to play an essential role in modulating Hox homeodomain amino-terminal arm contact with DNA in the core of the Hox half site such that heterodimers of different compositions could distinguish single nucleotide alterations in the Hox half site both in vitro and in cellular assays measuring transactivation. When complexed with Pbx, Hox proteins B1 through B9 and A10 showed stepwise differences in their preferences for nucleotides in the Hox half site core (TTAT to TGAT, 5′ to 3′) that correlated with the locations of their respective genes in the *Hox* cluster. These observations demonstrate previously undetected DNA-binding specificity for the amino-terminal arm of the Hox homeodomain and suggest that different binding activities of Pbx-Hox complexes are at least part of the position-specific activities of the *Hox* genes.

Many of the transcriptional regulatory proteins that function during development have similar types of a DNA-binding motif known as the homeodomain. Some of the most thoroughly studied homeodomain proteins are the Hox proteins of the fruit fly *Drosophila melanogaster*, which have been shown to play critical roles in regulating regional specification along the anterior-posterior axis of the body plan (see reference 49 for a review). In vertebrates, Hox proteins have many features in common with their *Drosophila* counterparts, including primary sequence, chromosomal organization of their respective genes, and regionally and developmentally restricted expression profiles (2, 49). Vertebrate Hox proteins also appear to specify identity along the anterior-posterior axis, as demonstrated by homeotic transformations in mice with targeted disruptions of *Hox* genes (36). Hox homeoproteins may also play important lineage-specific functions in a variety of somatic tissues, including the hematopoietic system (39, 72). The diverse in vivo functions attributed to Hox proteins are thought to reflect their selective transcriptional properties on appropriate target genes. In support of this model, elegant studies with flies have shown that the developmental specificities of several Hox proteins are mediated by sequences within or directly flanking their DNA-binding homeodomains (10, 20, 37, 43, 48, 86).

In spite of the important functional roles attributed to the homeodomain, the diverse developmental specificities of Hox proteins are not accounted for by their in vitro DNA-binding specificities as monomeric proteins. Although modest differences in DNA-binding specificity in vitro have in some cases been observed to correlate with selective functions in vivo (15), Hox proteins with disparate effects on regional identity bind DNA in vitro with disappointingly similar specificities (9, 13, 14, 25, 38, 59). These and other observations strongly suggest that combinatorial interactions with heterologous cofactors are required for functional specificity (23). Such cooperative actions are well established for homeodomain proteins of the POU class (27, 50, 69, 74, 79, 80, 84) and for yeast homeodomain proteins **a**1 and α2, whose combinatorial interactions modulate their recognition of specific operators in genes regulating mating types (30). Although homo- and heterotypic interactions influence the transcriptional properties of homeodomain proteins (3, 21, 85), the mechanisms that increase their specificity for target sites remain to be elucidated.

Recent studies have shown that the DNA-binding and developmental properties of Hox proteins can be modulated by interactions with a distinctive subset of non-Hox homeodomain proteins. The latter include *Drosophila* extradenticle (exd), identified genetically to act in parallel with Hox proteins in the determination of segmental morphologies (58, 65), and its mammalian homologs, the Pbx proteins (51), first discovered at the sites of chromosomal translocations in a subset of human leukemias (32, 55). In vitro, exd cooperatively binds DNA with Ubx and abd-A (11, 77) and in flies displays an indispensable role in the coordinate regulation of downstream genes with these Hox proteins (47, 67, 75). Similarly, the mammalian Pbx proteins cooperatively bind synthetic DNA probes in vitro with Hox proteins from several different paralog groups (12, 44). Cooperative interactions between Hox and Pbx/exd proteins appear to be mediated at least in part by the highly conserved YPWM motif upstream of the Hox home-

* Corresponding author. Phone: (415) 723-5471. Fax: (415) 498-6222.

odomains (12, 31, 35, 54, 61). But not all Hox proteins with a YPWM motif bind cooperatively to the probes used, and many of those that do bind form highly unstable complexes (12, 73). These observations suggest that different Hox-containing complexes may have distinct sequence specificities, a possibility consistent with the ability of exd to modulate the binding of engrailed to a DNA site recognized poorly by Ubx (77). However, it was not clear from these studies what role, if any, exd/Pbx-like cofactors may play in target site selectivity by Hox proteins, which have extensive similarities in their DNA-binding homeodomains, particularly since ablation of the putative Hox binding site (TAAT) in several synthetic targets did not appear to prevent cooperative DNA binding in the context of a canonical Pbx/exd site (12, 44, 77).

We report here that interactions with Pbx cofactors facilitate the discriminative binding of Hox proteins to bipartite DNA sites consisting of a conserved Pbx half site directly adjacent to specific Hox half sites. Pbx modulates the ability of Hox homeodomain N-terminal arms to recognize DNA in the core of the Hox half site. This in turn induces a transition in the DNA-binding specificities of heterodimeric Pbx-Hox complexes that varies in a stepwise gradient across the *HoxB* cluster. These observations demonstrate previously undetected specificity for the homeodomain N-terminal arm and provide a molecular framework for understanding the functional contributions of this variable region of the homeodomain.

MATERIALS AND METHODS

Plasmid constructions. In vitro expression clones for wild-type and chimeric Pbx proteins under control of the SP6 promoter have been described in previous studies (12, 51, 55). In vitro expression clones for FLAG-tagged HoxB2, -B3, -B5, -B6, -B7, and -B8 have been reported earlier (12, 73). FLAG-tagged forms of HoxB1 and HoxB4 were constructed by substituting MDYKDDDKSS in place of the first 2 and 13 amino-terminal amino acids, respectively, using full-length human cDNAs reported previously (1, 60). A FLAG-tagged version of HoxA10 contained an additional 21 amino acids (MDYKDDDKSREFPFAKLSHNV) at its amino terminus. HoxB9 (46) containing a six-His tag in place of its first 12 amino acids was expressed from the T7 promoter in pET28a. Hox domain swap mutants were constructed by using PCR and standard cloning techniques. The hexapeptide, linker, and N-terminal arm of a given Hox homeodomain were fused to helices 1, 2, and 3 of a heterologous homeodomain by means of synthetic *Eco*RI sites which were engineered into helix 1 of HoxB1, -B7, and -A10 without altering the amino acid sequence of the respective proteins. Fusion of hexapeptide/linker elements onto heterologous homeodomains was accomplished by the use of synthetic linkers and PCR.

EMSA and binding site selections. Proteins for DNA binding assays and electrophoretic mobility shift assay (EMSA) were produced in vitro from SP6 expression plasmids by use of a coupled reticulocyte lysate system as described previously (12). DNA-binding reactions were performed at 4°C for 30 min, using conditions reported earlier (12), and subjected to EMSA (29) using 6% polyacrylamide gels (0.75-mm thickness) in 0.25× Tris-borate-EDTA buffer. DNA probes (50,000 cpm per binding reaction) consisted of gel-purified, end-labeled, double-stranded oligonucleotides that had the same backbone (5′-CTGC GX_{11}CCGC-3′, where X_{11} represents the binding site of interest). Sequences of binding sites are indicated in the text, tables, and figure legends.

A modification of the selective amplification and binding (SAAB) assay (7) was used to determine consensus binding sites for various Pbx-Hox heterodimers. A single-stranded oligonucleotide containing 30 internal degenerate positions (5′-GAGGATCCAGTCAGCATGN_{30}CTCAGCCTCGAGATCTCG-3′) 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 in vitro-translated Pbx and FLAG-tagged Hox proteins [10 μl of each translate in 100 μl of total volume containing 14 μg of poly(dI-dC), 75 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris-HCl (pH 7.5), 6% glycerol, 1% bovine serum albumin (BSA), and 1% Nonidet P-40]. Protein-DNA complexes were precipitated with an anti-FLAG monoclonal antibody (1 μg/100 μl) and protein G-Sepharose beads. Pellets were washed eight times with washing buffer (75 mM NaCl, 15 mM Tris-HCl [pH 7.5], 0.15% Triton X-100, 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.

Transient transfections and transcriptional assays. For transfection experiments, the cDNAs for various Pbx (40, 52) or Hox proteins were cloned into the mammalian expression vector pCMV1. Reporter constructs contained three copies of a particular Pbx-Hox binding site and associated backbone flanking sequences cloned immediately upstream of a minimal promoter [liver/bone/kidney alkaline phosphatase, pLΔ44cat(X) (33)] and a chloramphenicol acetyltransferase (CAT) reporter gene (26). The human B-precursor acute lymphoblastic leukemia cell line REH was transfected by electroporation with DEAE-dextran as described previously (40). An internal control consisting of a plasmid expressing the luciferase gene under control of the Rous sarcoma virus long terminal repeat was included in each transfection. CAT and luciferase assays were performed as described previously (40) 48 h following transfections. For each experiment, transfections were performed in duplicate on at least three separate occasions with similar results.

Molecular modeling. The homeodomain regions of HoxB7 and Pbx1 were modeled on the basis of the nuclear magnetic resonance structure of the Antennapedia-DNA complex (6, 57). Homology modeling was performed by the segment matching and optimization method (41, 42). The two protein-DNA complexes were assembled on TAATGG sites, which were then replaced with the Pbx-HoxB7 consensus 5′-TGATTTATGG-3′. The hexapeptide and linker of HoxB7 were manually modeled to produce the desired contacts with the carboxy terminus of Pbx, which was modeled on its predicted structure by using the PHD program for secondary structure prediction by neural network (70). The model was finally optimized by using the software program Discover (version 2.9.5; Biosym Technologies, San Diego, Calif.) with the AMBER forcefield (81, 82).

RESULTS

Determination of high-affinity DNA-binding sites for various Pbx-Hox heterodimeric complexes. A modified SAAB procedure was used to determine the compositions of DNA sequences preferentially bound with high affinity by various Pbx-Hox heterodimeric complexes. The experimental approach involved incubation of in vitro-translated Pbx and Hox proteins with an oligonucleotide containing 30 degenerate nucleotides flanked by PCR "handles." DNA-protein complexes were affinity purified by using antibodies directed against an epitope tag expressed at the N terminus of the Hox proteins. This approach ensured that all complexes contained a Hox protein component, thereby excluding the selection of sites bound by Pbx alone. 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.

The consensus DNA-binding sites determined for heterodimeric complexes consisting of Pbx1a and one of several possible Hox proteins (HoxB1, -B4, -B6, -B7, or -A10) are shown in Table 1. For each Pbx-Hox heterodimer, a clear consensus in which the nucleotide composition at each of 11 contiguous positions showed a strong bias for a single nucleotide could be determined. Furthermore, an overall consensus for the heterodimer pairs, consisting of an 11-nucleotide sequence, 5′-ATGATTNATNN-3′, in which nucleotides 1 to 6, 8, and 9 were highly conserved with little variation, could be derived. The three remaining positions, 7, 10, and 11, varied between the individual consensus sequences, although for a given Pbx-Hox pair, specific nucleotides were favored at these three positions, resulting in a distinct consensus for each heterodimer. Variability in the 3′ half of the overall consensus sequence suggested that this portion may be contacted by the Hox component of each heterodimer. The consensus for this half (TNATNN) was very similar to those determined previously (TAATNN) for monomeric Hox proteins (9, 17, 38, 53, 56, 57, 59, 68). Although no Pbx-Hox pair actually preferred TAATNN as a high-affinity site under our selection conditions, most selected a TAAT core at positions 6 to 9 in a minority (5% [HoxB1], 14% [HoxB4], 8% [HoxB6] and 28% [HoxB7]) of the sequences analyzed, which contrasted with the strong preferences observed for TGAT or TTAT cores. All of the

TABLE 1. Consensus DNA-binding sites for Pbx1-Hox heterodimers

Pbx-Hox complex	n^a	Consensus DNA site at position[b]:										
		1	2	3	4	5	6	7	8	9	10	11
GST-Pbx		A/T	T	G	A	T	T	G	A	T		
Pbx1-HoxB1	19	A (89)	T (100)	G (100)	A (100)	T (100)	T (100)	G (95)	A (100)	T (100)	C (47)	G (53)
Pbx1-HoxB4	28	A (89)	T (100)	G (100)	A (100)	T (100)	T (100)	G (86)	A (100)	T (100)	G (59)	A (56)
Pbx1-HoxB6	26	A (69)	T (100)	G (100)	A (100)	T (100)	T (100)	T (92)	A (100)	T (100)	T (69)	A (54)
Pbx1-HoxB7	42	A (54)	T (100)	G (100)	A (100)	T (100)	T (100)	T (57)	A (100)	T (100)	G (66)	G (42)
Pbx1-HoxA10	16	A (100)	T (100)	G (100)	A (100)	T (100)	T (100)	T (100)	A (100)	T (75)	G (93)	A (66)
Overall consensus	131	A	T	G	A	T	T	N	A	T	N	N

[a] n, number of selected sequences that were analyzed to obtain each consensus.
[b] Numbers in parentheses are percentages of sites containing the indicated nucleotides. Positions flanking those shown did not demonstrate an obvious selection bias. The overall consensus resulted from an analysis of 131 sequences from the five different site selections. The GST-Pbx consensus sequence is from reference 40.

consensus sites contained ATGAT at positions 1 to 5, which matches the 5′ half of the consensus site (5′-A/TTGATTGAT-3′) determined previously by site selections employing glutathione S-transferase (GS)–Pbx in the absence of Hox proteins (40). The site selection data suggested that all of the Pbx-Hox pairs preferred to bind a bipartite sequence consisting of two adjacent half sites in which the Pbx component of the heterodimer contacts the 5′ half (ATGAT) and the Hox component contacts the more variable 3′ half (TNATNN).

Pbx-Hox complexes display site-specific preferences in their DNA-binding properties. EMSAs were performed to study the specificity of DNA binding by various Pbx-Hox pairs on the consensus DNA sites. As shown in Fig. 1, different profiles of binding were observed with oligonucleotide probes containing the different consensus sites embedded in a standardized DNA backbone. Under these conditions, Pbx1 itself was incapable of binding to any of the probes in the absence of added Hox proteins (lane 1). Some Hox proteins weakly bound the probes in the absence of Pbx, but the resulting complexes were extremely unstable, with half-lives of less than 1 min (data not shown). With the B1 consensus probe, only Pbx1-HoxB1 showed substantial steady-state binding by a complex containing both Pbx and Hox components (Fig. 1). EMSA with the B4 consensus probe showed robust binding by Pbx1-HoxB1 and Pbx1-HoxB4 complexes, weaker binding by Pbx1-HoxB6 and Pbx1-HoxB7 heterodimers, and minimal binding by Pbx1-HoxA10. EMSA with the B7 consensus probe showed a markedly different profile of steady-state binding, with abundant shifted complexes for heterodimers Pbx1a-HoxB6, Pbx1a-HoxB7, and, unexpectedly, Pbx1a-HoxA10. Our previous studies indicated that HoxA10 was unable to effectively bind a synthetic DNA probe in cooperation with Pbx proteins as a result of the absence of a hexapeptide motif (12). We show below that the observed DNA binding by Pbx1a and HoxA10 is dependent on a different, newly discovered motif in HoxA10. Nevertheless, the potential for Pbx1 and HoxA10 cooperativity permitted the selection of a consensus Pbx1-HoxA10 site which matched the overall consensus described above (Table 1). With the A10 consensus probe, a profile of DNA binding by the various Pbx-Hox heterodimers was obtained which was very similar to that observed with the B6 and B7 consensus sites (Table 2). Taken together, the EMSA data indicated that individual Pbx-Hox heterodimers have specific, high-affinity binding site preferences, although there were various degrees of overlap in the spectrum of sites bound by each heterodimeric pair.

The DNA-binding properties of Pbx-Hox heterodimers were also assessed by kinetic analyses. Pbx1-HoxB1 heterodimers bound the B1 consensus site very stably, with a half-life longer than 30 min (Fig. 2A). This contrasted with Pbx1-HoxB1 binding to a synthetic probe containing nonselected consensus Pbx and Hox motifs, which we showed previously (73) to be extremely unstable, with a half-life of less than 3 min (Fig. 2). Half-lives for other Pbx-Hox complexes on the B1 site could not be determined because of their minimal steady-state binding. Dissociation studies using the B7 site showed that Pbx complexes containing HoxB6, -B7, or -A10 were very stable, whereas those containing HoxB1 and -B4 were less stable (Fig. 2B) but not as unstable as Hox proteins alone (data not shown). The dissociation data provide further support for distinct DNA-binding site preferences by the various Pbx-Hox heterodimers tested.

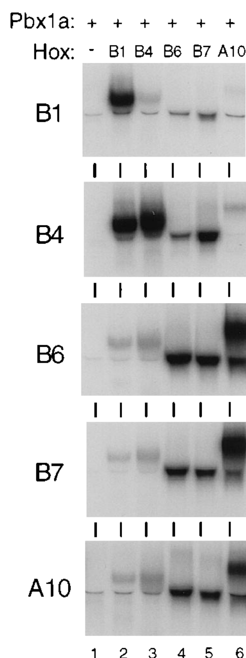

FIG. 1. EMSA of DNA binding by Pbx-Hox heterodimeric complexes on various consensus DNA sites. In vitro-translated proteins (as indicated above the lanes) were incubated in DNA-binding reaction mixtures in the presence of radiolabeled probe and then subjected to EMSA. The identities of consensus binding sites present in the oligonucleotide probes are indicated at the left and correspond to the sequences listed in Table 1. Binding reaction mixtures contained 2 μl of each specifically programmed lysate. HoxB6 and -B7 form complexes with Pbx which migrate at the same position as an endogenous band present in the reticulocyte lysate (lane 1).

TABLE 2. Steady-state DNA-binding results for Pbx-Hox heterodimers on various DNA sites

Binding site	Nucleotide sequence	Steady-state DNA binding[a]				
		B1	B4	B6	B7	A10
B1 consensus	ATGATTGATCG	++++	±	−	±	−
B4 consensus	ATGATTGATGA	+++	+++	++	++	±
B6 consensus	ATGATTTATTA	±	±	+++	+++	+++
B7 consensus	ATGATTTATGG	±	±	+++	+++	++++
A10 consensus	ATGATTTATGA	±	±	++	+++	+++
$B7_{T_7 \rightarrow G_7}$	ATGATTGATGG	++++	+++	++	++	±
Hox_{TAAT}	ATGATTAATGG	±	+++	+++	+++	±
$B7_{T_7 \rightarrow C_7}$	ATGATTCATGG	−	−	−	±	±
$B7_{G_{10} \rightarrow C_{10}}$	ATGATTTATCG	±	−	±	+	+
$B7_{space\ 1}$	ATGATGTTATGG	−	−	−	−	−
$B7_{space\ 2}$	ATGATGCTTATGG	−	−	−	−	−
$B7_{space\ 3}$	ATGATGCGTTATGG	−	−	−	−	−

[a] Steady-state DNA binding for the Hox component of each Pbx1-Hox heterodimer complex is indicated as follows: −, no binding; ±, very weak binding; + to +++, various intermediate levels of binding; ++++, maximal binding.

HoxA10 requires a tryptophan-containing motif to cooperatively bind DNA with Pbx proteins. The ability of HoxA10 to cooperatively bind DNA with Pbx1 was further investigated by site-directed mutagenesis to delineate a potential motif alternative to the hexapeptide that earlier studies showed was essential for HoxB6 and B7 interactions with Pbx proteins (12). A minimal fragment of HoxA10 containing its homeodomain and three upstream amino acids ($A10_{HD}$) was incapable of cooperative interactions with Pbx1a on the B7 site (Fig. 3, lane 4), which is bound avidly by wild-type HoxA10 (Table 2). A construct containing 10 upstream amino acids plus the homeodomain ($A10_{W\ +\ HD}$) displayed strong cooperativity with Pbx1a (Fig. 3, lane 2), suggesting the presence of an essential motif upstream of the HoxA10 homeodomain. Although this portion of HoxA10 does not display homology with the hexapeptide (YPWM) motifs found in Hox proteins of the

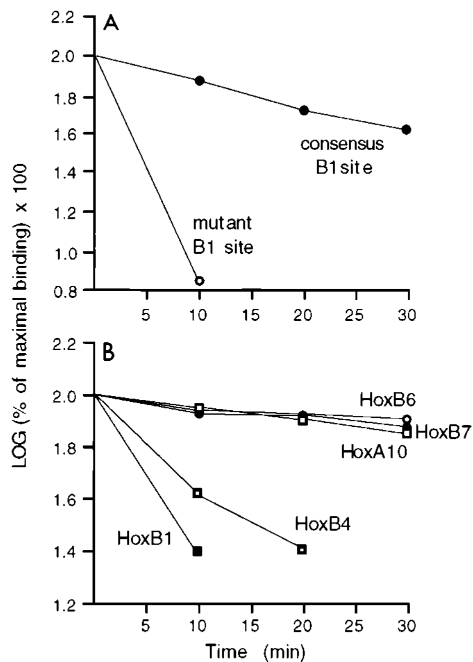

FIG. 2. Kinetic analysis of dissociation rates for various Pbx-Hox heterodimeric complexes on different consensus DNA sites. Preformed Pbx-Hox complexes were either subjected to immediate gel electrophoresis (time zero) or incubated in the presence of a 100-fold excess of unlabeled DNA for the times indicated prior to electrophoresis. Densitometric measurements of each Pbx-Hox complex were used to calculate the percentage of originally bound probe remaining in the complex at different time points. The dissociation of Pbx1a-HoxB1 complexes (A) was assessed on a B1 consensus site (ATGATTGATCG) or a synthetic probe (12) containing three substitutions in the B1 consensus site (TTGATTGATGC). The dissociation of various Pbx-Hox complexes (B) was assessed on an oligonucleotide containing the B7 consensus site (ATGATTTATGG).

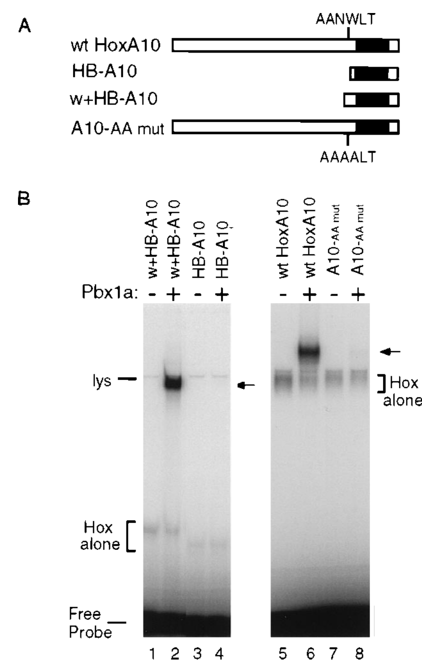

FIG. 3. Cooperative DNA binding of HoxA10 and Pbx1 is dependent on a tryptophan-containing motif upstream of the HoxA10 homeodomain. (A) Schematic representations of wild-type and mutant HoxA10 proteins. The HoxA10 homeodomain is depicted as a solid box. Letters indicate amino acids in the region of the tryptophan-containing motif. HB-A10 contained amino acids 313 to 390 of HoxA10, and w+HB-A10 contained amino acids 305 to 390 of HoxA10. (B) EMSA of in vitro-translated proteins whose identities are indicated above the gel lanes. Pbx1a-HoxA10 cooperative DNA binding was observed only with HoxA10 proteins containing an intact tryptophan-containing motif. The DNA probe contained the B7 consensus site which is bound avidly by wild-type (wt) HoxA10 and differs by one nucleotide from the A10 consensus site (Table 1). Arrows indicate cooperative Pbx-Hox complexes.

paralog groups 1 to 8, three amino acids (ANW) in this region are conserved in the Hox paralog groups 9 and 10 from human, mouse, chicken, and *Xenopus* sequences (8, 28), and a conserved tryptophan adjacent to the homeodomain is found in representatives of paralog group 12. To test the requirement for these amino acids in cooperative binding by HoxA10, they were mutated to alanines by site-directed mutagenesis. As shown in Fig. 3, the mutant HoxA10 construct (A10-$_{\text{AAmut}}$) was incapable of binding the B7 probe with Pbx1a. These data demonstrate that an alternative tryptophan-containing motif accounts for the ability of HoxA10, which lacks a hexapeptide, to cooperatively bind DNA with Pbx proteins. Given that the A10-$_{\text{AA}}$ mutation did not significantly affect binding by Hox alone (Fig. 3, lanes 5, 7, and 8) but did disrupt Pbx-Hox cooperativity almost completely, the tryptophan motif must mediate protein-protein interactions between Hox and Pbx.

Pbx modulates Hox homeodomain N-terminal arm interactions with DNA. The site selection data suggested that three nucleotide positions (7, 10, and 11) in the variable 3′ portion may influence the DNA-binding specificities of Pbx-Hox complexes. Several dinucleotide combinations were tolerated at positions 10 and 11, consistent with previous studies of DNA binding by homeodomain proteins in the absence of cofactors showing that the comparable nucleotide positions for single Hox sites (TAAT<u>NN</u>) modestly influenced binding affinities and specificities (22, 38, 59, 76). The dinucleotide combinations that were selected at positions 10 and 11 were virtually identical to those determined in previous selection experiments using homeodomain fragments of paralogous/orthologous proteins in the absence of cofactors (e.g., GG/TA for B6/B7, orthologs of Antp/Ubx; GA>TG for B4, ortholog of Dfd/HoxA4/Chox-1.4; and GG/GA for A10, ortholog of AbdB/HoxA10) (5, 16, 38, 71). These data provide further evidence that the Hox components of the Pbx-Hox heterodimers recognize the 3′ half site and indicate that the binding specificity of Hox helix 3 may not be modulated in the presence of Pbx cofactors.

Most importantly, in the presence of Pbx, position 7 of the consensus was found to critically influence DNA binding. For instance, conversion of the 3′ half site (T<u>T</u>ATGG) of the B7 consensus to T<u>A</u>ATGG, making it more similar to Hox sites with a canonical TAAT core, showed marked alterations in binding. Steady-state binding for Pbx1-HoxB4 went from negligible to robust, whereas that for Pbx1-HoxA10 was significantly reduced, in contrast to no apparent effects on binding by HoxB6- and HoxB7-containing complexes (Table 2, B7 consensus and Hox$_{\text{TAAT}}$). The importance of position 7 for binding specificity was further illustrated by its alteration within the context of the B1 and B7 consensus sites. For example, a $G_7 \rightarrow T_7$ change in the B1 site (ATGATT<u>G</u>ATCG→ATGATT<u>T</u>ATCG) markedly reduced steady-state binding by Pbx1-HoxB1 complexes (Table 2, B1 consensus and B7$_{G_{10}\rightarrow C_{10}}$). Similarly, a $T_7 \rightarrow G_7$ change in the B7 site (ATGATT<u>T</u>ATGG→ATGATT<u>G</u>ATGG) significantly reduced binding by HoxA10-containing complexes and increased binding by Pbx1-HoxB1 and Pbx1-HoxB4 complexes (Table 2, B7 consensus and B7$_{T_7\rightarrow G_7}$). A $T_7 \rightarrow C_7$ change in the B7 site (ATGATT<u>T</u>ATGG→ATGATT<u>C</u>ATGG) disrupted all cooperative binding (Table 2, B7$_{T7\rightarrow C7}$), showing that C_7 was not tolerated by any of the Pbx-Hox complexes. Separation of the half sites by one, two, or three nucleotides also abrogated all cooperative binding (Table 2, B7$_{\text{space 1}}$, B7$_{\text{space 2}}$, and B7$_{\text{space 3}}$).

Crystal and solution structures of homeodomain interactions with DNA predict a potential role for the N-terminal arm of the homeodomain contacting the 5′ portion of the TAATNN motif in the DNA minor groove (6, 34, 57). Comparison of the Pbx-Hox consensus sites suggested the possibility that the Hox homeodomain N-terminal arm contributes substantially to the binding specificity at position 7, perhaps modulated by interactions with Pbx which we showed earlier (12) to be dependent on the upstream hexapeptide. To test this, chimeric Hox proteins were constructed by replacing the hexapeptide, intervening linker, and N-terminal arm of one Hox protein with the comparable portions of another (Fig. 4), and the chimeric proteins were assessed for binding to DNA probes with different nucleotides at position 7 of the B7 consensus. Like the respective wild-type proteins, none of the chimeras bound with Pbx to C_7-containing sites (data not shown). Chimeras of HoxB1 and A10 showed that an exclusive preference for G_7 was conferred by the N-terminal arm/linker/hexapeptide cassette from HoxB1, whereas a restrictive preference for T_7 was conferred by the comparable portions of HoxA10 (Fig. 4, lanes 1 to 8). Additional experiments showed that the ability of HoxB7 to bind G_7-, T_7-, or A_7-containing sites (Figs. 4, lanes 15 to 17) could be transferred to HoxB1 (lanes 12 to 14) or HoxA10 (lanes 18 to 20) by the hexapeptide/linker/N-terminal arm of HoxB7. The comparable elements of HoxB1 or HoxA10 could transfer their stringent N_7 specificities to HoxB7 (lanes 9 to 11 and 21 to 23, respectively) providing

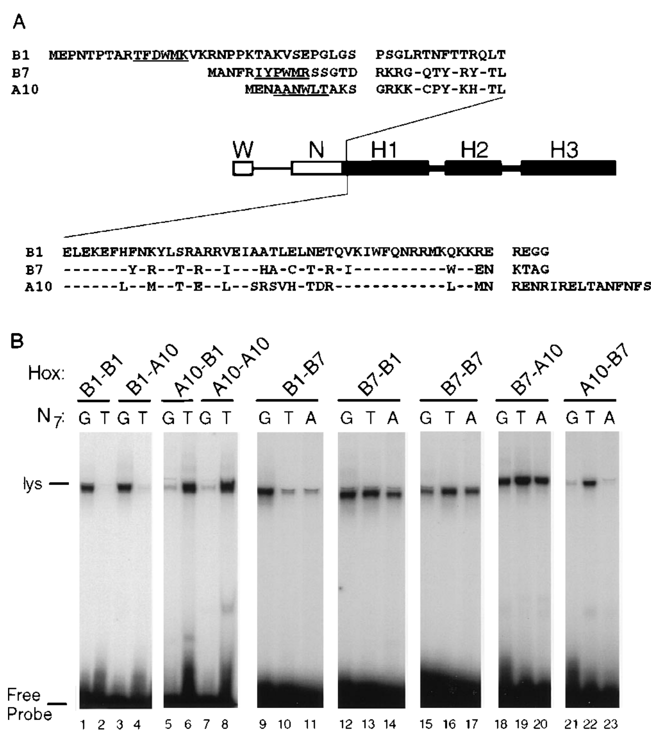

FIG. 4. The N-terminal arm of the Hox homeodomain, within the context of its associated tryptophan motif, substantially influences Hox DNA-binding specificity in the presence of Pbx. (A) The amino acid sequences of Hox protein fragments used for EMSA are shown in single-letter code. Residues identical to those in the HoxB1 homeodomain are shown as dashes. The tryptophan motifs are underlined. Chimeric Hox proteins were created by combining different upstream portions (tryptophan motif, linker, and N-terminal arm depicted above the schematic) with heterologous downstream portions (helices 1, 2, and 3 depicted below the schematic) of the respective homeodomains. Gaps in the sequences indicate amino- and carboxy-terminal limits of the homeodomains. (B) EMSA was performed with in vitro-translated chimeric Hox proteins whose identities are indicated above the lanes. Nomenclature indicates the source of the tryptophan motif, linker, and N-terminal arm followed by the source of helices 1, 2, and 3 (e.g., B1-A10). DNA probes consisted of oligonucleotides containing the B7 consensus site (T) or a mutant B7 site containing G_7 or A_7 (lanes labeled G or A, respectively).

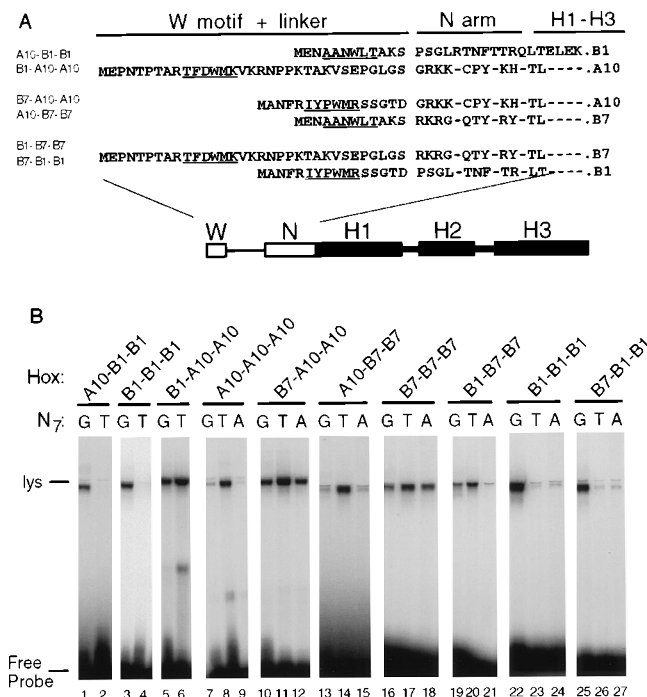

FIG. 5. The Hox N-terminal arm and associated Pbx dimerization motif function as an integral unit to determine DNA-binding specificity. (A) The amino acid sequences of Hox protein fragments used for EMSA are shown in single-letter code. Residues identical to those in the HoxB1 homeodomain are shown as dashes. The compositions of helices 1, 2, and 3 are indicated at the carboxy end of each sequence. Gaps in the sequences indicate amino-terminal limits of the homeodomains. Pbx dimerization motifs are underlined. Nomenclature for chimeric proteins indicates the composition of the tryptophan/linker motif, Hox N-terminal arm, and helices 1 to 3, respectively (e.g., $B1_{W/linker}$ $A10_{N\text{-}arm}A10_{H1\text{-}3}$). (B) EMSA was performed with in vitro-translated chimeric Hox proteins whose identities are indicated above the lanes. DNA probes consisted of oligonucleotides containing the B7 consensus site (T) or a mutant B7 site containing G_7 or A_7 (lanes labeled G or A, respectively).

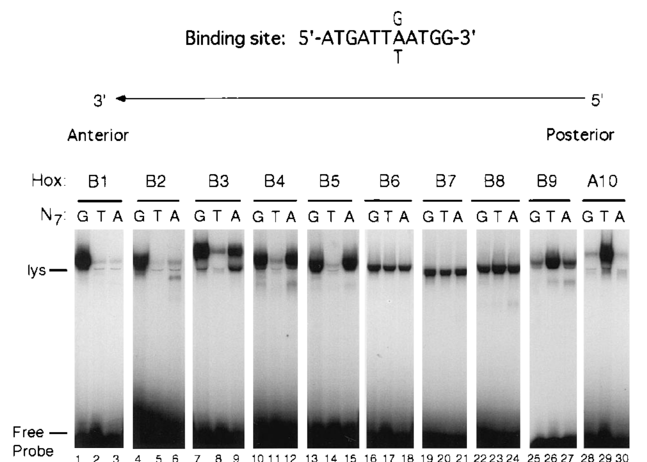

FIG. 6. A gradient of N_7 specificities across the *Hox* locus. EMSA was performed with in vitro-translated Pbx1a and Hox proteins whose identities are indicated above the lanes. DNA probes consisted of oligonucleotides containing the B7 consensus site (T) or a mutant B7 site containing G_7 or A_7 (lanes labeled G or A, respectively). The relative linkages and anatomical relationships of genes that code for the Hox proteins used in EMSA are shown at the top.

further evidence that binding preferences in the Hox half site cores were determined by N-terminal elements and not helices 1 to 3 of the respective homeodomains.

Both the hexapeptide/linker element and Hox homeodomain N-terminal arm contribute to DNA recognition in the core of the Hox half site. Additional domain swapping experiments addressed the relative contributions of the Hox homeodomain N-terminal arms versus the upstream Pbx dimerization motifs to binding specificities. Chimeric proteins containing the tryptophan motifs and associated linkers of HoxA10 or HoxB7 fused to the HoxB1 homeodomain (constructs A10-B1-B1 and B7-B1-B1, respectively) displayed N_7 specificities characteristic of HoxB1 (Fig. 5, lanes 1 to 4 and 25 to 27). By these criteria, the N_7 specificity of the HoxB1 N-terminal arm appeared relatively insensitive to upstream elements, suggesting that the amino acid sequence of the N-terminal arm primarily determined its specificity. However, when the hexapeptide/linker element from HoxB7 was grafted onto the homeodomain of HoxA10, the resulting chimera (B7-A10-A10) lost the exclusive preference for a TTAT core characteristic of HoxA10 and could bind probes with several different core sequences, a specificity more similar to that of HoxB7 (lanes 10 to 12 compared with lanes 7 to 9 and 16 to 18). Similarly, the N_7 specificity of HoxB7 homeodomain could be converted to that of HoxA10 by the tryptophan/linker element of HoxA10 (lanes 13 to 15). Thus, the tryptophan/linker elements of HoxB7 and A10 could completely alter the binding specificities of each other's N-terminal arms (which differ at 8 of 12 residues) but not the N-terminal arm of HoxB1. These findings, however, contrasted with the properties of the HoxB1 hexapeptide/linker, which could not confer HoxB1 specificity on the HoxB7 or HoxA10 homeodomain but, rather, created new N_7 binding specificities (Fig. 5, lanes 5, 6, and 19 to 21) in the chimeric proteins that did not fully reconstitute those of either constituent protein. Thus, hexapeptide/linker motifs have the potential to significantly modify the DNA-binding specificities of Hox homeodomain N-terminal arms, but their relative effects vary among different homeodomains, suggesting that the binding site preferences in the core of the Hox half site are determined by the combined properties of the Hox N-terminal arm, the linker, and the Pbx dimerization motif. Furthermore, within the context of Pbx1 cooperativity, DNA contact by the Hox homeodomain N-terminal arm appears to play a major role in binding specificity, considerably more than reported for Hox protein binding to DNA in the absence of Pbx-like cofactors (38, 59).

Stepwise differences in N_7 DNA-binding specificities across the *Hox* locus. The homeodomain N-terminal arm specificities of the entire HoxB cluster and HoxA10 proteins were comparatively assessed by EMSA. These studies employed N_7 variants of the B7 consensus probe whose $G_{10}G_{11}$ dinucleotide appeared to be well tolerated by all of the Hox proteins in the foregoing mutational analyses, thereby minimizing any potential effects of helix 3 on DNA-binding differences. As shown in Fig. 6, there was a very clear transition in N_7 specificities, 3′ to 5′ across the cluster. HoxB1 and B2 displayed virtually exclusive preferences for G_7 (Fig. 6, lanes 1 to 6). HoxB3, B4, and B5 strongly preferred sites containing G_7 or A_7 (lanes 7 to 15), although B3 showed an obvious bias for G_7 over A_7. HoxB6, -B7, and -B8 showed the broadest specificities by binding well to G_7-, A_7-, and T_7-containing sites (lanes 16 to 24). HoxB9 appeared to bind all three sites but displayed a very strong preference for the T_7-containing site rather than an A_7- or T_7-containing site (lanes 25 to 27). HoxA10, the most 5′ member examined, displayed a virtual exclusive preference for T_7 (lanes 28 to 30). Thus, under the EMSA conditions used, there is a stepwise alteration in binding specificities of Hox homeo-

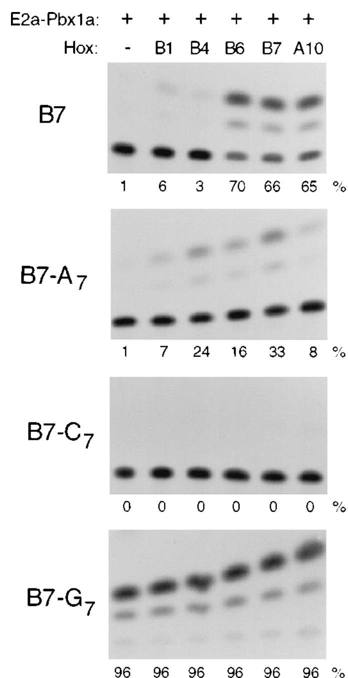

FIG. 7. Sequence-specific transcriptional activation by various Pbx-Hox heterodimers on reporter genes containing variations of the B7 consensus binding site. Representative results are shown for CAT assays assessing relative transcriptional properties of E2a-Pbx1a coexpressed with various Hox proteins whose identities are indicated at the top. The binding sites present in the different reporter genes used for transfections are indicated at the left and differ only at nucleotide position 7. The percentages of CAT conversion are indicated at the bottom. The results shown are representative of those obtained in at least three experiments performed in duplicate. Transfection efficiencies were standardized by reference to a cotransfected Rous sarcoma virus-luciferase construct.

proteins which allows their placement into at least four distinct categories based on their preference for G, G/A, G/A/T, or T at the N_7 position of the consensus Pbx-Hox sites. Furthermore, at least two Hox proteins (HoxB3 and HoxB9) displayed transitional specificities that appear intermediate between adjacent categories, further suggesting that there is a graduated progression in N-terminal arm specificities across the *Hox* locus.

Sequence-specific transcriptional activation by Pbx-Hox heterodimeric complexes. The abilities of various Hox proteins to modulate the DNA-binding properties of Pbx-Hox heterodimeric complexes were further tested in transient transcriptional assays. Since the transcriptional properties of wild-type Pbx proteins have not yet been established, these studies used the oncogenic Pbx chimeric protein E2a-Pbx1, which previous studies have demonstrated to be a potent transcriptional activator (40). The reporter gene for these studies contained three Pbx1-HoxB7 consensus binding sites upstream from a minimal promoter and CAT reporter gene. Cotransfection of the reporter and an E2a-Pbx1 expression construct into the REH lymphoid cell line showed no detectable activation of the reporter gene (Fig. 7), suggesting that the chimeric Pbx protein was incapable of binding and activating transcription in the absence of exogenously expressed Hox proteins. This finding correlated with the inability of E2a-Pbx1a to bind by itself to the B7 probe in EMSA (data not shown). Similarly, no reporter gene activation was observed when the individual Hox proteins were expressed in the absence of cotransfected E2a-Pbx1 (data not shown). In contrast, coexpression of either HoxB6, -B7, or -A10 with E2a-Pbx1a resulted in high-level activation of the reporter gene (Fig. 7), reflecting the abilities of these Hox proteins to cooperatively bind the B7 probe with Pbx1 in EMSA (Fig. 1 and Table 2). Coexpression of HoxB1 or B4 with E2a-Pbx1, however, showed virtually no reporter gene activation (6 or 3% conversion, respectively), consistent with the markedly reduced capacity of Pbx1-HoxB1 and Pbx1-HoxB4 heterodimers to bind the B7 site (Fig. 1).

The abilities of Hox proteins to modulate in vivo DNA-binding and transcriptional activities were further tested by altering the binding sites of the reporter gene (Fig. 7). A single nucleotide substitution, $T_7 \rightarrow A_7$, converted the site to AT GATTAATGG, which represents a canonical Hox site obtained as a minor product in our site selections and which binds well to HoxB3-B8 proteins (Table 2 and Fig. 6). Similar to the B7 reporter, no transcriptional activity was induced by Hox proteins alone or by the E2a-Pbx1 chimera in the absence of exogenous Hox proteins (Fig. 7). Coexpression of HoxB7 showed robust activation (33% conversion), whereas HoxA10 activity was reduced eightfold (8% versus 65% on the B7 site), reflecting its relative intolerance for A_7-containing sites. Most significantly, an eightfold increase in activation (24% versus 3% on the B7 reporter) was observed for the HoxB4-containing complex. In contrast, HoxB1 showed minimal activation with E2a-Pbx1 on both reporters. Another reporter with a C_7-containing site (ATGATTCATGG) showed no activation with all transfected E2a-Pbx1/Hox combinations, reflecting its inability to serve as a binding site in vitro. Similar analyses with a G_7-containing site (ATGATTGATGG) showed high-level activation under all conditions even in the absence of cotransfected Hox proteins (Fig. 7). Since E2a-Pbx1 does not dimerize on this site in EMSA (11a), the observed activation likely resulted from endogenous proteins capable of heterodimerizing with E2a-Pbx1 on the G_7-containing site which is bound by a broader array of Hox proteins than sites with A_7 or T_7 (Fig. 6). The transient transfection data illustrate the importance of position 7 in determining binding specificity and support the conclusion that the modes of DNA-binding in vivo and in vitro must be very similar.

DISCUSSION

The studies presented here clearly demonstrate that interactions with Pbx-like cofactors permit Hox proteins with highly similar homeodomains to distinguish single nucleotide differences in high-affinity binding sites and, further, that the differences in their binding specificities correlate with positions of the respective genes in the *Hox* cluster. These studies provide additional insight into the molecular mechanisms contributing to the functional specificity of Hox proteins and suggest a topological model for how Pbx-Hox heterodimeric complexes may dock on DNA. Although a number of earlier studies have addressed the preferred in vitro binding sites for Hox proteins (for a review, see reference 38; 5, 16, 59), they generally used subtotal protein fragments restricted to the homeodomains and were invariably conducted in the absence of cofactors which have recently been shown to markedly enhance the DNA-binding properties of several Hox proteins (11, 12, 44, 77). Our studies were conducted to establish general principles that may govern the sequence-specific DNA-binding properties of Hox proteins in the presence of Pbx cofactors independent of other promoter selective factors that may operate in vivo.

A topological model for Pbx-Hox heterodimeric interactions with DNA. An unexpected finding was that Pbx and Hox proteins cooperatively bind DNA through a consensus sequence

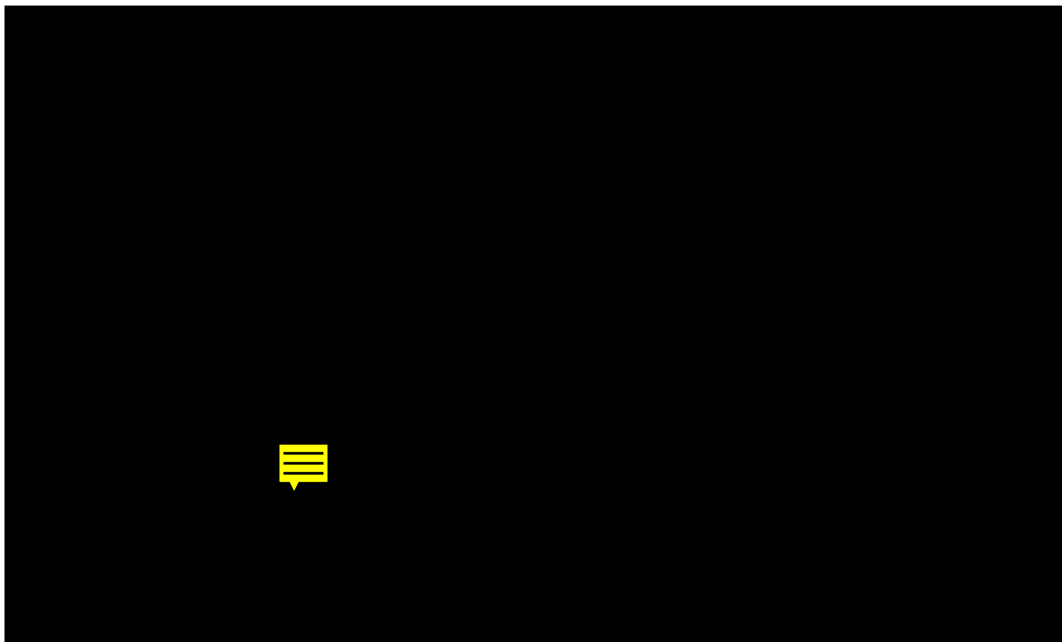

FIG. 8. Proposed molecular model of the Pbx-Hox heterodimer-DNA complex. Stereo views are shown, with ribbons drawn through the alpha carbons of the protein domains and DNA strands shown as a transparent space-filling model. Views are from the lateral aspect (A) and along the DNA longitudinal axis (B). The Pbx homeodomain is green, and the Hox homeodomain is blue. The DNA strand in yellow encodes the Pbx1-HoxB7 consensus site 5′-TGATTTATGG-3′, bottom to top. N-terminal and C-terminal extensions were added to the Hox and Pbx homeodomains, respectively, to illustrate that these regions, which are essential for Pbx-Hox cooperativity, exit the complex on the same side of the DNA helix (bottom center, A; lower right, B), allowing for potential intermolecular contacts. The homeodomain regions of HoxB7 and Pbx1 were modeled on the nuclear magnetic resonance structure of the Antennapedia-DNA complex (6, 57). The hexapeptide and linker of HoxB7 were manually modeled to produce the desired contacts with the carboxy terminus of Pbx, which was modeled on its predicted secondary structure. This figure was generated with Insight II software and optimized by using the Discover program (Biosym Technologies).

consisting of tandem half sites with no intervening spacer. This differs from the tandem but separated configuration of half sites in *hsg* operators cooperatively bound by **a**1 and α2, yeast proteins involved in mating-type determination whose cooperative interactions are a paradigm for how homeodomain proteins may effect high-specificity DNA-binding through combinatorial interactions (30). Recognition of a contiguous 11-bp site by Pbx-Hox heterodimers provides an explanation for earlier observations that Hox sites located a few nucleotides away from a consensus Pbx site were dispensable for Pbx-Hox cooperative binding in vitro (12, 44). It is now apparent that this occurred because the octamer cores of Pbx-Hox sites (TGAT TNAT) are very similar or identical to the consensus Pbx site (TGATTGAT) determined previously in studies using GST-Pbx proteins (40, 45, 78). The previously determined Pbx consensus therefore is likely to have resulted from GST-Pbx proteins binding as homodimers to adjacent TGAT half sites under conditions used for site selections. However, full-length wild-type Pbx proteins do not bind the consensus sites as homodimers under physiologic conditions. Rather, as demonstrated in this study, specific nucleotides within and flanking the octamer cores of the consensus sites influence the affinity and stability of binding by various Pbx-Hox heterodimeric complexes. Our findings may also explain the recent observation that a Pbx consensus-like site is required for HoxB1 autoregulation of its own expression with Pbx/exd cofactors in rhombomere 4 of the hindbrain (64). Repeat 3, the strongest element within the HoxB1 rhombomere 4 enhancer, adheres to the overall Pbx-Hox consensus determined here and contains a G at position 7 which is an important feature of the consensus site that we determined for Pbx1-HoxB1 heterodimers. This provides important support for the view that the consensus sites that we identified in vitro for Pbx-Hox DNA-binding may be relevant for their in vivo function as well.

The close apposition of Pbx and Hox proteins required to bind the consensus sites is likely to be accommodated by an unusual structural feature of Pbx proteins. In particular, residue 50 in recognition helix 3 of the homeodomain, which generally plays an important role in sequence-specific contacts in the major groove (22, 76), is a glycine in the Pbx/exd subfamily, in contrast to the long side chain amino acids in all other homeodomains reported to date (38). Gly_{50} may abbreviate DNA recognition by Pbx within the major groove by eliminating contact with bases at positions 6 and 7 of the consensus sequence, whose composition would be specified through the minor groove by the N-terminal arm of the adjacent Hox protein. A similar role in abbreviating base-specific contacts has been proposed for the Cys_{50} of POU homeodomains (24). Structural models of tandemly oriented Pbx and Hox homeodomains bound to the consensus sequence suggest that a helical motif downstream of the Pbx homeodomain and essential for cooperativity (12) would be favorably positioned to make contact with tryptophan-containing motifs upstream of the Hox homeodomain (Fig. 8). Such an interaction could potentially stabilize interaction with DNA by the Gly_{50}-containing helix 3 of Pbx while simultaneously modulating Hox homeodomain N-terminal arm specificity. It is currently unclear whether additional contacts between Pbx and Hox proteins may contribute to the stability of the complex. This model, which is based on site selection data and the differential effects of combined Hox and binding-site mutations on Pbx-Hox binding, will require confirmation by crystallographic studies.

During the course of determining optimal binding sites for

Pbx-Hox complexes, we discovered that Hox proteins from the Abd-B class can cooperatively interact with Pbx proteins. For HoxA10, this interaction requires a conserved tryptophan-containing motif upstream of the homeodomain. This motif is also present in Hox proteins from paralog group 9, and we showed that HoxB9 is capable of cooperating with Pbx, implying a mechanism similar to the one that we uncovered for HoxA10. These observations indicate that there is important heterogeneity in the domains Hox proteins can utilize to modulate interaction with Pbx proteins. Both HoxB9 and HoxA10 displayed very restrictive sequence requirements (T_7 in the consensus site) for cooperative binding with Pbx, which accounts for the lack of cooperativity between HoxA10 and Pbx observed in previous studies that used sites lacking T_7 (12). The diverged motif in the Abd-B class proteins appears to play a critical role in establishing this specificity, since grafting of the HoxA10 tryptophan motif and linker onto HoxB7 converts its DNA-binding specificity to that of HoxA10 (Fig. 5). The observed in vitro cooperativity between Abd-B class Hox proteins is consistent with recent in vivo studies implicating exd in modulating Abd-B function (66).

Pbx modulates the DNA-binding specificity of the Hox homeodomain N-terminal arm. Another unexpected observation was the infrequent appearance of TAAT core motifs in the Hox half sites determined by selections for high affinity-binding sites in the presence of Pbx. Although TAAT core half sites were compatible with robust binding for most Pbx-Hox complexes (Table 2 and Fig. 6), they may not be preferred, as suggested by the site selections and in vivo activation data for HoxB6 and HoxB7 (Fig. 7). For complexes containing HoxB1, -B2, and -A10, TAAT cores were clearly unfavorable. These and other Hox proteins, when complexed with Pbx1, showed strong or exclusive preferences for TGAT and TTAT cores. In contrast, none of the Hox complexes bound exclusively to TAAT half sites, nor did any recognize sites containing a TCAT core. Thus, the nucleotide composition at position 2 in the Hox half site (T<u>N</u>ATNN) plays a critical role in the ability of particular Pbx-Hox complexes to bind DNA, more than shown previously for DNA binding by monomeric Hox proteins. Our domain-swapping experiments showed that these preferences were conferred by the N-terminal arm of the Hox homeodomain within the context of upstream sequences (tryptophan-containing motifs and their linker arms) that mediate interactions with Pbx. The structural components establishing sequence specificity in the Hox half site core appear to vary for different Hox proteins. For HoxB1, the N-terminal arm is the major determinant, whereas for HoxB7 and -A10, upstream elements appear more dominant. These data suggest that the hexapeptide, linker, and N-terminal arm function together as an integral unit, a conclusion consistent with our previous studies of Hox-Pbx-DNA complex stabilities, using a single binding site (73).

It has long been recognized that the extreme N-terminal region of the homeodomain is highly variable (38) and, on the basis of X-ray crytallographic studies, appears to contact DNA in the minor groove (34, 83). However, little has been known about how the N-terminal arm achieves its specificity. Our data suggest that a major consequence of Pbx-Hox interactions is an enhancement and stabilization of Hox homeodomain N-terminal arm binding within the minor DNA groove, thereby conferring previously undetected specificity to this variable region of the homeodomain. This provides a new molecular framework for understanding the contributions of the N-terminal arm to in vivo specificity of homeodomain proteins, which previously has been most thoroughly investigated in *Drosophila* homeodomain proteins, such as Ubx, Scr, Antp, and Dfd, some of which are orthologs of the Hox proteins used in our studies (10, 18, 43, 48, 86). Functional differences have been ascribed to the homeodomain N-terminal arms for Hox proteins as well (62). Our findings caution that functional differences attributable to Hox N-terminal arm DNA-binding specificity may reflect the contributions of the tryptophan/linker motif as well, and future studies will need to focus on the individual contributions of specific residues in the N-terminal arms and whether the linker effects on DNA-binding specificity derive from differences in length or sequence.

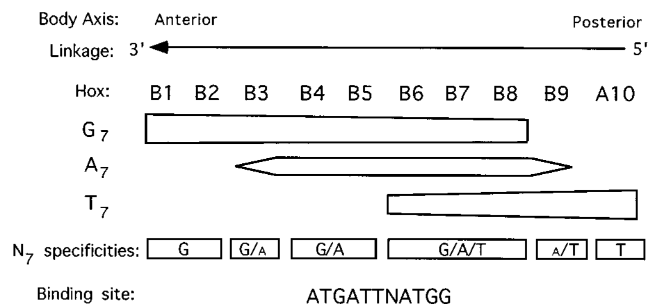

FIG. 9. Diagrammatic representation of the DNA-binding specificity differences for nucleotide composition at position 7 of the Pbx-Hox consensus site. Body axis and linkage relationships of the HoxB1 to -B9 and A10 genes are shown at the top. Binding of Pbx-Hox complexes to sites with G, A, or T at position 7 of the consensus site is indicated in the middle. Summation of the binding characteristics of each Pbx-Hox complex yields the various categories of N_7 specificities shown below.

In addition to providing further evidence that Hox proteins can discriminately bind DNA with high affinity, our studies show that they also exhibit differences in their DNA-binding specificities that correlate with the positions of their respective genes in the *Hox* locus. Within the resolution of our analyses, there appear to be three overlapping components to this phenomenon: an increasing preference for G_7 toward the 3′ end, an increasingly restrictive preference for T_7 at the 5′ end, and a recognition of A_7 throughout the middle of the locus (Fig. 9). The observed stepwise differences in DNA-binding properties could distinguish four categories of N_7 specificities with two additional transitional categories. It is likely that more sensitive analyses will reveal more subtle differences in specificity that may be functionally important in vivo and divide the categories even further and that other cofactors may fine-tune the specificity gradient. Our observations confirm and significantly extend earlier studies that provided evidence for distinct binding classes of Hox proteins (16) whose specificities were determined partially by residues in the homeodomain N-terminal arms. Site selections with the monomeric HoxA10 homeodomain have also provided evidence for a preferred TTAT core in its binding site (5). Our data further refine this by showing a stepwise gradation of specificities across the *HoxB* locus in which a more 3′ location confers a preference for TGAT cores, whereas a 5′ location confers a TTAT preference. The observed linkage-related differences appear to constitute a general Hox DNA recognition code and suggest that different binding activities of Pbx-Hox complexes are at least part of the position-specific activities of the Hox genes.

Our studies are consistent with a model that conceptualizes the homeodomain as a compact DNA-binding structure whose specificity can be modulated by means of flexible amino- and carboxy-terminal extenders, a model based on original studies of the yeast **a**1 and α2 homeodomain proteins (30). The biological specificity of Hox proteins has been shown to reside in the sequence of the homeodomain, primarily the amino-ter-

minal arm and helix 3 (20, 37, 48) but, as for α2, target site specificity does not reside solely in the homeodomain. The homeodomain N-terminal arm of Hox proteins which contacts bases in the minor groove has been shown to have a disordered conformation in crystal and solution structure determinations of the homeodomain in the absence of DNA and cofactors (19). Our findings suggest that the hexapeptide and other tryptophan-containing motifs may function like α2 flexible extenders to mediate interactions with cofactors such as Pbx. Intermolecular contact between the hexapeptide and Pbx may induce conformational changes in the N-terminal arm of the Hox homeodomain, perhaps analogous to those observed for the α2 C-terminal extender upon contact with **a**1 (63). However, this cannot entirely account for the functional specificity of Hox proteins since some continue to exhibit overlapping target site specificities in vitro even in the presence of Pbx. Additional specificity could be acquired through C-terminal flexible extenders that modulate the recognition properties of helix 3 or mediate interactions with other classes of transcriptional proteins. In support of this possibility, the C terminus of Ubx has been shown to be important for its target site selectivity and functional specificity (3, 10).

The importance of cofactor interactions for regulating Hox protein specificity and function. Interactions with Pbx cofactors provide several opportunities for regulatory control and refinement of Hox protein function. Multiple members of both the Hox and Pbx protein families allow for combinatorial control by Pbx-Hox complexes of differing compositions, which would not be possible if the DNA-binding motifs were covalently coupled as in POU proteins. In fact, at least biochemically, the interactive capabilities of Pbx-like cofactors appear to extend beyond the Hox family to non-Hox homeodomain proteins (73, 77), suggesting that the DNA-binding and potential transcriptional effector functions of Pbx-like cofactors can be exploited by a wide variety of homeodomain proteins. Another level of regulatory control may involve posttranslational modifications, in response to changes during the cell cycle or extracellular signals, that could abrogate or enhance the abilities of preformed Hox and Pbx proteins to assemble into highly specific DNA-binding complexes. Indeed, posttranslational regulation may account for some of the in vivo effects of Ubx and exd (4). Yet another level of regulatory control could be achieved by the various Hox isoforms that differ in the compositions of their linkers separating the hexapeptide from the N-terminal arm. Given our observations that some hexapeptide/linker elements could completely alter the N_7 binding preferences of heterologous homeodomains, the prediction is that various isoforms will display differences in their DNA-binding specificities on consensus DNA sites. Finally, although Pbx/exd family proteins are the only Hox cofactors to be identified to date by genetic screens in *D. melanogaster*, additional cofactors could serve analogous roles. Such hypothetical factors may, like Pbx/exd, bind DNA with low affinity by themselves but be significantly stabilized through interactions with the highly conserved tryptophan motifs and in turn modulate Hox N-terminal arm specificity. Furthermore, these hypothetical cofactors could bind DNA sites different from Pbx/exd and, as cooperative complexes with Hox proteins, target genes (or response elements) distinct from Pbx-Hox heterodimers.

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