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Cooperative Interactions between HOX and PBX Proteins Mediated by a Conserved Peptide Motif

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Homeoprotein products of the *Hox/HOM* gene family pattern the animal embryo through the transcriptional regulation of target genes. We have previously shown that the labial group protein HOXA-1 has intrinsically weak DNA-binding activity due to residues in the N-terminal arm of its homeodomain (M. L. Phelan, R. Sadoul, and M. S. Featherstone, Mol. Cell. Biol. 14:5066–5075, 1994). This observation, among others, suggests that HOX and HOM proteins require cofactors for stable interactions with DNA. We have demonstrated that a putative HOX cofactor, PBX1A, participates in cooperative DNA binding with HOXA-1 and the Deformed group protein HOXD-4. Three Abdominal-B class HOX proteins failed to cooperate with PBX1A. We mapped the interacting domain of HOXD-4 to the YPWMK pentapeptide motif, a conserved sequence found N terminal to the homeodomain of HOXA-1 and many other homeoproteins but absent from the Abdominal-B class. The naturally occurring fusion of the transcriptional activation domain of E2A with PBX1 creates an oncoprotein implicated in human pre-B-cell leukemias (M. P. Kamps, C. Murre, X.-H. Sun, and D. Baltimore, Cell 60:547–555, 1990; J. Nourse, J. D. Mellentin, N. Galili, J. Wilkinson, E. Starbridge, S. D. Smith, and M. L. Cleary, Cell 60:535–545, 1990). A pentapeptide mutation that abolished cooperative interaction with PBX1A in vitro also abrogated synergistic transcriptional activation with the E2A/PBX oncoprotein. The direct contact of PBX family members by the HOX pentapeptide is likely to play an important role in developmental and oncogenic processes.

Vertebrate embryos are patterned along multiple trunk and limb axes by the *Hox* gene complex (42). This family encodes homeodomain-containing transcription factors that confer positional identity through the differential regulation of target gene expression. The 38 *Hox* genes found in mice and humans are arranged in four genomic clusters, *Hoxa* to *Hoxd*, related to the *HOM* complex of *Drosophila melanogaster*. These complexes can be aligned such that more closely related genes (paralogs) occupy the same position in each complex, suggesting that they arose by duplications of an ancestral complex (42). Recent phylogenetic comparisons indicate that all animals share related *Hox/HOM*-type gene clusters (76). Expression of the *Hox* and *HOM* genes largely follows a colinear pattern, such that the more 3′ a gene is located in a cluster, the more anteriorly it is expressed. Thus, more closely related HOX proteins from the same paralogous group are expressed in similar fashions. Paralogous genes have both distinct and overlapping functions manifested in their most anterior domain of expression (7, 30).

DNA binding by HOX and HOM proteins is mediated by the homeodomain, a conserved 60-amino-acid sequence that consists of a flexible N-terminal arm followed by three alpha helices (25). Base-specific contacts are made by the N-terminal arm and helix 3 via the minor and major grooves, respectively. Structural studies have demonstrated that even highly diverged homeodomains interact with DNA in a conserved fashion (83). HOX/HOM homeodomains can be divided into major classes based on sequence homology. This division also serves to classify the proteins according to their DNA-binding preferences. The majority of HOX/HOM homeodomains belong to the Antennapedia (Antp) class (HOX paralogous groups 2 to 8), which includes a number of proteins shown to bind and activate through elements of 6 to 9 bp containing a TAAT core motif. All of the Antp class homeodomains contain arginine at position 3 in the N-terminal arm, which contacts the second position in the TAAT core. Abdominal-B (Abd-B) class homeodomains have a lysine at position 3, and Abd-B exhibits a preference for a TTAT core (17). The members of the labial subfamily (HOX paralogous group 1) are unique since none of their homeodomains encode basic residues at positions 2 and 3. At least for HOXA-1, this results in a decreased affinity for DNA rather than an alteration in DNA-binding specificity (63). This may necessitate cooperation with a cofactor for site-specific DNA-binding by HOXA-1.

Three other aspects of DNA-binding by the HOX/HOM family suggest that cofactor interactions may be generally employed. First, inspection of gene regulatory sequences reveals a high proportion of presumptive HOX binding sites, suggesting that not all of these sites are used. Second, most HOX or HOM proteins recognize similar binding sites with only modest preferences, suggesting that the HOX homeodomain alone could not effectively discriminate between targets (8, 13, 18, 61). Third, although the K_d for HOX binding to a specific recognition sequence can be in the nanomolar range, the affinity for nonspecific sites is also relatively strong (3, 19), implying a need for cofactors in site-specific recognition. Consistent with a requirement for protein interactions, chimeric and deletion analysis of HOM proteins in vivo implicates non-DNA-contacting regions both flanking and within the homeodomain in functional specificity (26, 43, 48, 50).

A potential cofactor for HOM proteins, extradenticle (exd), was identified genetically by its ability to alter segment identity without affecting *HOM* gene expression (60). Maternal overexpression of exd rescued this phenotype, demonstrating that regulation by the *HOM* genes is not responsible for its segment

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specification function. Furthermore, exd was shown to cooperate with HOM proteins genes such as Ultrabithorax (Ubx) for regulation of specific target genes (69). Cloning of this gene revealed that its product is a homeodomain-containing protein. It was predicted that heterodimer formation by exd and specific HOM proteins could contribute to homeotic selector specificity at the level of DNA binding by modifying target gene selection in a cooperative fashion (68). Recently, it has been confirmed that exd does cooperatively bind with Ubx and other homeodomain-containing proteins to specific DNA sequences (6, 79).

The mammalian homologs of *exd* are the *PBX* family members (55). The proto-oncogene *PBX1* was identified as one of the loci affected in the t(1;19) translocation found in a quarter of all pediatric pre-B-cell acute lymphoblastic leukemias (38, 57). This translocation results in expression of a fusion protein consisting of the activation domains of E2A and most of PBX1, including its homeodomain. The high homology between PBX and exd proteins (71%) suggests not only that PBX proteins can act as HOX cofactors but also that HOX proteins expressed in hematopoietic lineages are required for the oncogenic potential of E2A/PBX.

The experiments described here show that the vertebrate exd homolog PBX1A displays cooperative DNA binding with some HOX proteins. We map a domain of HOXD-4 required for interaction with PBX1A to the pentapeptide, a sequence N terminal to the homeodomain conserved in many homeoproteins (52, 66). The pentapeptide-containing HOXA-1 protein also cooperates with PBX1A, whereas three Abd-B homologs that naturally lack the pentapeptide fail to do so. An intact pentapeptide is required for a HOX protein to strongly activate transcription of a promoter bearing HOX and PBX binding sites. In addition, the oncogenic E2A/PBX fusion protein activates transcription with a HOX partner in a synergistic manner that is dependent on the pentapeptide. The sequence and context of the pentapeptide in different HOX proteins suggest mechanisms to increase the specificity of target site selection. A number of non-HOX homeoproteins also have a pentapeptide motif located N terminal to the homeodomain, revealing a role for PBX family members in a broad spectrum of developmental processes.

MATERIALS AND METHODS

Plasmid construction. T3-driven expression vectors for *Hoxd-4* and *Hoxa-1* used for in vitro translation were generated by subcloning the coding sequences for these proteins from p4.2 and pHoxa-1(HD+) (see below) into the *Sma*I site of pBS(+) (Stratagene). Though not relevant to the present study, alternative splice signals in the *Hoxa-1* cDNA sequence (44) were modified as follows to produce pHoxa-1(HD+), from which only the homeodomain-containing product is translated. PCR amplification of three overlapping fragments spanning the transcription start site up to the first codon of the homeobox disrupted the alternative 5′ splice donor and 3′ acceptor sequences and removed the common intron. These fragments were joined by using the technique of splicing by overlap extension, and the product, after verification by sequencing, was subcloned as a *Bam*HI-*Hin*cII insert into p4.2/1.6 (63) to produce pHoxa-1(HD+). Mutation of the alternative 5′ donor sequence required the introduction of a valine-to-isoleucine substitution at position 115; otherwise, the 331-amino-acid HOXA-1 product is identical to the one reported previously (44). The primer pairs used for each of the PCR reactions were O-144 (AGGATCCAAGAATGAACTCC TTTCTG) and O-145 (GGGTACCCACCACTTATGTCTGCTT), O-146 (CGA GTTGTGGTCCAAGC) and O-147 (GCTTCTTGGTGGGAGGCGTGGAG), and O-148 (CTCCCACCAAGAAGCTTGTCGTTC) and O-149 (GGTTGAC CCACGTAGCCGTACTCTCCAACTTTCCCTGTTTGGGAGGGTTT).

The *E2A/PBX1A* cDNA and the T3-driven expression vector, pSK-pbx1a, were generous gifts of Mark Kamps. The *E2A/PBX1A* cDNA was cloned into the eukaryotic expression vector pSG5 (27) for transfection experiments. pSG5-based expression vectors allowing T7-driven transcription of *Hoxd-9*, *Hoxd-10*, and *Hoxd-11* were kindly provided by Denis Duboule. The T3-driven expression vector for PBX1A ΔC-term was produced by removal of an *Nco*I-*Xho*I fragment extending into the pBSK polylinker. Construction of HOXD-4 mutants ΔGH, ΔAB, and ΔD is described elsewhere (67). Mutant ΔABCD was made by replacing a *Bam*HI-*Eco*RI fragment spanning exon 1 with the sequence GATCCAC CATGAGT (top strand). The two-amino-acid substitution WM to AA was made by replacing an *Acc*I-*Eco*RI fragment spanning region D with the sequence CTACCCTGCGGCGAAGAAGGTGCACGTG (top strand). The HOXD-4 ΔC mutation was created by deletion of a *Sty*I-*Pst*I fragment. In vitro-translated HOXD-4 ΔABCD, ΔGH, and ΔC proteins were prepared using the T3 or T7 promoter of pBS(+), while ΔAB, ΔD, and WM to AA were produced by using the T7 promoter of pET-3b (72).

The eukaryotic expression vector based on the murine phosphoglycerate kinase (*pgk*) regulatory elements (2) is described elsewhere (67). pPGK-HOXD-4/VP16 encoded the VP16 acidic activation domain fused to the C terminus of HOXD-4. To make this vector, the stop codon of *Hoxd-4* in p4.2 (67) was replaced with an *Xba*I site by PCR. Then, the 3′ 79 codons of the VP16 open reading frame were amplified by PCR using primers containing *Xba*I sites and subcloned in frame with the *Hoxd-4* coding region. The primers for PCR replacement of the *Hoxd-4* stop codon were O-83 (ACGTGAATTCGGCGAAC CCCAACTACACCGG) and O-77 (GGCCTCTAGATAAGGTCGTCAGGTC CGT). The primers for amplification of VP16 were O-98 (CTAGTCTAGAC TACCCACCGTACTCGTC) and O-99 (GATCTCTAGAACGGCCCCCCCG ACCGAT). The construct was verified by sequencing. pPGK-HOXD-4(WM to AA)/VP16 was generated by subcloning the *Bam*HI-*Eco*RI fragment of pPGK-HOXD-4(WM to AA) into the above-mentioned construct. Construction of pPGK-PBX1A involved subcloning the *Xba*I-*Eco*RV fragment of *pbx1a* into the *pgk*-based vector.

The luciferase reporter construct pML is described elsewhere (65). pML(5xHOX) and pML(5xHOX/PBX) were made by multimerization of oligonucleotides corresponding to the 3′ TAAT-containing HOXD-4 recognition site in the *Hoxd-4* promoter (65) (CCCTGCCCTTCACCATTAGCTCGACAGTCT CAGCC) and the two-site HOX/PBX probe (79) (see below), respectively, into the *Sma*I site of pML.

In vitro transcription and translation of expression vectors. Proteins were produced by using a TnT in vitro transcription-translation kit (Promega). Quantitation of unlabeled proteins was deduced from parallel [^{35}S]Met-containing reactions run on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and measured on a Fuji BioImaging Analyzer.

EMSA. The two-site DNA probe (79) having both HOX and PBX binding sites, GTCAATTAAAGCATCAATCAATCAATTTCG (one strand shown), was end labeled as described previously (65). Electrophoretic mobility shift assay (EMSA) binding and electrophoresis conditions were as previously described (63), with the following modifications. A total of 4 μl of in vitro-translated protein was preincubated on ice for 30 min with 200 ng of poly(dI-dC), except where otherwise indicated, in 24 μl. Then 1 μl containing 40,000 cpm of DNA probe was added. The binding reaction (final conditions: 100 mM KCl, 10 mM Tris-Cl [pH 7.5], 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 12% [vol/vol] glycerol) proceeded for 30 min at 23°C before electrophoresis at 4°C on a 5% (29:1) polyacrylamide gel.

P19 cell culture and transfection assays and Western blot (immunoblot) analysis. Maintenance of P19 embryonal carcinoma cells in tissue culture and transfection of retinoic acid-treated P19 cells have been described elsewhere (67). Differentiating P19 cells were used because they are efficiently transfected and express endogenous *Hox* genes (4). Briefly, 10 μg of total DNA was added to each 10-mm-diameter dish 48 h after addition of retinoic acid to 3×10^{-7} M. For the transfection assays, this included 1 μg of each of the appropriate expression vectors and 4 μg of the appropriate pML-based luciferase reporter construct. The effect of differences in transfection efficiency was normalized to β-galactosidase activity from cotransfected pRSV-lacZ (1 μg). For Western analysis, the amount of expression vector was raised to 4 μg. As a control, we used a mock transfectant in which no expression vector was added. Equivalent levels of β-galactosidase activity for each extract were separated by electrophoresis on an SDS–12% polyacrylamide gel, transferred to nitrocellulose, and probed with an antiserum directed against the HOXD-4 N terminus as described previously (67).

RESULTS

HOXA-1 and HOXD-4, but not the Abd-B homologs HOXD-9, HOXD-10, and HOXD-11, form multimeric complexes on DNA with PBX1A. Recently, cooperative DNA binding has been demonstrated for the *Drosophila* homeoproteins exd and Ubx (6, 79). Products of the mammalian *PBX* gene family are likewise implicated as cofactors of *Hox* gene products on the basis of their homology to exd (68). Using a DNA probe (79) containing a consensus HOX recognition site (5′-TTAATTG-3′) and an optimal binding site for PBX1A (5′-ATCAATCAA-3′) (80), we tested the ability of a number of in vitro-translated full-length HOX proteins to bind DNA cooperatively with PBX1A in an EMSA (Fig. 1). The proteins

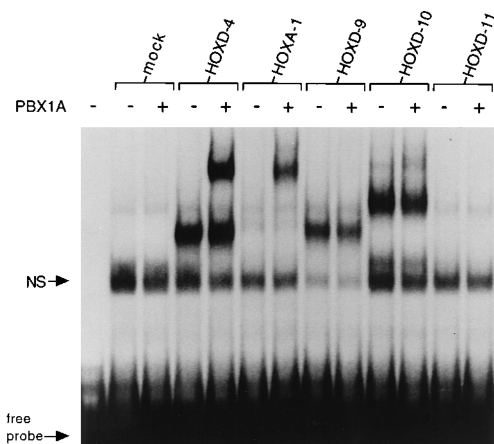

FIG. 1. Effect of PBX1A on DNA binding by murine HOX proteins as detected by EMSA. A ^{32}P-labeled DNA probe containing binding sites for both HOX and PBX1A proteins (79) was incubated with in vitro-translated HOX proteins alone or in the presence of in vitro-translated human PBX1A as shown. Bound DNA and unbound DNA were separated by gel electrophoresis. "Mock" refers to translation reactions in which no template was added; therefore, the nonspecific (NS) bands detected in these lanes are due to endogenous proteins in the reticulocyte lysate. Although not detected in this experiment, the faint complex of PBX1A and DNA runs slightly more slowly than the complex containing HOXD-4. For HOXD-4 and HOXA-1, monomer binding can be detected in the absence of PBX1A. This binding is weak for HOXA-1. Addition of PBX1A promotes the formation of a strong complex with slower mobility. PBX1A does not alter DNA binding in extracts containing the Abd-B homolog HOXD-9, HOXD-10, or HOXD-11.

tested are encoded by the *Deformed* homolog *Hoxd-4* (21, 65), the *labial* homolog *Hoxa-1* (4, 44), and three members of the large *Abd-B* subfamily: *Hoxd-9* (14), *Hoxd-10* (14), and *Hoxd-11* (32). HOXD-4, HOXD-9, and HOXD-10 each formed a strong complex in the absence of PBX1A, illustrating the overlap in sequence specificity between members of different subfamilies. As noted previously (63), HOXA-1 displayed poor DNA-binding activity. HOXD-11, despite containing a homeodomain very similar to that of HOXD-9 and -10, showed no DNA-binding activity. PBX1A alone also produced no measurable shift under our conditions, which used low amounts of a nonspecific competitor (see Materials and Methods). When PBX1A was included in the HOX/DNA-binding incubation mixtures, an additional, low-mobility complex was seen for both HOXD-4 and HOXA-1. For HOXA-1 and PBX1A, this complex represents high-affinity binding resulting from the combination of two homeoproteins that bind DNA poorly on their own. In contrast, the binding of the three Abd-B homologs to DNA was unaffected by the presence of PBX1A. This result suggests that multiprotein complexes are formed on this two-site DNA probe by PBX1A and either HOXD-4 or HOXA-1 but not HOXD-9, HOXD-10, or HOXD-11.

The multimeric protein-DNA complex contains both HOX and PBX1A proteins. To test whether both PBX1A and HOX proteins were present in the low-mobility complex, we substituted wild-type PBX1A and HOXD-4 with truncated versions of each protein. The HOXD-4 deletion mutant lacked amino acids 31 to 111 inclusive (region C in Fig. 3A), while the PBX1A mutant lacked the C-terminal 111 residues. These PBX1A residues represent more than two-thirds of all PBX1A sequences which have no homology with exd (68). The use of either truncated protein in the EMSA resulted in an increased mobility for the induced protein-DNA complex, and the use of both produced the fastest mobility (Fig. 2A). This observation strongly suggests that both wild-type proteins are present in the original low-mobility complex. That the unique PBX1A C-terminal sequence is dispensable for cooperative binding indicates that formation of this complex is related to the Ubx-exd interaction (79).

The interaction between HOX and PBX1A proteins increases total DNA binding by each of the three proteins and thus represents cooperative DNA binding. As mentioned, the effect of the PBX1A-HOX interaction on binding by HOXA-1 and PBX1A was most dramatic, whereas HOXD-4 bound the probe well even in the absence of PBX1A. To confirm whether the interaction between these proteins was cooperative, we

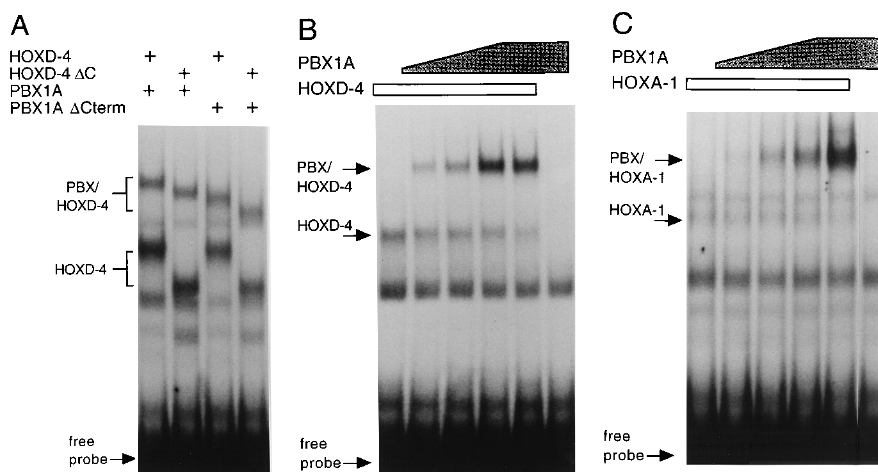

FIG. 2. The low-mobility complex is the result of cooperative DNA binding between PBX1A and HOX proteins. (A) The mobilities of complexes induced by truncated or full-length HOXD-4 and PBX1A proteins were compared. The HOXD-4 deletion mutant (HOXD-4 ΔC) lacks 81 residues spanning region C in Fig. 3A. PBX1A ΔC-term was truncated by 111 amino acids. The faster mobilities of the complexes formed with one or both truncated proteins reveal the presence of both PBX1A and HOXD-4. (B) The amount of binding by HOXD-4 alone (first lane) or in the presence of increasing amounts of PBX1A was resolved by EMSA. The amount of PBX1A was increased in twofold increments. The fourth lane from the left contains equivalent amounts of HOXD-4 and PBX1A. Therefore, this strong shift is occurring at equimolar concentrations of the two proteins. In this panel, the amount of poly(dI-dC) was 1 μg per reaction. (C) The effects of increasing amounts of PBX1A on binding by HOXA-1 were similarly determined and reveal strong cooperative interactions. Quantitation of autoradiographic signals was done on a Fuji Bio Imaging Analyzer.

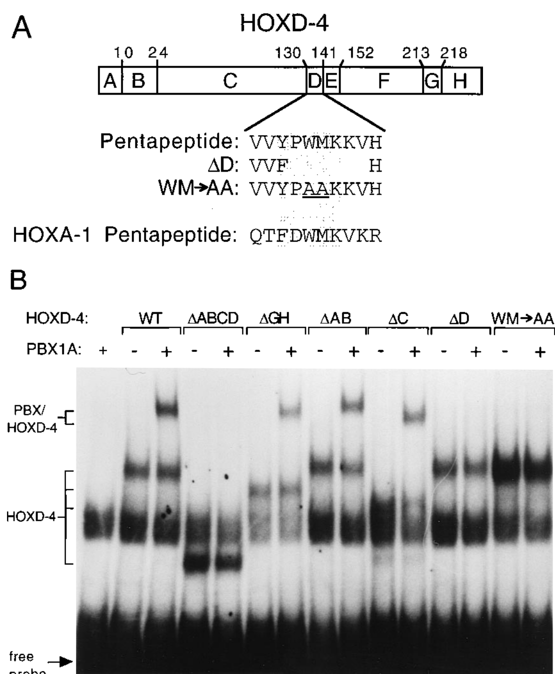

FIG. 3. Mapping of the PBX1A-interacting domain of HOXD-4. (A) The HOXD-4 sequence was divided into eight regions (67) based on conservation within the Deformed subfamily (regions B, E, and G) or across subfamilies (regions A, D, and F). Region F is the homeodomain. The codon number which marks the N-terminal boundary of each region and the sequence of region D (with the pentapeptide shaded) are shown above and below, respectively. The sequences of the pentapeptide deletion (ΔD), the two-residue substitution (WM to AA), and the HOXA-1 pentapeptide are also shown. (B) HOXD-4 proteins containing deletions corresponding to one or more regions defined in Fig. 3A, or carrying the WM-to-AA pentapeptide substitution, were tested for the ability to bind the DNA probe alone and in combination with PBX1A. Equimolar amounts of each HOXD-4 protein were used in a 4% (29:1) gel. Poly(dI-dC) was used at 1 μg per reaction. WT, wild type.

tested the ability of PBX1A to increase the total amount of binding produced by HOXD-4 and HOXA-1. Increasing amounts of PBX1A were added to a fixed amount of HOXD-4 (Fig. 2B) or HOXA-1 (Fig. 2C). Cooperative interactions increased DNA binding by 8.1-fold with respect to monomer binding by HOXA-1. Additionally, higher levels of PBX1A shifted the majority of HOXD-4 binding activity into the low-mobility complex and increased the total amount of binding by HOXD-4.

A conserved pentapeptide motif in HOXD-4 is required for interaction with PBX1A. The HOXD-4 amino acid sequence can be divided into eight regions (Fig. 3A) based on sequence conservation (67). This division served to guide a deletion analysis to map domains of HOXD-4 responsible for the interaction with PBX1A. We tested a number of HOXD-4 deletion mutants, each containing the homeodomain, for cooperative DNA binding (Fig. 3B). As expected, the presence of the homeodomain conferred on each mutant the ability to bind DNA as a monomer, albeit with an altered mobility commensurate with the size of each deletion. The HOXD-4 C terminus was dispensable for cooperative binding (Fig. 3B, ΔGH), while deletion of the N-terminal 140 amino acids abolished interactions with PBX1A (Fig. 3B, ΔABCD). We therefore tested finer mutations in the N terminus for their effects on the cooperative interaction. Deletion of regions A and B (ΔAB) or region C (ΔC) had no effect on cooperative binding. However, the HOXD-4 mutant ΔD, which was created by the removal of six residues spanning the pentapeptide motif (Fig. 3A), was defective for interaction with PBX1A. A similar sequence is found in many HOX/HOM proteins, including HOXA-1, which also cooperatively binds with PBX1A. This finding suggests that HOXA-1 may also interact with PBX1A via its pentapeptide. The HOXD-4 and HOXA-1 motifs are identical at only three pentapeptide positions (Fig. 3A). We tested the effect of converting two of these, the tryptophan and methionine in HOXD-4, to alanine residues (Fig. 3A, WM to AA). This substitution was sufficient to abolish HOXD-4–PBX1A cooperativity, demonstrating that the HOXD-4 pentapeptide motif is necessary for interaction with PBX1A.

The HOXD-4 pentapeptide is required for transcriptional activation of a reporter containing HOX and PBX binding sites. We wished to determine whether HOX/PBX1A interactions could influence the transcriptional activity of a test promoter. Preliminary cotransfection experiments in differentiated P19 cells revealed that transcriptional activation by wild-type HOXD-4 and PBX1A was weak in our system (data not shown), possibly because endogenous cellular PBX and HOX proteins are plentiful enough to mask the activity of additional protein produced from transfected vectors. We therefore performed cotransfection experiments with expression vectors for PBX1A and a fusion protein (HOXD-4/VP16) between HOXD-4 and the VP16 acidic transcriptional activation domain. We used luciferase reporter plasmids driven by a minimal adenovirus major late promoter. pML had no other transcriptional elements, whereas two additional reporters carried multiple upstream insertions of either a HOXD-4 recognition site (p5xHOX) or the HOX and PBX binding sites used for Fig. 1 to 3 (p5xHOX/PBX) (Fig. 4A).

The results of the transfections are shown in Fig. 4B. For all three reporter constructs, PBX1A alone had no measurable effect on luciferase levels. This result is consistent with previous reports on the weak transcriptional activation properties of this protein when it is not fused to the activation domains of E2A (46, 49, 80). In contrast, HOXD-4/VP16 activated expression of all three reporter genes. Weak activation seen with pML was likely due to binding to nonspecific or cryptic recognition sequences. The insertion of HOX binding sites in front of the minimal promoter (p5xHOX) resulted in a further fourfold increase in luciferase values above that seen for pML. The strongest activation by HOXD-4/VP16 was seen when both HOX and PBX binding sites were present upstream of the minimal promoter (p5xHOX/PBX) and resulted in a further 2.5-fold increase in transcription.

The effect of coexpression of PBX1A with HOXD-4/VP16 was dependent on the reporter construct tested. Coexpressed PBX1A had little additional effect on transcription from the pML and p5xHOX reporters but produced a 1.7-fold increase in activation of p5xHOX/PBX relative to HOXD-4/VP16 alone. Overall, there was a 170-fold transcriptional activation of p5xHOX/PBX by coexpressed HOXD-4/VP16 and PBX1A. From these results, it is apparent that the juxtaposition of PBX and HOX binding sites in the promoter has a strong effect on HOXD-4/VP16 activity that is further amplified in the presence of exogenous PBX1A.

To investigate the role of the pentapeptide in mediating transcriptional activation of these promoters, we tested a derivative of the HOXD-4/VP16 fusion protein, HOXD-4(WM to AA)/VP16, having the two-amino-acid substitution in the pentapeptide that abolished cooperative interactions with PBX1A in vitro. Western blot analysis of extracts of transfected cells showed that this mutation did not affect the amount of protein produced (Fig. 5). Conversion of WM to AA did not significantly alter transcriptional activation of the

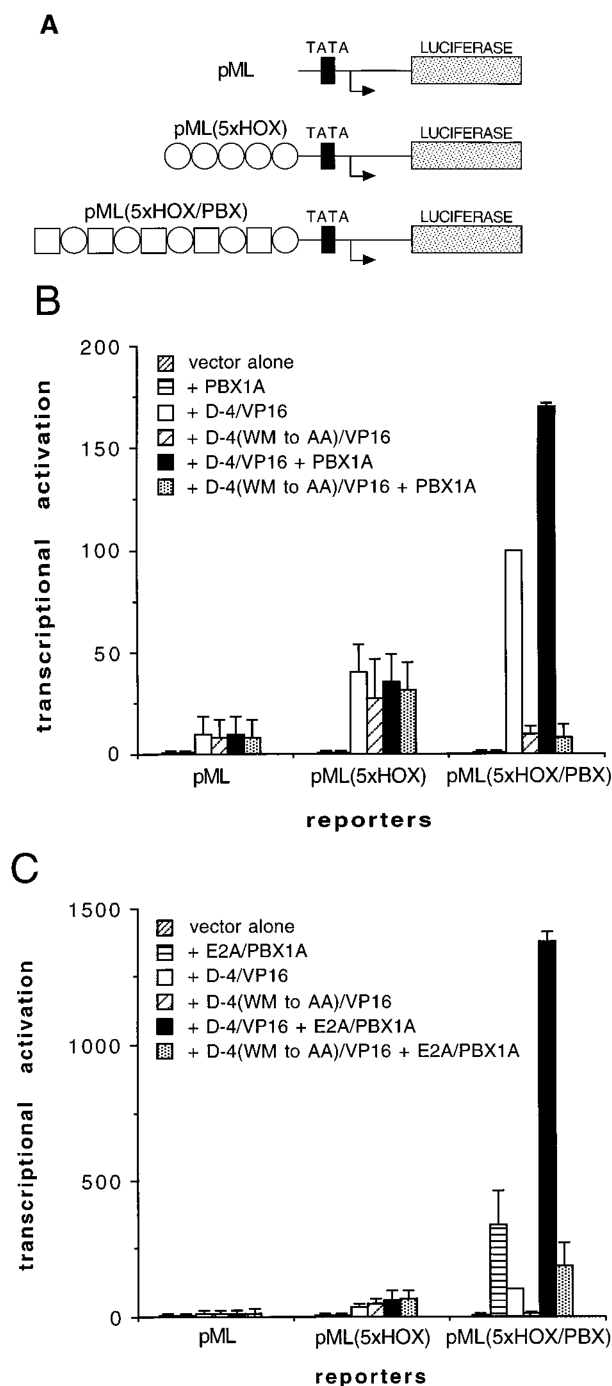

FIG. 4. The pentapeptide is required for strong transcriptional activation from a promoter bearing HOX and PBX binding sites. (A) Luciferase reporters contained either no upstream binding sites (pML), HOX binding sites [circles in pML(5xHOX)], or HOX and PBX binding sites [circles and squares in pML(5xHOX/PBX)]. (B) Results of cotransfections with expression vectors for PBX1A and HOXD-4/VP16 or HOXD-4(WM to AA)/VP16. (C) Results of cotransfections with expression vectors for E2A/PBX1A and HOXD-4/VP16 or HOXD-4(WM to AA)/VP16. Transcriptional activity from the three reporters was calculated as the fold increase over the activity of the reporter in the absence of cotransfected expression vectors. These values were then expressed relative to the activity of pML(5xHOX/PBX) in the presence of HOXD-4/VP16, which was set to 100. The standard deviations for two experiments are given. Note the difference in scale between panels B and C.

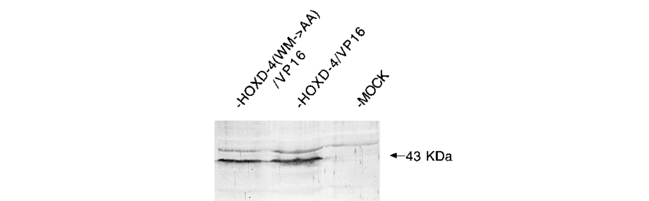

FIG. 5. Immunoblot detection of HOXD-4/VP16 fusion proteins. Extracts of cells transfected with expression vectors for HOXD-4/VP16 or HOXD-4(WM to AA)/VP16 were assayed by Western blot analysis for levels of the two proteins. A mock transfection was included. Amounts of cell protein were normalized to β-galactosidase activity from a cotransfected vector. HOXD-4/VP16, which has a predicted molecular mass of approximately 36 kDa, runs just below the 43-kDa marker. HOXD-4 and other proline-rich homeoproteins have been seen previously to run with slower than expected mobilities by SDS-polyacrylamide gel electrophoresis (67, 73). Comparable amounts of the two proteins accumulate in transfected cells. We have made similar observations for a HOXD-4 protein carrying a deletion of the pentapeptide (67).

pML and p5xHOX reporter plasmids which do not carry PBX binding sites. By contrast, mutation of the pentapeptide markedly decreased transcriptional activation of the p5xHOX/PBX reporter. Thus, the pentapeptide is required for strong transcriptional activation from a promoter that carries both HOX and PBX binding sites. The results of our EMSA experiments strongly suggest that this is due to the formation of cooperative complexes with endogenous PBX proteins. Mutation of the pentapeptide may result in exclusion of HOXD-4(WM to AA)/VP16 by endogenous cooperative HOX-PBX complexes that can form tighter associations with the HOX and PBX binding sites but have weaker transcriptional activation functions (see below).

Finally, we tested the effect of exogenous PBX1A on transcriptional activation by HOXD-4(WM to AA)/VP16. No rescue of activity was observed on any reporter, consistent with a requirement of the pentapeptide for cooperative interaction with PBX1A. We conclude that the loss of the HOXD-4 pentapeptide sequence has a dramatic effect on transcriptional regulation when HOX and PBX binding sites are both present in the promoter. The results further suggest that there are endogenous PBX-like cofactors expressed in differentiated P19 cells that are available for interaction with HOXD-4/VP16.

The E2A/PBX oncoprotein activates transcription in a synergistic manner dependent on the HOXD-4 pentapeptide. The E2A/PBX1 fusion protein acts as a potent transcriptional activator (46, 49, 80). Moreover, transcriptional activation by E2A/PBX is strongly correlated with oncogenic potential (56). We therefore tested whether E2A/PBX activity could be modulated by cooperative interaction with a HOX protein. We performed cotransfection experiments with the same three luciferase reporters described above. While transcriptional activation of pML and pML(5xHOX) was weak, we observed greater than 300-fold transcriptional activation of the pML (5xHOX/PBX) reporter cotransfected with an expression vector for E2A/PBX (Fig. 4C). This result was consistent with the presence of PBX binding sites in the latter reporter only (46, 49, 80). We next examined whether activation by E2A/PBX was modulated by interaction with HOX proteins. Cotransfection with the HOXD-4/VP16 expression vector did not increase transcriptional activation by E2A/PBX on the pML and pML(5xHOX) reporters. By contrast, strong synergistic activation was observed with pML(HOX/PBX), resulting in a 1,400-fold increase in reporter activity.

To determine whether this synergistic effect was mediated by the HOXD-4 pentapeptide, we cotransfected in parallel the expression vector for HOXD-4(WM to AA)/VP16. Mutation

of the pentapeptide abolished the synergistic effect with E2A/PBX (Fig. 4C). We conclude that the E2A/PBX oncoprotein can undergo pentapeptide-dependent interactions with HOX proteins in the transcriptional activation of target genes.

DISCUSSION

We have investigated the ability of PBX1A, the human homolog of exd, to mediate cooperative DNA binding with HOX proteins. HOXA-1 has intrinsically weak DNA-binding activity, and we suggested that it may be particularly dependent on cofactor interactions for effective site recognition (63). We now show that a cooperative HOXA-1/PBX1A complex does indeed show greatly increased DNA binding activity over either protein alone (Fig. 2C). As argued in the introduction, HOX proteins in addition to HOXA-1 may also require cofactor interactions. Supporting this contention, HOXD-4 also undergoes cooperative DNA binding with PBX1A (Fig. 2B). Such cooperative interactions are likely to contribute to HOX and PBX function in vivo. Consistent with this view, we observed stronger transcriptional activation by a HOXD-4/VP16 fusion protein through multimerized HOX and PBX recognition sites than with HOX sites only. Moreover, this effect was enhanced by cotransfection with PBX1A or E2A/PBX expression vectors (Fig. 4).

Our results further demonstrate the requirement of the HOXD-4 pentapeptide for cooperative interaction with PBX1A both in vitro and in vivo. The pentapeptide is a conserved sequence found N terminal to the homeodomain in many HOX/HOM proteins (52, 66) and in other homeoproteins (31, 33, 40, 59). How does the pentapeptide promote cooperative DNA binding with PBX1A? The most straightforward models would propose a primary role either in DNA binding or in the direct contact of PBX proteins. Several pieces of evidence argue for the latter. First, structural and biochemical studies have shown that the presence of the pentapeptide does not alter the DNA-binding activity of the homeodomain (18, 66). Our own work shows that the pentapeptide does not directly influence the recognition of HOX binding sites, even when adjacent to PBX binding sites (Fig. 3B). While binding by PBX1A could alter the conformation of DNA to promote direct DNA recognition by the pentapeptide, this should not necessitate the retention of PBX1A in the complex. The observation that overall binding by PBX1A is increased cooperatively in a pentapeptide-dependent manner is more easily explained by direct protein-protein contact. Second, transcriptional activation is affected by the pentapeptide only in the context of a promoter carrying both HOX and PBX binding sites, suggesting that protein-protein contacts stabilize the occupancy of adjacently bound PBX and HOX proteins. Third, the analogous region of the yeast homeoprotein Matα2 appears to contact directly the MCM1 cofactor, providing a precedent for cooperative interactions through a domain located N terminal to the homeodomain (82). Fourth, we have shown that the invariant tryptophan and methionine residues of the pentapeptide are essential for function. These hydrophobic residues could readily contribute to an appropriate interface for protein interactions. Fifth, a motif in the globin β chain (VYPW) that contacts both α chains in the hemoglobin tetramer is identical to four adjacent residues overlapping the pentapeptide of HOXD-4. There is thus a well-described precedent for the involvement of a motif closely related to the pentapeptide in protein-protein interactions (22). Last, recent experiments have shown that the Ubx homeodomain and pentapeptide are required for efficient interaction with exd in a yeast two-hybrid assay, a system that relies on protein-protein interactions (34). None of these arguments excludes additional roles for the pentapeptide in DNA recognition (see below).

The region spanning the pentapeptide is conserved between HOX and HOM homologs. Additionally, PBX family members are highly homologous (90%) to exd over an approximately 270-amino-acid stretch (68). The C-terminal 111 amino acids of PBX1A are diverged from exd and are dispensable for interaction with HOXD-4 (Fig. 2A). It therefore seems likely that the domains required for PBX-HOX and exd-HOM protein interactions are conserved between insects and vertebrates. Consistent with this prediction, deletion of the nonconserved 53-residue C terminus of exd has a negligible effect on interactions with Ubx (79). Fusion to E2A deletes the first 88 residues from PBX1 (38, 57), yet this molecule displays synergistic transcriptional activation with a HOXD-4/VP16 fusion protein that is dependent on the HOXD-4 pentapeptide (Fig. 4C). Together, these results map domains required for cooperativity within regions of PBX1 that are highly conserved with exd.

Multiprotein DNA-binding complexes may serve to increase the occupation of particular sites by altering the rate of association or dissociation of a protein with its DNA target. In the case of the exd-Ubx interaction, exd was shown to decrease the rate of dissociation of Ubx from DNA (6). We have observed a very rapid rate of dissociation for the HOXD-4 homeodomain from its recognition site, indicating that the stability of the HOXD-4–DNA interaction is a limiting factor (our unpublished results). Complex stability may be achieved through one or more mechanisms. By analogy to Antp, the region of HOXD-4 containing the pentapeptide and N-terminal arm is unstructured. Interaction with PBX1A may structure the pentapeptide and flanking sequences, analogous to the induced structuring of the Matα2 homeoprotein C terminus upon interaction with Mat**a**1 (64). This ordered configuration could impart a specific spacing requirement on binding sites for the two proteins, similar to that obtained through Matα2-MCM1 interaction (77). An additional possibility is the induction of extended DNA contacts through residues spanning the adjacent N-terminal arm and pentapeptide domains. Interestingly, the region of the yeast Matα2 homeoprotein that contacts MCM1 is likewise unordered and has been suggested to adopt a structure through protein-protein contacts that may promote further contacts to DNA as well (82). By analogy, these authors correctly predicted a role for the pentapeptide in cooperative DNA binding. As noted by others (24), an unstructured domain may provide for moderately strong protein-protein interactions that contribute substantially to DNA binding while allowing partners to be shuffled in a multicomponent regulatory network.

The identification of a function for the pentapeptide allows us to suggest a number of ways in which members of the HOX/HOM family might differentially exploit such interactions to produce functional specificity in both insects and mammals (Fig. 6). First, within the HOX/HOM subfamily, only the Abd-B class proteins (HOX paralogous groups 9 to 13) lack a pentapeptide (20). This is consistent with our observation that HOXD-9, HOXD-10, and HOXD-11 do not cooperate with PBX1A for binding to the two-site probe and with the previous observation that Abd-B is unable to cooperate with exd (79). In total, full-length proteins from four different pentapeptide-containing subfamilies have now been shown to interact with exd or PBX1A on the two-site probe: HOXA-1 (labial), HOXD-4 (Deformed), Ubx (79), and Abd-A (79). This may represent a significant functional distinction between Abd-B and non-Abd-B subfamily proteins, consistent with the effect of *exd* on loss-of-function *HOM* gene mutations: *Abd-B* does not

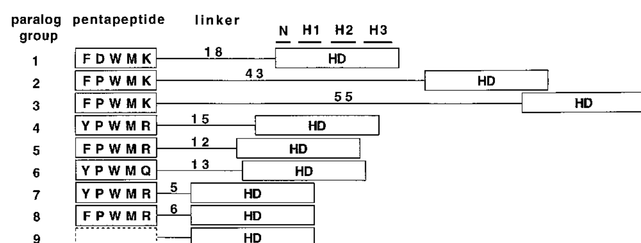

FIG. 6. Variation in pentapeptide sequence and pentapeptide-homeodomain linker length between HOX/HOM subfamilies. The pentapeptide sequences and structures of HOXB proteins, where available (paralogous groups 1 through 7), and HOXD-8 and HOXD-9 are shown. *HoxB* is the only complex with genes encoding members of all subfamilies from 1 to 9. The lack of a pentapeptide in Abd-B class proteins (paralogous groups 9 to 13) is represented by an empty dashed box. Values in the linker region indicate the number of residues between the pentapeptide and homeodomain (HD). Only the linkers are drawn to scale. N, homeodomain N-terminal arm; H1, H2, and H3, α helices 1, 2, and 3. For HOXD-4, the linker corresponds to region E (Fig. 3A).

display overlapping or synergistic transformations in *exd* larvae (60). Second, subfamily-specific sequence variation exists both within and flanking the pentapeptide (Fig. 6). Such variations may affect the affinity of HOM proteins for exd and of HOX proteins for the different PBX family members. Third, spacing between the pentapeptide and the homeodomain also varies between subfamilies (Fig. 6). The region of Antp containing both the pentapeptide and the homeodomain N-terminal arm has been shown to be flexible in solution (66). The length of this flexible linker may affect the ability of a homeoprotein to present the pentapeptide to PBX/exd-like cofactors on differentially spaced or oriented binding sites. Interestingly, proboscipedia (9), Ubx (41, 58), and Antp (5) isoforms can have variable spacing between the two domains through alternative splicing. In at least one case, this appears to create isoforms with distinct functions (50). Recent results obtained with the yeast two-hybrid assay show that differences in the Ubx linker region can strongly modulate interactions with exd (34). Additionally, minor groove contacts made by the homeodomain N-terminal arm may act to restrict the flexibility of the adjacent linker region. Such a model has been proposed to explain the effect of N-terminal arm differences on DNA binding by the bipartite POU domains of Brn-2 and Brn-3 (47). Analogous interactions between Matα2 and MCM1 (82) result in a substantial increase in specificity for a particular spacing of Matα2 recognition sites.

If pentapeptide sequence or spacing confers differential site-specific recognition of DNA, then how are HOX and HOM proteins from four different subfamilies able to interact with PBX1A or exd on the same DNA probe? One possibility is that the positioning of the sites in this probe happens to fall within a range of overlapping specificity. Another nonexclusive explanation is that optimal DNA recognition by PBX1A or exd lowers the specific recognition requirements of its HOX/HOM cofactor. Our transcriptional data clearly show that the PBX/exd site is required for synergy, while others have shown that the HOX/HOM site in this same probe is not required for cooperative interactions (79). This finding suggests that optimal or specific binding by the HOX or HOM protein may not be required in the presence of an optimal site for PBX or exd. The Mat**a**1-Matα2 interaction has been shown to be unaffected by Matα2 mutations which disrupt site-specific monomer binding (81). This provides a precedent for the action of homeoprotein heterodimers in which one partner can provide most or all of the binding specificity through interaction with the second. Specific recognition by the HOX or HOM protein may be critical when both sites are suboptimal, such as those seen in the enhancer of *dpp* (6). It is via such elements that the pentapeptide-homeodomain spacing may impose specificity in vivo. Consistent with this, neither HOXD-4 nor HOXA-1 cooperated with PBX1A on an enhancer element (dpp80a) (6) containing sites which were oriented differently from the probe used here (data not shown). Perhaps binding to these suboptimal sequences requires specific recognition by both proteins, and HOXD-4 and HOXA-1 are unable to interact effectively both with DNA and PBX1A in this particular context.

With a DNA probe in which the orientation of the HOX/HOM and PBX/exd binding sites was different from that used here, Ubx proteins lacking the pentapeptide-containing N terminus were suggested to interact with exd through the Ubx homeodomain and C terminus (6). It was shown that neither of the corresponding domains of Antp was able to substitute for this interaction effectively. In contrast, our results indicate that the pentapeptide, located N terminal to the homeodomain, is a critical mediator of HOXD-4/PBX1A interactions and that the HOXD-4 C terminus is not required. Moreover, HOXD-4 has identity with Antp at five of the six homeodomain positions which differ between Ubx and Antp (39), and yet it cooperates with PBX1A in our assay. Another group has demonstrated the importance of the Ubx pentapeptide for binding to exd (34). We also note that a 20- to 50-fold excess of exd was required in the previous study (6) to produce significant cooperative binding with a pentapeptide-deficient Ubx protein, whereas equimolar levels of HOXD-4 and PBX1A produced cooperative binding in our assay (Fig. 2B). This variance may be due to differences between the two DNA probes and experimental conditions, in addition to a requirement for the pentapeptide for high-affinity interactions with exd. Similarly, the homeodomain of Matα2 interacts weakly with MCM1, but strong interactions require the region N terminal to the homeodomain (82).

While results of an in vivo analysis of Ubx-exd interactions agree with the conclusions regarding the importance of the Ubx homeodomain and C terminus in vitro, the pentapeptide was encoded by the Ubx constructs tested in flies (6). Therefore, this experiment does not address the importance of the pentapeptide for functional interactions in vivo. Likewise, an ectopically expressed UbxΔNN construct, whose activity was shown to be sensitive to the presence of exd, retained the Ubx pentapeptide (68). To date, engrailed is the only protein that lacks a pentapeptide and yet undergoes significant cooperative interactions with exd (60, 79). Taken together, the available information on HOX/HOM interactions with PBX/exd suggests that the pentapeptide is required, while other regions, such as the homeodomain, also play a role in determining the strength or specificity of these interactions.

The t(1;19) translocation, present in many pediatric pre-B-cell leukemias (38, 57), results in an oncogenic fusion protein between E2A and PBX1 (12, 36, 37, 56). Fusion to E2A converts PBX proteins from weak to strong transcriptional activators (37, 46, 49, 80). Our transfection results indicate that this activity may depend on cooperative interactions with HOX proteins mediated by the pentapeptide (Fig. 4C). Interestingly, it was recently demonstrated that the homeodomain of PBX1A is not required for the transforming potential of E2A/PBX (56). Previous reports on the activity of homeodomainless homeoproteins suggest that protein-protein interactions are sufficient for correct target recognition in the absence of direct DNA binding (23). In the case of homeodomainless E2A/PBX1A, this transforming potential might occur through interactions with particular HOX proteins via the pentapeptide. *Hox* genes are widely expressed in the adult mouse, including

hematopoietic lineages (45), and several have been shown to have transforming potential (1, 51, 62). Our results likewise suggest that the oncogenicity of HOX proteins may be dependent on cooperative interactions with PBX family members.

Besides the HOX/HOM family, the pentapeptide is found N terminal to the homeodomain in the products of the *msh/Msx* family (11, 29, 54, 70), the *caudal/cdx* family (31, 33, 53), *ems/emx* family (10, 75), *HOX11* (15, 28, 40), and insulin promoter factor 1 (IPF1) genes (59). The latter two genes are required for the development of the spleen (71) and pancreas (35), respectively. Misexpression of *HOX11* has been implicated in some human T-cell acute lymphoblastic leukemias, and IPF1 also directs insulin gene expression. *cdx-1* and *cdx-2* are expressed specifically in the intestinal epithelium (16, 78), while *Msx-1* has been implicated in mesenchymal-epithelial interactions of craniofacial and tooth development (74). *ems/emx* members are expressed in anterior structures, whereas *caudal* is initially posterior. Thus, PBX/exd family members, which are widely expressed (55), could cooperate with a variety of homeoproteins in a broad spectrum of developmental and oncological processes, in addition to positional specification by *Hox* and *HOM* gene products.

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