THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 30, Issue of July 25, pp. 19081–19087, 1997
Printed in U.S.A.

Residues Flanking the HOX YPWM Motif Contribute to Cooperative Interactions with PBX*

(Received for publication, April 18, 1997, and in revised form, May 19, 1997)

Kandavel Shanmugam‡§¶, Mark S. Featherstone‡§‖, and H. Uri Saragovi‡ ‡‡§§**

From the ‡McGill Cancer Centre and Departments of ‖Oncology, §Medicine, and ‡‡Pharmacology and Therapeutics, McGill University, Montreal, Quebec H3G 1Y6, Canada

***Hox* genes encode transcription factors that are major determinants of embryonic patterning. Recently, we and others have shown that specific recognition of target sites in DNA is partly achieved through cooperative interaction with the extradenticle/pre-B-cell transformation-related gene (EXD/PBX) family of homeodomain-containing proteins. This interaction is mediated by the YPWM motif present N-terminal to the homeodomain in HOX proteins. In the present study, we use YPWM peptides to confirm the importance of this motif for mediating HOX/PBX interactions. We also used a novel monoclonal antibody directed against the YPWM to show that occlusion of this motif abrogates cooperativity with PBX. In addition, we present evidence that residues flanking the YPWM, both N-terminally and C-terminally, stabilize the HOX·PBX cooperative complex. Because these flanking residues are also conserved among paralogs, they are likely to help distinguish the specificity of HOX/PBX interactions. Our data further show that the relative importance of individual residues within and flanking the YPWM is dependent on the identity of position 6 of the cooperative binding site (TGATTNATGG). These results suggest that interactions between PBX and the YPWM motif are modified by a base pair predicted to contact the N-terminal arm of the HOX homeodomain.**

The patterning of the animal embryo along the anteroposterior axis is a tightly regulated developmental process in which *Hox* genes play a major role (1). In *Drosophila*, the *Hox* genes are represented by a single cluster, Hom-C. There are 39 *Hox* genes identified to date in mice and humans, grouped into four clusters, A to D (2). *Hox* genes occupying the same relative position in each cluster form a group of paralogous genes that are more closely related to each other than to their neighbors in other paralogous groups (2). In addition, paralogs tend to be expressed in similar domains along the anteroposterior axis (1). Accordingly, paralogs possess both unique and overlapping functions as shown by gene targeting studies (3). There exists a fine correlation between the position of a given *Hox* gene on the chromosome and its spatio-temporal expression pattern with respect to the anteroposterior axis (2, 4, 5). Accordingly, genes at the 3′-end of the cluster are turned on earlier and have their boundaries of expression set more anteriorly (6). Genes more 5′ in the cluster have a more posterior boundary of expression and are activated later (6).

HOX proteins bind DNA through the conserved homeodomain, encoded by the homeobox (7). The homeodomain consists of three α-helices and an N-terminal arm that is unstructured in unbound proteins. Site-specific DNA binding is achieved by interaction of the third helix with the major groove and interaction of the N-terminal arm with the minor groove (7). *Hox* genes from paralog groups 1–8 also encode a highly conserved motif present N-terminal to the homeodomain (7, 8). This motif, variously called the pentapeptide, hexapeptide, or YPWM motif, is connected to the homeodomain through a flexible linker (9). NMR analysis of the antennapedia (ANTP) HOX protein of *Drosophila* revealed that the conserved YPWM motif is unstructured in solution (9). HOX proteins derive functional specificity to regulate target gene expression by interacting with the homeodomain-containing cofactor extradenticle (EXD) in *Drosophila* (10–15) or PBX (pre-B-cell transformation-related gene) (16) in mammals (17–21). This interaction is dependent on the YPWM motif (10, 17–19, 22–24). PBX residues contacted by the YPWM motif of HOX proteins are located within and immediately following the homeodomain in PBX (18, 25, 26). Although abdominal-B (ABD-B) members (paralogous groups 9–13) do not possess a classical YPWM motif, some form cooperative complexes with PBX through conserved tryptophan residues. Similar to the position of the YPWM motif in HOX proteins, the conserved tryptophan in ABD-B members is present N-terminal to the homeodomain (27, 28). Inhibition of DNA binding by the *Drosophila* HOX protein labial (LAB) is a second function attributed to the YPWM motif. This inhibition is relieved by interaction with EXD or PBX (23). Thus far, studies focused on the YPWM motif have demonstrated it to be the key PBX/EXD interacting motif. Recent work has shown that peptides bearing this motif are sufficient to induce PBX monomer binding (22).

Mutational and biochemical analyses of the HOX/PBX binding site (5′-TGATTNATGG-3′) demonstrate that PBX occupies the 5′ half-site (TGAT) while the HOX partner occupies the 3′ half-site (TNATGG) (26, 29, 30). Individual HOX proteins, upon heterodimerization with PBX, acquire different specificities for target DNA recognition (28). These binding differences are achieved in part through the modulation of the HOX N-terminal arm by PBX (28, 29, 31, 32). One of the residues that mediates DNA binding by the N-terminal arm is the arginine at position 3 of the homeodomain in paralogs 2–8, while the same position is occupied by a conserved lysine in ABD-B class HOX proteins (33). Studies from our laboratory (32) have

* This work was funded by grants from the Medical Research of Canada (to M. S. F.) and from the National Cancer Institute of Canada and the Medical Research Council (to H. U. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by a Fonds de la Recherche en Santé du Québec Ph.D. studentship.

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

§§ Recipient of a Scholar Award from the Pharmaceutical Manufacturers Association of Canada-Medical Research Council of Canada.

shown that residue 3 is important for the binding of HOX monomers but does not contribute to DNA binding in heterodimers with PBX. Hence, N-terminal arm residues that normally mediate minor groove contacts by HOX monomers are displaced in the cooperative complex (32).

Residues flanking the YPWM core are well conserved among proteins of the same paralogous group but vary significantly between proteins from different groups (Fig. 1*C*), suggesting a role in the specificity of HOX function. The present study demonstrates that YPWM-dependent interactions are stabilized by the residues flanking the core motif. We further show that this modulation of HOX/PBX interactions leads to differential target site recognition.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pTrcHisA was generated by subcloning the *Pst*I/*Hin*dIII fragment of *Hoxd4* (34) into the same sites in pTrcHisA. For the alanine scanning mutagenesis of the YPWM and the N- and C-terminal flanking residues, a *Pst*I/*Xba*I fragment of Hoxd4 (34) was first subcloned into identical sites in M13 mp19 phagemid. Single-stranded (sense) DNA was produced from the M13 phagemid carrying the Hoxd4 insert, and mutagenesis was performed using the Sculptor mutagenesis kit (Amersham Corp.). As a final step toward generation of the mutants, a *Pst*I/*Eco*RI fragment of M13 phagemid DNA carrying the different alanine scanning mutations were subcloned into the same restriction sites in both the pTrcHisAHoxd4 and pPGK-Hoxd4-vp16 (17) backgrounds. Construction of the luciferase reporters pML (5 × Hox) and pML (5 × HOX/PBX) are described elsewhere (17, 35).

Protein Expression and Purification—HOXD4, HOXD4 (WM-AA) and the alanine scanning mutants were expressed as N-terminal histidine-tagged fusion proteins and purified as described (32). The purity and concentration of the purified proteins were estimated as described earlier (32). PBX1A and E2A-PBX1A were synthesized *in vitro* using a TnT *in vitro* transcription/translation coupled kit (Promega).

Generation of Hybridomas and Selection of Positive Clones—A synthetic peptide (KLH-CAVVYPWMKKVHVNSVNPNY-CO_2H) spanning the YPWM and flanking N- and C-terminal residues coupled to KLH (Peptide Innovations Inc.) was used as an immunogen to generate monoclonal antibodies against the YPWM region using a strategy described previously (36). To screen for positive clones that would recognize the YPWM of HOXD4, bacterially expressed and purified pTrcHisAHoxd4 (explained above) was used as the source of antigen in an enzyme-linked immunosorbent assay (ELISA)[1] as explained elsewhere (37).

Isotyping, Purification, and Epitope Mapping of the 10D11 mAb by ELISA—mAb 10D11 was isotyped using a mouse immunoglobulin isotyping kit from Serotec. It was found to be IgG 1A class and hence was purified to homogeneity on a protein G-Sepharose column (38). Histidine-tagged alanine-scanning mutants of the YPWM and the flanking residues of HOXD4 (explained above) served as the source of antigen for the epitope mapping of mAb 10D11 by ELISA.

EMSA and Dissociation Rate Experiments—EMSA and dissociation rate experiments were performed as described previously (32). Equal amounts of wild type and mutant HOXD4 proteins were used based on estimates from Coomassie Blue-stained polyacrylamide gels. Labeled DNA probe 0–262, TGATTNATGG (32), used in this study contained A, G, or T at the sixth position. 0–160 (32) was used as the cold competitor in dissociation rate experiments. YPWM and nonspecific peptides were purchased from Peptide Innovations Inc. Prior to use they were resuspended in distilled water. Quantification of the labeled DNA-bound HOX·PBX cooperative complexes at various time points and estimation of the half-lives of the complexes were carried out as described previously (32).

The figures showing the EMSA data were produced electronically in Freehand 5.0 for Macintosh. Autoradiographs were scanned as reflective grayscale images using a Umax UC 1260 scanner and the Autodensity 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.

Cell Culture and Transfection Assay—Transient transfection was performed in HEK293 cells as described previously (17). HEK293 cells were cultured in α-MEM supplemented with 10% fetal calf serum and antibiotics (Sigma). Mouse 10D11 hybridoma was cultured in RPMI containing 5% fetal calf serum.

[1] The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; mAb, monoclonal antibody.

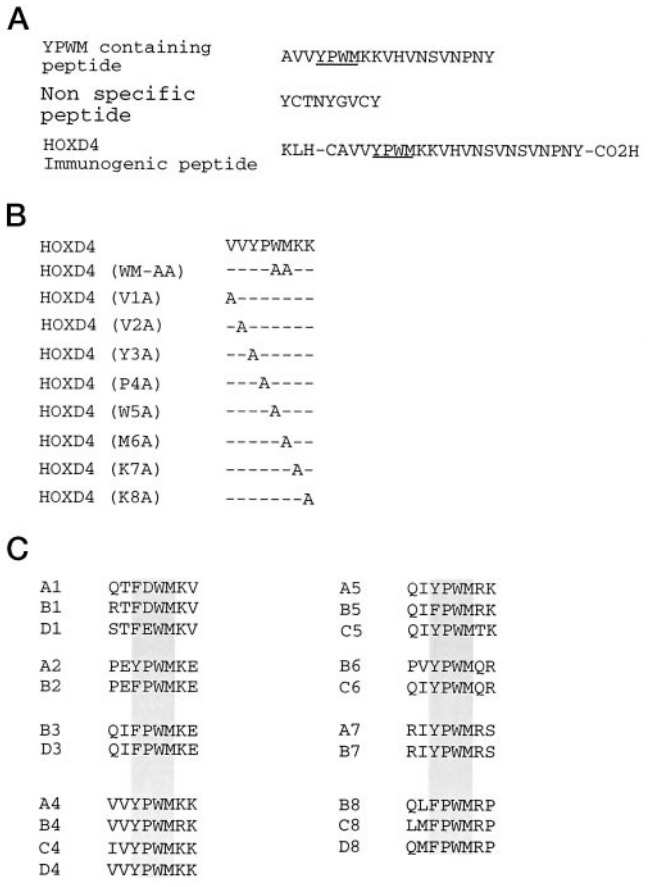

FIG. 1. *A*, amino acid sequence of the YPWM (19-mer), nonspecific peptide (12-mer), and the immunogenic peptide. The YPWM motif is *underlined* in both the YPWM and the immunogenic peptide. The immunogenic peptide is N-terminally conjugated to keyhole limpet hemocyanin (*KLH*), a carrier molecule. *B*, YPWM and N- and C-terminal flanking residues of HOXD4 subjected to alanine scanning mutagenesis. Residues are numbered 1–8, N to C terminus. Alanines in the scan are indicated. *Dashes* show identity. WM to AA substitution in HOXD4 (WM-AA) (17) is shown. *C*, alignment of the region containing the YPWM and the flanking residues of HOX proteins from paralogous groups 1–8.

RESULTS

A YPWM Peptide Prevents HOX/PBX Interactions—Previously, we have shown that HOXD4 and PBX1A cooperatively bind DNA through a motif present in HOX proteins called the pentapeptide or YPWM motif (17). This YPWM motif is a key region for mediating interactions with PBX, since mutating residues WM to AA is sufficient to prevent the formation of a HOX·PBX cooperative complex (17). We tested the ability of a synthetic peptide (YPWM peptide; Fig. 1*A*) containing the HOXD4 YPWM core and flanking residues to prevent cooperative complex formation between HOXD4 and PBX1A. Using increasing concentrations of the synthetic YPWM peptide, a 50% reduction of the starting HOXD4·PBX1A complex was observed at 160 μM concentration (Fig. 2, *lane 5*). A further increase in the concentration of the synthetic peptide to 320 and 640 μM reduced the amount of the starting complex to 25 and 5%, respectively (Fig. 2, *lanes 6* and *7*). A complete loss of the starting complex (Fig. 2, *lane 8*) resulted when the synthetic peptide reached a concentration of 800 μM. To confirm the specificity of the HOXD4 YPWM-containing peptide to prevent HOX·PBX complexes, we also tested a nonspecific syn-

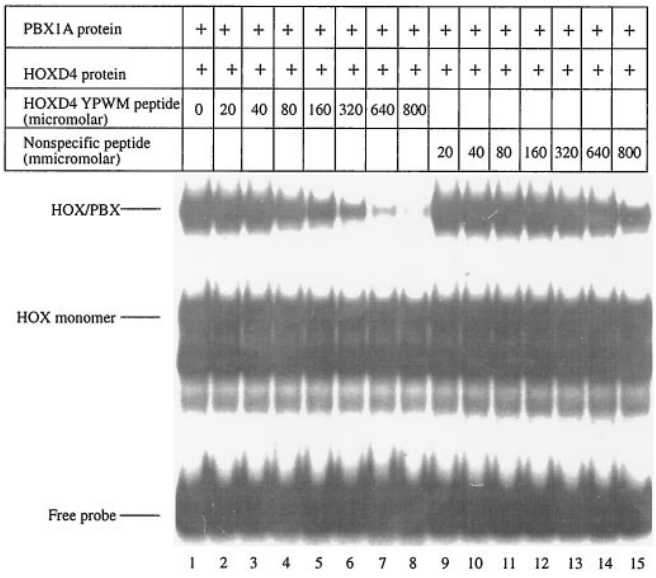

FIG. 2. **YPWM peptide prevents HOX·PBX interactions in EMSA.** A dose-dependent inhibition of HOXD4·PBX1A cooperative complex formation is caused by the YPWM peptide (see Fig. 1*A*) (*lanes 2–8*), while a nonspecific peptide under similar conditions had no profound effect (*lanes 9–15*). Peptide concentrations are indicated *above* the respective *lanes*. *Lane 1*, which contains no peptide, served as a positive control that was set to 100 for densitometric quantitation of the HOXD4·PBX1A cooperative complexes.

thetic peptide (Fig. 1*A*) in a band shift assay to prevent HOXD4·PBX1A complex formation. At comparable concentrations, there is little reduction in complex formation (Fig. 2, *lanes 9–15*).

Production of a Monoclonal Antibody against the HOXD4 YPWM Motif—Using the YPWM peptide (Fig. 1*A* and see "Experimental Procedures"), we produced a B cell hybridoma whose monoclonal antibody, designated 10D11, specifically recognizes the YPWM motif of HOXD4 protein. To finely map the epitope recognized by the mAb 10D11, bacterially purified histidine-tagged alanine scanning mutants spanning the YPWM motif and flanking residues of HOXD4 (Fig. 1*B*) were used in ELISA. The key residues in the YPWM motif of HOXD4 recognized by 10D11 mAb are the tyrosine and tryptophan, with complete loss of recognition if either one of the residues is mutated to alanine (Fig. 3*A*). The proline and methionine also contribute (Fig. 3*A*), defining the pentapeptide core (YPWM) as the 10D11 epitope.

Epitope mapping of 10D11 mAb was confirmed functionally by the ability of the antibody to supershift wild type and alanine scanning mutants of HOXD4 (Fig. 1*B*). In this assay, the antibody did not supershift the tyrosine and tryptophan mutants (Fig. 3*B*, *lanes 2*, *8*, and *12*) and only weakly supershifted the methionine and proline mutants (Fig. 3*B*, *lanes 10* and *14*). As seen by ELISA (Fig. 3*A*), flanking residues N-terminal and C-terminal to the YPWM core did not contribute to the epitope with the possible exception of V2 (Fig. 3*B*, *lane 6*). Taken together, the results of the epitope mapping show that the 10D11 mAb is specific against the core YPWM motif. Binding of the antibody to HOXD4 protein does not interfere with HOXD4 monomer binding to the DNA probe; nor does the mAb spuriously interact with the DNA probe (Fig. 3*B*, *lane 19*).

10D11 mAb Is Able to Prevent Cooperative Complexes of HOXD4 with PBX1A and E2A-PBX1A—Since 10D11 mAb is highly specific for the YPWM region of HOXD4, we tested its ability to prevent or disrupt HOX/PBX interactions. We co-incubated 10D11 mAb with HOXD4 and either PBX1A or E2A-PBX1A (39, 40) in EMSA. We found that cooperative complex

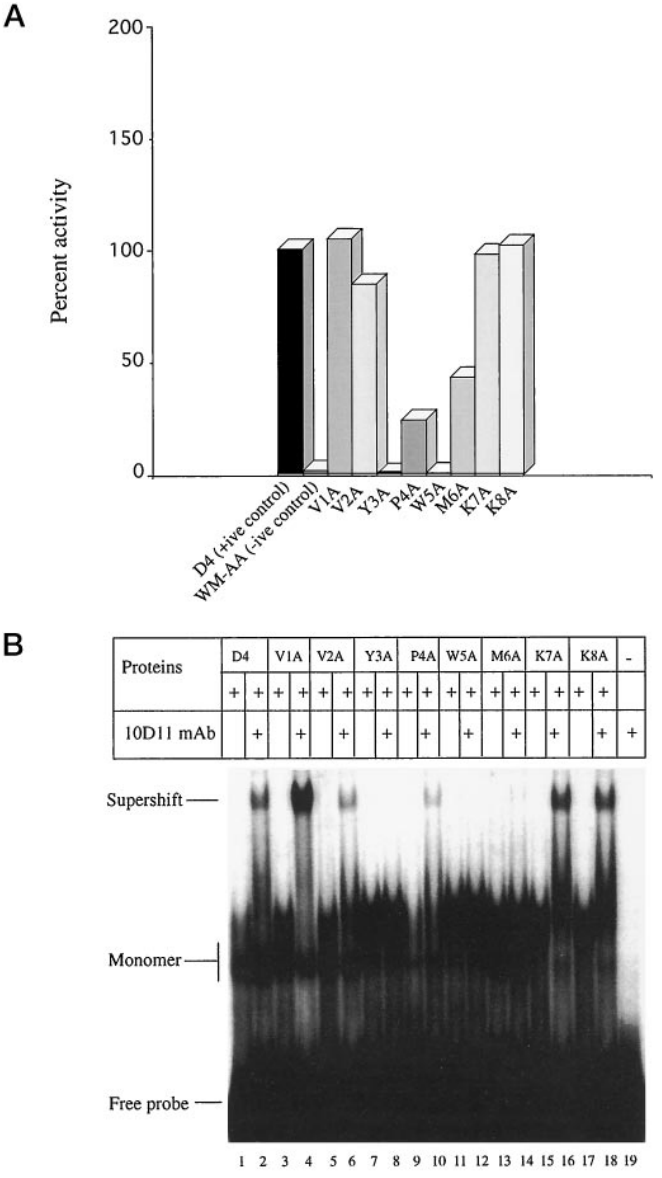

FIG. 3. **Epitope mapping of 10D11 mAb.** *A*, histogram representing the epitopes recognized by 10D11 mAb as seen in ELISA. The sources of antigens are explained under "Experimental Procedures." HOXD4 served as a positive control. A WM-AA derivative of HOXD4 was used as a negative control. The key residues recognized by the mAb are the tyrosine and the tryptophan (Y3A, W5A) followed by proline and methionine (P4A, M6A). The optical density at 416 of HOXD4 was taken as 100% activity of the 10D11 mAb. *B*, epitope mapping by supershift analysis in EMSA. 2 μg of purified 10D11 mAb was added to a standard steady state EMSA reaction containing bacterially purified HOXD4 and labeled DNA probe 0–260 (TGATTGATGG). As shown by ELISA, key residues recognized by the mAb in this assay are tyrosine (*lane 7 versus lane 8*), tryptophan (*lane 11 versus lane 12*), and methionine (*lane 13 versus lane 14*) followed by proline (*lane 9 versus lane 10*). *Lane 2*, supershift of the wild-type HOXD4. *Lane 19* shows that the mAb 10D11 has no DNA binding property.

formation of PBX1A or E2A-PBX1A with HOXD4 (Fig. 4*A*, *lanes 2* and *6*) was abrogated by co-incubation with 10D11 mAb (Fig. 4*A*, *lanes 4* and *8*). Similarly, when HOXD4 alone was preincubated with 10D11 mAb before the addition of PBX1A, the cooperative complex failed to form (Fig. 4*A*, *lane 9*). A cooperative complex could be recovered by preblocking the 10D11 monoclonal antibody with nearly equimolar concentration of YPWM peptide to an *in vitro* reaction containing HOX, PBX1A, and 10D11 (Fig. 4*A*, *lane 5*). We made use of a monoclonal antibody against the E2A portion of E2A-PBX1A to

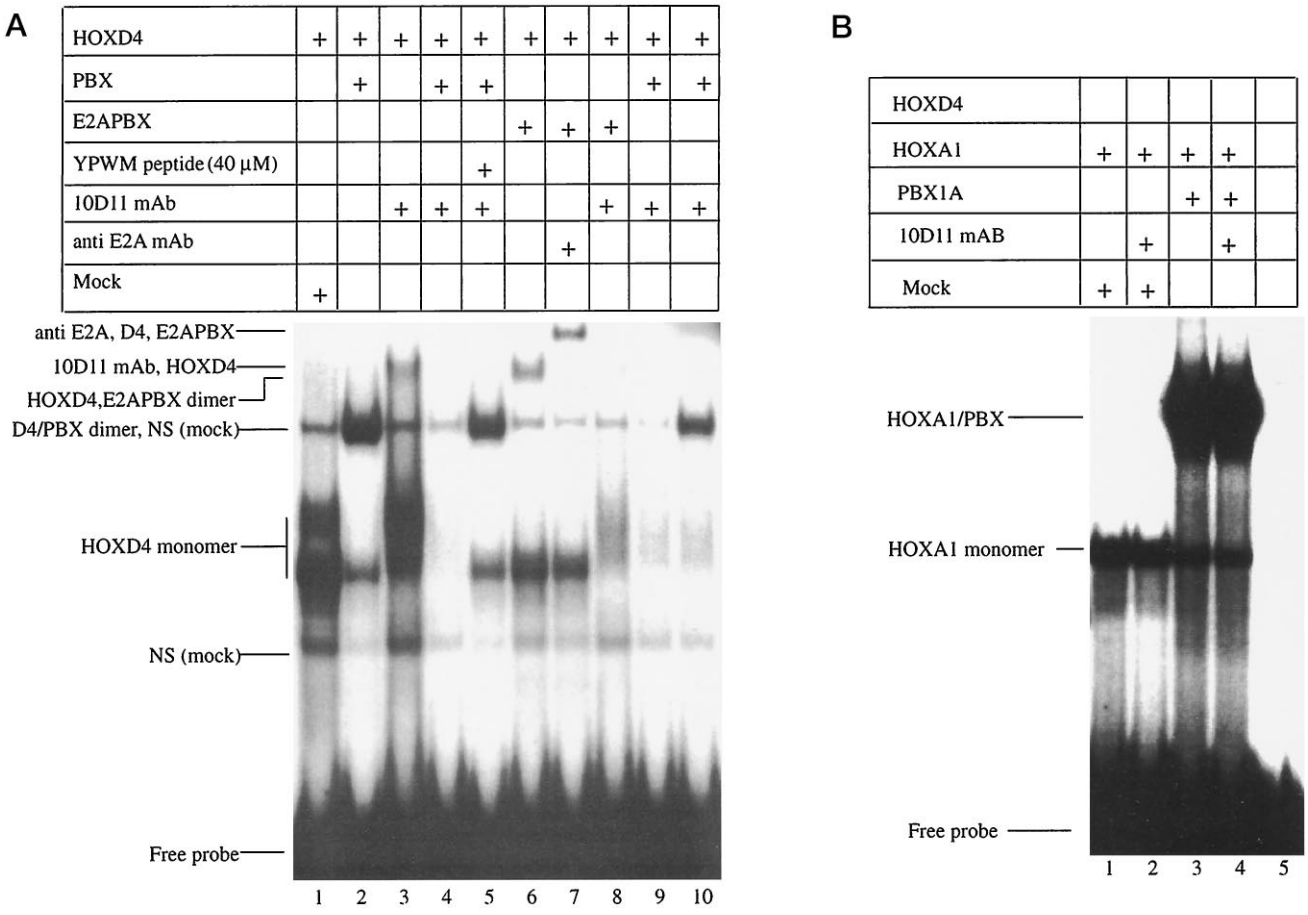

FIG. 4. *A*, 10D11 mAb prevents HOXD4·PBX1A and HOXD4·E2A-PBX1A cooperative complexes. 2 μg of 10D11 mAb was added to an *in vitro* reaction containing HOXD4 (*lane 3*, monomer supershift), HOXD4 and PBX1A (*lane 4*, ablation of the HOXD4·PBX1A cooperative complex), or HOXD4 and E2A-PBX1A (*lane 8*, ablation of the HOXD4·E2A-PBX1A cooperative complex). *Lane 1* represents a HOXD4 monomer control. It also shows the presence of a nonspecific (*NS*) mock band, which arises from the reticulocyte lysate used to transcribe/translate PBX1A or E2A-PBX1A. This nonspecific band migrates at the same position as the HOXD4·PBX1A cooperative complex (compare *lane 2* (HOXD4·PBX1A cooperative complex) with *lane 1*). The addition of YPWM peptide (*lane 5*) recovers the HOXD4·PBX1A cooperative complex. The HOXD4·E2A-PBX1A cooperative complex (*lane 6*) is supershifted in the presence of 2 μg of anti-E2A mAb (*lane 7*). Preincubating HOXD4 with 10D11 mAb prior to the addition of PBX1A and the DNA probe results in the loss of the cooperative complex (*lane 9*), while the addition of 10D11 mAb to a preformed HOXD4·PBX1A cooperative complex results in partial disruption (*lane 10*). *B*, the addition of 10D11 mAb to an *in vitro* reaction containing HOXA1 does not supershift the A1 monomer (compare *lanes 1* and *2* with *lanes 1* and *3* of *panel A*). The HOXA1·PBX1A cooperative complex (*lane 3*) is unaltered in the presence of 10D11 mAb (*lane 4*). The labeled DNA probe used in both *panels A* and *B* was 0–260, with G at the sixth position.

supershift the DNA-bound HOXD4·E2A-PBX1A cooperative complex (Fig. 4*A*, *lane 7*). By contrast, 10D11 does not form a supershifted complex, consistent with its ability to prevent the formation of HOX·E2A-PBX1A heterodimers on DNA. The addition of the 10D11 mAb to a preformed HOXD4·PBX1A complex resulted in disruption of more than 50% of the cooperative complex (Fig. 4*A*, compare *lanes 10* and *2*). Thus, 10D11 mAb can prevent and partially disrupt HOX·PBX complexes, emphasizing the importance of the YPWM for HOX/PBX interactions.

The pentapeptide of HOXA1 differs from that of HOXD4 at two positions within the 10D11 mAb epitope (Fig. 1*C*). Unlike results with HOXD4, 10D11 mAb was unable to supershift HOXA1 bound to DNA as a monomer (Fig. 4*B*, *lanes 1* and *2*); nor could 10D11 mAb interfere with HOXA1·PBX1A cooperative complex formation *in vitro* (Fig. 4*B*, *lanes 3* and *4*). We conclude that abrogation of HOXD4·PBX1A complex formation by 10D11 is specific and dependent on the YPWM epitope.

Importance of Residues Flanking the HOXD4 YPWM Motif for Interaction with PBX1A—Residues flanking the YPWM motif in HOX proteins are well conserved among members of the same paralogous group but can vary significantly between proteins of two different paralogous groups (Fig. 1*C*). We used alanine scanning mutants of HOXD4 (Fig. 1*B*) to test the importance of residues within and flanking the YPWM for the modulation of HOX/PBX interactions. Steady state EMSA was performed to look for both HOX monomer and HOX·PBX cooperative complex binding defects on two different probes, A6 and G6, that differ at the sixth position of the cooperative binding site in DNA (see "Experimental Procedures"). We and others have previously shown the sixth position to affect complex stability and the specificity of HOX·PBX binding (28, 32). Although there was no difference in the monomer binding to either probe (Fig. 5, *A* and *B*, *lanes 5*, *7*, *9*, *11*, *13*, *15*, *17*, and *19*), the tyrosine, tryptophan, and methionine of HOXD4 were each required to form a cooperative complex with PBX1A on both of the probes (Fig. 5, *A* and *B*, *lanes 10*, *14*, and *16*). The ability of P4A to cooperate with PBX1A was significantly affected on a G6 probe (Fig. 5*B*, *lane 12*), and mutation of the first valine reduced cooperation with PBX1A by 80% on the G6 probe (Fig. 5*B*, *lane 6*). Conversion of the second valine to alanine had a more dramatic effect, with more than 90% reduction in the cooperative complex on both A6 and G6 probes (Fig. 5, *A* and *B*, *lane 8*). The flanking lysines C-terminal to the YPWM did not show a difference in their interaction with PBX1A on either probe (Fig. 5, *A* and *B*, *lanes 18* and *20*).

Residues Flanking the YPWM Motif Contribute to the Stability of Complexes with PBX—To further investigate the contribution of residues within and flanking the YPWM to HOX/PBX

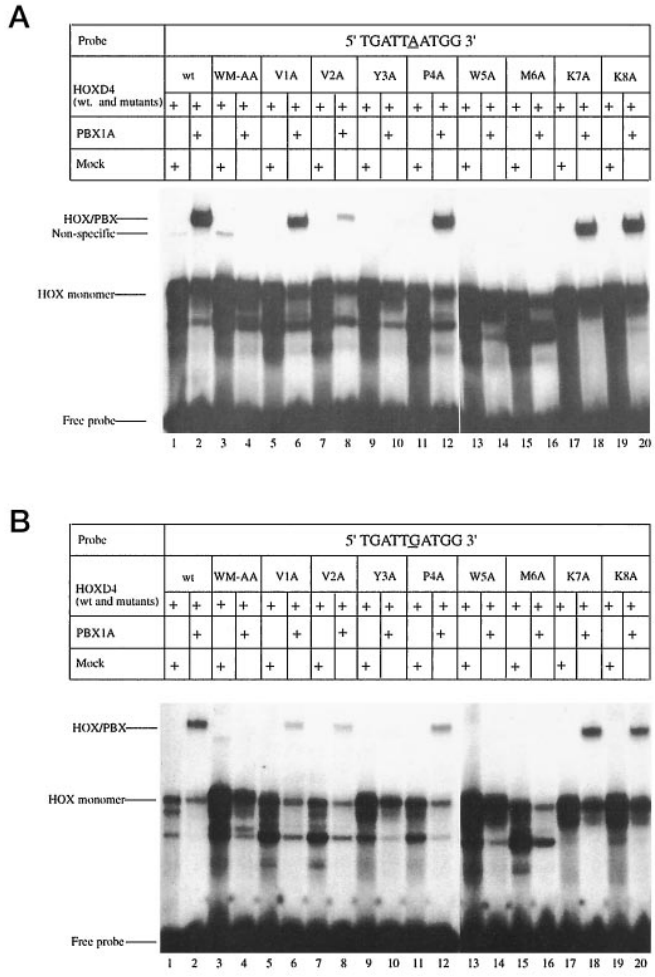

FIG. 5. **Role of YPWM and the flanking residues in the formation of HOX·PBX complexes as seen in steady state EMSA.** *A*, formation of HOXD4·PBX1A cooperative complexes on a binding site having an A at the sixth position. Wild type HOXD4 served as a positive control, and HOXD4 (WM-AA) served as negative control for cooperative interaction with PBX1A. Wild type, HOXD4 (WM-AA), and alanine scanning mutants were examined for both monomer binding (*lanes 1*, *3*, *5*, *7*, *9*, *11*, *13*, *15*, *17*, and *19*) and cooperative interactions with PBX1A (*lanes 2*, *4*, *6*, *8*, *10*, *12*, *14*, *16*, *18*, and *20*). HOXD4 and the mutant derivatives were bacterially expressed and purified, while PBX1A was *in vitro* transcribed and translated (see "Experimental Procedures"). Mock rabbit reticulocyte lysate was added to the reactions performed to observe HOX monomer binding to control for the absence of PBX1A. A nonspecific band was observed in *lanes 1* and *3*. *B*, formation of HOXD4·PBX1A cooperative complexes on a G6 binding site. The reaction conditions were identical to those mentioned for *panel A*.

interactions, we measured the dissociation rates of the cooperative complexes. Tryptophan and methionine mutants were not tested, since there was no observable complex formed. Tyrosine to alanine was tested, since a very weak complex was observed on longer exposures. Dissociation rate EMSAs were performed to study the stability of HOX·PBX cooperative complexes on a heterodimeric site with position 6 occupied by A, G, or T. The half-lives of the HOX·PBX1A complexes for the A6, G6, and T6 probes are summarized in Table I. There are two striking observations. First, all residues made a significant contribution to stability on at least one of the three probes tested. Second, the importance of a given residue for complex stability is dependent on base identity at position 6.

As seen earlier (32), wild type HOXD4·PBX1A was most stable on heterodimeric probes G6 and T6, while the cooperative complex had a shorter half-life on an A6 probe. Although the proline to alanine change (P4A) did not seem to affect steady state binding (Fig. 5), there was some loss of stability on

TABLE I
Measure of the stability of HOX · PBX complexes on a consensus HOX · PBX site that differs at position 6

S.E. is shown for each value. Experiments were performed two or four times.

Proteins	Stability		
	TGATTAAT	TGATTGAT	TGATTTAT
		%	
HOXD4	100[a]	100[a]	100[a]
HOXD4 (V1A)	55 ± 1	73 ± 6	74 ± 8
HOXD4 (V2A)	27 ± 3	39 ± 1	31 ± 5
HOXD4 (Y3A)	12 ± 4	6 ± 0	40 ± 1
HOXD4 (P4A)	61 ± 6	77 ± 12	81 ± 5
HOXD4 (K7A)	46 ± 3	86 ± 3	63 ± 7
HOXD4 (K8A)	36 ± 1	79 ± 6	45 ± 0

[a] Half-lives in minutes for wild type HOXD4 are as follows: 16 (A6 probe); 32 (G6 probe); 31 (T6 probe).

the A6 probe (Table I). While mutation of tyrosine (Y3A) reduced complex stability 8- and 17-fold on A6 and G6 sites, there was only a 2.5-fold drop on the T6 probe. Conversion of the first valine (V1A) caused a 2-fold decrease in complex stability on an A6 probe, while mutation of the second valine (V2A) decreased stability by 4-fold on this same site. The V2A mutant also displayed 2–4-fold reduced stability on the remaining two probes. The two flanking lysines contribute significantly to the stability of HOX·PBX complexes with 2–3-fold reductions in the half-lives of K7A and K8A on A6 and T6 probes. By contrast, there were no major effects observed on the half-lives of the lysine mutants with a G6 probe. In summary, flanking residues and the internal proline and tyrosine contribute to the fine modulation of HOX/PBX interactions. Moreover, the relative contribution of most residues depends on the identity of the base pair at position 6 in the heterodimer binding site.

Transcriptional Activation by Alanine Scanning Mutants of HOXD4—The *in vivo* importance of residues within and flanking the YPWM motif was further assessed by examining the ability of the alanine scanning mutants to activate transcription through a G6 cooperative binding site. Since HOXD4 is a poor transcriptional activator, a HOXD4-VP16 fusion protein was used as described previously (17). HOXD4-VP16 and its mutant derivatives depend on endogenous PBX proteins for their cooperative interactions. As seen *in vitro* on a G6 probe, residues tyrosine, tryptophan, and methionine within the YPWM core were important for cooperativity (Fig. 6). A dramatic effect was seen upon mutation of the flanking valine immediately N-terminal to the YPWM motif (V2A) with a decrease of 80% of transcriptional activation (Fig. 6). This was consistent with decreased stability of V2A·PBX complexes *in vitro* (Fig. 5). Both of the lysines C-terminal to the YPWM motif also contribute to cooperative interactions with PBX by this assay (Fig. 6). Mutation of the proline within the YPWM core (P4A) had a modest effect on transcriptional activation. Similar levels of transcriptional activation through a HOX monomer binding site were observed for all of the alanine scanning mutants, demonstrating that all of these proteins were expressed at the same level (data not shown). Together, our results show that residues flanking the YPWM core have a role in modulating HOX/PBX interactions.

DISCUSSION

There are three aspects to the current study. First, we have shown the importance of the residues in the YPWM core for cooperativity with PBX by using a mAb, 10D11, specific to the YPWM motif. Second, in addition to the YPWM core, we demonstrate a role for the flanking residues in stabilizing HOX·PBX cooperative complexes. Last, we show that the importance of a given residue either in the YPWM core or in the

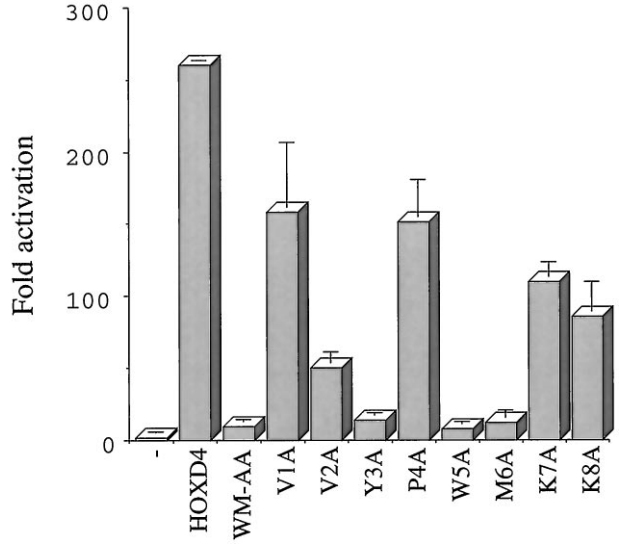

FIG. 6. **YPWM and flanking residues modulate the transcriptional activation from a promotor bearing HOX and PBX binding sites with G at position 6.** The *histogram* depicts the results of cotransfection with expression vectors for HOXD4 (WM-AA)-VP16 or the alanine scanning mutants fused to VP16. The luciferase reporter is driven by G6 cooperative binding sites and has been described previously (17). Transfected HOX proteins depend on the endogenous PBX proteins for synergistic activation. The -fold activation of HOXD4-VP16 that served as a positive control in both of the experiments was set to 100. The S.E. values for four experiments are given.

N- and C-terminal flanking regions, is determined by position 6 of a TGATTNAT HOX·PBX binding site.

YPWM-containing Peptide Prevents HOX/PBX Interactions—We have shown that a peptide spanning the YPWM motif and flanking residues of HOXD4 is able to interfere with HOX/PBX interactions. In other studies, peptides derived from EN2 and HOXB7 could completely abolish EN2·PBX or HOXB7·PBX complex formation at concentrations as low as 20 μM (41). Interference with HOXA5·PBX (22) or HOXD4·PBX complex formation (Fig. 2) required higher concentrations of 500 μM HOXA5 peptide or 640 μM HOXD4 peptide, respectively. The requirement for higher peptide concentrations could be attributed to the residues flanking the YPWM core. These flanking residues are conserved among paralogs but vary between paralogous groups within the HOX cluster (Fig. 1). In contrast to the peptides spanning the YPWM motif of HOXB3, HOXB7, HOXC6, and HOXA5 (22, 41), we did not observe induction of PBX monomer binding to DNA by a HOXD4-YPWM-containing peptide; nor does an EN2 peptide induce DNA binding by a PBX monomer (41). It is possible that residues flanking the YPWM in the fourth paralogous group make less stable contacts with PBX.

10D11 Is Able to Prevent Cooperative Complexes of HOXD4 with PBX1A and E2A-PBX1A—We (Fig. 2) and others (22, 41) have shown that by masking the HOX interaction domain on PBX using YPWM-containing peptides, the formation of HOX·PBX cooperative complexes can be prevented. In a complementary approach, we show that a monoclonal antibody directed against the YPWM motif of HOXD4 protein can likewise prevent the formation of a cooperative complex with PBX or E2A-PBX (Fig. 4*A*). While co-incubation of 10D11 with HOXD4 and PBX abrogates complex formation, the addition of the mAb to a preformed HOXD4·PBX complex resulted in only a 50% reduction (Fig. 4*A*, *lane 10*). Based on the above, we conclude that once a HOX·PBX·DNA trimeric complex is formed, it is significantly refractory to disruption. To assess the specificity of 10D11, we examined cross-reactivity with other HOX proteins. One such HOX protein, HOXA1, has FDWM instead of the YPWM in HOXD4. HOXA1 forms a stable complex with PBX in the presence of the mAb, proving that deviation from the recognized epitope allows cooperative complex formation in the presence of the antibody.

The Contribution of the Conserved Tyrosine of the YPWM Core Is Dependent on the Binding Site in DNA—HOX and PBX proteins interact to form stable cooperative complexes when bound to DNA through a YPWM motif present in HOX proteins from paralogous groups 1–8 (10, 17–19, 22). This motif is present N-terminal to the homeodomain and is connected to it through a linker region that varies between 5 and 56 amino acids (9, 42). We have examined the contribution of residues within and flanking the YPWM for cooperative interaction with PBX using three assays and up to three variant binding sites. The tryptophan and the methionine in the YPWM core are the most conserved residues, and mutating either to alanine is sufficient to abrogate HOX/PBX interactions *in vitro* and *in vivo* (present study and Refs. 19 and 24). The first position in the YPWM core is a tyrosine or phenylalanine, depending on the HOX protein examined (Fig. 1*C*). Mutating the tyrosine of HOXD4 to alanine also significantly abrogated cooperative complex formation on most probes in all three assays. Similar results were obtained with HOXB8 and HOXB4 proteins when phenylalanine or tyrosine were mutated to aspartic acid or leucine, respectively (19, 24). An interesting exception to our findings is the dissociation rate of Y3A on a T6 binding site. While the dissociation rates increased more than 10-fold on A6 and G6 probes, cooperative complexes with Y3A on a T6 probe were only 2.5-fold less stable.

Position 2 in the YPWM core, normally occupied by proline or aspartic acid seems to play some role in cooperative interactions. Mutating this residue in HOXD4 to alanine had a significant effect on the formation of the cooperative complex in all of our assays. Again the effect was dependent on the binding site assayed. There was less of an effect on dissociation rates on a G6 or T6 than on an A6 probe. The reduction in the HOXD4(P4A)·PBX complex on a G6 probe in steady state EMSA could be attributed to a defect in the association rate, since stability of the cooperative complex was not significantly altered. Mutation of the proline in HOXB4 and HOXB8 had little consequence in other studies (19, 24), and our results are modest. This is at odds with the high conservation of this proline in paralogous groups 2–8, and suggests a function in addition to contacting PBX. A more important role in contacting additional cofactors is one possibility.

Residues Flanking the YPWM Motif Are Important for Modulating HOX/PBX Interactions—Apart from the YPWM core, we demonstrated that residues flanking the core motif are important in the modulation of HOX·PBX cooperative complexes. Valines N-terminal to the YPWM core, when individually mutated to alanine, showed a significant reduction in the cooperative complex in one or more of the assays used here. Most strikingly, the V2A substitution markedly reduced cooperativity in all three experiments, suggesting that this residue makes a major contribution to interactions with PBX. This same position is occupied by leucine in the HOXB8 protein. Mutation of this residue to aspartic acid had no effect in EMSA (19). However, steady state EMSA often does not reveal changes in complex stability. Hence, it would be of interest to determine the stability of complexes formed between PBX and HOXB8 mutated at residues flanking the YPWM core. Differences in stability could well be used by the different HOX proteins to differentially cooperate with PBX in the regulation of their respective effector genes.

The other flanking residues, rather than playing an absolute

role in stabilizing the cooperative complex, exhibit a significant dependence on the sixth position of a HOX·PBX consensus binding site. Mutation of the first valine (V1A) also affected cooperativity in all three assays, although less dramatically than V2A protein. V1A reduced the stability of the cooperative complex significantly on an A6 probe and to a lesser extent on a G6 probe. This contrasts with the steady state analysis, which showed very poor complex formation of V1A on the G6 site (Fig. 5*B*, *lane 6*). This may suggest a greater effect of V1A on association rate than the stability of the complex once formed. By dissociation rate experiments, the C-terminal lysine K7 was shown to be important for the stability of cooperative complexes on A6 and T6 probes. Likewise, K8 played a role on A6 and possibly on T6 probes as well. Neither lysine played a role on a G6 probe. The *in vivo* activity of both lysines was reduced by half on a G6 probe, contrasting with the results of the dissociation rate experiments. It is possible that the difference in the *in vivo* and *in vitro* results for K7A and K8A could arise from differences in the proteins used in the different assays. For *in vitro* assays, N-terminally truncated HOXD4 was bacterially expressed as a His-tagged fusion protein (see "Experimental Procedures"), while full-length VP16 fusion proteins were used for the transcriptional activation assays. It is also hard to rule out the possibility that other factors might be involved in stabilizing the HOX/PBX interactions *in vivo* and may be dependent on the C-terminal lysines.

It has been shown that PBX, upon interaction with HOX, modulates the role of the HOX homeodomain N-terminal arm (28, 29, 31, 32). This results in subtle but discrete differences in target site recognition through position 6 of the binding site (28). Based on this, it was concluded that HOX proteins at the 3′-end of the cluster had a preference for G at position 6 of a HOX·PBX consensus binding site, and T at the same position for proteins at the 5′-end (28). Proteins in the middle of the cluster have a broader specificity at position 6 (28). The structure of the engrailed/DNA co-crystal shows that this position is contacted by the third residue in the N-terminal arm, which is an arginine in the case of HOXD4 (43). Studies from our lab have shown that although residue 3 is important for HOX monomers to contact position 6, this is not true in the case of a HOX·PBX heterodimer (32). The present study shows that position 6, which is still expected to contact the HOX N-terminal arm, determines the relative importance of amino acids within and flanking the YPWM for cooperativity. This could be due to conformational changes in HOX or PBX as the result of interactions with DNA. This is consistent with our observation that the interaction of the HOX N-terminal arm with position 6 is altered in the cooperative complex (32).

The present study shows that both the YPWM and the flanking residues determine the affinities of HOX·PBX complexes for DNA targets that differ at the sixth position. It is important to note that these flanking residues differ between paralogous groups but are well conserved in the same group. This may provide a mechanism for establishing the specificity of proteins within and between paralogous groups. Similar to the YPWM motif in HOX proteins, yeast Matα2 has a C-terminal tail that is unfolded as a monomer bound to DNA and takes up a defined structure upon interaction with a second homeodomain protein, a1 (44). Three leucine residues in the C terminus of Matα2 form an amphipathic helix that hydrophobically interacts with residues spanning helices 1 and 2 in a1 (44). A fourth isoleucine flanks the other three N-terminally and stabilizes the short amphipathic helix by packing intramolecularly against Leu-65 and Leu-69 (44). The crystal structure of a1/α2 has also revealed another functional role for the residues flanking the amphipathic helix in stabilizing the heterodimer interaction through hydrogen bonds with the a1 homeodomain. Analogous to the a1/α2 hydrophobic interactions, the YPWM motif must make hydrophobic contacts with PBX. As for Matα2, residues flanking the YPWM, namely the N-terminal valines and the C-terminal lysines, may be involved in stabilizing the YPWM interactions via intra- and intermolecular interactions.

Acknowledgments—We thank Isabel Rambaldi for help throughout this work, Michael L. Phelan for bacterially purified HOXA1, and members of the laboratory for useful discussions and critical reading of the manuscript.

REFERENCES

1. McGinnis, W., and Krumlauf, R. (1992) *Cell* **68,** 283–302
2. Krumlauf, R. (1994) *Cell* **78,** 191–201
3. St-Jacques, B., and McMahon, A. P. (1996) *Curr. Opin. Genet. Dev.* **6,** 439–444
4. Duboule, D., and Dollé, P. (1989) *EMBO J* **8,** 1497–1505
5. Graham, A., Papalopulu, N., and Krumlauf, R. (1989) *Cell* **57,** 367–378
6. Duboule, D. (1994) *Development* (suppl.) 135–142
7. Gehring, W. J., Qian, Y. Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A. F., Resendez-Perez, D., Affolter, M., Otting, G., and Wüthrich, K. (1994) *Cell* **78,** 211–223
8. Mavilio, F., Simeone, A., Giampaolo, A., Faiella, A., Zappavigna, V., Acampora, D., Poiana, G., Russo, G., Peschle, C., and Boncinelli, E. (1986) *Nature* **324,** 664–668
9. Qian, Y. Q., Otting, G., Furukubo-Tokunaga, K., Affolter, M., Gehring, W. J., and Wüthrich, K. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10738–10742
10. Johnson, F. B., Parker, E., and Krasnow, M. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 739–743
11. Peifer, M., and Wieschaus, E. (1990) *Genes & Dev.* **4,** 1209–1223
12. Chan, S.-K., Jaffe, L., Capovilla, M., Botas, J., and Mann, R. S. (1994) *Cell* **78,** 603–615
13. Rauskolb, C., Peifer, M., and Wieschaus, E. (1993) *Cell* **74,** 1101–1112
14. Rauskolb, C., and Wieschaus, E. (1994) *EMBO J.* **13,** 3561–3569
15. van Dijk, M. A., and Murre, C. (1994) *Cell* **78,** 617–624
16. Kamps, M. P., Look, A. T., and Baltimore, D. (1991) *Genes & Dev.* **5,** 358–368
17. Phelan, M. L., Rambaldi, I., and Featherstone, M. S. (1995) *Mol. Cell. Biol.* **15,** 3989–3997
18. Chang, C.-P., Shen, W.-F., Rozenfeld, S., Lawrence, H. J., Largman, C., and Cleary, M. (1995) *Genes & Dev.* **9,** 663–674
19. Neuteboom, S. T. C., Peltenburg, L. T. C., van Dijk, M. A., and Murre, C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 9166–9170
20. Pöpperl, H., Bienz, M., Studer, M., Chan, S.-K., Aparacio, S., Brenner, S., Mann, R., and Krumlauf, R. (1995) *Cell* **81,** 1031–1042
21. Lu, Q., Knoepfler, P. S., Scheele, J., Wright, D. D., and Kamps, M. P. (1995) *Mol. Cell. Biol.* **15,** 3786–3795
22. Knoepfler, P. S., and Kamps, M. P. (1995) *Mol. Cell. Biol.* **15,** 5811–5819
23. Chan, S., Popperl, H., Krumlauf, R., and Mann, R. (1996) *EMBO J.* **15,** 2476–2487
24. Shen, W., Chang, C., Rozenfeld, S., Sauvageau, G., Humphries, R., Lu, M., Lawrence, H., Cleary, M., and Largman, C. (1996) *Nucleic Acids Res.* **24,** 898–906
25. Chang, C.-P., de Vivo, I., and Cleary, M. L. (1997) *Mol. Cell. Biol.* **17,** 81–88
26. Lu, Q., and Kamps, M. P. (1996) *Mol. Cell. Biol.* **16,** 1632–1640
27. Izpisua, B. J., Falkenstein, H., Dollé, P., Renucci, A., and Duboule, D. (1991) *EMBO J.* **10,** 2279–2289
28. Chang, C.-P., Brocchieri, L., Shen, W.-F., Largman, C., and Cleary, M. L. (1996) *Mol. Cell. Biol.* **16,** 1734–1745
29. Chan, S., and Mann, R. (1996) *Proc. Nat. Acad. Sci. U. S. A.* **93,** 5223–5228
30. Knoepfler, P., Lu, Q., and Kamps, M. (1996) *Nucleic Acids Res.* **24,** 2288–2294
31. Lu, Q., and Kamps, M. (1997) *Oncogene* **14,** 75–83
32. Phelan, M., and Featherstone, M. (1997) *J. Biol. Chem.* **272,** 8635–8643
33. Ekker, S. C., Jackson, D. G., von Kessler, D. P., Sun, B. I., Young, K. E., and Beachy, P. A. (1994) *EMBO J.* **13,** 3551–3560
34. Featherstone, M. S., Baron, A., Gaunt, S. J., Mattei, M.-G., and Duboule, D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 4760–4764
35. Pöpperl, H., and Featherstone, M. S. (1992) *EMBO J.* **11,** 3673–3680
36. Reed, J., Tanaka, S., Cuddy, M., Cho, D., Smith, J., Kallen, R., Saragovi, H., and Torigoe, T. (1992) *Anal. Biochem.* **205,** 70–76
37. Catty, D. (1988) *Antibodies: A Practical Approach*, Vol. I, pp. 1–203, IRL Press, Oxford
38. LeSauteur, L., Maliartchouk, S., Jeune, H., Quirion, R., and Saragovi, H. (1996) *J. Neurosci.* **16,** 1308–1316
39. Kamps, M. P., Murre, C., Sun, X.-H., and Baltimore, D. (1990) *Cell* **60,** 547–555
40. Nourse, J., Mellentin, J. D., Galili, N., Wilkinson, J., Starbridge, E., Smith, S. D., and Cleary, M. L. (1990) *Cell* **60,** 535–545
41. Peltenburg, L., and Murre, C. (1996) *EMBO J.* **15,** 3385–3393
42. Bürglin, T. R. (1994) in *Guidebook to Homeobox Genes* (Duboule, D., ed) pp. 44–49, Oxford University Press, Oxford
43. Kissinger, S. R., Liu, B., Martin-Blanco, E., Kornberg, T. B., and Pabo, C. O. (1990) *Cell* **63,** 579–590
44. Li, T., Stark, M. R., Johnson, A. D., and Wolberger, C. (1995) *Science* **270,** 262–269