

# Distinct HOX N-terminal Arm Residues Are Responsible for Specificity of DNA Recognition by HOX Monomers and HOX·PBX Heterodimers\*

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Michael L. Phelan<sup>†§¶</sup> and Mark S. Featherstone<sup>‡§¶\*\*</sup>

From the <sup>‡</sup>McGill Cancer Centre, and Departments of <sup>§</sup>Medicine (Division of Experimental Medicine) and <sup>¶</sup>Oncology, McGill University, Montréal, Québec H3G 1Y6, Canada

**Dimerization with extradenticle or PBX homeoproteins dramatically improves DNA binding by HOX transcription factors, indicating that recognition by such complexes is important for HOX specificity. For HOX monomeric binding, a major determinant of specificity is the flexible N-terminal arm. It makes base-specific contacts via the minor groove, including one to the 1st position of a 5'-TNAT-3' core by a conserved arginine (Arg-5). Here we show that Arg-5 also contributes to the stability of HOX·PBX complexes, apparently by forming the same DNA contact. We further show that heterodimers of PBX with HOXA1 or HOXD4 proteins have different specificities at another position recognized by the N-terminal arm (the 2nd position in the TNAT core). Importantly, N-terminal arm residues 2 and 3, which distinguish the binding of HOXA1 and HOXD4 monomers, play no role in the specificity of their complexes with PBX. In addition, HOXD9 and HOXD10, which are capable of binding both TTAT and TAAT sites as monomers, can cooperate with PBX1A only on a TTAT site. These data suggest that some DNA contacts made by the N-terminal arm are altered by interaction with PBX.**

Many developmental events are under the control of homeobox genes, whose products regulate the transcription of effector genes by binding to specific DNA sequences. The classic example involves proteins encoded by the homeotic selector complex (*Hom-C*) of the fruit fly *Drosophila melanogaster*, which determine the identity of body segments along the anteroposterior axis. A similar function is performed in mammals by the homologous *Hox* genes. These genes specify the identity of mesodermal and ectodermal derivatives such as vertebrae and rhombomeres (reviewed in Ref. 1), as well as contributing to limb patterning. Homologous genes are expected to be found in all organisms with an anteroposterior axis. The 39 *Hox* genes of mice and humans are located in four clusters, termed *HOXA* to *HOXD*, homologous to the *Hom-C* of insects. Genes that encode similar products occupy the same position in each cluster, and this permits the functional partitioning of the *Hox* family into 13 paralog groups based on the relative position of a particular gene. The expression boundaries of the *Hox* genes

during embryogenesis, which are critical for patterning, are colinear with genomic position (2, 3). With few exceptions, the smaller the paralog group number, the earlier and more anteriorly the genes of that group are expressed.

Despite a wealth of genetic and biochemical data on the *in vivo* and *in vitro* activities of *Hox* gene products, relatively little is known about how *Hox* genes from different paralog groups produce the discrete regulatory effects required at particular axial positions. HOX proteins have a single DNA binding domain, the homeodomain, that consists of a flexible N-terminal arm and three  $\alpha$ -helices. When bound to DNA the third helix, or recognition helix, makes a number of DNA contacts in the major groove. The N-terminal arm extends from the packed helical structure and lies along the minor groove. The consensus HOX binding site consists of a 5'-TAAT-3' core followed by a selection against C at the next two 3'-positions. Sequences 5' and 3' to the midpoint of the TAAT are recognized by the N-terminal arm in the minor groove and helix three in the major groove, respectively. There are clear deficiencies in the monomer binding activity of HOX proteins. The HOX consensus site is short and somewhat degenerate and so statistically far too common to direct a given HOX protein to select the promoters it is intended to regulate. Furthermore, by comparison to the crystal structure of the related engrailed (EN) homeodomain-DNA complex (4), the TAAT core is specified by residues of which all but one, residue three in the N-terminal arm, are completely conserved across the HOX family. As a result, there are predominantly only three variations of HOX monomer binding. Proteins from paralog groups 2–8, as well as EN, have an arginine at position 3 and exhibit a preference for TAAT-containing sites. HOX proteins from the large ABDOMINAL-B (ABD-B) subfamily (paralog groups 9–13), which have Lys-3, recognize both TTAT and TAAT. HOX proteins from paralog group one are not conserved at residue three and, as demonstrated for HOXA1 (5) and HOXB1 (6), exhibit weak DNA-binding activity. While weaker preferences for flanking sequences have been observed *in vitro* (7–9), this specificity has not been shown to account for differences in regulatory ability. Paradoxically, chimeric studies in *Drosophila* demonstrate that the homeodomain and immediate flanking regions are major determinants of functional specificity (10–14). The flexible N-terminal arm, for which several functions have been described (15–18), is implicated in a number of these studies (12, 19, 20). Many of the implicated residues are not predicted to contact DNA in monomeric binding, suggesting they contribute to specificity by contacting other proteins, including cofactors (17, 21–23). Alternatively, the DNA binding conformation may be altered in these higher order complexes, thereby allowing these residues to contact DNA.

The product of another *Drosophila* homeobox gene, *extradenticle* (*exd*), has been shown to cooperate with several HOM

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<sup>\*\*</sup> Chercheur-Boursier of the Fonds de la Recherche en Santé du Québec. To whom correspondence should be addressed: McGill Cancer Centre, 3655 Drummond St., Montréal, Québec, Canada H3G 1Y6. Tel.: 514-398-8937; Fax: 514-398-6769; E-mail: featherstone@medcor.mcgill.ca.

proteins for binding to specific DNA elements (24, 25) and regulation of target genes *in vivo* (26). This is consistent with genetic evidence that *exd* mutations affect the ability of *HOM* genes to specify segment identity (27). The three *exd* homologues in humans comprise the *PBX* family (28). Mammalian HOX and PBX proteins have also been shown to be able to cooperatively bind DNA (29–32). A critical HOX determinant for interaction is the pentapeptide, found N-terminal to the homeodomain of many HOX proteins (29, 30, 33–35). It has the consensus (Y/F)PWM(K/R). As well, a diverged tryptophan-containing sequence found in a number of ABD-B subfamily members has been shown to permit cooperative interactions with PBX (36). On its own, the pentapeptide is capable of increasing PBX DNA-binding activity (33), which suggests that it may stabilize or induce a particular conformation of PBX. In PBX, regions within and immediately C-terminal to the homeodomain are important for cooperativity (29, 37). Although the homeodomains of the HOX-PBX heterodimer are not predicted to contact one another, they appear to act as protein interfaces (25, 34, 37, 38). Most of the *in vitro* studies to date have used an experimentally derived heterodimer binding site (39, 40) containing a 5'-TGATTGAT-3' core. Such heterodimer sites are functional *in vivo*, as the *Hoxb1* autoregulatory enhancer (ARE)<sup>1</sup> contains three related sequences that are required to direct *Hoxb1*-like expression of a *lacZ* transgene in the hind-brain (6). This sequence is recognized as adjacent TGAT half-sites, with the PBX and HOX partners binding the 5' and 3' half-sites, respectively, in a head-to-tail arrangement (37, 41). The close proximity of the two homeodomains in the HOX-PBX heterodimer raises the possibility of an overlap in base-specific and DNA backbone contacts. Overlaps have been suggested to contribute to the DNA binding cooperativity of Oct-1 POU subdomains (42, 43) and may even dictate specificity.

Interaction with PBX molecules dramatically increases the strength and selectivity of DNA binding by HOX proteins (24, 25, 29, 30, 33), both of which are likely to contribute to HOX functionality. However, the ability of these interactions to produce functional specificity between HOX proteins is poorly understood. In this paper, we further characterize the HOX-PBX complex on DNA in an attempt to elucidate mechanisms by which this specificity may arise. As an initial step, the importance of the diverged HOXA1 pentapeptide was confirmed. We also show that different HOX-PBX complexes exhibit differences in stability based on sequence variations at the 2nd position in the 3' half-site. This position is predicted to be contacted by the HOX N-terminal arm via the minor groove. The same relative position distinguishes the three major HOX monomer binding types. Although we provide evidence that the HOX N-terminal arm remains in the minor groove in the cooperative complex, the residues responsible for differences in the binding characteristics of HOXA1 and HOXD4 monomers are not responsible for heterodimer specificity. This is consistent with the observation that monomer and heterodimer preferences at this position are not the same. These results suggest that conformational changes in the N-terminal arm of the HOX partner result from the HOX-PBX interaction. The ability of different residues to confer N-terminal arm DNA binding specificity in different contexts adds an important dimension to the functional versatility of the homeodomain N-terminal arm.

#### EXPERIMENTAL PROCEDURES

**Plasmid Construction**—An initial step in generating all the *Hoxa1*-containing plasmids used in this study was the generation of pPGK-Hoxa1(HD+), a eukaryotic expression vector encoding only the home-

odomain-containing product of *Hoxa1* (44). This was made by inserting the *Bam*HI/*Hinc*II fragment of pHoxa1(HD+) (30) into pCEV-1 (5). pPGK-Hoxa1-vp16 was created by first introducing an *Xba*I site at the 3' end of the open reading frame by inserting a double-stranded oligonucleotide, O-220 (CTACCTCAGACTCTAGA, top strand only), into an *Acl*I site. Then an *Xba*I fragment from pPGK-Hoxd4-vp16 (30) encoding the activation domain of VP16 was ligated into this site. This replaced the last six codons of *Hoxa1* with codons 412–490 of *vp16*. The vector for bacterial expression of His-tagged HOXA1, pTrcB-Hoxa1, was generated by subcloning the *Hind*III fragment of pPGK-Hoxa1(HD+) into pTrcB (Invitrogen). The product of this vector contains HOXA1 sequences from 16 amino acids N-terminal to the pentapeptide to the C terminus. The two amino acid substitution in the pentapeptide (WM to AA) was created by site-directed mutagenesis using oligonucleotide O-176 (AGGGTTTCTTTTAACTTTTCGCCGCTCAAAGGTCTGCG). The N-terminal arm R5A substitution was created by polymerase chain reaction amplification of pPGK-Hoxa1(HD+) using the oligonucleotides O-245 (GTGGGTCAACCCCAACGAGTGGCCACCAATTTC) and 1.6–2 (AAGAAGTCTAGATCAGTGGGAGGTAGTCAG). The N-terminal arm N2K,A3R substitution is described elsewhere (5). These mutant *Hoxa1* sequences were subcloned into both pPGK-Hoxa1(HD+)-vp16 and pTrcB-Hoxa1 using convenient restriction sites. pTrcA-Hoxd4 was created by subcloning a *Pst*I/*Hind*III fragment from p4.2 (45) directly into pTrcA. The product of this construct contained HOXD4 sequences from 22 amino acids N-terminal to the homeodomain to the C terminus. All constructs were confirmed by sequencing.

**Protein Expression and Purification and Transfections**—HOXA1 and HOXD4 used in these experiments were expressed as His-tagged fusion proteins and batch-purified from MC1061 bacteria using an imidazole elution protocol (Invitrogen). These were stored at –80 °C in 200 mM Na<sub>2</sub>PO<sub>4</sub> (pH 7.4), 500 mM NaCl, 10 mM  $\beta$ -mercaptoethanol, 0.01% Triton X-100, 300 mM imidazole, and 20% glycerol. Amounts of the partially purified proteins were estimated by Coomassie staining and the Bradford assay. PBX1A, HOXD9, and HOXD10 were produced as described previously (30) using a TnT *in vitro* transcription/translation kit (Promega). Transient transfections into retinoic acid-differentiating P19 embryonal carcinoma cells were as described previously (30).

**Electrophoretic Mobility Shift Assay (EMSA) and Dissociation Rate Experiments**—EMSA was conducted as described previously (30) with the following modifications. Approximately 1.5–2.5 ng of His-tagged HOX protein and 1.7  $\mu$ l of reticulocyte lysate expressing full-length PBX1A or a mock translation were used per 10- $\mu$ l reaction. Final buffer conditions were 10 mM Tris (pH 7.5), 75 mM NaCl, 30 mM imidazole, 1 mM dithiothreitol, 1 mM EDTA, 540 ng/ml bovine serum albumin, 12% glycerol, and 80 (Figs. 2A, 3, and 5) or 320 (Figs. 5 and 6) ng of poly(dI-dC)/10  $\mu$ l. The DNA probes used were as follows: O-160, CGAATTTGATTGATTGATGCTTTAATTGAC; O-209, GCGGTGATGATGGGCGCTG; O-262, CCCATGATTNATGGCCCCCCCC.

O-160 was subcloned into the *Sma*I site of pBS<sub>+</sub>, whereas O-209 and O-262 were subcloned into the *Srf*I site of pCR-Script (Stratagene). This facilitated verification of the probe sequence and the isolation of all four variations of O-262. The resulting plasmids were digested with *Bam*HI/*Kpn*I, *Bam*HI/*Not*I, and *Bam*HI/*Sma*I, respectively, and labeled with [ $\alpha$ -<sup>32</sup>P]dATP by Klenow. The labeled fragment was purified using the Crush and Soak protocol (46).

For dissociation rate experiments, 400 ng/ $\mu$ l unlabeled double-stranded competitor DNA (O-160) was added at time 0 following preincubation of proteins with labeled DNA. 5- $\mu$ l samples were loaded at the indicated time points. The fraction of labeled DNA bound at each time was determined using a Fuji Bas2000 Imager. The log of this value was plotted against time to get a line of best fit where the slope is equivalent to the negative of  $K_d$ , the dissociation rate constant. Half-lives were determined from the equation  $t_{1/2} = -\log(0.5)/K_d$ .

The figures showing EMSA data were produced electronically in Freehand 5.0 for Macintosh. Autoradiographs were scanned as reflective gray scale images using a Umax UC1260 scanner and the Auto-density function. The resulting images were saved as PICT files in Adobe Photoshop 3.0 for Macintosh and then placed into Freehand for labeling. Other than uniform size changes, the images were unmodified.

#### RESULTS

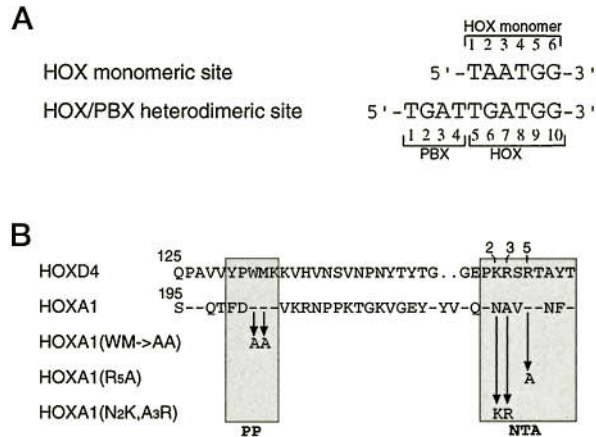
**The Pentapeptide of HOXA1 Is Required for Interaction with PBX1A Both *In Vitro* and in Transfected Cells**—We have previously shown that both HOXD4 and HOXA1 can cooperatively bind DNA with PBX1A and that for HOXD4 this ability is dependent on an intact pentapeptide motif (5). Specifically, mutation of the HOXD4 pentapeptide from YPWMK to YPAAK

<sup>1</sup> The abbreviations used are: ARE, autoregulatory enhancer; EMSA, electrophoretic mobility shift assay.

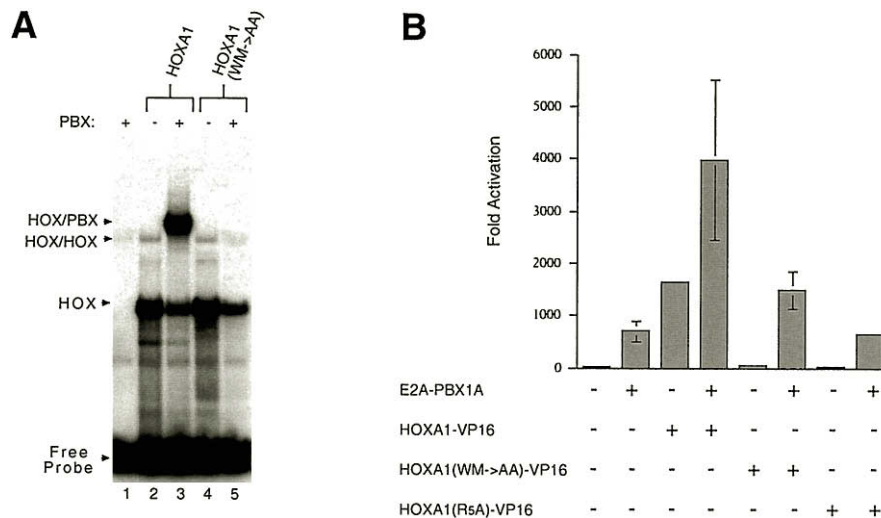
is sufficient to abrogate interaction with PBX1A. HOXA1 also contains a pentapeptide; however, the motifs of HOXA1 and HOXD4 share only the sequence WMK and have no flanking homology (Fig. 1B). To determine the importance of the HOXA1 pentapeptide for interaction with PBX, we tested the effect of the same WM to AA pentapeptide conversion, HOXA1(WM to AA), on the formation of the HOXA1-PBX complex *in vitro*. To assess whether the weak DNA-binding activity of HOXA1 monomers was affected by substitutions in the pentapeptide, we used conditions that revealed monomer binding activity (see

"Experimental Procedures") and a DNA probe containing binding sites for both heterodimeric and HOX monomeric complexes. Although the WM to AA substitution in the HOXA1 pentapeptide had no apparent effect on the level of monomer binding (Fig. 2A, lanes 2 and 4), it completely abolished detectable HOX-PBX complex formation (compare lanes 3 and 5).

To confirm this result, we used a luciferase reporter assay to look at the ability of transfected HOXA1 and PBX1A to cooperatively bind DNA *in vivo*. Because unmodified HOX and PBX proteins are poor transcriptional regulators, versions of HOXA1 and PBX1A proteins containing strong activation domains were used. These were the E2A-PBX1A oncoprotein and a HOXA1 protein to which the transcriptional activation domain of VP16 was fused (HOXA1-VP16). Endogenous HOX and PBX proteins are broadly expressed in differentiated cell types and are thus available to cooperate with proteins expressed from transfected vectors in the experiments reported below. As seen previously, HOXA1 has intrinsically weak DNA-binding activity (5). It was therefore not surprising to find that the HOXA1-VP16 fusion protein was completely unable to affect the activity of either a minimal promoter or a promoter containing five copies of the consensus HOX monomer binding site of Fig. 1 (data not shown). In contrast, HOXA1-VP16 produced a 1650-fold activation of a reporter that contained five copies of a PBX/HOX cooperative binding site, pML(5xHOX/PBX) (Fig. 2B). The activity of the E2A-PBX1A oncoprotein was likewise dependent on the presence of these heterodimer binding sites and produced a 700-fold activation. When transfected together, HOXA1-VP16 and E2A-PBX1A were able to generate a 4000-fold increase in luciferase activity. Thus, HOX and PBX proteins appear to participate in the cooperative DNA binding interaction seen in the *in vitro* binding assay to achieve synergistic transcriptional activation *in vivo*. If this is the case, this activity also should be dependent on an intact pentapeptide. Introduction of the WM to AA pentapeptide mutation into the HOXA1-VP16 construct abolished its ability to cooperate with E2A-PBX1A, thus confirming this hypothesis (Fig. 2B). Additionally, the observation that point mutations in the pentapeptide reduced the activity of HOXA1-VP16 alone from 1650- to



**FIG. 1. Comparison of DNA binding sites and the sequences of HOX proteins used in this study.** A, consensus sites for HOX monomeric DNA binding (*top*) and HOX-PBX cooperative complexes (*bottom*). The sequences recognized by the HOX homeodomain in the monomeric and heterodimeric sites are aligned vertically. DNA binding by the PBX partner extends the recognition sequence in the 5' direction (37, 41). B, pentapeptide, linker, and N-terminal arm sequences of HOXD4 and HOXA1. The pentapeptides and N-terminal arms are indicated by shaded boxes labeled PP and NTA, respectively. Dashes indicate identity, and periods represent positions not present in HOXD4. The amino acid positions of the sequences shown, relative to the start of each protein, are shown above the most N-terminal residue. The pentapeptide and N-terminal arm point mutations of HOXA1(WM to AA), HOXA1(R5A), and HOXA1(N2K,A3R) are indicated by arrows. Positions 2, 3, and 5 of the HOX N-terminal arm are noted.



**FIG. 2. Conserved residues in both the pentapeptide and N-terminal arm of HOXA1 are required for interaction with PBX1A both *in vitro* and in transfected P19 cells.** A, comparison of the ability of HOXA1 and HOXA1(WM to AA) to bind DNA as a monomer or as a heterodimer with PBX1A. Specific bands corresponding to HOX-PBX heterodimer, HOXA1 homodimer, and HOXA1 monomer are identified. The low level of HOXA1 homodimer relative to the HOX-PBX heterodimer indicates that it is poorly cooperative. Unlike the HOX-PBX complex, it is also not dependent on an intact HOXA1 pentapeptide. The DNA probe corresponds to O-160 (see "Experimental Procedures"). B, the effect of transfected expression vectors on luciferase expression from a reporter containing five copies of both the HOX monomeric and the HOX-PBX heterodimeric sites shown in Fig. 1A. Transfections were performed using retinoic acid-differentiating P19 cells. Plus signs next to the products of the expression vectors indicate which plasmids were added. Values are expressed as fold activation over transfection of the reporter plasmid alone with carrier DNA. Within each experiment, the fold activation for HOXA1-VP16 was set to the average for all the experiments (1648-fold), and the values for other conditions are expressed relative to this value. Bars indicate the standard deviation.



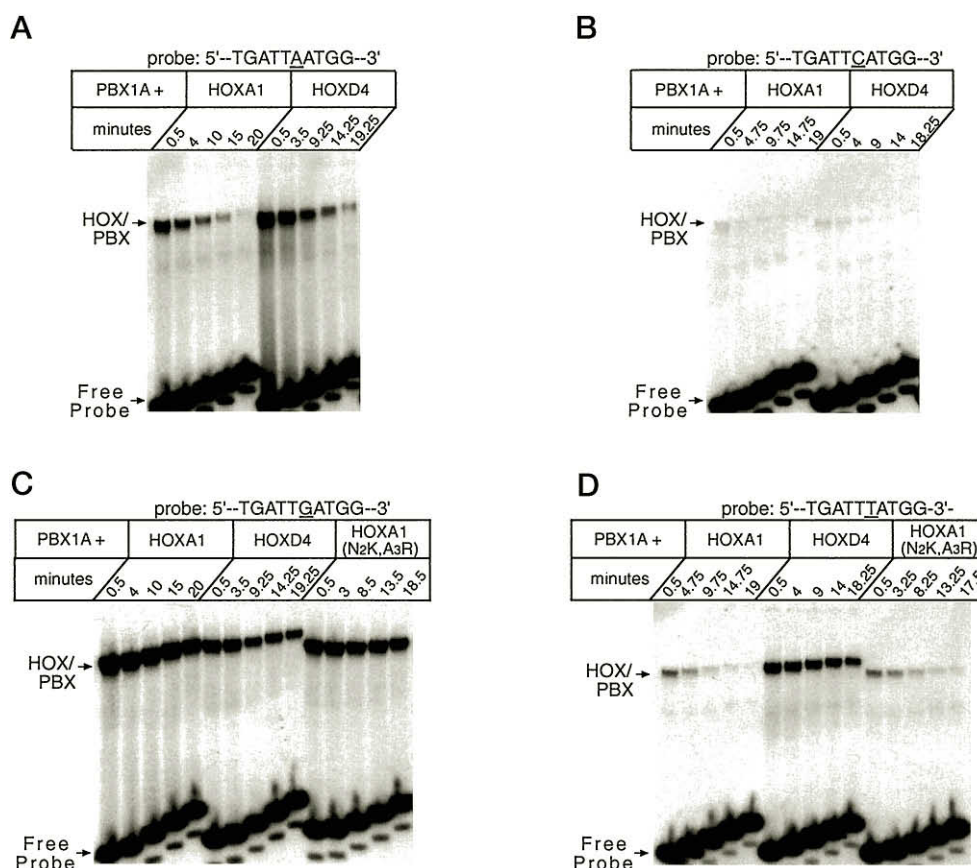


FIG. 3. **Differences in HOX-PBX complex stability are revealed on heterodimeric binding sites that vary at position 6.** The rate of dissociation of HOX:PBX1A complexes containing HOXD4, HOXA1, or HOXA1(N2K,A3R) were measured using all four variations at position 6. The heterodimeric site (derived from O-262) is shown at the top of each panel, and the time at which each lane was loaded following addition of unlabeled specific competitor is indicated. These data were used to calculate the half-lives expressed in Table I.

37-fold suggests that it is no longer recruited to the cooperative binding sites by endogenous PBX proteins.

Collectively these data demonstrate that, as seen previously for HOXD4, HOXA1 requires an intact pentapeptide for its ability to interact with PBX partners.

*The Relative Stability of a Given HOX-PBX Complex Cannot Be Predicted by the Ability of the HOX Partner to Bind DNA as a Monomer*—Given the cooperativity between HOX and PBX partners in binding DNA (24, 25, 29, 30, 33), it is reasonable to expect that differences in the activity of HOX-PBX heterodimers are exploited for HOX functional specificity *in vivo*.

To determine whether HOXA1 and HOXD4 differentially interact with PBX, we compared the binding kinetics of HOX:PBX1A heterodimers containing either HOXD4 or HOXA1. The relative stabilities of these complexes can be estimated by comparing the rates of dissociation of the proteins from a labeled DNA probe upon addition of a vast excess of unlabeled competitor. The complex containing HOXA1 was more stable than its HOXD4-containing counterpart (Fig. 3C, Table I). The measured half-life of the HOXD4-PBX1A complex at 23 °C was 15 min, while the half-life of the HOXA1-PBX1A complex was 25 min. In comparison, HOXD4 as a monomer dissociates completely from a consensus HOX site in less than 2 min (data not shown). This demonstrates that interaction with PBX partners can dramatically increase the stability of interactions with DNA of different HOX proteins. However, this increase is not necessarily uniform.

Since the HOXA1 homeodomain has been shown to bind DNA much more poorly than HOXD4, the greater stability of the heterodimer containing HOXA1 is somewhat surprising. The difference in monomeric binding activity has been shown

TABLE I  
Half-lives (in minutes) of HOX-PBX1A complexes on heterodimeric sites that vary at position 6

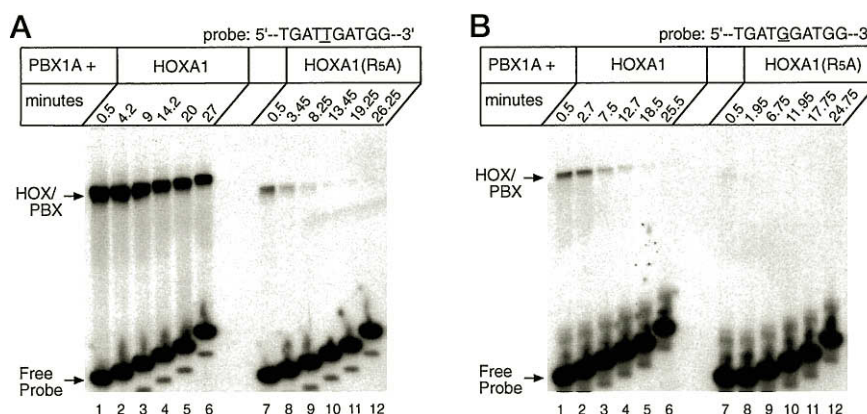
	Position 6 <sup>a</sup>			
	Adenine	Cytosine	Guanine	Thymine
HOXA1	7.9	2.6	25.2 ± 4.1 <sup>b</sup>	4.9 ± 0.7
HOXD4	6.9	3.1	15.1 ± 2.6	14.2 ± 2.6
HOXA (N2K, A3R)			24.2 ± 7.2	5.5 ± 0.6

<sup>a</sup> Numbering according to Fig. 1.

<sup>b</sup> Standard deviation ( $n = 3$ , denominator =  $n - 1$ ).

to result from the lack of conservation in HOXA1 of N-terminal arm residues that contribute to HOXD4 DNA binding via the minor groove (5). Specifically, the HOXD4 residues Lys-2 and Arg-3 are substituted by Asn-2 and Ala-3 in HOXA1, greatly reducing HOXA1 monomeric DNA-binding activity. The greater stability of HOXA1-PBX over HOXD4-PBX complexes suggests that other factors control the level of heterodimer activity. While the N-terminal arms of distantly related homeodomains can be similarly oriented in monomeric DNA binding complexes (47), this region is inherently unstructured (48). Thus, it cannot be assumed to adopt the same conformation in higher order complexes as in monomer binding. As a result, several models can be proposed for how HOXA1 manages to form more stable HOX-PBX heterodimers. These are based on three conceivable conformations of the HOX N-terminal arm. In the first model, the same contacts between the N-terminal arm and DNA that are formed in the monomer are maintained in the heterodimer. In this case, HOXA1 must compensate through interactions with either PBX1A or DNA via sequences outside the N-terminal arm. In a second model, the N-terminal

**FIG. 4. Dissociation rate experiments demonstrate that the conserved Arg-5 residue in the HOXA1 N-terminal arm contributes to the stability of the HOXA1-PBX1A heterodimer at the level of DNA binding.** A, preformed complexes on a labeled DNA probe (O-262 with G6, see "Experimental Procedures") containing the sequence 5'-TGATTGATGG-3' were competed by a vast excess of unlabeled DNA (O-160) containing both HOX and HOX-PBX binding sites. The time at which samples were loaded following addition of the cold competitor is indicated above each lane. These data were used to calculate the half-lives expressed in Table I. B, the identical experiment to that shown in A, except that the labeled probe used (O-209) contained the sequence 5'-TGATGGATGG-3'.



arm remains in the minor groove but adopts new DNA contacts that are favorable for HOXA1. The third model proposes that the interaction of the adjacent pentapeptide with PBX may displace the HOX N-terminal arm from the minor groove.

**The HOX N-terminal Arm Contributes to DNA Binding in Heterodimers with PBX**—The divergence (Fig. 1A) between the core of the heterodimer half-site recognized by the HOX partner, 5'-TGAT-3', and the canonical HOX recognition site, 5'-TAAT-3', suggests that the first model cannot be correct. By comparison to the crystal structure of the related EN homeodomain (4), Arg-3 of the HOXD4 homeodomain is predicted to contribute to monomer binding by contacting the second base pair in the TAAT core. In the heterodimeric half-site recognized by the HOX homeodomain, the analogous position is occupied by a G:C base pair. Thus, the combination of predicted HOXD4 N-terminal arm contacts in the heterodimer is of necessity not the same as in the HOXD4 monomer. The loss of this contact could at least partly explain the PBX-dependent convergence of HOXA1 and HOXD4 binding activities.

This observation does not distinguish between the second and third models. Therefore, we asked whether the HOX N-terminal arm still contributes to DNA binding in the heterodimer. An N-terminal arm-DNA contact seen in numerous homeodomain-DNA crystal (4, 49, 50) and solution (51) structures occurs between Arg-5 and O-2 of the thymine at the 1st position of the TAAT core. Arg-5 is conserved in all HOX proteins (Fig. 1B), whereas the correct A:T base pair is found in both the HOX monomer (position 1) and HOX-PBX heterodimer (position 5) sites (Fig. 1A). If the HOX N-terminal arm retains this minor groove contact in the heterodimer, then mutation of this arginine to alanine (R5A) will result in a decrease in DNA binding stability.

The effect of the R5A mutation on HOXA1 was tested first by EMSA. The dissociation of the HOXA1-PBX1A and the HOXA1(R5A)-PBX1A heterodimers from this probe is shown in Fig. 4A. Half-lives for the two complexes were just under 27 and under 5 min, respectively (Table II). Thus, more than a 5-fold decrease in stability is produced by the R5A mutation. A similar decrease in stability for the HOXA1-PBX1A complex is seen when the binding site used contains a G at position 5 rather than a T (Fig. 4B, lanes 1-6; Table II). To determine if Arg-5 of HOXA1 contributes to heterodimer complex stability by recognition of the T at position 5, we tested the effect of using both the mutated HOXA1 and the DNA probe with G at position 5. The half-life of this complex was not further reduced (Table II), as would be expected for two independent functions. The most straightforward conclusion is that the role of Arg-5 in contacting T5 is retained in the heterodimeric complex.

In addition, the effect of the R5A mutation was tested in the

**TABLE II**  
Half-lives (in minutes) of HOX-PBX1A complexes on heterodimeric sites that vary at position 5

	Position 5 <sup>a</sup>	
	Thymine	Guanine
HOXA1	26.8 ± 2.3 <sup>b</sup>	4.4 ± 2.6
HOXA1 (R5A)	4.3 ± 2.5	4.7 ± 2.7

<sup>a</sup> Numbering according to Fig. 1.

<sup>b</sup> Standard deviation ( $n = 3$ , denominator =  $n - 1$ ).

transfection assay. This mutation reduces activation by HOXA1-VP16 alone from 1650- to 8.5-fold and when cotransfected with E2A-PBX1A from 4000- to 640-fold (Fig. 2B). The latter value is similar to that obtained by transfection of E2A-PBX1A alone. Thus, cooperativity between HOXA1(R5A) and PBX1A is severely attenuated both *in vitro* and *in vivo*.

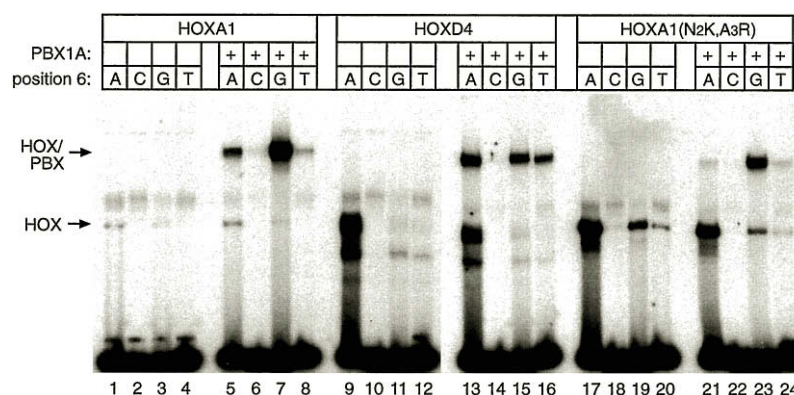
From the above results we conclude that minor groove contacts by the HOXA1 N-terminal arm are important for complex stability. Since Arg-5 is conserved in all HOX homeodomains, these observations can be extended to other PBX-interacting HOX proteins, such as HOXD4. This interpretation is consistent with the second model, in which the N-terminal arm adopts at least a subset of the minor groove contacts formed in the monomer. This subset includes that of a residue which is conserved between HOXA1 and HOXD4 (Arg-5).

**HOXA1 and HOXD4-containing Complexes Exhibit DNA Sequence Preferences at Positions Predicted to Be Recognized by the HOX N-terminal Arm**—While the above results indicate that Arg-5 of the HOX N-terminal arm retains its minor groove contact in HOX-PBX heterodimers, the greater stability of HOXA1-PBX over HOXD4-PBX complexes suggests that the roles of residues two and three have been altered. Arg-3 of EN has been shown to contact the A:T base pair at position 2 of the TAAT monomer site (4); however, this corresponds to the G:C base pair at position 6 of the heterodimeric site (Fig. 1A). The presence of G6 in the heterodimeric site used to this point may thus not permit contacts with residues two and three of HOXD4.

To address this hypothesis, we tested the effect of all four bases at position 6 on the activity of the HOX-PBX complexes. First, steady state binding was analyzed for both the HOX monomers and the HOX-PBX heterodimers. As expected, HOXD4 alone has a preference for the A6 probe, since this contains the sequence 5'-TAATGG-3' (Fig. 5, lane 9). Under the same conditions, HOXA1 alone shows little or no binding to any site (Fig. 5, lanes 1-4). To see whether these monomer preferences could be transferred to the higher order complexes, we also tested HOX-PBX heterodimers. Very strong binding for HOXA1-PBX1A was seen with the G6 probe (lane 7), strong



**FIG. 5. HOX proteins exhibit distinct DNA binding preferences between monomeric binding and PBX1A-mediated heterodimeric binding at a position predicted to be contacted by the N-terminal arm.** DNA probes (derived from O-262) containing the heterodimeric binding site shown in Fig. 1A, but with the indicated variations at position 6, were used in EMSA. HOXD4 and HOXA1, and the chimeric HOXA1(N2K,A3R), were tested for their ability to bind these four sites singly and in combination with PBX1A.



binding was seen on A6 (lane 5), and there was weaker binding on T6 (lane 8). The relative binding to each probe was  $G6 \gg A6 > T6 > C6$ . For the HOXD4-PBX1A complex, similar levels of binding were seen for A6 and G6, whereas binding to T6 was somewhat lower (lanes 13–16) ( $A6 \sim G6 > T6 \gg C6$ ).

The effect of variations in the heterodimer site at position 6 on complex stability was also studied (Fig. 3). The calculated half-lives are presented in Table I. The stability of both HOX-PBX complexes on A6 is similar, despite the fact that the complex containing HOXD4 shows stronger steady state binding. This discrepancy is most likely due to the ability of HOXD4 monomer binding to accelerate the formation of the heterodimer on this particular sequence (data not shown). Binding to the C6 probe, which was extremely weak, was also similar for the two HOX-PBX complexes. In contrast, the stability of the complexes formed on both G6 and T6 was clearly different (Fig. 3, C–D, Table I). Both HOXA1-PBX1A and HOXD4-PBX1A complexes were very stable on binding sites bearing G6, although the former showed two-thirds greater stability (25 versus 15 min). Substitution of G6 with T6 reduced the stability of HOXA1-PBX1A 5-fold relative to G6, whereas the stability of HOXD4-PBX1A was essentially unchanged.

In conclusion, the two HOX-PBX complexes have distinct preferences at position 6. Numerous lines of evidence indicate that this corresponds to the 2nd position in the 3' HOX half-site. For homeodomains binding DNA as monomers, this position is known to be contacted in the minor groove by residue three (4).

**Residues Two and Three of the HOX N-terminal Arm Do Not Contribute to the Specificity of HOX-PBX Heterodimers**—The above observations prompted us to test whether the same residues that confer monomer preferences between HOXA1 and HOXD4 may also be responsible for conferring the position 6 preferences of the corresponding HOX-PBX complexes. For this, we used a HOXA1 protein in which the Asn-2 and Ala-3 residues were replaced with Lys-2 and Arg-3 found in HOXD4. This chimera, referred to as HOXA1(N2K,A3R) (5), showed a strong preference for the A6 probe (Fig. 5, lane 17). These observations are consistent with our previous results showing that these residues distinguish the ability of HOXD4 versus HOXA1 to recognize sites containing a TAAT core (5) and confirm the prediction that they do so by specifying the 2nd position in the TAAT core.

When HOXA1(N2K,A3R) was incubated with PBX1A, the binding preferences of the resulting heterodimer ( $G6 \gg A6 \sim T6 > C6$ ) were much more similar to the heterodimer containing HOXA1 (Fig. 5, compare lanes 21–24 with lanes 5–8) than to that containing HOXD4 (lanes 13–16), despite the fact that this chimeric HOX protein acts like HOXD4 as a monomer. This is consistent with the observation that the binding preference of the HOXD4 monomer (A6) did not deter-

mine the preference of the HOXD4-PBX1A heterodimer (A6, G6, and T6).

We also tested whether the N-terminal arm substitutions responsible for differences in monomer binding between HOXA1 and HOXD4 were involved in differences in the stability of the corresponding HOX-PBX complexes. As seen for the steady state binding, the substitution of Lys-2 and Arg-3 into HOXA1 had little or no effect on complex stability (Fig. 3 and Table I). The half-lives of the HOXA1(N2K,A3R)-PBX1A complex on G6 and T6 were 24 and 6 min, respectively.

Thus, while our results implicate Arg-5 of the HOX N-terminal arm in binding site selection by HOX-PBX heterodimers, here we see that positions 2 and 3 of this structure do not influence this process. This is in striking contrast to the importance of these residues for HOX monomer binding.

**ABD-B Subfamily Members Can Interact with PBX1A on a Heterodimeric Site That Contains a TTAT Core**—ABD-B subfamily members, which lack a recognizable pentapeptide, fail to cooperate on the heterodimeric site shown in Fig. 1A (24, 29, 30). Given the above results, we wished to test HOXD9 and HOXD10 on the probes with variations at position 6.

HOXD10 (Fig. 6A) and HOXD9 (data not shown) bind both the A6 and T6 heterodimer probes as monomers, which was expected since the 3' half-sites are TAAT and TTAT, respectively. Additionally, they heterodimerize with PBX1A on the site containing T6 (Fig. 6A, lane 8). This demonstrates that the previously reported inability to cooperate with PBX1A (30) is due to the DNA site tested. The stability of these complexes bound to T6 was tested in the same manner as the complexes in Fig. 3. While most of the bound HOXD10 monomer dissociated within 2 min (Fig. 6B), the HOXD10-PBX1A heterodimer was considerably more long-lived, with a calculated half-life of 11.5 min. Under the same conditions, HOXD9-PBX1A had a half-life of 16.7 min. Thus, for ABD-B subclass members, the preference for thymine at position 6 is shared by the HOX monomer and the HOX-PBX complex.

## DISCUSSION

There are three major conclusions from the work presented here. First, we show that the highly conserved Arg-5 residue in the HOXA1 N-terminal arm retains a minor groove contact in a heterodimeric complex with PBX. Thus, the HOX N-terminal arm remains in close proximity to the minor groove in both monomeric and cooperative complexes. Second, this raises the possibility that, as for HOX monomer binding, this region can contribute to the binding site preferences of various HOX-PBX complexes. We confirm this by showing that binding preferences at one of the positions predicted to be contacted by the HOX N-terminal arm varies depending on the HOX protein in the complex. Finally, the N-terminal arm residues responsible for this specificity are not the same as those which distinguish

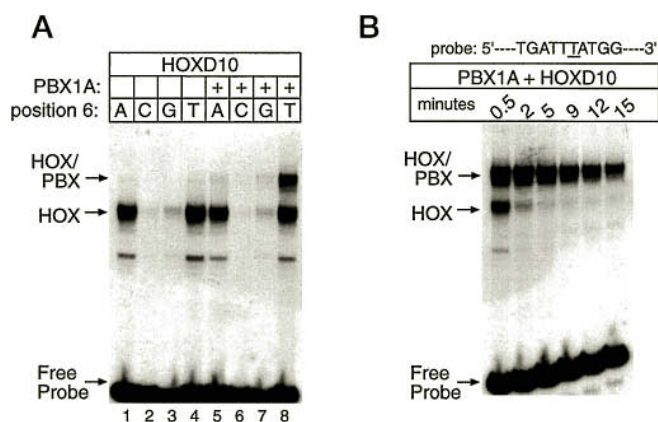


FIG. 6. **HOXD10 can cooperate with PBX1A, but only on a heterodimeric site which contains thymine at position 6.** **A**, steady state binding by HOXD10 to each of the position 6 variants (derived from O-262) in the absence (lanes 1–4) or presence (lanes 5–8) of PBX1A. **B**, the stability of the HOXD10-PBX1A complex on the T6 probe was measured in the same manner as described for Figs. 3 and 5.

HOXA1 and HOXD4 monomer activities.

**The Pentapeptide of HOXA1 Is Required for Cooperative Interaction with PBX1A**—The first observation reported here is the requirement of the HOXA1 pentapeptide for cooperative interaction with PBX. It has been shown recently that the divergent pentapeptide of labial (LAB), the *Drosophila* HOXA1 homologue, appears to block DNA binding by a bacterially purified LAB protein and that this can be abrogated by the presence of EXD or mutation of the LAB pentapeptide (52). Although HOXA1 monomer binding is weak, it is not improved by mutation of the pentapeptide or by the presence of PBX1A (Fig. 2A). Additionally, the low transcriptional activity of HOXA1-VP16 on a promoter containing HOX monomer sites was not increased by mutation of the pentapeptide or by cotransfection of a vector expressing E2A-PBX1A (data not shown). These data argue against the use of such a mechanism by all LAB homologues. Rather, the lack of basic residues at homeodomain positions 2 and 3 appears to be sufficient to explain HOXA1 dependence on cofactors (5). HOXB1, another LAB subfamily member, is likewise dependent on EXD-PBX cooperativity for strong DNA binding (6), but the effect of its pentapeptide on monomeric and cooperative binding has not been reported. Interestingly, negative effects of N-terminal sequences on monomeric binding have been reported for exd and EN (52, 53).

**A Conserved Residue in the HOX N-terminal Arm, Arg-5, Makes a Base-specific Contact in the Heterodimeric Binding Site**—Several structural studies have shown that Arg-5, a residue found in all HOX proteins, performs a conserved function for diverged homeodomains by forming a stable, specific interaction with a thymine in the minor groove upon monomeric binding (4, 42, 49–51). Our results show that this DNA binding function is conserved upon interaction with PBX. We note that two of the three heterodimer sites in the murine *Hoxb1* autoregulatory enhancer (ARE) contain a guanine at position 5 (one of three sites in chicken and pufferfish), and yet this enhancer is responsive to HOXB1 in mice (6) and LAB in flies (6, 52) in what appears to be an EXD-PBX-dependent fashion. As well, a transgenic *Hoxb1-lacZ* reporter construct containing the ARE is responsive to ectopically expressed HOXA1 (54). How might this relatively poor site be functional *in vivo*? One possibility is that multiple degenerate sites act synergistically to achieve effective transcriptional regulation. Synergy has been demonstrated *in vivo* for both the *Hoxb1* ARE (6) and for EXD-dependent *dpp* enhancer elements (55–57). However, transfection studies with the *Hoxb1* ARE show it responds poorly to

HOXA1-VP16 and E2A-PBX1A.<sup>2</sup> This discrepancy may be due to a requirement for additional spatial and temporal cues, including post-translational modifications and the presence of other transcription factors (57). Consistent with such a requirement, the *Hoxb1* ARE is responsive to ectopically expressed HOXB1 only in restricted domains, and it generates an incomplete *lab*-like expression pattern in *Drosophila* (6), despite the fact that *exd* and *PBX* genes are widely expressed (28, 58, 59).

**HOX Proteins Confer Specific Binding Preferences at a Position Predicted to be Recognized by the N-terminal Arm**—The apparent proximity of the HOX N-terminal arm to the minor groove in the heterodimer prompted us to test the effect of base substitutions at position 6 on the stability of the HOX-PBX complexes. Four HOX proteins suitably representative of the three monomer subclasses (see Introduction) were used and displayed distinct preferences in complexes with PBX. These data suggest that variations at position 6 alone could sufficiently discriminate between different HOX-PBX complexes. This could be combined with subtle preferences for positions 3' to the TNAT core (8, 9, 36, 60) and magnified by the ability of clustered heterodimeric sites to act cooperatively *in vivo* (6, 25).

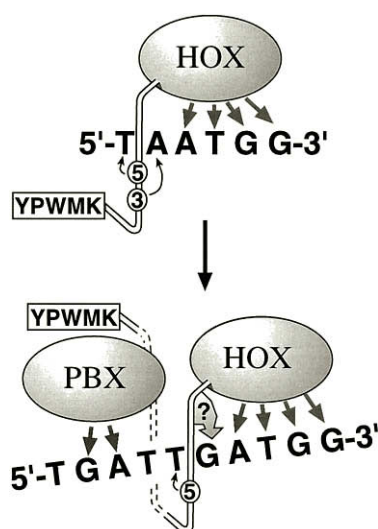
Similar results implicating position 6 in distinguishing different HOX-PBX complexes have been reported recently (36). However, there are a number of distinctions. Although HOXD4 showed stable binding to T6 in our hands (Table I), poor T6 binding was reported for its paralog, HOXB4. Our conclusions are based primarily on dissociation rates, which showed similar half-lives on T6 and G6, whereas the HOXB4 result is based on steady state binding. It is reasonable to expect that stability of binding is a critical determinant for the ability of a HOX protein to compete with family members for a particular site and thus that HOXD4 will be active on at least a subset of T6 sites *in vivo*. Additionally, for both steady state and dissociation experiments, we see poor cooperative binding by both HOXD9 and HOXD10 with PBX1A on any probe other than T6, whereas HOXB9 showed much stronger steady state binding on A6 and G6 relative to HOXA10 (36). This may represent real differences between highly related paralogs or may be the result of variations in experimental protocol.

**Binding Preferences of the HOX Protein, and the N-terminal Arm Residues That Confer These Preferences, Differ Between the Monomer and the Heterodimer**—Although HOXA1 and HOXD4 dictated the specificity of HOX-PBX heterodimers at position 6, these preferences did not reflect the DNA binding characteristics of the HOX monomers (Fig. 5). While Arg-3 is common to ANTP, EN, even-skipped (EVE), and paired homeodomains, it forms different DNA contacts in each structure (49, 50) despite the conservation of the Arg-5-DNA contact (see above). It was thus possible that these N-terminal arm residues were reconfigured to form different contacts between monomeric and heterodimeric DNA binding. However, our results demonstrate that the position 6 preferences conferred by the HOX partner do not reside in the same residues that distinguish HOX monomer binding. Consistent with this observation, LAB subfamily members are not conserved at homeodomain residues two and three, and yet HOXA1 (our results) and HOXB1 (36) both heterodimerize with PBX1A most effectively on the G6 probe. Similar differences in HOX monomer *versus* heterodimeric binding have been noted by others who have also implicated the N-terminal arm in the specificity of monomeric and heterodimeric complexes (36, 41).

Between HOX paralog groups, N-terminal arm sequences are more variable than the remainder of the homeodomain. These differences are well-conserved within paralog groups,

<sup>2</sup> M. L. Phelan and M. S. Featherstone, unpublished results.





**FIG. 7. Heterodimerization between HOX and PBX alters the DNA contacts made by the HOX N-terminal arm.** This diagram summarizes aspects of HOXD4 binding DNA as a monomer (*top*) and in a heterodimer with PBX (*bottom*). N-terminal arm residues which make base-specific contacts via the minor groove are numbered, and their contacts are represented by curved arrows. Predicted recognition between homeodomains and bases in the major groove are indicated by large black arrows. These assignments are based primarily on the structures of yeast  $\alpha 2/a 1$  (63) and EN (4) homeodomain-DNA co-crystals. The predicted contacts for the PBX recognition helix involve the conserved Asn-51-adenine contact predicted for all homeodomains, and an Arg-55-guanine contact seen for  $\alpha 1$  (63), as previously noted (41). Our results demonstrate that residue five of the HOX homeodomain retains its DNA contact in heterodimers with PBX, and the identity of residues two and three no longer affects specificity. We speculate that other residues in the HOX N-terminal arm may be recruited to confer specificity via minor groove or phosphate backbone contacts (arrow with question mark) as a result of interactions with PBX. Consistent with this suggestion, residues 6, 7, and 8 of the N-terminal arm are highly conserved within, but not between Hox paralog groups, and have previously been implicated in distinguishing homeodomain DNA-binding specificity (9, 64).

however, suggesting a functional role confirmed by studies *in vivo* (12, 20). Nonetheless, these N-terminal arm differences have moderate, if any, effect on monomeric DNA-binding activity *in vitro* (8). Results presented here provide an explanation for this discrepancy in that interaction with PBX (or EXD) significantly alters the interaction of the HOX N-terminal arm with DNA.

What induces the N-terminal arm to alter its DNA contacts? Presumably, this is the result of a PBX-mediated change in the conformation of the N-terminal arm and/or the minor groove (Fig. 7). In one scenario, interaction of the pentapeptide with PBX may impart a structure on the adjacent N-terminal arm. The sequence N-terminal to the HOX homeodomain, including the pentapeptide or its equivalent in ABD-B subfamily members, has been shown to influence the recognition of position 6 by the N-terminal arm in some instances (36). Alternatively, changes in N-terminal arm-minor groove interaction may result from the distortion of DNA topography. Interaction with DNA has already been shown to induce N-terminal arm conformation in monomeric complexes (61, 62).

**ABD-B Class Proteins Share a Subset of Their Monomeric Binding Activity with the Heterodimer**—HOXD9 and HOXD10 appear to cooperate with PBX via a motif immediately N-terminal to the homeodomain which, other than having a tryptophan, is diverged from the pentapeptide consensus (36). Unlike HOXA1 and HOXD4, complexes of HOXD9 and HOXD10 with PBX1A bind a subset of their monomeric binding site preferences. Both proteins are active on TTAT and TAAT sites as monomers and on TTAT during heterodimeric binding. The

source of the TTAT preference exclusive to the ABD-B subfamily monomers has been mapped to N-terminal arm residues Lys-3, Lys-6, and Pro-7 (9). In contrast, most HOX monomers can bind TAAT, including HOXA1(N2K,A3R), suggesting that position 6 and 7 identity is usually unimportant for monomeric recognition. Thus, the positioning of the N-terminal arm of ABD-B subfamily members in the context of a TTAT monomeric site may be distinct from recognition of TAAT and may be more similar to the N-terminal arm conformation occurring in HOX-PBX heterodimers. Alternatively, T6 may be specified by different N-terminal arm residues in monomeric *versus* heterodimeric complexes. We note that HOXA1 and HOXD4 also differ in the N-terminal arm at positions 4, 7, and 8, in addition to positions 2 and 3 (Fig. 1B). One or more of these residues may therefore be brought into play in complexes with PBX.

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