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Pbx Raises the DNA Binding Specificity but Not the Selectivity of Antennapedia Hox Proteins

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We have used a binding site selection strategy to determine the optimal binding sites for Pbx proteins by themselves and as heterodimeric partners with various Hox gene products. Among the Pbx proteins by themselves, only Pbx3 binds with high affinity, as a monomer or as a homodimer, to an optimal binding site, TGATTGATTTGAT. An inhibitory domain located N terminal of the Pbx1 homeodomain prevents intrinsic Pbx1 binding to this sequence. When complexed with Hoxc-6, each of the Pbx gene products binds the same consensus sequence, TGATTTAT, which differs from the site bound by Pbx3 alone. Three members of the Antennapedia family, Hoxc-6, Hoxb-7, and Hoxb-8, select the same binding site in conjunction with Pbx1. The affinities of these proteins as heterodimeric partners with Pbx1 for the selected optimal binding site are similar. However, the binding specificity of Hox proteins for optimal binding sites is increased, compared to nonspecific DNA, in the presence of Pbx proteins. Thus, while cooperative DNA binding involving heterodimers of Pbx and Hox gene products derived from members within the Antennapedia family does not increase binding site selectivity, DNA binding specificity of the Hox gene products is significantly enhanced in the presence of Pbx.

Embryonic differentiation involves the action of a diverse set of transcriptional regulators. One class of such regulators, the homeotic selector proteins, has been particularly well studied. The homeotic proteins control embryonic development along the anterior-posterior axis in a wide variety of organisms, including *Drosophila melanogaster* and mammals (22, 34). In *Drosophila*, the homeotic selector genes are organized into two clusters, the Antennapedia complex and the Bithorax complex, together referred to as the homeotic gene complexes, HOM-C. Mutations both in the *Drosophila* HOM-C genes and in their vertebrate counterparts, the Hox genes, that interfere with their expression patterns, resulting in morphological transformations, have been identified (7, 19, 25, 31, 48).

The homeotic proteins share a region of 60 amino acids, designated the homeodomain, which is a DNA binding motif recognizing AT-rich sequences and consisting of a flexible N-terminal arm followed by three α-helices, two of which fold into a helix-turn-helix configuration (9, 24, 51). The homeotic proteins show great functional specificity during development, and this high degree of biological specificity has been mapped to regions within and immediately flanking the homeodomain (4, 23, 28, 33). However, in vitro, the homeodomain proteins bind to similar or identical DNA sequences containing a TAAT core motif (9, 12, 14). Thus, Hox proteins with quite divergent biological activities display similar DNA binding properties. To account for their high degree of specificity, it has been proposed that other factors contribute to and/or enhance the biological specificity of the Hox proteins.

Recently, homeodomain proteins of a new class, including the *Drosophila* gene product extradenticle (exd), have been shown to function as such cofactors (43, 49). exd acts in parallel with some of the homeotic proteins to regulate common downstream target genes, including *decapentaplegic* (*dpp*) and *wingless* (43, 49, 50). In vitro binding assays demonstrated that exd has the ability to bind cooperatively with Ubx to sites present in the *dpp* enhancer (3). Furthermore, exd and either Ubx or abd-A, have the ability to bind as heterodimers to a distinct synthetic site (52).

In mammals, three homologs of exd, designated Pbx1, Pbx2, and Pbx3, have been identified (35). Pbx1 was originally identified as a proto-oncogene which is translocated in pre-B acute lymphoblastoid leukemias (18, 41). The chromosomal translocation involving chromosome 1 and 19 results in a fusion protein in which the N-terminal portion of E2A, a helix-loop-helix (HLH) protein, is fused to the homeodomain of Pbx1, converting a nontranscriptional activator (Pbx1) into a transcriptional activator (E2A-Pbx1) (26, 30, 54). Pbx1, Pbx2, and Pbx3 are closely related to each other, with a 97% identity at the amino acid level within their homeodomains. Whereas Pbx2 and Pbx3 are widely expressed, Pbx1 is not expressed in cells of the lymphoid lineage (35).

Cooperative interactions involving Hox proteins and Pbx or exd proteins require a highly conserved hexapeptide sequence located immediately upstream of the Hox homeodomain (6, 17, 20, 40, 46). The Hox homeodomain is also important for cooperativity, since grafting of the hexapeptide onto a heterologous homeodomain abolishes the ability to bind DNA as heterodimers (40, 42).

Both exd and Pbx also have the ability to bind cooperatively to synthetic DNA templates with members of the engrailed family of proteins (52, 53). A conserved tryptophan motif located immediately N terminal of the engrailed homeodomain and related to the hexapeptide is required for cooperative DNA binding (45). engrailed-Pbx heterodimers bind a different site than Hox-Pbx complexes, indicating that Pbx enhances the binding site selectivity of Hox and engrailed. Recent studies have indicated that Pbx1 has the ability to modulate the DNA binding selectivity of divergent Hox gene products (5).

The DNA binding properties of heterodimeric interactions involving two homeodomain proteins that control cell type determination in yeast, **a**1 and α2, have been extensively studied (16). **a**1-α2 heterodimers exhibit significantly higher affinities for operator binding sites present in haploid-specific pro-

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moters than do α2 homodimers. Furthermore, the specificity of an **a**1-α2 heterodimer is greatly increased compared to an α2 homodimer for binding sites present in haploid-specific promoters (13, 16). In this study we show that Hox and Pbx proteins have DNA binding affinities and specificities that are distinct from those of either protein alone.

MATERIALS AND METHODS

Plasmids. pSP65-Pbx1 and pSP64-Pbx2 were kindly provided by M. L. Cleary. The cDNA of Pbx3 was isolated from an Epstein-Barr virus-transformed human B-cell library via PCR using Pbx3-specific primers and was cloned into the vector pSS (40), a modified form of pSP64-ATG (52). Sequencing of the entire Pbx3 coding region showed that we have isolated a new isoform of Pbx3. The sequence is identical to the Pbx3 sequence published by Monica et al. (35) except for the replacement of a 40-amino-acid stretch in the C terminus (amino acid 338 Ser to amino acid 377 Thr) by two amino acids (Gly and Cys). The 543-bp *Pvu*II fragment of Pbx3, which encodes amino acids 134 to 314 and includes the complete homeodomain (35), was cloned into pSS to generate Pbx3 134–314. The Pbx1 N-terminal and C-terminal deletion constructs were generated by PCR. PCR products Pbx1 80–430, Pbx1 119–430, Pbx1 172–430, and Pbx1 233–430 were cloned into pSP64-ATG, while the Pbx1 1–295 PCR product was cloned into pSS. The 615-bp *Pvu*II fragment of Pbx1 encoding amino acids 105 to 309 was cloned into pSS. The plasmid containing the Hoxb-7 cDNA was generously provided by F. Meijlink and R. de Jong and is derived from an incomplete cDNA originally cloned by R. Krumlauf and collaborators (8). pSP64-ATG–Hoxb-8 was constructed by inserting an 822-bp *Sac*I cDNA fragment containing the entire Hoxb-8 coding region (21) without the first methionine into the *Sac*I site of pSP64-ATG. The Hoxc-6 full-length coding region was amplified from a cDNA obtained from E. Boncinelli (32) and cloned in pSP64-ATG. Hoxd-4 was kindly provided by W. McGinnis. The plasmid containing the Hoxb-3 coding region was a kind gift from S. Guazzi. For in vitro transcription and translation, the Hoxb-3 sequence was cloned into pSP64-ATG.

In vitro translation and EMSA. All proteins used in this study were produced with the SP6 TNT rabbit reticulocyte lysate coupled transcription-translation system (Promega, Madison, Wis.) according to the manufacturer's protocol. Control reactions using [^{35}S]methionine were performed and analyzed on a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel to verify that proteins of the correct size were produced in similar quantities. Electrophoretic mobility shift assays (EMSAs) were performed as described previously (52). Briefly, double-stranded ^{32}P-end-labeled oligonucleotides (30,000 cpm) were incubated with in vitro-translated proteins in a buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol, in the presence of 1 μg of poly(dI-dC) · poly(dI-dC) and 1 μg of bovine serum albumin for 30 min at room temperature (RT). Bound and free probes were separated on a 5% 36:1 polyacrylamide gel. After drying the gel, results were visualized by autoradiography.

Dissociation rates of the various Pbx-Hox heteromeric complexes were determined as follows. After 30 min of preincubation of the in vitro-translated proteins and the ^{32}P-radiolabeled binding site, 500 ng (a 1,000-fold excess) of the same unlabeled binding site was added to the binding reaction and incubation continued for different times (as indicated in the figure legends) prior to electrophoresis. The zero time points refer to binding mixtures to which H_2O instead of 500 ng of competitor DNA was added and which also continued incubation for 15 min prior to electrophoresis. Intensities of the shifted Pbx-Hox protein-DNA complexes were determined by using a PhosphorImager. Due to very stable complexes formed by Pbx1 with either Hoxb-7, Hoxb-8, or Hoxc-6 on their corresponding selected high-affinity site, their dissociation rates were determined at 37°C instead of at RT.

Competition experiments were performed as follows. In vitro-translated proteins were incubated with increasing amounts (as indicated in the figure legends) of either specific competitor DNA (unlabeled binding site) or nonspecific competitor DNA (unlabeled, unrelated oligonucleotide with the same size as the binding site) for 15 or 30 min at RT (as indicated in the figure legends). Subsequently, probe was added to each binding reaction and incubation continued for another 15 or 30 min (as indicated in the figure legends). Samples were subjected to electrophoresis. Intensities of shifted complexes were determined by using a PhosphorImager.

The ^{32}P-radiolabeled probes used were P1b7 (GAGATGATTTATGACTTT GTC), P1b8 (GAGATGATTTATTACTTTAGTC), P1c6 (GAGATGATTTAT TACTTTGTC), P3 (GTTGATTGATTTGATGGGTC), and P1+b7 (CGAAATTGATTGATCATTTAATTGAC).

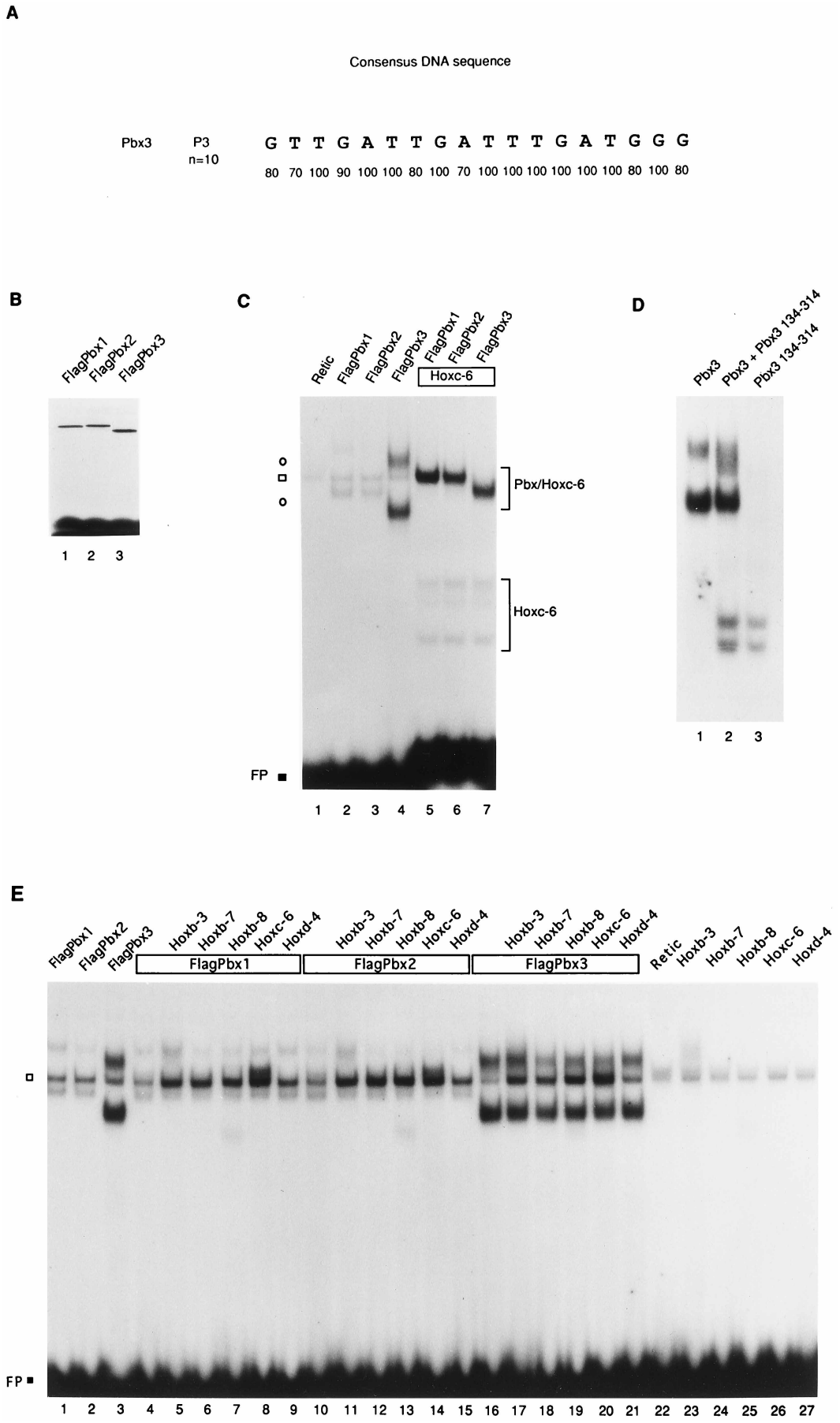
Binding site selection. A combination of affinity purification and PCR amplification was used to determine consensus binding sites for various Pbx-Hox heterodimers. Five hundred nanograms of a single-stranded oligonucleotide pool containing 16 internal random nucleotides (5′ CGC GGA TCC TGC AGC TCG AG N_{16} GTC GAC AAG CTT CTA GAG CA 3′) was annealed to an oligonucleotide primer complementary to the 3′ arm (5′ CAG CTG TTC GAA GAT CTC GT 3′) and converted to double-stranded DNA by using the Klenow fragment of *Escherichia coli* DNA polymerase I. One hundred fifty nanograms of the resultant double-stranded DNA was incubated with 10 μl of in vitro-translated FLAG-tagged Pbx and 10 μl of in vitro-translated Hox proteins in 100 μl of total volume containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 15 μg of poly(dI-dC), 10 μg of bovine serum albumin, 5% (vol/vol) glycerol, and 1% Nonidet P-40 for 30 min at RT. Protein-DNA complexes were immunoprecipitated by using the anti-FLAG M2 monoclonal antibody coupled to agarose beads (Eastman Kodak, New Haven, Conn.). Pellets were washed five times with 1 ml of ice-cold washing buffer containing 10 mM Tris-HCl (pH 7), 75 mM KCl, and 0.1% Nonidet P-40. Subsequently, the pellets were resuspended in 50 μl of H_2O and boiled for 10 min and 10 μl of the supernatants, containing the DNA, was used for PCR (10 cycles) using primers complementary to the 5′ arm (5′ GCG CCT AGG ACG TCG AGC TC) and the 3′ arm (shown above) at 55°C. After phenol-chloroform extraction, about 15% of the amplified DNA was used for a subsequent round of selection and amplification. After six rounds of selection and amplification, enrichment for specifically bound DNA oligonucleotides was verified by EMSA. Part of the finally obtained amplified PCR products was digested with *Hin*dIII and *Xho*I and cloned into pBluescriptSK (pBSK) (Stratagene, San Diego, Calif.). Nucleotide sequences of randomly selected clones were determined and visually aligned to establish a consensus DNA binding site.

RESULTS

Pbx3 has the ability to bind as a monomer or as a homodimer to a high-affinity binding site. The Pbx members Pbx1, Pbx2, and Pbx3 have closely related sequences (35). Truncated forms of Pbx1 recognize an optimal binding site that contains the sequence TTGATTGAT (26, 30, 54). To determine the optimal binding sites for full-length Pbx1, Pbx2, and Pbx3, we used a modified version of binding site selection. Each of the Pbx proteins was N-terminally tagged with a FLAG epitope. The tagged Pbx proteins were in vitro translated and incubated with degenerate double-stranded oligonucleotides containing 16 random base pairs. The Pbx-DNA complexes were affinity purified using a monoclonal antibody directed against the FLAG epitope. Bound DNA was amplified by PCR and used for subsequent selection and amplification steps. After six rounds of selection, oligonucleotides bound to the Pbx proteins were isolated, amplified, cloned into pBSK, and sequenced. A consensus sequence for Pbx3 was readily apparent and consisted of three direct repeats of the Pbx consensus half site TGAT, designated P3 (Fig. 1A). Pbx2 and Pbx3, however, did not allow the selection of a high-affinity binding site. To compare the relative DNA binding affinities of Pbx1, Pbx2, and Pbx3 for P3, we analyzed their binding properties. Each of the FLAG-tagged Pbx proteins was efficiently translated in vitro as verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1B). The in vitro-translated proteins were incubated with the radiolabeled P3 binding site and analyzed by EMSA. While in vitro-translated Pbx3 binds with high affinity to the P3 probe, Pbx1 and Pbx2 exhibit weak DNA binding (Fig. 1C, lanes 2 to 4). We noticed that two Pbx complexes are present (Fig. 1C). Since the P3 probe contains three repeats of the Pbx1 consensus half site, the most simple explanation is that these different complexes represent various oligomeric states of Pbx binding to DNA.

To rule out the possibility that the difference in DNA binding affinity between Pbx3 and Pbx1 and Pbx2 was caused by differential activities of the preparations, we mixed identical amounts of Pbx1, Pbx2, or Pbx3 with Hoxc-6. The protein mixtures were incubated with a radiolabeled DNA sequence, previously shown to allow Pbx–Hoxc-6 cooperative binding, and analyzed by EMSA. Pbx1, Pbx2, and Pbx3 bind with Hoxc-6 to this binding site with similar affinities (Fig. 1C, lanes 5 to 7), indicating that the proteins are equivalently active. Taken together, these observations show that Pbx3 exhibits an intrinsic DNA binding activity that is clearly distinct from those of Pbx1 and Pbx2.

To determine whether Pbx3 binds as a monomer or as an oligomer, we designed a truncated form of Pbx3 consisting of amino acids 134 to 314, including the homeodomain. The trun-


A
Consensus DNA sequence
Pbx3
P3
n=10
G T T G A T T G A T T T G A T G G G
80 70 100 90 100 100 80 100 70 100 100 100 100 100 100 80 100 80
B
FlagPbx1
FlagPbx2
FlagPbx3
1 2 3
C
Retic
FlagPbx1
FlagPbx2
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FlagPbx1
FlagPbx2
FlagPbx3
Hoxc-6
Pbx/Hoxc-6
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FP
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Pbx3
Pbx3 + Pbx3 134-314
Pbx3 134-314
1 2 3
E
FlagPbx1
FlagPbx2
FlagPbx3
Hoxb-3
Hoxb-7
Hoxb-8
Hoxc-6
Hoxd-4
FlagPbx1
FlagPbx2
FlagPbx3
Retic
FP
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

FIG. 1. Pbx1, Pbx2, and Pbx3 exhibit different intrinsic DNA binding affinities. (A) The consensus DNA binding site (P3) for Pbx3 as determined by binding site selection is shown. n indicates the number of sequences analyzed to determine the consensus sequence. The number under each nucleotide represents the percentage of sequences containing the indicated nucleotide. (B) FLAG-tagged Pbx1, Pbx2, and Pbx3 were transcribed and translated in vitro in the presence of [^{35}S]methionine and analyzed by SDS-PAGE. (C) One microliter of in vitro-translated FLAG-tagged Pbx1, Pbx2, or Pbx3 was incubated with radiolabeled probe P3 (lanes 2 to 4) or mixed with 1 μl of in vitro-translated Hoxc-6 and incubated with a radiolabeled DNA sequence, GTCAATTAAATGCGCATCAATCAATTTCG, to which Pbx and Hox bind cooperatively (lanes 5 to 7). Binding reactions were analyzed by EMSA. ○, shifted Pbx complex; □, nonspecific complex; FP, free probe. (D) One microliter of in vitro-translated full-length Pbx3 (lane 1), 1 μl of a truncated form of Pbx3 (Pbx3 134–314) (lane 3), or a combination of full-length (1 μl) and truncated (2 μl) Pbx3 proteins (lane 2) was incubated with radiolabeled probe P3 and analyzed by EMSA. (E) One microliter of in vitro-translated FLAG-tagged Pbx1, Pbx2, or Pbx3 was incubated with radiolabeled probe P3 in the absence or presence of different in vitro-translated Hox proteins (1 μl) as indicated on the figure and analyzed by EMSA. Binding of the various Hox proteins (1 μl) by themselves to probe P3 is shown in lanes 23 to 27. □, nonspecific complex; FP, free probe. The lanes marked Retic (C and E) represent incubation of 2 μl of the unprogrammed reticulocyte lysate only.

cated form (Pbx3 134–314) and the full-length Pbx3 were incubated with the optimal Pbx3 binding site (P3) as a probe. Both full-length and truncated forms of Pbx3 showed two complexes when analyzed by EMSA (Fig. 1D, lanes 1 and 3). To determine whether the two complexes represent different oligomeric states of Pbx3, we mixed the short and full-length forms and analyzed the complexes by EMSA. When both full-length and truncated Pbx3 were present, an additional complex migrating with intermediate mobility could be detected (Fig. 1D, lane 2). The presence of a complex with intermediate mobility is best explained by the presence of a homodimer. The data suggest that Pbx3 has the ability to bind the selected Pbx3 binding sequence either as a monomer (lower complex) or as a homodimer (upper complex). The absence of a complex with a mobility that is intermediate between the lower form of full-length Pbx3 and the short forms indicates that the lower form of full-length Pbx3 migrates as a monomer. Furthermore, the data show that the Pbx3 region extending from amino acids 134 to 314, including the homeodomain, is sufficient for homodimeric DNA binding.

To determine whether the P3 probe allows cooperative DNA binding involving Hox and Pbx gene products, Hoxb-3, Hoxb-7, Hoxb-8, Hoxc-6, and Hoxd-4 were incubated with the three Pbx members as indicated and analyzed by EMSA (Fig. 1E). With the exception of weak binding of Pbx–Hoxc-6 heterodimers, the Hox proteins tested did not show significant cooperative DNA binding with Pbx to the selected Pbx3 consensus site (Fig. 1E, lanes 4 to 27).

Pbx1 contains an inhibitory domain that prevents intrinsic DNA binding. Since Pbx1 and Pbx2 exhibit affinities for the selected P3 binding site lower than that of Pbx3, it is conceivable that these proteins contain an inhibitory domain, blocking their intrinsic DNA binding activity. To determine whether inhibitory domains are present in Pbx1, we generated several truncated Pbx1 forms (Fig. 2A). SDS-PAGE of the in vitro-translated proteins showed that the various truncated forms of

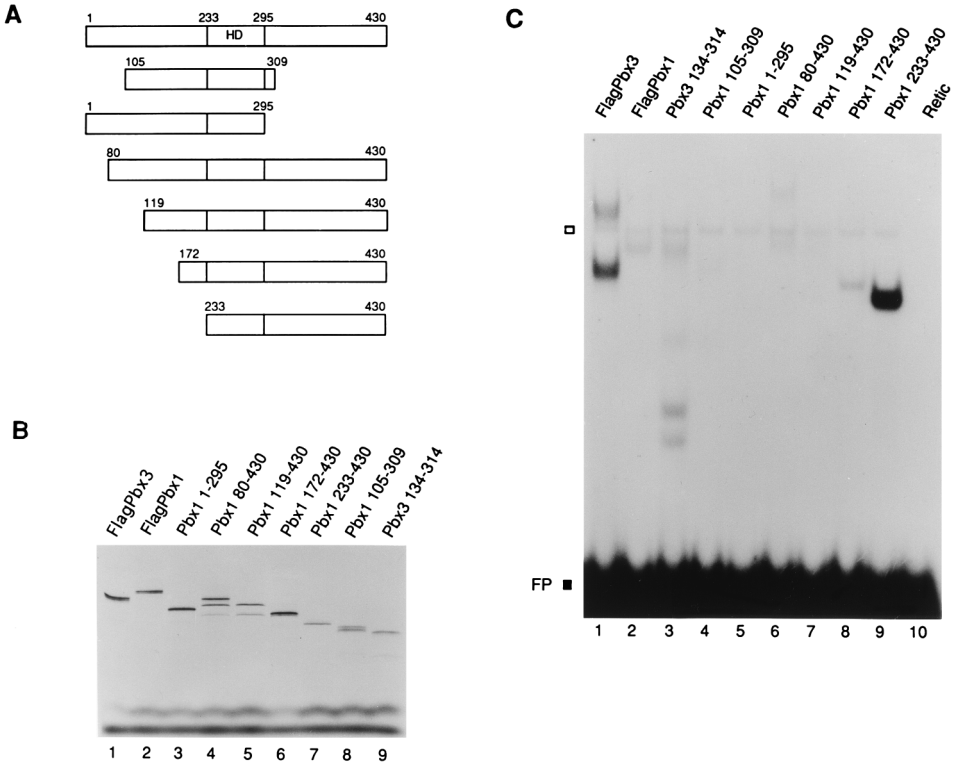

FIG. 2. Pbx1 domain in the N terminus prevents intrinsic DNA binding activity. (A) Schematic representation of the full-length Pbx1 protein and various truncated derivatives. Numbers are amino acid residues. HD, homeodomain. (B) SDS-PAGE analysis of the various Pbx1 and Pbx3 protein derivatives that are transcribed and translated in vitro in the presence of [^{35}S]methionine. (C) One microliter of the indicated in vitro-translated full-length or truncated versions of Pbx1 and Pbx3 was incubated with radiolabeled probe P3 and analyzed by EMSA. The lane marked Retic represents incubation of 2 μl of the unprogrammed reticulocyte lysate only. □, nonspecific complex; FP, free probe.

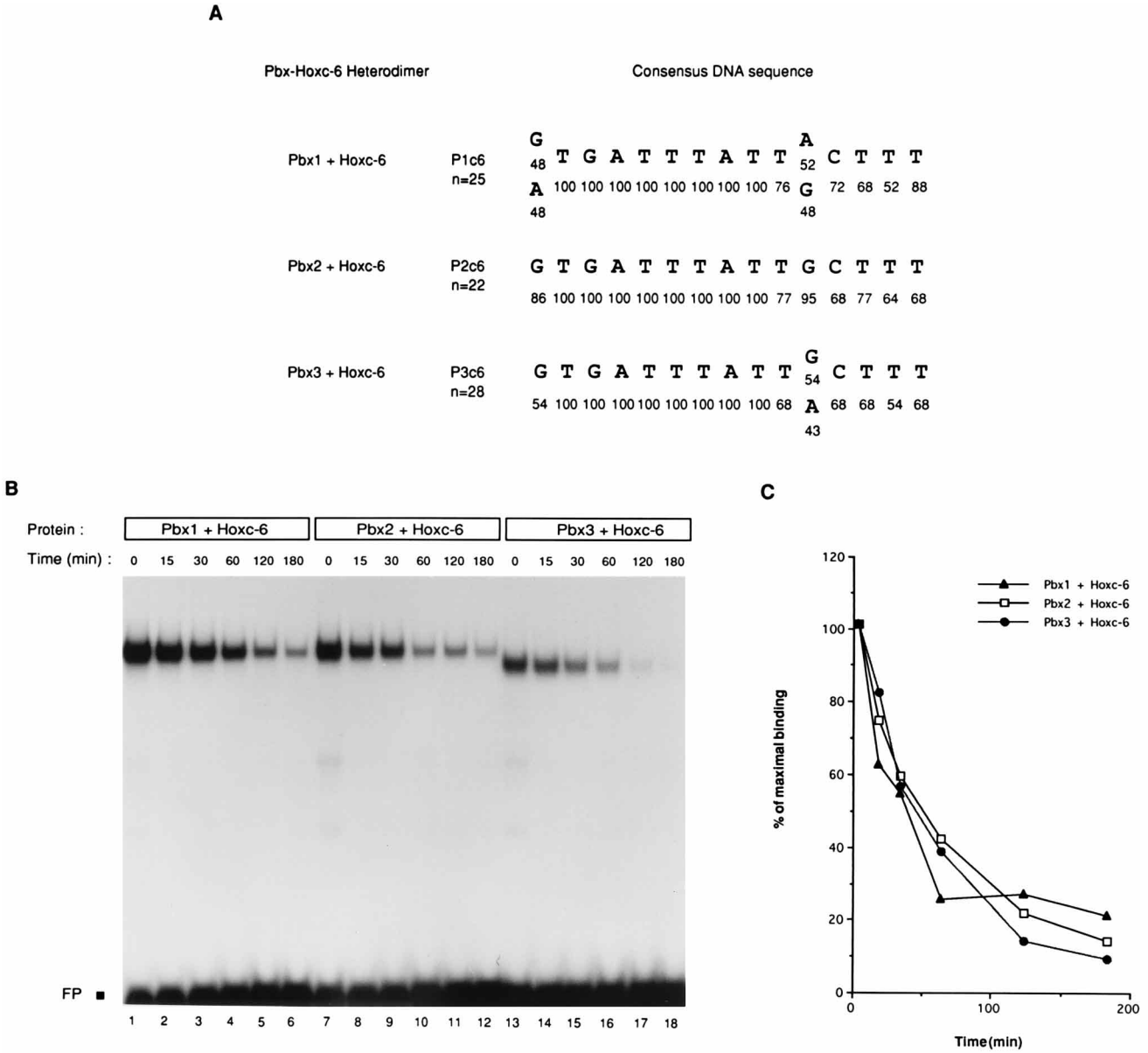

FIG. 3. Pbx1, Pbx2, and Pbx3 heterodimers with Hoxc-6 select identical binding sites. (A) The selected consensus DNA binding sites, P1c6, P2c6, and P3c6, for the various Pbx–Hoxc-6 heterodimer combinations are shown. n indicates the number of sequences used to determine the consensus DNA binding site. The number under each nucleotide represents the percentage of sequences containing the indicated nucleotide. (B) Dissociation rates of the various Pbx–Hoxc-6 complexes on the P1c6 binding site. One microliter of in vitro-translated Hoxc-6 and either 1 μl of Pbx1, Pbx2, or Pbx3 were incubated with radiolabeled probe P1c6 for 30 min. Subsequently, 500 ng of specific competitor DNA (unlabeled P1c6) was added to the binding reaction mixtures, and incubation continued for the times indicated on the figure prior analysis by EMSA. FP, free probe. (C) Quantitative representation of the results of the EMSA (B) as determined by PhosphorImager. The intensity of the shifted protein-DNA complex at time point zero is taken as 100% (maximal binding). The intensities of the shifted complexes at the time points 15, 30, 60, 120, and 180 min (*x* axis) are plotted as percentages of the maximal bound probe P1c6 (*y* axis).

Pbx1 were equally well translated (Fig. 2B). Full-length and truncated Pbx1 proteins were incubated with probe P3 and analyzed by EMSA. Deletion of the C-terminal domain of Pbx1 did not allow intrinsic DNA binding activity (Fig. 2C, lanes 4 and 5). Deletion of the Pbx1 N-terminal portion of the protein, removing 171 amino acids, also did not allow intrinsic DNA binding activity of Pbx1 (Fig. 2C, lanes 6 to 8). However, further deletion of 61 amino acids located immediately N terminal of the homeodomain allowed Pbx-1 to bind by itself with relatively high affinity (Fig. 2C, lane 9). These data indicate the presence of an inhibitory domain preventing intrinsic Pbx1 DNA binding.

Pbx1, Pbx2, and Pbx3 select identical binding sites with Hoxc-6. Previous studies indicated that Pbx proteins have the ability to differentially modulate the DNA binding activities of Hox and engrailed proteins (53). These studies showed that engrailed-Pbx heterodimeric complexes recognize a different target site than do Hox-Pbx heterodimers. The data described above indicate that Hoxc-6 has the ability to bind, albeit weakly, as a heterodimeric complex to an optimal Pbx3 binding

site. To determine whether Pbx1, Pbx2, and Pbx3 have the ability to differentially modulate the DNA binding activities of Hoxc-6, we performed a binding site selection using heteromeric complexes consisting of tagged Pbx and Hoxc-6. As a control, we also incubated Hoxc-6 by itself with the degenerate oligonucleotide pool.

In vitro-translated FLAG-tagged Pbx1, Pbx2, or Pbx3 was mixed with in vitro-translated Hoxc-6, incubated with a population of randomized oligonucleotides, and selected for high-affinity binding sites by immunoprecipitation as described above. After five rounds of selection and amplification, the binding sites were radiolabeled and used for further enrichment by EMSA. The shifted complexes were gel purified and DNA was isolated, cloned into pBSK, and analyzed by sequencing. The optimal binding site for each of the Pbx combinations was identical, containing a TGATTTATT sequence (Fig. 3A). A Pbx half-site, TGAT, was invariably present in all of the sequences. The consensus for the 3′ half of the sequence TTATT is closely related to the canonical Hox sequence (TAT[G/T]) identified previously for *Drosophila* and mammalian Hox gene products (1, 10, 44). Thus, Pbx-Hox heterodimers bind a bipartite sequence which contains a Pbx half site directly adjacent to a Hox site.

To determine the dissociation rates for the various Pbx–Hoxc-6 heterodimers, the Pbx-Hox complexes were incubated in the presence of probe P1c6, which contains the optimal binding site, TGATTTATT, until equilibrium for 30 min (Fig. 3B). The complexes were subsequently incubated with unlabeled DNA containing the same sequence, and aliquots were removed at different time points. These experiments were performed at 37°C, since the Pbx-Hox heterodimers did not dissociate from the DNA within 1 h at RT, suggesting a relatively high affinity for the selected binding site. The various samples were analyzed by EMSA, and the binding activities of the complexes at various time points were plotted (Fig. 3C). The data indicate that the dissociation rates of Pbx1, Pbx2, and Pbx3 when complexed to Hoxc-6 are similar (Fig. 3C). We also assayed for association rates but could not detect significant differences between the various combinations (39). In summary, the data indicate that the Pbx members select the same binding sites in the presence of Hoxc-6.

Binding site selection for Pbx1-Hox heterodimers. To examine whether various Hox gene products have distinct binding site preferences in the presence of Pbx, we performed binding site selections. In vitro-translated Hoxb-7, Hoxb-8, and Hoxc-6 were incubated in the presence of FLAG-tagged Pbx1, with a degenerate oligonucleotide pool containing 16 random base pairs. The binding site selection strategy was performed as described above. Three consensus sites, designated P1b7, P1b8, and P1c6, were established for Pbx1–Hoxb-7, Pbx1–Hoxb-8, and Pbx1–Hoxc-6 heterodimers, respectively (Fig. 4). All of the sequences showed a Pbx half-site, TGAT, and a Hox half-site, TTAT (Fig. 4). The consensus sites for Pbx1–Hoxc-6, Pbx1–Hoxb-7, and Pbx1–Hoxb-8 complex only differ in nucleotides located 3′ of the TTAT core (Fig. 4). To determine whether the differences in nucleotides flanking the core motif affect the DNA binding affinity of the Hox-Pbx1 complexes, we determined the dissociation rates for each of the complexes, using the various binding sites as probes as indicated (Fig. 5). The dissociation rates were determined under the same conditions described above. No significant differences in the dissociation rates could be detected with the various combinations of Pbx1-Hox heterodimers and probes P1b7, P1b8, or P1c6 (Fig. 5). Thus, Hox gene products derived from members within the Antennapedia family, in conjunction with Pbx1, select identical consensus binding sites.

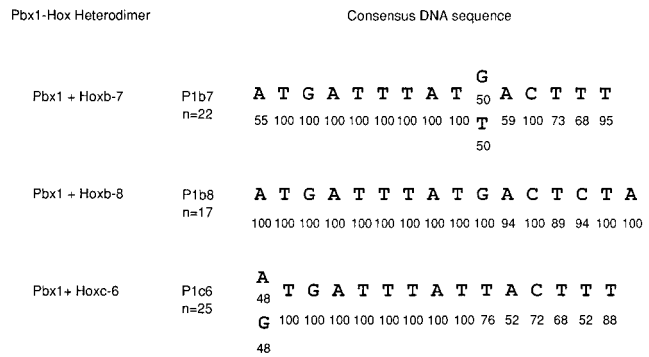

FIG. 4. Hoxb-7, Hoxb-8, and Hoxc-6 select identical binding sites in conjunction with Pbx1. The selected consensus DNA sequences, P1b7, P1b8, and P1c6, for the different Pbx1-Hox heterodimer combinations are shown. n indicates the number of sequences used to establish a consensus sequence. The number under each nucleotide indicates the percentage of sequences containing the indicated nucleotide.

To determine whether Pbx1 when complexed with Hox proteins derived from other families selects different binding sites, we used the same strategy for heterodimers consisting of Hoxb-3 or Hoxd-4 and Pbx1. Using these combinations, we were not able to identify a consensus binding site (39). To examine if Hoxb-3 and Hoxd-4 are capable of binding as heterodimers to the sites selected for DNA binding with Hoxb-7 (probe P1b7), Hoxb-8 (probe P1b8), and Hoxc-6 (probe P1c6), the Hox gene products in the presence of Pbx1 were analyzed by EMSA. As a comparison, we also analyzed the dissociation rates of Pbx1–Hoxb-7 (probe P1b7), Pbx1–Hoxb-8 (probe P1b8), and Pbx1–Hoxc-6 (probe P1c6) under the same conditions. Since the steady-state binding of Pbx1–Hoxb-3 and Pbx1–Hoxd-4 complexes was greatly reduced compared to those of Hox proteins derived from within the Antennapedia family, these experiments were performed at RT. When complexed to Pbx1, both Hoxb-3 and Hoxd-4 rapidly dissociate from each of the probes tested, in contrast to heterodimers between Pbx1 and Hoxb-7, Hoxb-8, or Hoxc-6 (Fig. 6). Pbx1–Hoxd-4 binding to probe P1b8 was barely detectable and was not assayed for dissociation rate. Taken together, these data indicate that whereas members of the Antennapedia family display similar affinities for selected binding sites, other families of Hox gene products exhibit distinct DNA binding properties.

Pbx proteins raise the DNA binding specificity of Hox gene products. Previous studies indicated that Pbx gene products greatly enhance the DNA binding affinity of Hox proteins (5, 6, 29, 53). One question that arises is whether Pbx proteins have the ability to increase the DNA binding specificity of the Hox proteins. To quantify DNA binding specificity, we used a strategy previously described for demonstrating an increase in **a**1-α2 DNA binding specificity compared to α2 DNA binding by itself (13). Briefly, increasing concentrations of unlabeled specific and nonspecific competitor DNA were incubated either in the presence of in vitro-translated Hoxb-8 by itself or in the presence of both Pbx-1 and Hoxb-8. After 30 min, ^{32}P-labeled probe was added, and the mixture was incubated for an additional 30 min and analyzed by EMSA. We used one of the selected optimal consensus binding sites, P1b8, as a probe. Two distinct, unlabeled competitor DNA fragments of the same size were used to compete for DNA binding activity with the labeled optimal binding site, P1b8. One of the unlabeled fragments was that of the optimal binding site, P1b8 (specific competitor); the other was a randomized sequence unrelated to the P1b8 sequence (nonspecific competitor).

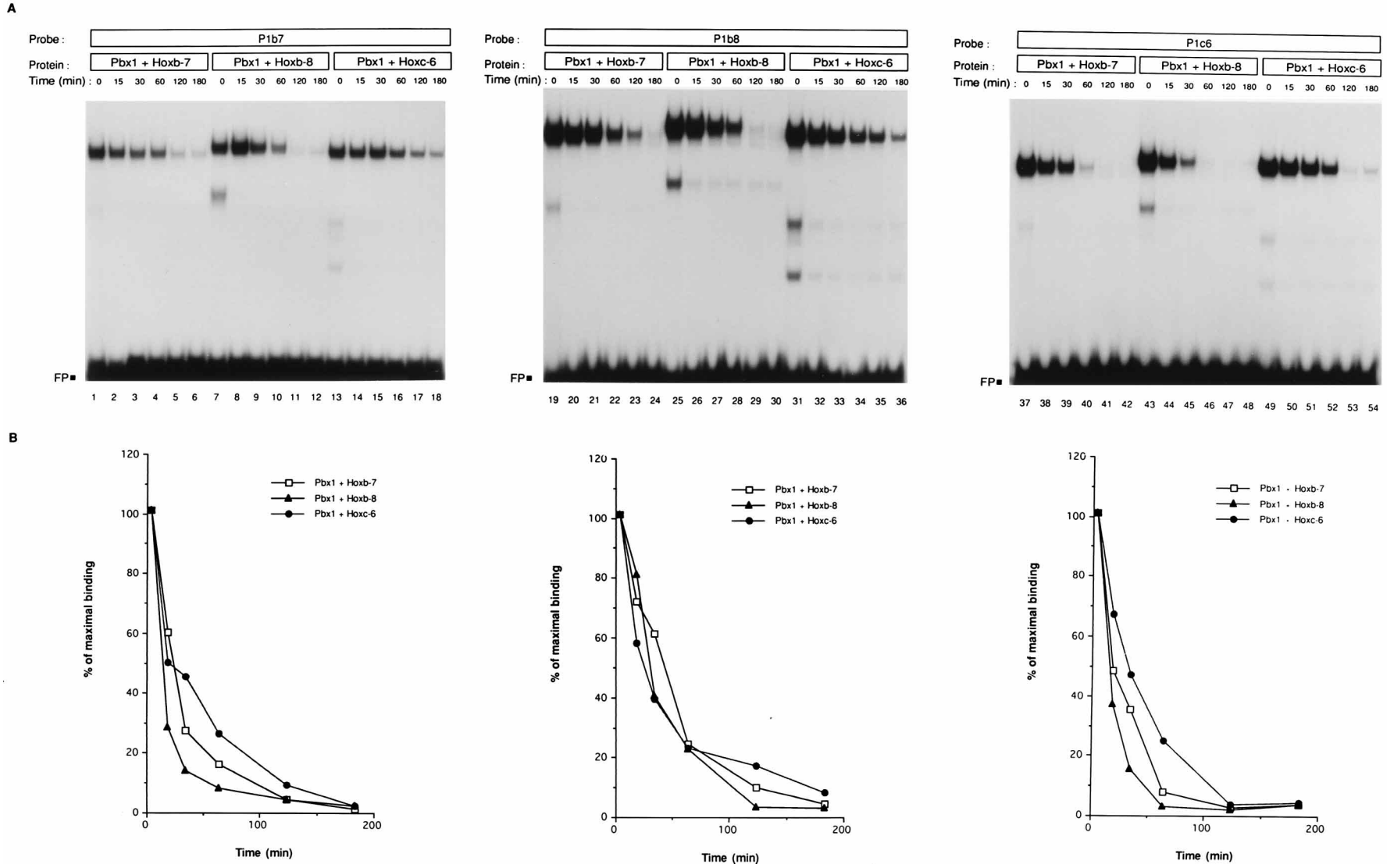

FIG. 5. Dissociation rates for Hoxb-7, Hoxb-8, and Hoxc-6 proteins as heterodimers with Pbx1. (A) One microliter of in vitro-translated Pbx1 and either 1 μl of in vitro-translated Hoxb-7, 1 μl of Hoxb-8, or 1 μl of Hoxc-6 were incubated with radiolabeled probe P1b7, P1b8, and P1c6 for 30 min. Subsequently, 500 ng of specific competitor DNA (unlabeled P1b7, P1b8, or P1c6, respectively) was added to the binding reaction mixtures and incubated for the times indicated on the figure prior analysis by EMSA. FP, free probe. (B) Quantitative representation of the results of the EMSA (A) as determined by PhosphorImager. The intensity of the shifted protein-DNA complex at time point zero is taken as 100% (maximal binding). The intensities of the shifted complexes at the various time points (*x* axis) are plotted as percentages of the maximal bound probe (*y* axis).

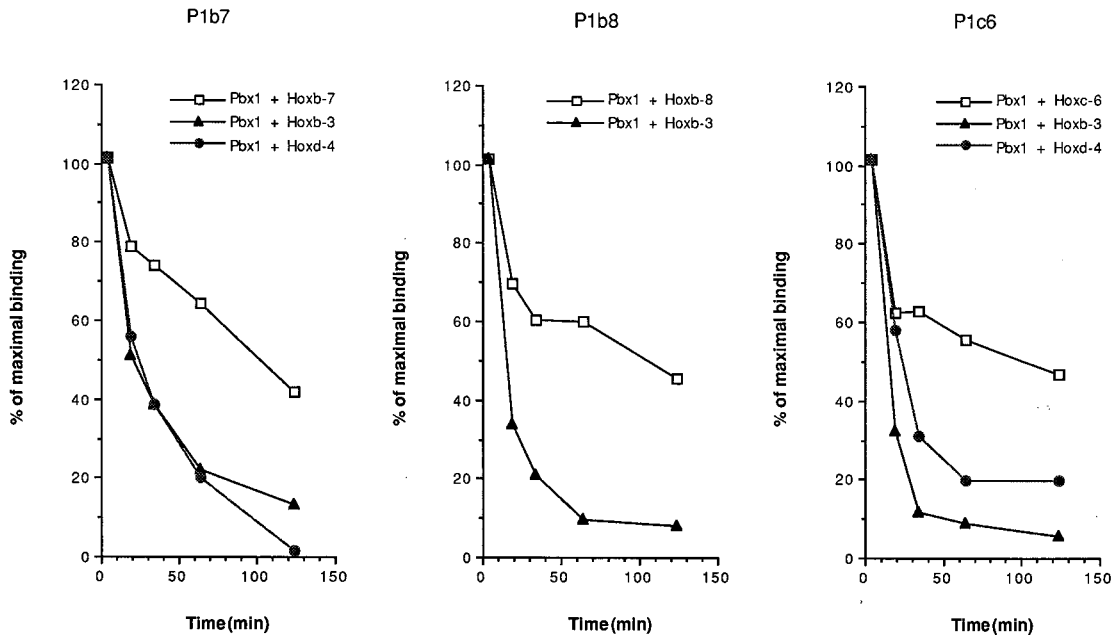

FIG. 6. Heterodimers between Pbx1 and Hoxb-3 or Hoxd-4 display DNA binding affinities for the selected binding sites different from those of Pbx1–Hoxb-7, Pbx1–Hoxb-8, or Pbx1–Hoxc-6 heterodimers. Various Pbx1-Hox heterodimers (mixture of 1 μl of in vitro-translated Pbx1 and 1 μl of in vitro-translated Hox) were incubated with radiolabeled probe P1b7, P1b8, or P1c6 for 30 min. Subsequently, 500 ng of specific competitor DNA was added to the binding reaction mixture and incubation continued for 15, 30, 60, or 120 min prior to analysis by EMSA. The shifted protein-DNA complexes were quantitated by using a PhosphorImager. The intensity of the shifted complex at time point zero was taken as 100% (maximal binding). Intensities of the shifted complexes at the time points 15, 30, 60, and 120 min (*x* axis) were plotted as percentages of the maximal bound probe (*y* axis).

Hoxb-8 was incubated, in the presence or absence of Pbx1, with increasing amounts of either specific competitor DNA (P1b8) or nonspecific competitor DNA (Fig. 7A). To quantify DNA binding, the complexes were examined by PhosphorImager. A twofold molar excess of specific competitor was required to reduce Pbx1–Hoxb-8 DNA binding by twofold (Fig. 7A, lanes 1 to 8). In contrast a 1,250-fold molar excess of nonspecific competitor DNA is required to achieve a 50% reduction for a Pbx1–Hoxb-8 heterodimer (Fig. 7A, lanes 9 to 18). Thus, a Pbx1–Hoxb-8 heterodimer can distinguish between the optimal binding site P1b8 and an unrelated site by a factor of approximately 600-fold.

A 50-fold molar excess of specific competitor was required to decrease Hoxb-8 DNA binding by itself twofold (Fig. 7A, lanes 19 to 25), whereas a 1,250-fold molar excess of nonspecific competitor was needed to compete for 50% of the Hoxb-8 DNA binding activity (Fig. 7A, lanes 26 to 33). Thus, in contrast to Pbx1–Hoxb-8 heterodimers, Hoxb-8 by itself can distinguish between the optimal binding site and an unrelated site by a factor of only 25-fold. Similar results were obtained by using Pbx2 and Pbx3 as heterodimeric partners (39).

In addition to P1b8, the selected binding site optimal for the Pbx1-Hoxb-8 heterodimer, we also used a DNA sequence containing an optimal Hoxb-7 binding site. This optimal Hoxb-7 binding site was established by performing a binding site selection assay as described above. The selected consensus for Hoxb-7 contained the core sequence TTTAATTG (data not shown). To determine the DNA binding specificity as described above we designed a template containing a Pbx1 binding site located adjacent the selected Hoxb-7 site, 5′CGAAA TTGATTGATCATTTAATTGAC 3′, referred to as P1+b7. A 10-fold molar excess of specific competitor DNA was required to achieve a 50% reduction in Pbx1–Hoxb-7 binding, while a 500-fold molar excess of nonspecific competitor DNA was needed to reduce Pbx1–Hoxb-7 binding by twofold (Fig. 7B, lanes 1 to 17). These data indicate that the Pbx1–Hoxb-7 heterodimer can distinguish between a template containing an optimal Hoxb-7 binding site and an unrelated site by a factor of 50-fold. To reduce Hoxb-7 binding by itself to 50%, a 50 fold-molar excess of specific competitor DNA was required (Fig. 7B, lanes, 18 to 24), while a 700-fold molar excess of nonspecific competitor DNA was needed to compete for 50% of Hoxb-7 DNA binding (Fig. 7B, lanes 25 to 32). Thus, Hoxb-7 by itself distinguishes between the DNA binding site P1+b7 and an unrelated site by a factor of 14. Taken together, the data show that Pbx proteins have the ability to raise the DNA binding specificity of Hox proteins for selected binding sites.

DISCUSSION

Members of a new class of atypical homeodomain proteins that includes extradenticle, Pbx1, Pbx2, and Pbx3 have recently been shown to function as partners for Hox proteins to bind cooperatively to DNA (3, 6, 30, 46, 47, 52, 53). These studies indicate that various combinations of Hox-Pbx and engrailed-Pbx heterodimers bind to distinct binding sites (5, 52, 53). We have now shown that the various Pbx gene products in conjunction with Hox proteins derived from within the Antennapedia family select identical optimal binding sites.

The question arises as to whether the selected binding sites bear any resemblance to target sites present in genes normally regulated by Pbx and Hox like proteins. Recently, one such target, the somatostatin gene, has been shown to contain a binding site for a Pbx-1–STF-1 heterodimer (ATGATTAATT ACT). Interestingly, this target site is identical over 12 out of 13 nucleotides present and surrounding the selected binding site of Pbx1 and Hoxb-7 (ATGATTTATTACT) (42).

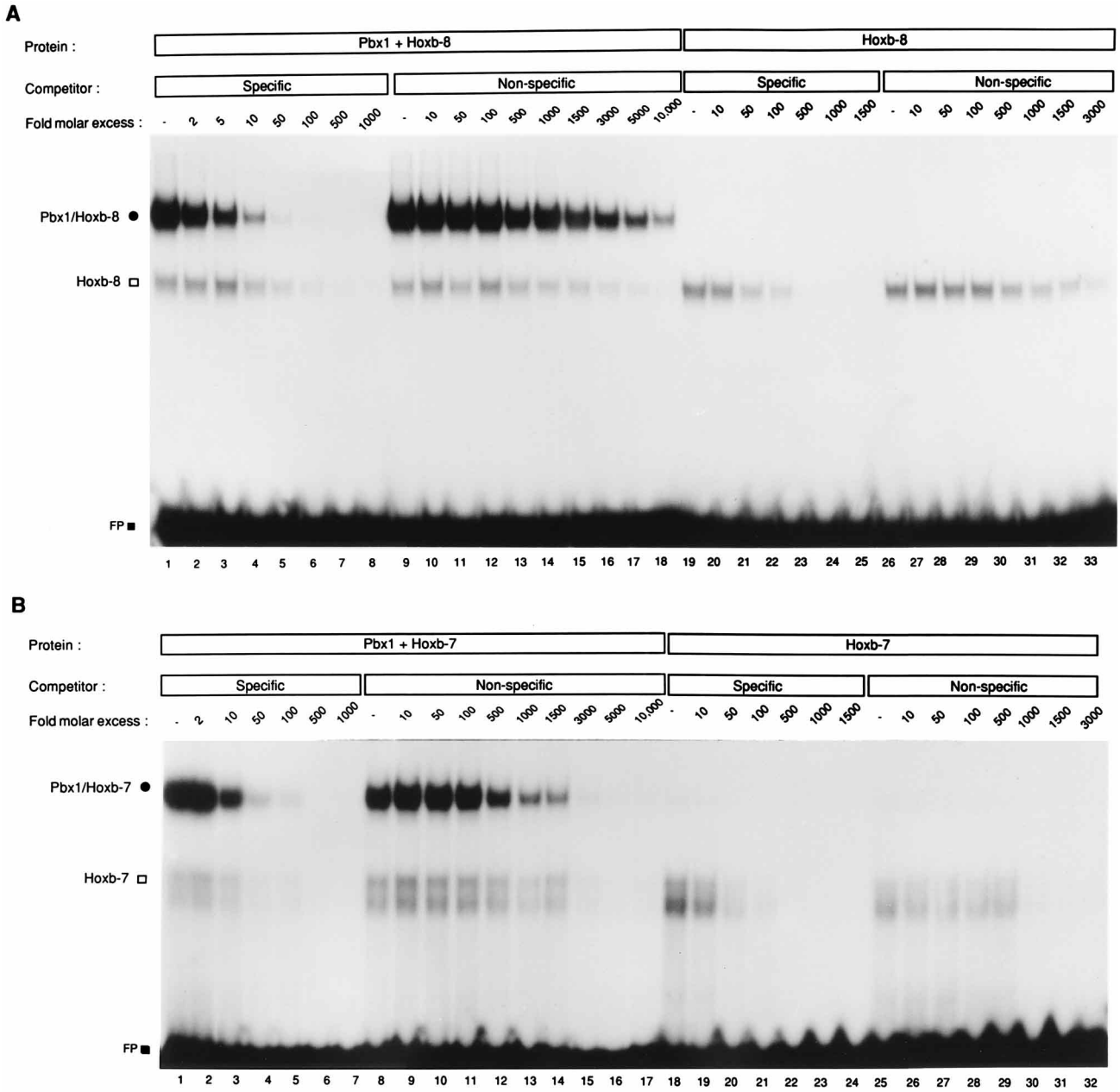

FIG. 7. Pbx1 increases the DNA binding specificity of Hox proteins. (A) One microliter of in vitro-translated Pbx1 and 1 μl of in vitro-translated Hoxb-8 or 1 μl of only Hoxb-8 were incubated for 30 min with increasing amounts of specific or nonspecific competitor DNA as indicated. Subsequently, radiolabeled probe P1b8 was added to each binding reaction mixture and incubated for an additional 30 min. Samples were subsequently analyzed by EMSA. (B) One microliter of in vitro-translated Pbx1 together with 1 μl of in vitro-translated Hoxb-7 or 1 μl of Hoxb-7 alone was incubated for 15 min with increasing amounts of specific or nonspecific competitor DNA as indicated. Radiolabeled probe P1+b7 was then added to each binding reaction mixture, and incubation continued for another 15 min. Samples were analyzed by EMSA. FP, free probe.

Pbx1, Pbx2, and Pbx3 display distinct intrisic DNA binding activities. The studies described above show that Pbx proteins in the presence of Hox gene products select different optimal binding sites than do Pbx proteins by themselves. Although previous studies have indicated that Pbx1, Pbx2, and Pbx3 by themselves select identical sites, we now show that Pbx1 and Pbx2 exhibit DNA binding properties different from those of Pbx3. The previous studies used Pbx protein fragments lacking the N and/or C terminus that were generated in *E. coli* and did not reveal significant differences in DNA binding affinities between the various members of the Pbx family (26, 30). In the studies described here, using full-length gene products and relatively low concentrations of protein, the data indicate that Pbx3 shows an intrinsic DNA binding activity which is clearly distinct from those of Pbx1 and Pbx2.

Pbx3 selects a binding site containing a tandem repeat of a bipartite TGATTGAT sequence. Since only Hoxc-6, from all the Hox proteins tested in vitro for cooperativity with Pbx3 on this selected binding site, showed weak cooperativity, it will be interesting to determine whether the same site in nuclear ex-

tracts is occupied by Pbx3 alone or by heterodimers between Pbx3 and Hox gene products or other protein partners.

Specificity of Hox proteins. The studies described here indicate that Pbx does not raise the selectivity of Hox proteins within the Antennapedia family for high-affinity binding sites. These data suggest that Pbx proteins do not allow these Hox proteins to discriminate between high-affinity binding sites and thus do not provide an explanation for the high biological specificity that is displayed by these gene products.

This raises the following question: how can one particular binding site recognized by various Hox gene products be involved in specific gene regulation? One hypothesis suggests itself. Hox proteins interact with various combinations of enhancer and promoter binding proteins to achieve specific gene regulation. A similar dilemma has been previously described for members of the HLH family. Heterodimers of HLH proteins, involving E2A and myogenic regulators, bind a common DNA sequence, designated the E2 box (38). The E2 box is found in the immunoglobulin enhancers (11, 27), in a number of pancreatic specific enhancers (36), and in a battery of muscle-specific promoters (15). The E2 box was also identified as the optimal binding site by binding site selection for E2A homodimers and E2A-MyoD heterodimers (2), similar to results described here for Hox-Pbx heterodimers. For HLH proteins, a number of these coactivators or repressors have been identified. For example, they include NF-κB and members of the ETS family, which together allow developmental-stage-specific expression of the immunoglobulin genes (37). For the Hox-Pbx heterodimers, it is less clear and will require a further characterization of promoter and enhancer regions that control Hox responsive genes.

While there is no clear difference in selectivity, Pbx-Hox heterodimers exhibit enhanced DNA binding specificity for a selected binding site compared to that of Hox proteins by themselves. For example, while a Pbx1–Hoxb-8 heterodimer has the ability to distinguish between a selected binding site and an unrelated sequence by a factor of 600-fold, Hoxb-8 by itself can distinguish between the same optimal binding site by a factor of only 25-fold. We have also tested whether Pbx2 and Pbx3 have the ability to enhance the DNA binding specificity of Hoxb-8 and obtained similar results (39). Even when a DNA sequence containing a Pbx1 site next to a selected site optimal for Hoxb-7 is used, Pbx1 enhances the DNA binding specificity of Hoxb-7.

In summary, previous studies have indicated that the formation of Hox-Pbx heterodimers increases the DNA binding affinity compared to Hox gene products by themselves. The studies addressed here indicate that the formation of Hox-Pbx heterodimers also enhances the specificity for DNA binding sites compared to the DNA binding specificity of Hox gene products by themselves. However heterodimers formed between Pbx and various Hox members within the Antennapedia family do not increase selectivity.

Biochemical similarities between a1-α2 and Pbx-Hox heterodimeric complexes. A wide variety of families of DNA binding proteins is known to interact to bind DNA as heterodimers. These include the leucine zipper, steroid receptors, and HLH proteins. The best-studied example of homeodomain heterodimeric proteins involves **a**1 and α2 (16). **a**1 and α2 are expressed in two different cell types in yeast, **a** and α cells respectively. They are coexpressed only in diploid cells. The **a**1-α2 heterodimer is thus present only in diploid cells, repressing a set of haploid-specific genes. α2 has the ability to bind DNA as a homodimer (16). The DNA binding properties of **a**1-α2 are distinct from those of α2 and a1 alone (13). The DNA binding affinity of an **a**1-α2 heterodimer is much higher than that of an α2 homodimer (13), and the specificity of an α2 homodimer for a haploid-specific operator, is much weaker than that of an **a**1-α2 heterodimer. Taken together, Pbx-Hox heterodimers have biochemical characteristics that are very similar to those of **a**1 and α2. Heterodimerization of homeodomain proteins may very well be a common mechanism, in both yeast and multicellular organisms, for modulating the individual DNA binding properties of homeotic proteins.

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