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The hexapeptide LFPWMR in Hoxb-8 is required for cooperative DNA binding with Pbx1 and Pbx2 proteins

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ABSTRACT **The *Hox* gene products are DNA-binding proteins, containing a homeodomain, which function as a class of master control proteins establishing the body plan in organisms as diverse as *Drosophila* and vertebrates. Hox proteins have recently been shown to bind cooperatively to DNA with another class of homeodomain proteins that include extradenticle, Pbx1, and Pbx2. *Hox* gene products contain a highly conserved hexapeptide connected by a linker of variable length to the homeodomain. We show that the hexapeptide and the linker region are required for cooperativity with Pbx1 and Pbx2 proteins. Many of the conserved residues present in the Hoxb-8 hexapeptide are required to modulate the DNA binding of the Pbx proteins. Position of the hexapeptide relative to the homeodomain is important. Although deletions of two and four residues of the linker peptide still show cooperative DNA binding, removal of all six linker residues strongly reduces cooperativity. In addition, an insertion of 10 residues within the linker peptide significantly lowers cooperative DNA binding. These results show that the hexapeptide and the position of the hexapeptide relative to the homeodomain are important determinants to allow cooperative DNA binding involving *Hox* and *Pbx* gene products.**

Recently a class of developmental regulators has been identified, the Hox proteins, that is important for the formation of body structures in developing organisms (1–7). The Hox proteins contain a DNA-binding domain, the homeodomain, consisting of three helices recognizing a specific TAAT-like DNA sequence (4, 6, 7). Although the DNA-binding activities of Hox proteins bind to similar sites (TAAT), their biological functions are highly specific. The *Hox* gene products can be further grouped into three classes: Antennapedia, abd-B, and labial (8). These classes are closely related to each other within the homeodomain. Proteins of the Antennapedia and labial class also contain a conserved hexapeptide separated from the homeodomain by a linker of variable length. Recently, another class of homeodomain proteins, including extradenticle and the *Pbx* gene products, has emerged that have the ability to raise the DNA-binding specificity of the *Hox* gene products (9, 10). For example, extradenticle has been shown to bind cooperatively with Ubx to sites present in the decapentaplegic enhancer (dpp) (9). extradenticle has also been shown to bind cooperatively to DNA with Ubx and abd-A using synthetic binding sites as a template (10) and to interact with Ubx and abd-A using a yeast two-hybrid approach (11). The interaction involving Ubx and extradenticle, in the two-hybrid assay, requires a region N-terminal of the homeodomain containing a conserved hexapeptide. Functional studies have demonstrated that extradenticle functions in parallel with some of the *Hox* gene products to regulate common target genes (12–14).

In mammals, three homologs of extradenticle have been identified, designated Pbx1, Pbx2, and Pbx3 (15). Each of these proteins is closely related to extradenticle, 85% over most of the coding sequence. *Pbx1* was originally identified as a protooncogene, which is translocated in pre-B acute lymphoblastoid leukemia (ALL) (16, 17). Specifically, in pre-B acute lymphoblastoid leukemia, the Pbx1 homeodomain is fused to the transactivation domain of transcription factor E2A, converting a nonactivator into a transcriptional activator (18–20). Both Pbx1 and Pbx2 have the ability to bind DNA in a cooperative manner with Hoxb-7 and Hoxb-8, as described for extradenticle (21).

Here we map the Hoxb-8 residues that are important for cooperative DNA binding with Pbx1 and Pbx2. The homeodomain and a highly conserved hexapeptide located N-terminal of the homeodomain are required for cooperative DNA binding involving Hoxb-8 and Pbx proteins.

MATERIALS AND METHODS

Plasmids. Plasmid pSP64-ATG (10) or a modified form, pSS, containing three out-of-frame stop codons cloned into the *Eco*RI site of the pSP64-ATG polylinker, were used to clone the various Hoxb-8 truncations used in this study. Full-length pSP64-ATG/Hoxb-8 was constructed by inserting an 822-bp *Sac* I cDNA fragment containing the entire *Hoxb-8* coding region (22) without the first methionine into the *Sac* I site of pSP64-ATG. The various Hoxb-8 truncations were generated by PCR using the full-length *Hoxb-8* gene as template. The Engrailed homeodomain was also generated by PCR and cloned into expression vector pSS. The Hoxb-8/Engrailed chimaeric protein was made by using two-step PCR (23). In the first PCR reaction two products were generated that encode the Hoxb-8 linker and the Engrailed homeodomain and partially overlap in sequence at the desired junction between the Hoxb-8 linker and the Engrailed homeodomain. These primary PCR products were gel-purified and combined in the second PCR reaction. The heteroduplex formed after annealing of the two primary products at their overlapping sequence served then as a template for *Taq* polymerase, and by using primers specific for the 5′ and 3′ end of this formed Hoxb-8/Engrailed heteroduplex, the chimaeric DNA fragment was amplified. The insertion and deletions within the Hoxb-8 linker peptide were introduced with two-step PCR using primers spanning the *Hoxb-8* linker sequence but containing the specific deletion or insertion. All constructs were verified by sequencing.

Mutagenesis. Mutations within the Hoxb-8 hexapeptide were introduced via two-step PCR by using primers bearing the specific hexapeptide mutation. In the first PCR reaction two products were made: one encoding the 5′ part of Hoxb-8 and the mutated hexapeptide and the other one encoding the mutated hexapeptide and the 3′ part of Hoxb-8. These primary PCR products were gel-purified and combined in the second PCR reaction. Annealing of these two PCR products at their overlapping region (which is the mutated hexapeptide) results

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Abbreviation: EMSA, electrophoretic mobility-shift assay.

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in the full-length mutated *Hoxb-8* sequence, which is extended and amplified by *Taq* polymerase using primers specific for the 5′ and 3′ end of the *Hoxb-8* sequence. Amplified fragments were cloned into pSS and verified by sequencing.

***In Vitro* Translation and Electrophoretic Mobility-Shift Assay (EMSA).** All proteins used in this study were produced using the Promega SP6 TNT rabbit reticulocyte lysate-coupled transcription/translation system according to the manufacturer's protocol (Promega). Control reactions using [^{35}S]methionine were done and analyzed on a SDS/15% polyacrylamide gel to verify that proteins of the correct size were produced in similar quantities. EMSAs were done as described (10). Briefly, double-stranded ^{32}P-end-labeled oligonucleotides (30,000 cpm) were incubated with *in vitro* translated proteins in a buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% (vol/vol) glycerol, in the presence of 2 μg of poly(dI·dC) for 30 min at room temperature. All reactions were adjusted to contain equal amounts of reticulocyte lysate. Bound and free probe were separated on a 5% polyacrylamide gel. After drying the gel, results were visualized by autoradiography.

RESULTS AND DISCUSSION

Recently, both *Drosophila* and vertebrate *Hox* gene products have been shown to bind cooperatively to DNA with cofactors that include extradenticle and Pbx proteins (9, 10, 21). To determine the Hox domains required for interaction with Pbx, C-terminal and N-terminal deletions of Hoxb-8 were generated (Fig. 1*A*). The deletion constructs were transcribed *in vitro* and tested for translation by SDS/gel electrophoresis (Fig. 1*B*). The *in vitro* translation was efficient for each of the products, and equal amounts of the truncated versions were tested for their ability to bind to DNA, in the absence or presence of Pbx1 and Pbx2, by EMSA. As a probe we used a template that was previously shown to allow cooperative DNA binding involving Hoxb-8 and Pbx1 (21). Each of the truncated products contained the homeodomain and could bind DNA by itself (Fig. 1*C*, lanes 2–6). Although the Hoxb-8 homeodomain did not have the ability to interact with Pbx (Fig. 1*C*, lanes 11 and 16), the presence of 21 residues N-terminal of the homeodomain allowed cooperativity with Pbx1 and Pbx2 (Fig. 1*C*, lanes 10 and 15). These data indicate that a short peptide immediately N-terminal of the Hoxb-8 homeodomain, including the hexapeptide, is important for cooperative DNA binding with Pbx1 and Pbx2.

To test whether the region located N-terminal of the homeodomain and containing the hexapeptide is sufficient for cooperativity, a polypeptide containing these residues was grafted onto the *Drosophila* Engrailed homeodomain (Fig. 2*A*). Previously we demonstrated that Engrailed and Pbx1 interact to bind cooperatively to DNA (21). However, the Engrailed homeodomain by itself does not show cooperative DNA binding involving Pbx1 and Pbx2 (Fig. 2*B*, lanes 5 and 8). Although the Hoxb-8/Engrailed fusion protein is capable of binding to DNA by itself, no cooperative DNA binding could be observed when incubated with Pbx1 or Pbx2 (Fig. 2*B*, lanes 3, 6, and 9). Thus the Hoxb-8 hexapeptide, although required for cooperativity, is not sufficient for cooperativity with the Pbx proteins. This result suggests that the Hoxb-8 hexapeptide and the Hoxb-8 homeodomain are both required for cooperative DNA binding with Pbx. Engrailed, however, does not contain a hexapeptide N-terminal of its homeodomain, but Engrailed can bind DNA cooperatively with Pbx (21). Preliminary results indicate that a region located immediately N-terminal of the Engrailed homeodomain is required for cooperative DNA binding (L.T.C.P. and C.M., unpublished results).

In Antennapedia, the region located N-terminal of the homedomain, including the hexapeptide, has been shown to be

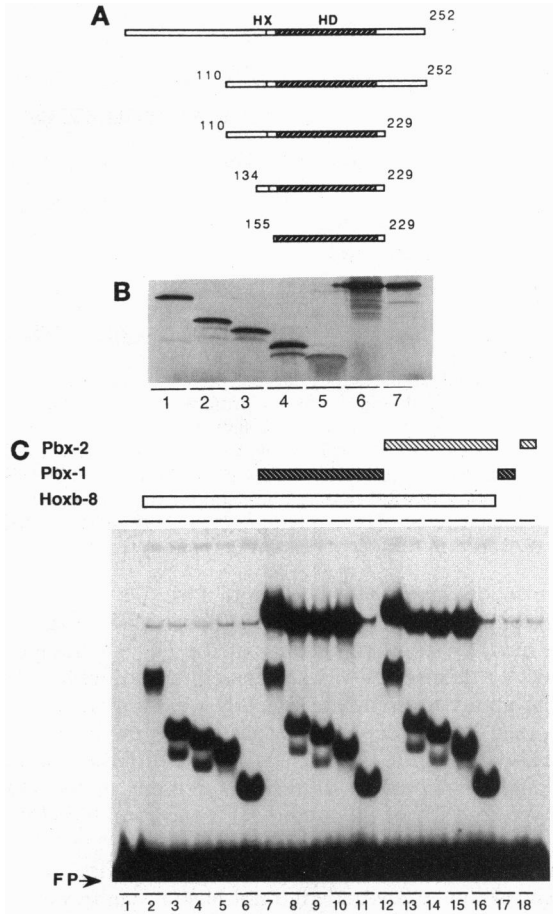

FIG. 1. A region immediately upstream of the Hoxb-8 homeodomain is required for cooperative DNA binding with Pbx1 and Pbx2. (*A*) Schematic representation of full-length Hoxb-8 and various deletion derivatives. The size of each protein is indicated. The hatched box represents the homeodomain (HD); HX indicates the hexapeptide. (*B*) Full-length and truncated forms of *Hoxb-8* were transcribed and translated *in vitro* in the presence of [^{35}S]methionine and analyzed by SDS/gel electrophoresis. Lanes: 1, full-length Hoxb-8-(1–252); 2, Hoxb-8-(110–252); 3, Hoxb-8-(110–229); 4, Hoxb-8-(134–229); 5, Hoxb-8-(155–229); 6, Pbx1; 7, Pbx2. (*C*) Full-length or truncated Hoxb-8 proteins were incubated with ^{32}P-end-labeled probe (5′-GTCAATTAAAGCATCAATCAATTTCG-3′) in the presence or absence of *in vitro*-translated Pbx1 or Pbx2 and analyzed by EMSA. Proteins (1 μl) added to each lane were as follows. Lanes: 1, free probe; 2, full-length Hoxb-8-(1–252); 3, Hoxb-8-(110–252); 4, Hoxb-8-(110–229); 5, Hoxb-8-(134–229); 6, Hoxb-8-(155–229); 7, full-length Hoxb-8-(1–252) and Pbx1; 8, Hoxb-8-(110–252) and Pbx1; 9, Hoxb-8-(110–229) and Pbx1; 10, Hoxb-8-(134–229) and Pbx1; 11, Hoxb-8-(155–229) and Pbx1; 12, full-length Hoxb-8-(1–252) and Pbx2; 13, Hoxb-8-(110–252) and Pbx2; 14, Hoxb-8-(110–229) and Pbx2; 15, Hoxb-8-(134–229) and Pbx2; 16, Hoxb-8-(155–229) and Pbx2; 17, Pbx1; 18, Pbx2. FP, free probe.

unstructured (24). Although the hexapeptide is extremely well conserved in *Hox* gene products (Table 1), other residues present in the region N-terminal of the homeodomain are not conserved. For example, Hoxb-8 and Hoxb-7 show no homology in this region, except for the hexapeptide (22, 25). However, both Hoxb-8 and Hoxb-7 have the ability to modulate the DNA-binding activity of Pbx1 (21), suggesting that the hexapeptide may be a crucial determinant allowing cooperative binding to occur. To determine the importance of the hexapeptide, we systematically mutated the six amino acids in a truncated version of Hoxb-8 containing residues 110–229 (Fig. 3*A*). The mutants were transcribed and translated *in vitro* and analyzed by SDS/gel electrophoresis (Fig. 3*B*). Mutant products were translated efficiently and examined for their ability

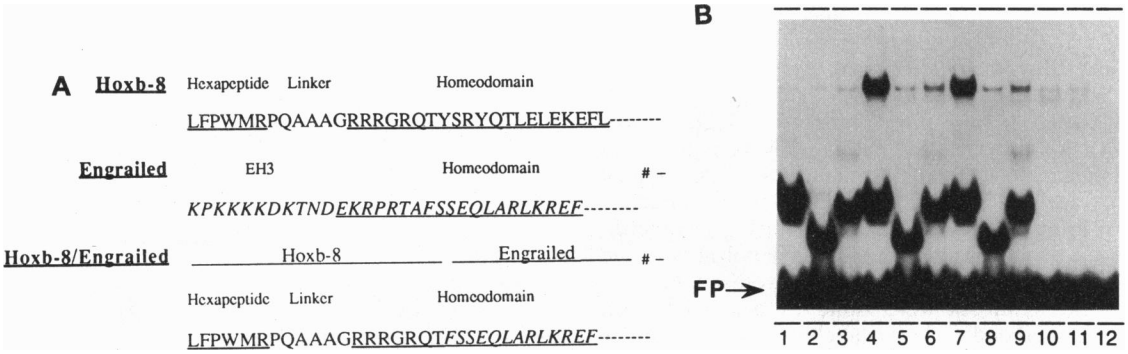

FIG. 2. The Hoxb-8 hexapeptide and linker upstream of the homeodomain are not sufficient for cooperative binding (*A*). A schematic diagram of Hoxb-8, Engrailed, and the fusion protein containing the Hoxb-8 linker peptide and the Engrailed homeodomain. The amino acid sequences of the predicted protein products are shown. The hexapeptide and the homeodomain are underlined. EH3, Engrailed homology region 3 (8). (*B*) DNA-binding analysis of the Engrailed homeodomain and the Hoxb-8/Engrailed chimeric proteins in the absence or presence of Pbx1 or Pbx2 using ^{32}P-end-labeled probe (5′-GTCAATTAAATGATCAATCAATTTCG-3′). The proteins (1 μl) added to each lane were as follows. Lanes: 1, Hoxb-8-(134–229); 2, Engrailed homeodomain; 3, Hoxb-8/Engrailed; 4, Hoxb-8-(134–229) and Pbx1; 5, Engrailed homeodomain and Pbx1; 6, Hoxb-8/Engrailed and Pbx1; 7, Hoxb-8-(134–229) and Pbx2; 8, Engrailed homeodomain and Pbx2; 9, Hoxb-8/Engrailed and Pbx2; 10, Pbx1; 11, Pbx2; 12, 2 μl of reticulocyte lysate. FP, free probe.

to bind cooperatively to DNA with Pbx. We evaluated the effects of double substitutions in the Hoxb-8 hexapeptide. While the DNA-binding activity of the Hoxb-8 hexapeptide mutants by themselves was unaffected, both double mutants lost the ability to modulate Pbx DNA-binding activity, indicating that the hexapeptide residues play an important role in cooperativity (Fig. 3*C*, lanes 2, 3, 12, and 13). To determine the individual contributions of residues present in the Hoxb-8 hexapeptide, each of the amino acids was mutated (Fig. 3*A*) and evaluated for its ability to modulate the DNA-binding activity of Pbx1 and Pbx2. Mutation of the first and sixth residues of the hexapeptide did not have a significant impact on cooperative DNA binding with Pbx1 (Fig. 3*C*, lanes 14, 19, and 20) and Pbx2 (data not shown). Similarly, substitution of the conserved proline with a valine did not alter the ability of Hoxb-8 to modulate Pbx-binding activity (Fig. 3*C*, lane 16). However, replacements of phenylalanine, tryptophan, and methionine with aspartate, tyrosine, and glutamate, respectively, abolished cooperativity (Fig. 3*C*, lanes 15, 17, and 18). To test the role of the hexapeptide in the context of full-length Hoxb-8 protein, the phenylalanine was replaced with an aspartate residue. Wild-type and mutant full-length Hoxb-8 were translated efficiently, as shown by SDS/gel electrophoresis (Fig. 3*B*, lanes 11 and 12). Although full-length Hoxb-8 containing the hexapeptide mutation is capable of binding to DNA by itself, it has lost the ability to modulate the DNA-binding activity of Pbx1 (Fig. 3*C*, lanes 21–24). These data demonstrate that the Hoxb-8 hexapeptide is required for cooperative DNA binding.

The number of residues that separate the hexapeptide from the homeodomain vary dramatically between different Hox polypeptides (8, 24) (Table 2). We considered the possibility that the linker peptide might be required to keep the hexapeptide distinct from the homeodomain. To determine the length of the Hoxb-8 linker peptide sufficient for cooperativity with the Pbx proteins we removed various portions of the Hoxb-8 linker peptide (Fig. 4*A*). The constructs were transcribed and translated *in vitro* and analyzed by SDS/gel electrophoresis (Fig. 4*B*). Each of the products was translated efficiently and analyzed by EMSA. While a two- and four-amino acid deletion does not affect the ability of Hoxb-8 to interact with Pbx1 and Pbx2, a deletion of all six residues strongly reduces cooperativity (Fig. 4*C*, lanes 7–9 and 12–14). To determine whether an insertion into the linker sequence of Hoxb-8 affects coopera-

Table 1. Conservation of the LFPWMR hexapeptide in various classes of homeodomain proteins

Class	Hexapeptide	Class	Hexapeptide
Antennapedia		Sex combs reduced	
D. melanogaster Antp	LYPWMR	*D. melanogaster* Scr	IYPWMK
D. melanogaster Ubx	FYPWMA	*M. musculus* Hoxa-5	IYPWMR
D. melanogaster abd-A	RYPWMT	*X. laevis* Hoxb-5	IFPWMR
Xenopus laevis Hoxc-6	IYPWMQ	*M. musculus* Hoxb-5	IFPWMR
Mus musculus Hoxc-6	IYPWMQ	*Homo sapiens* Hoxc-5	IYPWMT
M. musculus Hoxb-6	VYPWMQ	Proboscipedia	
M. musculus Hoxb-7	IYPWMR	*D. melanogaster* pb	EYPWMK
M. musculus Hoxb-8	LFPWMR	*M. musculus* Hoxb-3	IFPWMK
X. laevis Hoxb-8	LFPWMR	*H. sapiens* Hoxb-2	EFPWMK
M. musculus Hoxd-8	MFPWMR	*H. sapiens* Hoxd-3	IFPWMK
M. musculus Hoxc-8	MFPWMR	Empty spiracles	
Deformed		*D. melanogaster* ems	LYPWLL
D. melanogaster Dfd	IYPWMK	*H. sapiens* EMX1	FYPWVL
M. musculus Hoxd-4	VYPWMK	*H. sapiens* EMX2	FYPWLI
M. musculus Hoxa-4	VYPWMK		
X. laevis Hoxb-4	VYPWMK		
M. musculus Hoxb-4	VYPWMR		
M. musculus Hoxc-4	VYPWMK		

Antp, Antennapedia; Ubx, Ultrabithorax; abd-A, abdominal-A; Dfd, Deformed; Scr, Sex combs reduced; pb, proboscipedia; ems, empty spiracles; EMX, empty spiracles (*Drosophila*) homolog.

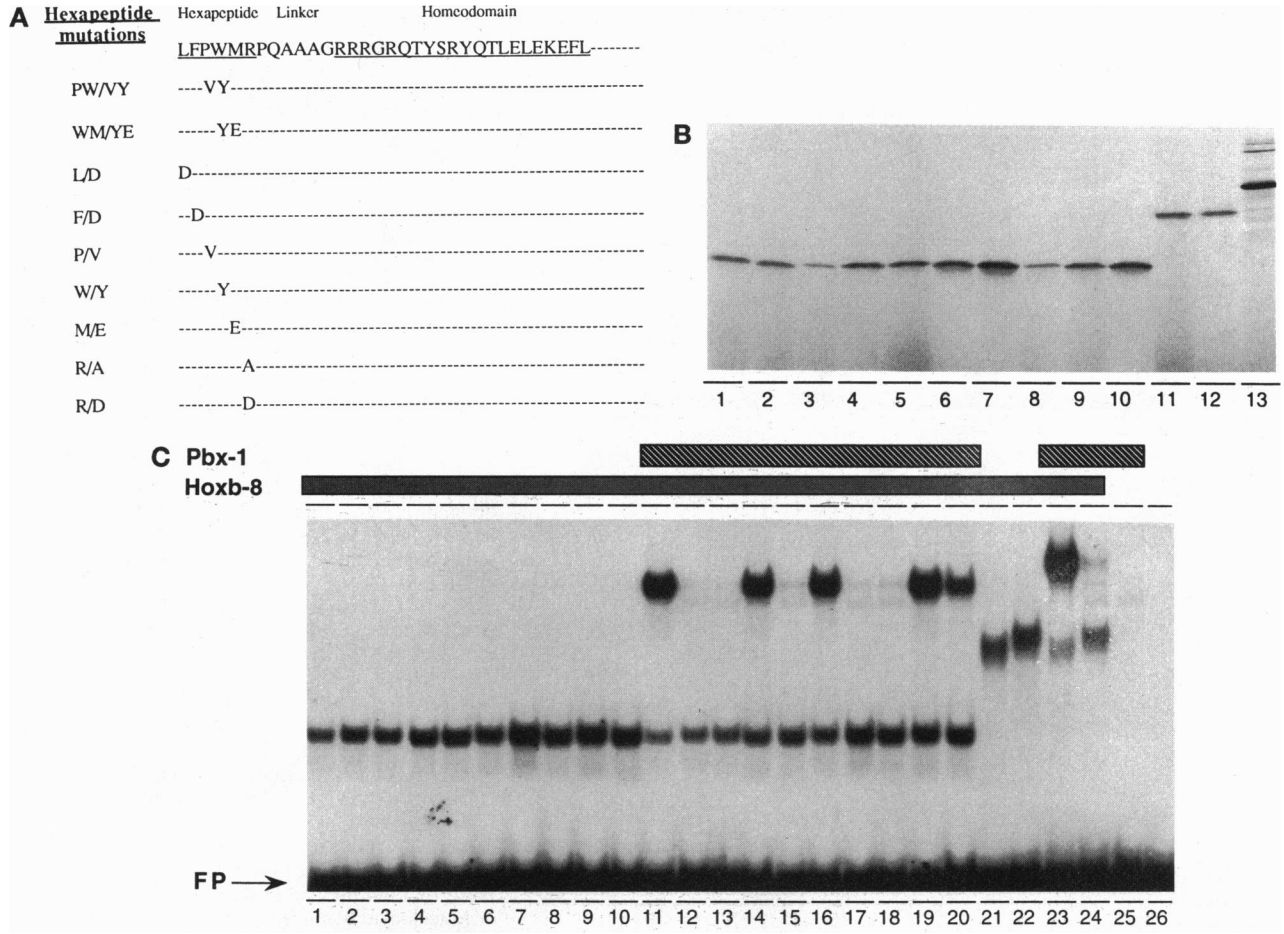

FIG. 3. The Hoxb-8 hexapeptide is required for cooperative DNA binding with Pbx1. (*A*) A schematic diagram is shown of the wild-type and mutant Hoxb-8 hexapeptide sequences. Amino acid substitutions in the hexapeptide are indicated. The hexapeptide and homeodomain are underlined (*B*). Wild-type and mutant forms of *Hoxb-8* were transcribed and translated *in vitro* in the presence of [^{35}S]methionine and analyzed by SDS/gel electrophoresis. Lanes: 1, Wild-type Hoxb-8-(110–229); 2, PW/VY; 3, WM/YE; 4, L/D; 5, F/D; 6, P/V; 7, W/Y; 8, M/E; 9, R/A; 10, R/D; 11, full-length Hoxb-8-(1–252); 12, full-length Hoxb-8 F/D; 13, Pbx1. (*C*) DNA-binding analysis of Hoxb-8 proteins containing wild-type or mutant hexapeptide sequences, in the absence or presence of Pbx1 using ^{32}P-end-labeled probe (5′-GTCAATTAAAGCATCAATCAATTTCG-3′). The *in vitro* translated proteins (1 μl) added to each lane were as follows. Lanes: 1, wild-type Hoxb-8-(110–229); 2, PW/VY; 3, WM/YE; 4, L/D; 5, F/D; 6, P/V; 7, W/Y; 8, M/E; 9, R/A; 10, R/D; 11, wild-type Hoxb-8-(110–229) and Pbx1; 12, PW/VY and Pbx1; 13, WM/YE and Pbx1; 14, L/D and Pbx1; 15, F/D and Pbx1; 16, P/V and Pbx1; 17, W/Y and Pbx1; 18, M/E and Pbx1; 19, R/A and Pbx1; 20, R/D and Pbx1; 21, full-length Hoxb-8-(1–252); 22, full-length Hoxb-8 F/D; 23, full-length Hoxb-8-(1–252) and Pbx1; 24, full-length Hoxb-8 F/D and Pbx1; 25, Pbx1; 26, 2 μl of reticulocyte lysate.

tive DNA binding we inserted 10 amino acids, derived from the linker peptide of abd-A, into the linker of Hoxb-8 (Fig. 4*A*). The *Hoxb-8* derivative was translated *in vitro* and analyzed by EMSA. The Hoxb-8 protein containing the insertion can still modulate, although relatively weakly, the DNA-binding activity of Pbx1 and Pbx2 (Fig. 4*C*, lanes 10 and 15). These data indicate that the length of the nonconserved peptide linking the hexapeptide to the homeodomain is important but flexible and needs to be distinct from the homeodomain to allow cooperativity.

Table 2. Length of the linker peptide separating the hexapeptide from the homeodomain in *Hox* gene products

Hox gene product	Linker peptide	*Hox* gene product	Linker peptide
Antp	6–10	cad	17
Dfd	20–30	Hoxb-8	6
Scr	16	Hoxb-7	5
pb	20–30	Hoxb-4	15
Ubx	9–52	Hoxc-6	13
abd-A	26	Hoxd-8	6
lab	106–112	Hoxc-8	5
ems	24		

Linker peptide refers to the numbers of residues that separate the homeodomain from the hexapeptide (8, 22, 24, 25). See Table 1 for proteins; lab, labial.

The cooperative interactions involving *Hox* and *Pbx* gene products bear many similarities to α2, a homeodomain protein involved in cell-type determination in *Saccharomyces cerevisiae* (26). α2 contains two unstructured peptides attached to the homeodomain, one specifying interaction with another homeodomain protein a1, the other specifying interaction with MCM1, a MADS domain-containing protein (23, 27). The regions are located either immediately N-terminal or C-terminal of the α2 homeodomain. We show here that a hexapeptide located six residues N-terminal of the Hoxb-8 homeodomain is required to modulate the DNA-binding activity of Pbx1 and Pbx2. Although still to be proven, the high degree of conservation of six residues within the flexible region in vertebrate *Hox* gene products suggests that they all may interact with the Pbx proteins (8, 24). We show here that, while the hexapeptide is important, it is not sufficient to allow cooperativity. When fused to the Engrailed homeodomain it does not allow cooperativity, suggesting that residues located in the homeodomain are important as well.

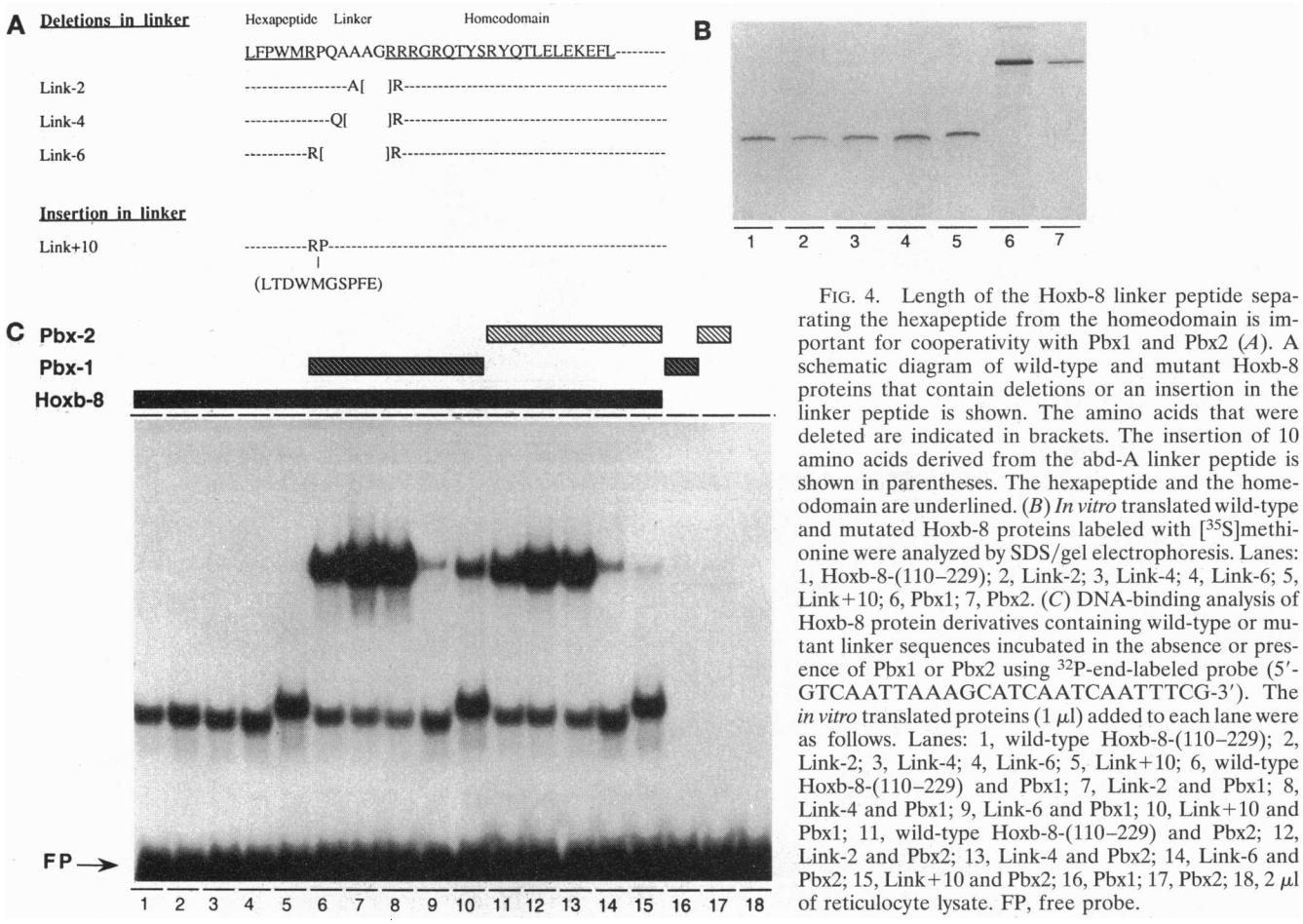

FIG. 4. Length of the Hoxb-8 linker peptide separating the hexapeptide from the homeodomain is important for cooperativity with Pbx1 and Pbx2 (*A*). A schematic diagram of wild-type and mutant Hoxb-8 proteins that contain deletions or an insertion in the linker peptide is shown. The amino acids that were deleted are indicated in brackets. The insertion of 10 amino acids derived from the abd-A linker peptide is shown in parentheses. The hexapeptide and the homeodomain are underlined. (*B*) *In vitro* translated wild-type and mutated Hoxb-8 proteins labeled with [^{35}S]methionine were analyzed by SDS/gel electrophoresis. Lanes: 1, Hoxb-8-(110–229); 2, Link-2; 3, Link-4; 4, Link-6; 5, Link+10; 6, Pbx1; 7, Pbx2. (*C*) DNA-binding analysis of Hoxb-8 protein derivatives containing wild-type or mutant linker sequences incubated in the absence or presence of Pbx1 or Pbx2 using ^{32}P-end-labeled probe (5′-GTCAATTAAAGCATCAATCAATTTCG-3′). The *in vitro* translated proteins (1 μl) added to each lane were as follows. Lanes: 1, wild-type Hoxb-8-(110–229); 2, Link-2; 3, Link-4; 4, Link-6; 5, Link+10; 6, wild-type Hoxb-8-(110–229) and Pbx1; 7, Link-2 and Pbx1; 8, Link-4 and Pbx1; 9, Link-6 and Pbx1; 10, Link+10 and Pbx1; 11, wild-type Hoxb-8-(110–229) and Pbx2; 12, Link-2 and Pbx2; 13, Link-4 and Pbx2; 14, Link-6 and Pbx2; 15, Link+10 and Pbx2; 16, Pbx1; 17, Pbx2; 18, 2 μl of reticulocyte lysate. FP, free probe.

In addition to the vertebrate Hox proteins the hexapeptide can also be found in *Drosophila* and other organisms (Table 1). For example, it is present in the *Caenorhabditis elegans Hox* gene product *pal-1* (VWPFMD) (8) (Table 1). It will be interesting to determine whether the hexapeptide in *Drosophila* and *C. elegans* Hox proteins also has the ability to interact with the extradenticle and *ceh-20* gene products, the equivalents of Pbx in *Drosophila* and *C. elegans*, respectively (28). The hexapeptide may well be a universal interaction motif that mediates cooperative DNA binding between two ancient classes of homeodomain proteins, the *Hox* and *Pbx* gene products.

Note. While this paper was submitted, it was reported that deletion of the hexapeptide in Hoxb-6 abolished cooperative DNA binding with Pbx1, consistent with the observations shown here (29).

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