Development 124, 1089-1098 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV5105

Specific residues in the Pbx homeodomain differentially modulate the DNA-binding activity of Hox and Engrailed proteins

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SUMMARY

Two classes of homeodomain proteins, Hox and Engrailed, have been shown to act in concert with the atypical homeodomain proteins Pbx and extradenticle. We now show that specific residues located within the Pbx homeodomain are essential for cooperative DNA binding with Hox and Engrailed gene products. Within the N-terminal region of the Pbx homeodomain, we have identified a residue that is required for cooperative DNA binding with three Hox gene products but not for cooperativity with Engrailed-2 (En-2). Furthermore, there are similarities between heterodimeric interactions involving the yeast mating type proteins MATa1 and MATα2 and those that allow the formation of Pbx/Hox and Pbx/En-2 heterodimers. Specifically, residues located in the a1 homeodomain that were previously shown to form a hydrophobic pocket allowing the α2 C-terminal tail to bind, are also required for Pbx/Hox and Pbx/En-2 cooperativity. Furthermore, we show that three residues located in the turn between helix 1 and helix 2, characteristic of many atypical homeodomain proteins, are required for cooperative DNA binding involving both Hox and En-2. Replacement of the three residues located in the turn between helix 1 and helix 2 of the Pbx homeodomain with those of the atypical homeodomain proteins controlling cell fate in the basidiomycete *Ustilago maydis*, bE5 and bE6, allows cooperative DNA binding with three Hox members but abolishes interactions with En-2. The data suggest that the molecular mechanism of homeodomain protein interactions that control cell fate in *Saccharomyces cerevisiae* and in the basidiomycetes may well be conserved in part in multicellular organisms.

Key words: cooperative DNA binding, Engrailed, homeodomain proteins, Hox, Pbx

INTRODUCTION

Homeodomain proteins play key roles in controlling cell fate and morphogenesis. In a number of regulatory networks, homeodomain interactions have been shown to enhance the DNA-binding affinity and specificity of relatively non-specific DNA-binding proteins. In *Saccharomyces cerevisiae* such cooperative interactions have been described in great detail (Johnson, 1992). Three cell types exist for *S. cerevisiae*, a cells, α cells and a/α cells. The a and α cells are haploid, whereas the a/α cell is diploid. In diploid cells, repression of haploid-specific genes is mediated by two homeodomain proteins, designated MATa1 and MATα2. a1 and α2 are both homeodomain proteins that bind to DNA as cooperative heterodimers. This cooperativity enhances both the affinity and specificity of the heterodimeric complex as compared to a1 and α2 alone (Goutte and Johnson, 1993). The interaction between a1 and α2 involves two domains. An unstructured peptide located immediately C-terminal to the α2 homeodomain interacts with residues present in the a1 homeodomain (Vershon and Johnson, 1993). Specific residues present in the turn between helix 2 and helix 3 present on a1 are required for a1/α2 cooperativity (Stark and Johnson, 1994).

In *Drosophila* and vertebrates, similar homeodomain protein interactions have been described. Morphogenesis in *Drosophila* is in part controlled by a family of proteins, designated as the homeotic selector proteins. The homeotic proteins are sequence-specific DNA-binding proteins that contain a region of homology, named the homeodomain (Laughon and Scott, 1984; McGinnis et al., 1984). In mammals, a class of closely related genes are present, the Hox genes, whose products are similarly involved in morphogenesis (Duboule and Dollé, 1989; Wilkinson et al., 1989; Krumlauf, 1994; Lawrence and Morata, 1994). Many *Drosophila* and vertebrate Hox gene products act in concert with another class of homeodomain proteins, extradenticle (exd) and its three mammalian homologues, Pbx1, Pbx2 and Pbx3 (Peifer and Wieschaus, 1990; Chan et al., 1994; van Dijk and Murre, 1994). exd and the Pbx gene products are closely related in their amino acids sequence and have similar biochemical properties (Flegel et al., 1993; Rauskolb et al., 1993). They belong to a class of homeodomain proteins that is referred to as atypical homeodomain proteins (Bürglin, 1994). The atypical homeodomain proteins are distinct from other homeodomain proteins in that their homeodomains contain extra amino acids, often a three amino acid insertion located in the turn between helix 1 and helix 2 (Bürglin, 1994). Other members of the atypical homeodomain proteins include a family of proteins, bE, which are involved in controlling cell fate in a number of fungi, including *Ustilago maydis*, *Coprinus cinereus* and *Schizophyllum commune* (Banuett, 1992).

Both exd and Pbx have the ability to modulate the DNA-binding activity of the Hox proteins (Chan et al., 1994; van Dijk and Murre, 1994; Chang et al., 1995; Johnson et al., 1995; Lu et al., 1995; Phelan et al., 1995; van Dijk et al., 1995). The cooperative DNA binding of Hox and Pbx proteins results in heterodimeric complexes that show increased DNA-binding affinity and specificity, similar to described for a1 and α2 (Goutte and Johnson, 1993). In addition, Pbx gene products also modulate the DNA-binding activity of both *Drosophila* Engrailed and mammalian Engrailed-2 (En-2) (van Dijk and Murre, 1994; van Dijk et al., 1995; Peltenburg and Murre, 1996). The Pbx/En-2 complexes prefer different binding sites, as compared to Pbx/Hox heterodimers (van Dijk and Murre, 1994; van Dijk et al., 1995).

The requirements for Hox and Engrailed cooperativity with the Pbx members have been well documented. A conserved hexapeptide located immediately N-terminal of the Hox homeodomain has been shown to be required for cooperative DNA binding (Chang et al., 1995; Knoepfler and Kamps, 1995; Neuteboom et al., 1995; Phelan et al., 1995). In Engrailed, a similar domain, EH2, which is closely related to the hexapeptide, is required for interaction with exd and the Pbx gene products (Peltenburg and Murre, 1996). Both the Engrailed and Hox homeodomain themselves are important for cooperativity, since chimeric proteins containing either the Hox hexapeptide or the EH2 domain when grafted onto a heterologous DNA-binding domain do not show cooperative DNA binding (Neuteboom et al., 1995; Peltenburg and Murre, 1996).

The structural determinants of Pbx for cooperative DNA binding with the Hox gene products have also been examined (Chang et al., 1995; Lu and Kamps, 1996). Residues located within the Pbx homeodomain and a region immediately C-terminal of the homeodomain are required for cooperativity with the Hox proteins (Chang et al., 1995; Lu and Kamps, 1996). Here we demonstrate that the Pbx homeodomain is required for cooperative DNA binding with both Hox and En-2 proteins. In addition, we show that distinct residues located within the Pbx homeodomain are required for cooperative DNA binding with either Hox or En-2. Substitutions of specific residues previously shown to be involved in contacts between a1 and α2 are required for cooperativity involving Hox and En-2. Substitution of Pbx residues located in the turn separating helix 1 and helix 2 for amino acids located in the same postion in bE, a protein controlling cell fate in the fungus *Ustilago maydis*, conserves the cooperativity with Hox, but abolishes cooperative DNA binding with En-2. The data suggest that the molecular mechanism underlying cooperative DNA binding between two homeodomain proteins may in part be conserved throughout evolution.

MATERIALS AND METHODS

Plasmids

The full-length Pbx1 protein was in vitro transcibed and translated from a derivative of pSP64 containing the full-length cDNA (Peltenburg and Murre, 1996). Pbx 23-430 and 51-430 were constructed by inserting the *Apa*I-digested appropriate PCR products in the full-length Pbx1 vector with *Apa*I and blunt *Nco*I ends. PCR products spanning Pbx 80-430, 119-430 and 172-430 were cloned into the *Eco*RV site of pSP64-ATG (van Dijk and Murre, 1994). To construct Pbx 1-320, the big *Nco*I fragment of the full-length Pbx1 construct was cloned in pSS, which contains three out-of-frame stop codons (Neuteboom et al., 1995). Similarly, a partial *Pvu*II fragment was used for Pbx 1-309 and a partial *Ssp*I fragment for Pbx 1-304. The C-terminal deletion construct Pbx 1-297 contains a PCR fragment spanning the N terminus down to the C-terminal end of the homeodomain (amino acid 297), cloned in pSS (Neuteboom et al., 1995).

Deletion construct Pbx 105-309 consists of a Pbx1 internal *Pvu*II fragment cloned in the *Eco*RV site of pSS. To equip the three smallest combined deletions with an extra methionine, which is necessary to visualize the labeled protein on SDS-PAGE gels, PCR products starting at the 5′ end of the homeodomain of templates Pbx 1-309, 1-304 and 1-297 were cloned in pSS-Flag, which encodes an N-terminal Flag tag (Peltenburg and Murre, 1996).

To exchange the Pbx1 homeodomain for the Hoxb-8 homeodomain in Pbx/b-8/Pbx, we generated hybrid fragments generated by PCR with oligonucleotides spanning the junctions and cloned them into the backbone of the Pbx1 full-length plasmid. The used Hoxb-8 translation construct was described in van Dijk et al. (1995). All mutant Pbx1 constructs were generated by two-step PCR. For these, oligonucleotides spanning each strand of the desired mutation(s) were used in the first round of PCR, followed by combination of the products and subsequent PCR with two distal primers. In all cases, cloned PCR inserts were verified by DNA sequencing.

In vitro translation and electrophoretic mobility shift assays (EMSA)

All proteins used in these experiments were synthesized using the SP6 TNT rabbit reticulocyte lysate-coupled transcription/translation system (Promega, Madison, WI). EMSAs were performed as described (van Dijk and Murre, 1994). Incubations and electrophoresis were done at room temperature. Reactions were adjusted to contain equal amounts of reticulocyte lysate. 1-2 μl of each translated protein, to a maximum of 4 μl, were used per reaction.

The ^{32}P end-labeled probes were 5′-GAGATGATTTATTACTTTAGTC-3′ and 5′-GTCAATTAAATGCGCATCAATCAATTTCG-3′ to detect Hox-Pbx cooperative binding, and 5′-GTCAATTAAATGATCAATCAATTTCG-3′ for Engrailed-Pbx complexes.

RESULTS

The Pbx1 N-terminal region contributes to cooperative DNA binding with Hox and Engrailed proteins

To determine the role of the N-terminal region of Pbx1 in allowing cooperative DNA binding with Hox and En-2, a series of N-terminal deletion constructs were designed and analyzed for cooperative DNA binding (Fig. 1A). The constructs were in vitro transcribed and translated using reticulocyte lysate and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1B). The various products were next analyzed for DNA binding in the presence or absence of in vitro translated Hoxb-7 by electrophoretic mobility shift assays (EMSA). As a probe, we used an optimal binding site for Pbx1/Hoxb-8 heterodimers identified by binding site selection (S. T. C. Neuteboom and C. M., unpublished data). Incubation of the Pbx1 N-terminal deletion proteins by themselves did not show any detectable specific shifted complexes (Fig. 1C, lanes 2-8). Upon addition of Hoxb-7, most truncated Pbx proteins bind efficiently to the DNA probe (Fig. 1C, lanes 10-16). N-terminal deletion of 50 amino acids results in a five-fold decrease (Fig. 1C, lanes 10-13) and DNA binding drops to almost undetectable levels upon deletion of 118 amino acids (Fig. 1C, lane 14). Further truncation leads to a partial restoration of the Hoxb-7/Pbx1 DNA binding (Fig. 1C, lanes 15 and 16), suggesting the presence of an inhibitory domain in the N-terminal region. Similar results were obtained

with Hoxb-8 and Hoxc-6 proteins (unpublished data). The truncated proteins were also tested for cooperative DNA binding with murine En-2. As a probe, we used a binding site that previously was shown to allow cooperativity involving Pbx1 and either *Drosophila* Engrailed or murine En-2 (van Dijk and Murre, 1994; van Dijk et al., 1995). DNA binding of truncated forms of Pbx or En-2 by themselves is barely detectable (Fig. 1D, lanes 2-9). However, cooperative binding is readily detectable when both Pbx1 and En-2 are present (Fig. 1D, lanes 10-16). Cooperative DNA binding is not influenced by deletion of the N-terminal 79 amino acids (Fig. 1D, lanes 10-13), but decreases dramatically upon removal of an additional 40 amino acids (Fig. 1D, lane 14). Further deletion restores cooperative DNA binding to levels characteristic of the full-length protein (Fig. 1D, lanes 15 and 16), again suggesting the presence of an inhibitory activity in the medial N-terminal region. These experiments show that the distal N-terminal domain is required for full cooperativity with Hox proteins, but not for cooperativity involving En-2. Deletion of an inhibitory region partly restores cooperative DNA binding with Hoxb-7 and almost completely restores the cooperativity with En-2.

The distal carboxy terminus of Pbx1 is not required for cooperative DNA binding with Hoxb-7 and En-2

To assess the role of the C-terminal flanking regions of Pbx1 for cooperativity with Hox and Engrailed proteins, four con-

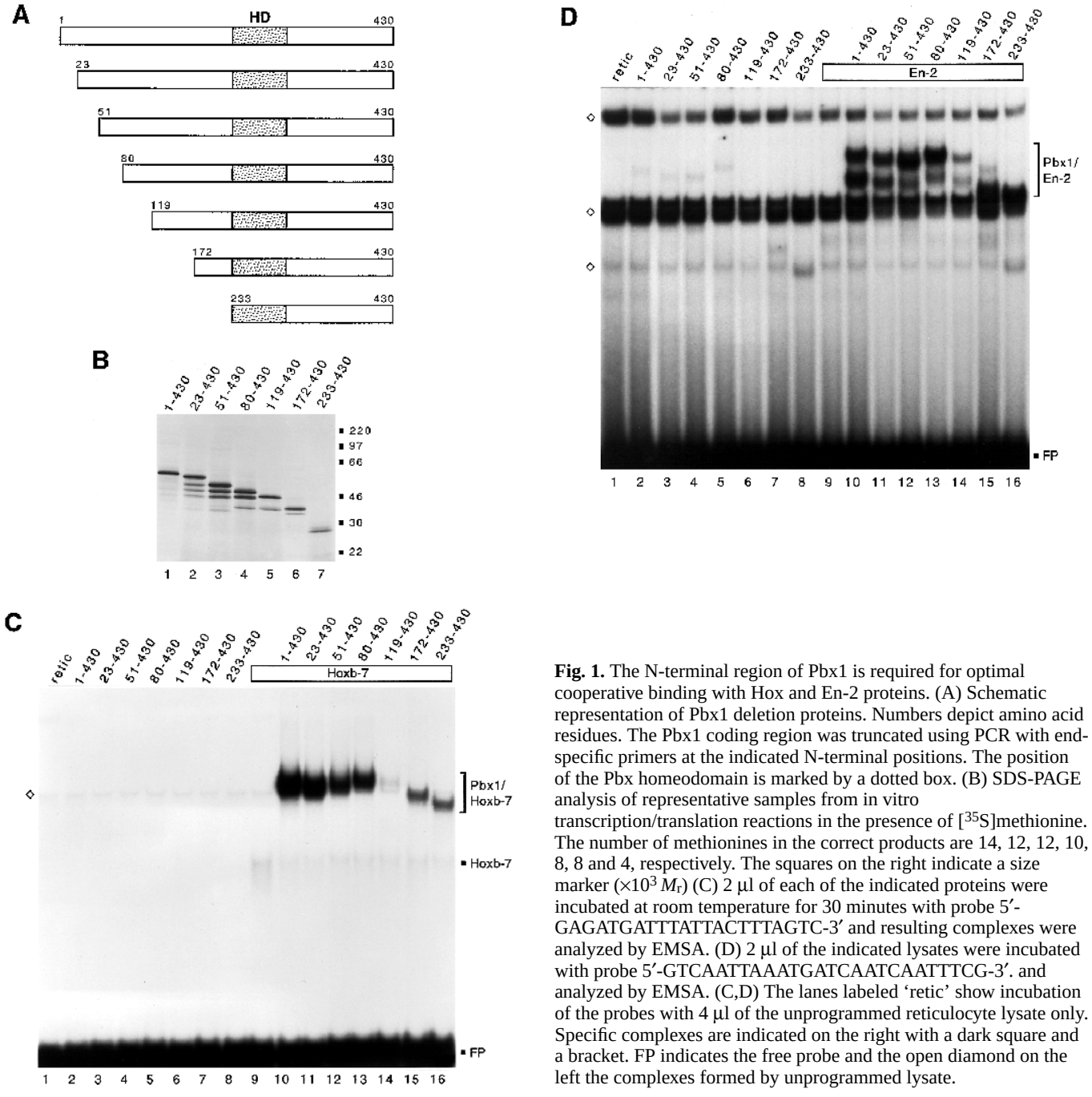


Fig. 1. The N-terminal region of Pbx1 is required for optimal cooperative binding with Hox and En-2 proteins. (A) Schematic representation of Pbx1 deletion proteins. Numbers depict amino acid residues. The Pbx1 coding region was truncated using PCR with end-specific primers at the indicated N-terminal positions. The position of the Pbx homeodomain is marked by a dotted box. (B) SDS-PAGE analysis of representative samples from in vitro transcription/translation reactions in the presence of [^{35}S]methionine. The number of methionines in the correct products are 14, 12, 12, 10, 8, 8 and 4, respectively. The squares on the right indicate a size marker ($\times 10^3$ M_r) (C) 2 μl of each of the indicated proteins were incubated at room temperature for 30 minutes with probe 5′-GAGATGATTTATTACTTTAGTC-3′ and resulting complexes were analyzed by EMSA. (D) 2 μl of the indicated lysates were incubated with probe 5′-GTCAATTAAATGATCAATCAATTTCG-3′. and analyzed by EMSA. (C,D) The lanes labeled ‘retic’ show incubation of the probes with 4 μl of the unprogrammed reticulocyte lysate only. Specific complexes are indicated on the right with a dark square and a bracket. FP indicates the free probe and the open diamond on the left the complexes formed by unprogrammed lysate.

secutive truncated coding regions were designed (Fig. 2A). To verify that comparable quantities were produced, representative samples of the in vitro translated proteins were analyzed on SDS-PAGE gels (Fig. 2B, lanes 1-5). EMSA analyses demonstrate that by themselves these proteins barely bind DNA (Fig. 2C, lanes 1-5). Upon addition of Hoxb-7, heterodimeric complexes are formed that bind with similar affinity as compared to wild-type Pbx1, except for the truncation lacking all residues C-terminal of the homeodomain (Fig. 2C, lanes 7-11). Cooperative DNA binding involving En-2 and truncated versions of Pbx1 decreases upon deletion to amino acid 304 (Fig. 2D, lanes 7-10) and is completely lost when all of the residues C-terminal of the homeodomain have been deleted (Fig. 2D, lane 11). These experiments indicate that the Pbx1 C-terminal 121 amino acids are not required for cooperative DNA binding with either Hoxb-7 or Engrailed, but residues immediately C-terminal of the homeodomain are essential.

The Pbx structural determinants for cooperative DNA binding with Hoxb-7 and Engrailed-2 are located in the homeodomain and the proximate C-terminal flanking amino acids

To determine the minimal region of Pbx1 required for cooperative DNA binding with Hox and En-2, we deleted both termini of the Pbx coding region (Fig. 3A). SDS-PAGE of the in vitro translated products showed that the various forms are equally well translated (Fig. 3B, lanes 1-5). When analyzed by EMSA, the truncated proteins do not exhibit DNA binding by themselves (Fig. 3C). However, in the presence of Hoxb-7, heterodimeric complexes are formed with full-length and

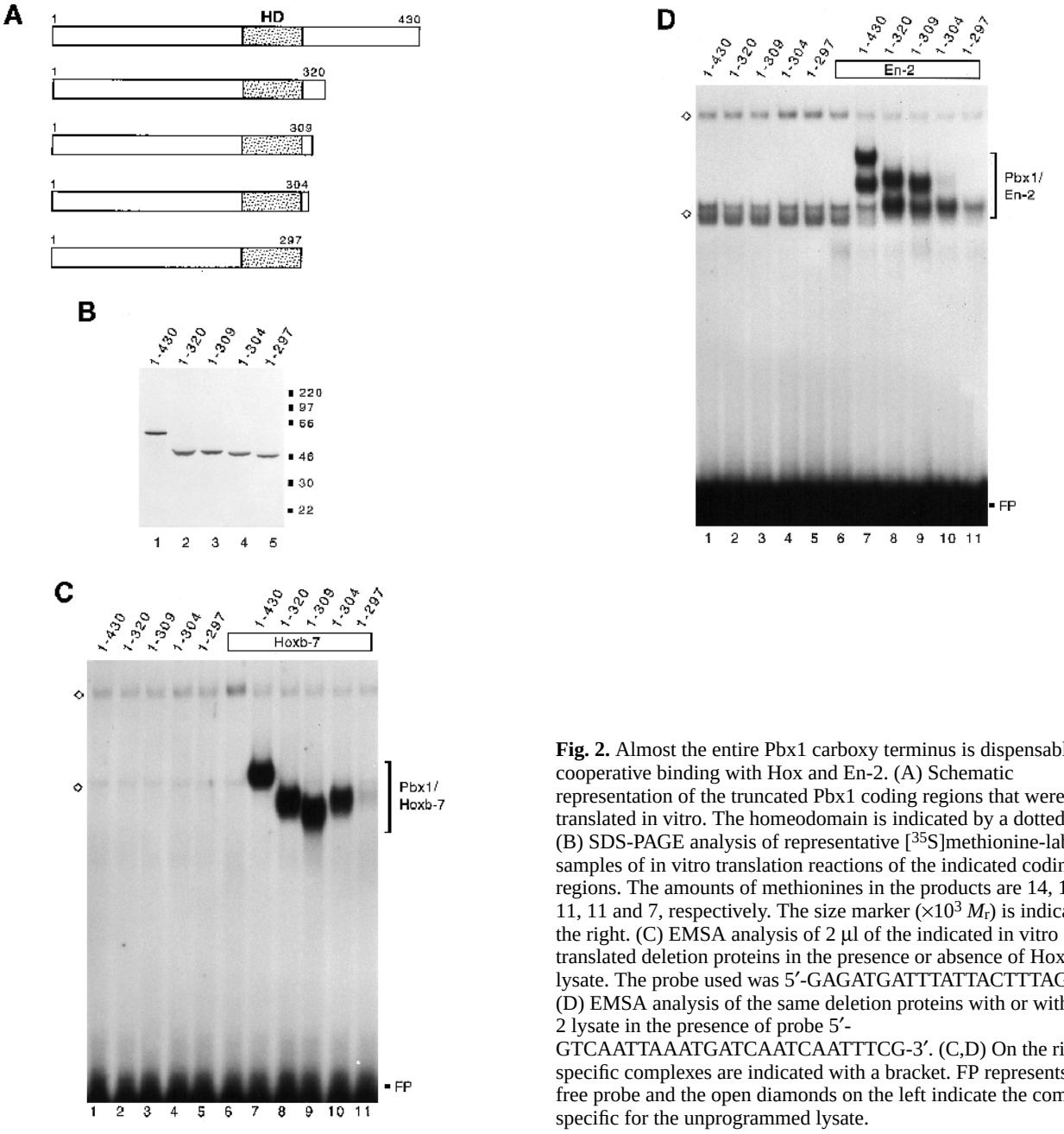


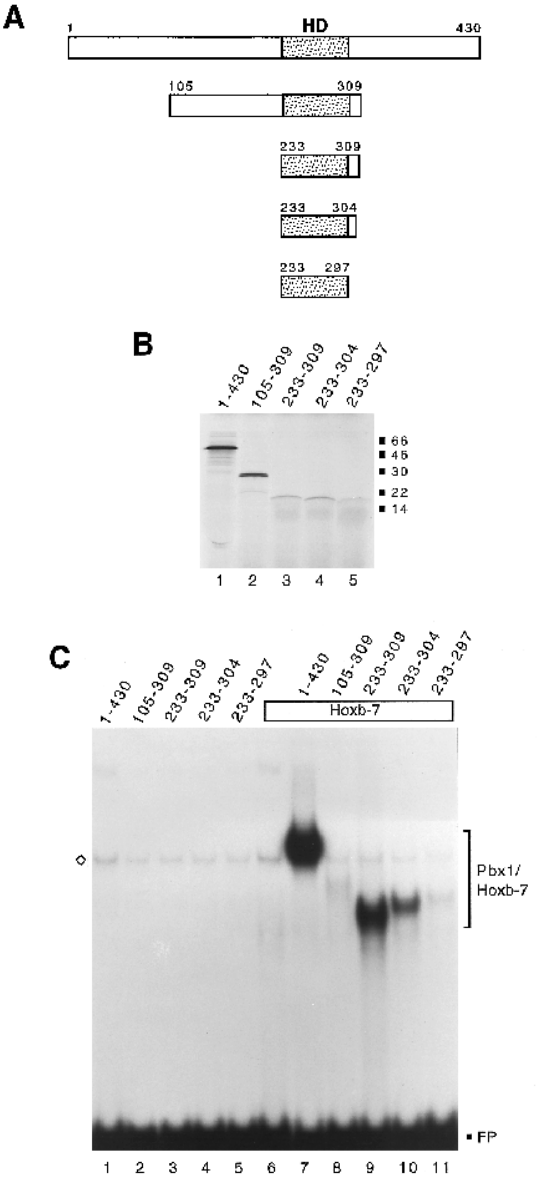
Fig. 2. Almost the entire Pbx1 carboxy terminus is dispensable for cooperative binding with Hox and En-2. (A) Schematic representation of the truncated Pbx1 coding regions that were translated in vitro. The homeodomain is indicated by a dotted box. (B) SDS-PAGE analysis of representative [^{35}S]methionine-labeled samples of in vitro translation reactions of the indicated coding regions. The amounts of methionines in the products are 14, 11, 11, 11 and 7, respectively. The size marker ($\times 10^3$ M_r) is indicated on the right. (C) EMSA analysis of 2 μl of the indicated in vitro translated deletion proteins in the presence or absence of Hoxb-7 lysate. The probe used was 5′-GAGATGATTTATTACTTTAGTC-3′. (D) EMSA analysis of the same deletion proteins with or without En-2 lysate in the presence of probe 5′-GTCAATTAAATGATCAATCAATTTCG-3′. (C,D) On the right, specific complexes are indicated with a bracket. FP represents the free probe and the open diamonds on the left indicate the complexes specific for the unprogrammed lysate.

truncated versions of Pbx1 (amino acids 233-309 and 233-304; Fig. 3C, lanes 7, 9, 10). When a portion of the N terminus is present, *i.e.* in deletion protein 105-309, Pbx1/Hoxb-7 cooperativity is almost abolished, again indicating the release of an inhibitory domain upon truncation of the 105 N-terminal residues (Fig. 3C, lane 8). The Pbx1 homeodomain by itself is not suffcient for cooperativity with Hoxb-7 (Fig. 3C, lane 11).

We next analyzed the truncated forms of Pbx1 for their ability to bind as multimeric complexes with En-2, using a probe containing sites that allow Pbx1/En-2 DNA binding complexes. Binding of full-length Pbx1 and a truncated form of Pbx1 (105-309) by themselves to this probe is barely detectable (Fig. 3D, lanes 1 and 2). However, the shorter products bind more efficiently (Fig. 3D, lanes 3-5 and see also Fig. 1D), indicating the presence of a domain preventing intrinsic DNA binding of Pbx1. The various truncated forms of Pbx1 bind as multimers with En-2 in a similar pattern as described above for Hoxb-7 (Fig. 3D, lanes 7, 9-10), except for the Pbx1 homeodomain that is still able to cooperate with En-2 (Fig. 3D, lane 11). Collectively, these data indicate that the Pbx1 homeodomain and a short C-terminal extension are required for cooperative DNA binding involving Hox proteins. Cooperative binding of Pbx1 with En-2 requires the Pbx1 homeodomain.

The homeodomain-flanking regions are not sufficient for cooperative DNA binding

The experiments described above indicate that the Pbx1 homeodomain and a C-terminal extension are required for binding to DNA as a heterodimer with Hoxb-7. To establish whether the Pbx1 C- and N-terminal domains flanking the Pbx1 homeodomain allow cooperative DNA binding with Hoxb-7 when fused to a heterologous homeodomain, a chimeric protein was generated. Within the context of full-length Pbx1, the Pbx1 homeodomain was exchanged for the Hoxb-8 homeodomain (Fig. 4A). The proteins were in vitro translated and analyzed by SDS-PAGE, showing that the correct products were translated with similar efficiencies (Fig. 4B, lanes 1-3). The hybrid protein was tested for cooperative binding with Hoxb-7 using either an optimal Hoxb-8/Pbx1-binding site or a probe originally shown to bind abd-A/exd heterodimers (S. T. C. Neuteboom and C. M.,



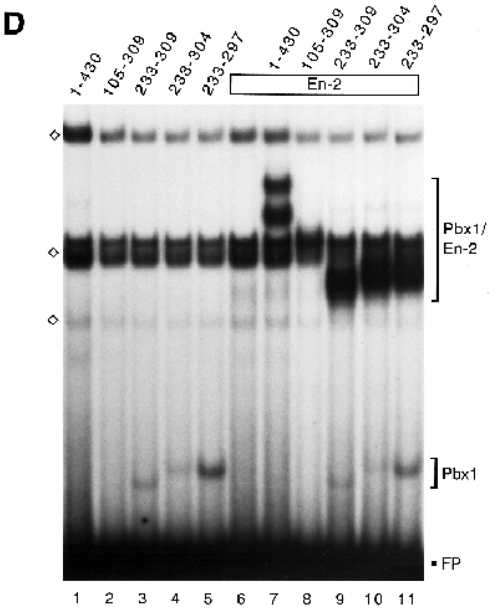


Fig. 3. The Pbx homeodomain plus a short C-terminal stretch are sufficient for Hox and En-2 cooperative binding. (A) Schematic representation of the C- and N-terminally deleted Pbx1 coding regions used for in vitro translation. The dotted box depicts the Pbx homeodomain. (B) Samples of the in vitro translation reactions were incubated with [^{35}S]methionine and analyzed by SDS-PAGE. To provide the short Pbx1 deletion proteins 233-309, 233-304 and 233-297 with an extra methionine, an N-terminal 'Flag' tag (Peltenburg and Murre, 1996) was linked to these coding regions. The number of incorporated methionines are per deletion protein 14, 7, 2, 2 and 2, respectively. On the right the size marker is depicted ($\times 10^3$ M_r). (C) 2 μl of the indicated lysates were analyzed by EMSA in the presence or absence of Hoxb-7, as indicated. The used probe was 5′-GAGATGATTTATTACTTTAGTC-3′. (C,D) EMSA analysis with probe 5′-GTCAATTAAATGATCAATCAATTTCG-3′ and the indicated Pbx1 lysates with and without En-2, as indicated. Brackets on the right correspond to specific complexes, FP indicated the free probe and the open diamonds mark the presence of complexes originating from the unprogrammed reticulocyte lysate.

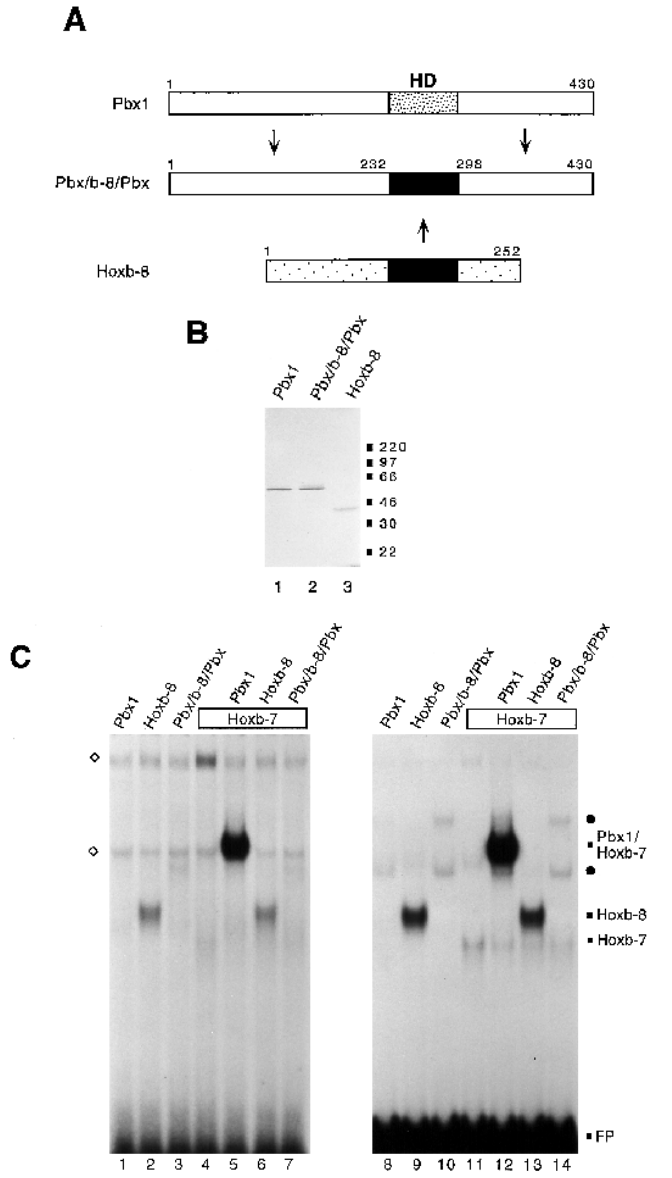


Fig. 4. The Pbx homeodomain-flanking sequences are not sufficient to support cooperative binding with Hoxb-7. (A) Schematic drawing of the fusion of the Hoxb-8 homeodomain to the Pbx1 flanking regions. Open boxes indicate Pbx1 flanking sequences, the lightly dotted box corresponds to Hoxb-8 flanking regions, the black box to the Hoxb-8 homeodomain and the densely dotted box indicates the Pbx1 homeodomain. (B) SDS-PAGE analysis of ^{35}S-labeled samples of the indicated translated constructs. A size marker is positioned on the right ($\times 10^3$ M_r). (C) EMSA analysis of the indicated proteins in the presence or absence of Hoxb-7 lysate. Left panel: incubation with probe 5′-GAGATGATTTATTACTTTAGTC-3′, right panel: incubation with probe 5′-GTCAATTAAATGCGCATCAATCAATTTCG-3′. Specific complexes are marked with a filled square, open diamonds correspond to complexes specific for the unprogrammed lysate, FP indicates the free probe and the filled circles mark DNA binding by the Pbx proteins themselves.

unpublished data and van Dijk et al., 1994). The hybrid Pbx/b-8/Pbx protein binds with higher affinity to both probes as compared to wild-type Pbx1 by itself, but with lower affinity than Hoxb-8 by itself (Fig. 4C, lanes 1-3 and 8-10). Thus, the Pbx flanking regions reduce the intrinsic DNA-binding activity of Hoxb-8 to these probes. The hybrid protein, however, does not show any cooperative binding to these probes with Hoxb-7 (Fig. 4C, lanes 5-7 and 12-14). These data suggest that the Pbx flanking regions are not sufficient for heterodimeric DNA binding with Hoxb-7, implying an important function for the Pbx homeodomain in cooperative DNA binding.

Some specific structural determinants involved in a1/α2 interactions are also required for Pbx/Hox and Pbx/En-2 cooperativity

The data described above confirmed a role for the Pbx1 homeodomain in allowing cooperative DNA binding with Hox and Engrailed proteins. To precisely define residues within the homeodomain, we pursued the following strategies. First, as described above, there are striking similarities between Pbx/Hox cooperative DNA binding and two homeodomain proteins a1 and α2. Recent structural data derived from the crystal structure of an a1/α2 heterodimer complexed to DNA have indicated a number of contacts between α2 and the homeodomain of a1 (Li et al., 1995). Interactions between these transcription factors are modulated through contacts in the a1 homeodomain, specifically in the pocket between helices 1 and 2 which is flanked by hydrophobic residues (Li et al., 1995) . Similar positions in the Pbx homeodomain are occupied by hydrophobic residues. Two hydrophobic residues in the Pbx1 homeodomain, an isoleucine and a tyrosine, were changed into arginine and lysine residues, designated as HD[15,19] (Fig. 5A). Both residues are commonly used at the same position in a wide variety of homeodomain-containing proteins (Bürglin, 1994) and thus are not expected to change the overall structure of the homeodomain. In addition, within the a1 homeodomain, one side of this pocket is flanked by a salt bridge between the N-terminal residue of the loop between helix 1 and helix 2 and the N terminus of helix 2. In HD[28,29], the two glutamates in helix 2 were changed to arginines, both of which are commonly found at the amino terminus of helix 2 (Fig. 5A).

By mutational analysis, it has been shown that residues located in the loop between helix 2 and helix 3 in a1 are involved in interaction with α2 (Stark and Johnson, 1994). At those positions, the Pbx1 homeodomain contains identical residues which are rarely present in other homeodomain-containing proteins. To assess whether these residues are required for cooperativity with Pbx1, they were changed into common leucines, designated as mutant HD[38,40].

Each mutant was efficiently translated in vitro (Fig. 5B, lanes 1, 5 and 6) and tested for their ability to bind DNA as heterodimers with Hoxb-7, Hoxb-8 and Hoxc-6 by EMSA. DNA binding by the Pbx proteins themselves was undetectable (unpublished data). Both mutants HD[15,16] (helix 1) and HD[28,29] (helix 2) are severely affected in their ability to modulate cooperative interactions with each of the Hox proteins (Fig. 5C, lanes 2, 6 and 7). Cooperative DNA binding involving mutant HD[38,40], located in the loop between helix 2 and helix 3 with the Hox proteins was not affected (Fig. 5, lane 8). Cooperative DNA binding of the mutants in conjunction with En-2 showed an identical pattern (Fig. 5D, lanes 12, 16, 17 and 18). Interestingly, mutant HD[28,29] showed significant intrinsic DNA binding to the En-2/Pbx1 probe, whereas with En-2 cooperative DNA binding is barely detectable (Fig. 5C, lanes 7 and 16). Taken together, these data suggest that there are similarities and clear differences

between a1/α2 interaction and Pbx/Hox or Pbx/En-2 cooperativity. In particular, it is striking that specific residues required for the formation of a1/α2 heterodimers are similarly required for cooperative DNA binding involving Pbx, Hox and En-2.

Differential structural requirements for Pbx/Hox and Pbx/Engrailed cooperativity

We also examined whether changes in amino acids within the region immediately N-terminal of helix 1 of the Pbx homeodomain have an impact on cooperativity involving either Hox or En-2. Mutations HD1 and HD6, substituting a rare alanine and arginine for an arginine and threonine, respectively, are changes to common residues found in homeodomain proteins. HD^{2} is a nonconservative substitution of an arginine into a glutamine residue. We also introduced two substitutions in helix 3 of the Pbx1 homeodomain. The first residue of helix 3 has not been implicated in base-specific contacts (Bürglin, 1994) and is in the Pbx homeodomain a valine, rarely present at that position in typical homeodomain proteins. We mutated V^{42} in Pbx1 into a common glutamate in mutant HD^{42} (Fig. 5A). Mutant HD^{50} contains a change from an uncommon G^{50} to the consensus glutamine residue, known to contact bases in the major groove of the DNA (Bürglin, 1994).

The mutants were transcribed and translated in vitro (Fig. 5B) and analyzed by EMSA for cooperative DNA binding with both Hox and En-2. HD^{1}, HD^{6}, HD^{42} and HD^{50} each were able to bind cooperatively to DNA in the presence of Hoxb-7, Hoxb-8 and Hoxc-6 (Fig. 5C, lanes 3, 5, 9 and 10). The same mutants were capable of cooperative DNA binding with En-2 (Fig. 5D, lanes 13, 15, 19 and 20). However, while mutant HD^{2} was severely affected in cooperativity with the Hox proteins (Fig. 5C, lanes 4), this mutaton did not affect cooperativity with En-2 (Fig. 5D, lane 14). Taken together, these data indicate that Pbx1 DNA binding in concert with the Hox gene products requires different structural determinants as compared to Pbx1/En-2 multimeric complexes.

The three-amino acid atypical insertion in the Pbx homeodomain is an important structural determinant for cooperative binding in conjunction with both Hox and Engrailed

The group of atypical homeodomain proteins are characterized by the abnormal length of their homeodomains (Bürglin, 1994). Pbx1, Pbx2 and Pbx3 are members of this family, since they contain insertions of three residues in the turn separating helix 1 from helix 2 (Fig. 6A, Bürglin, 1994). It is particularly

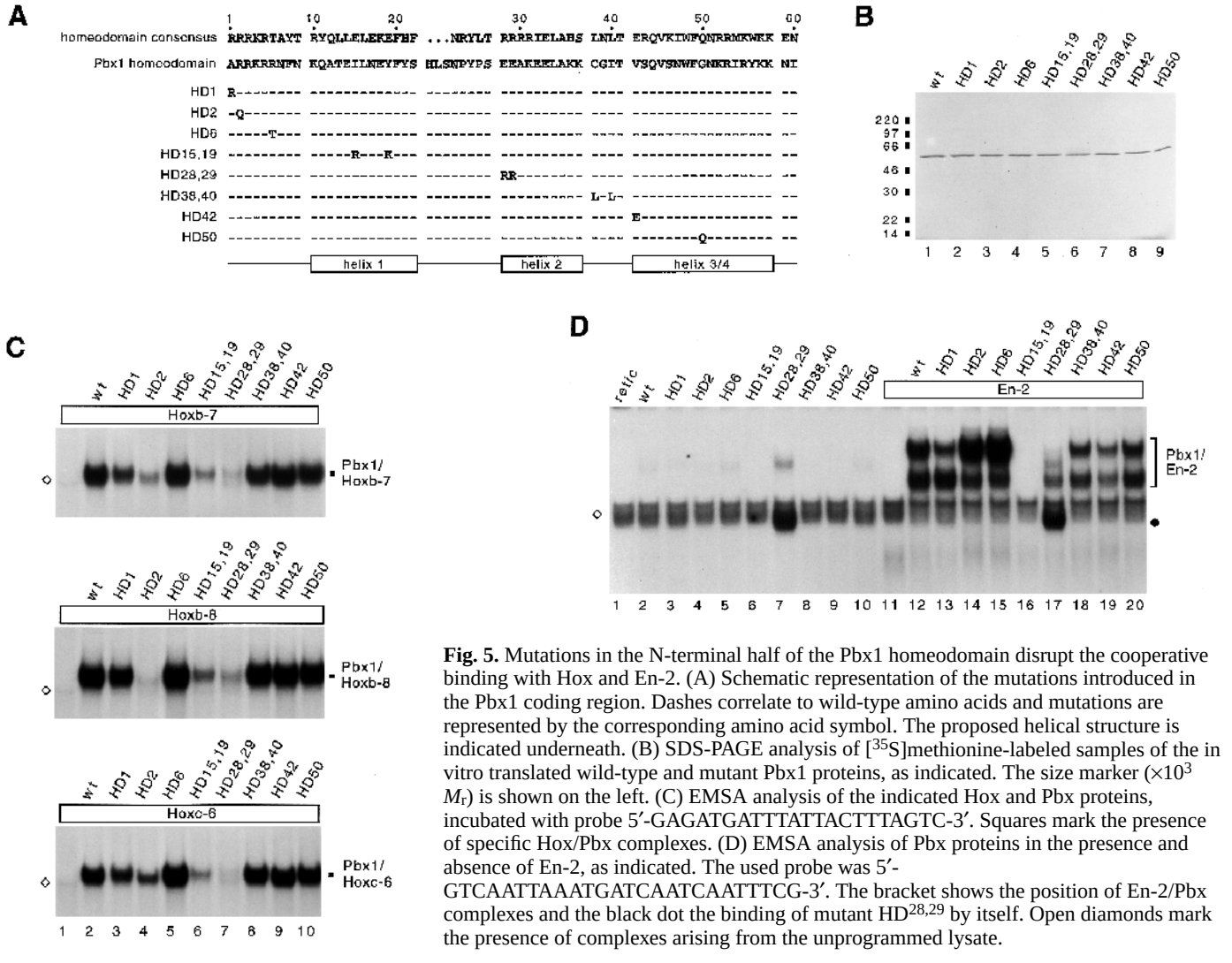


Fig. 5. Mutations in the N-terminal half of the Pbx1 homeodomain disrupt the cooperative binding with Hox and En-2. (A) Schematic representation of the mutations introduced in the Pbx1 coding region. Dashes correlate to wild-type amino acids and mutations are represented by the corresponding amino acid symbol. The proposed helical structure is indicated underneath. (B) SDS-PAGE analysis of [^{35}S]methionine-labeled samples of the in vitro translated wild-type and mutant Pbx1 proteins, as indicated. The size marker ($\times 10^{3}$ M_r) is shown on the left. (C) EMSA analysis of the indicated Hox and Pbx proteins, incubated with probe 5′-GAGATGATTTATTACTTTAGTC-3′. Squares mark the presence of specific Hox/Pbx complexes. (D) EMSA analysis of Pbx proteins in the presence and absence of En-2, as indicated. The used probe was 5′-GTCAATTAAATGATCAATCAATTTCG-3′. The bracket shows the position of En-2/Pbx complexes and the black dot the binding of mutant $HD^{28,29}$ by itself. Open diamonds mark the presence of complexes arising from the unprogrammed lysate.

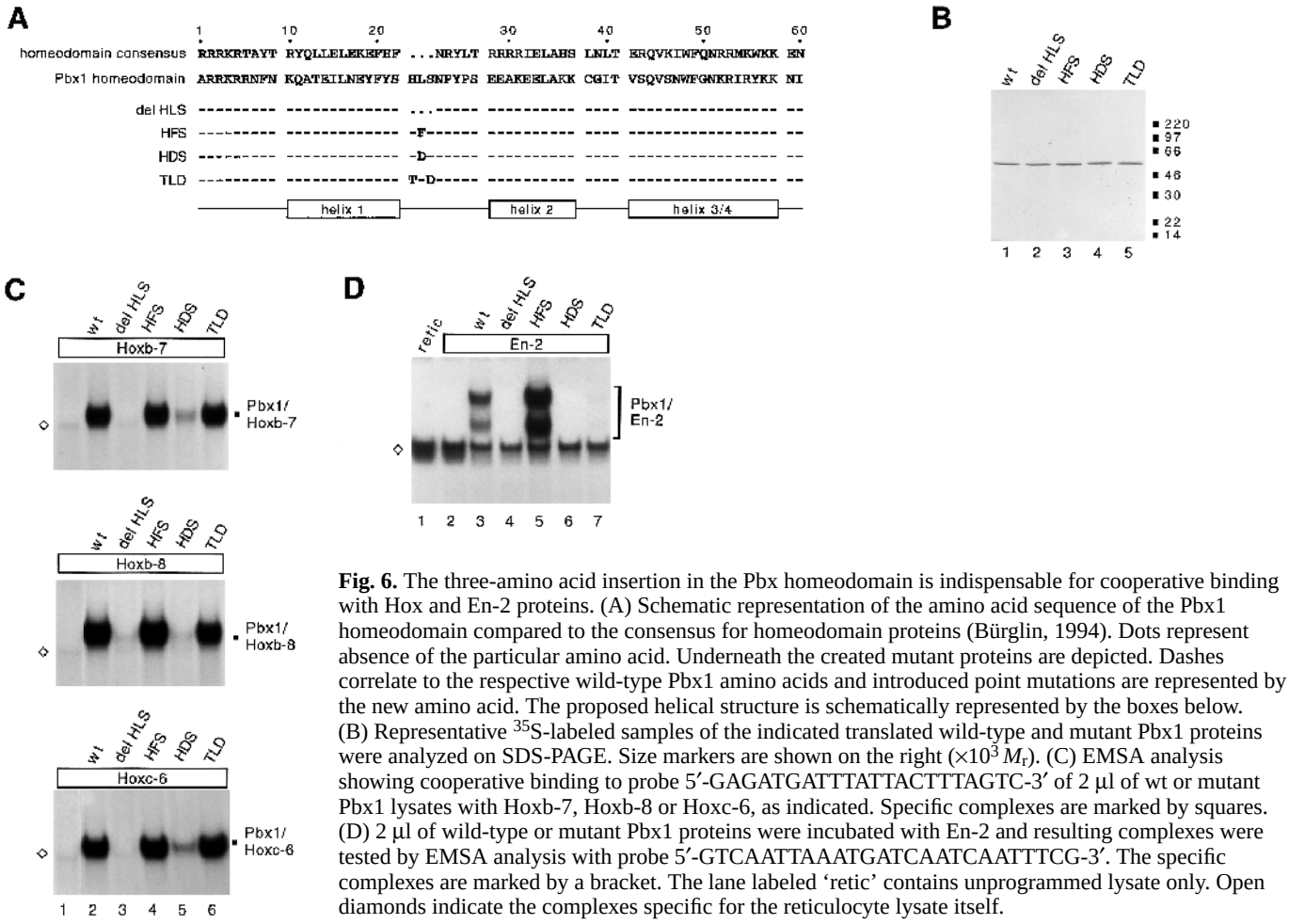


Fig. 6. The three-amino acid insertion in the Pbx homeodomain is indispensable for cooperative binding with Hox and En-2 proteins. (A) Schematic representation of the amino acid sequence of the Pbx1 homeodomain compared to the consensus for homeodomain proteins (Bürglin, 1994). Dots represent absence of the particular amino acid. Underneath the created mutant proteins are depicted. Dashes correlate to the respective wild-type Pbx1 amino acids and introduced point mutations are represented by the new amino acid. The proposed helical structure is schematically represented by the boxes below. (B) Representative ^{35}S-labeled samples of the indicated translated wild-type and mutant Pbx1 proteins were analyzed on SDS-PAGE. Size markers are shown on the right ($\times 10^3$ M_r). (C) EMSA analysis showing cooperative binding to probe 5′-GAGATGATTTATTACTTTAGTC-3′ of 2 μl of wt or mutant Pbx1 lysates with Hoxb-7, Hoxb-8 or Hoxc-6, as indicated. Specific complexes are marked by squares. (D) 2 μl of wild-type or mutant Pbx1 proteins were incubated with En-2 and resulting complexes were tested by EMSA analysis with probe 5′-GTCAATTAAATGATCAATCAATTTCG-3′. The specific complexes are marked by a bracket. The lane labeled ‘retic’ contains unprogrammed lysate only. Open diamonds indicate the complexes specific for the reticulocyte lysate itself.

intriguing that a family of homeodomain proteins controlling cell fate in fungi, named bE, have an insertion of three amino acids located between helix 1 and helix 2 (Gillissen et al., 1992; Bürglin, 1994) (Table 1). To determine whether the three-amino acid insertion is required for cooperative DNA binding with the Hox gene products and En-2, we deleted these three amino acids from the Pbx1 coding region (Fig. 6A, del HLS). del HLS was efficiently translated in vitro (Fig. 6B). To test for cooperative DNA binding, the deleted Pbx1 form was incubated with Hoxb-7, Hoxb-8, Hoxc-6 or En-2 and analyzed by EMSA. For all combinations of proteins, the results are similar and demonstrate that deletion of the triplet completely disrupts the cooperative binding (Fig. 6C, lanes 3; Fig. 6D, lane 4). We next examined whether specific residues within the triplet are essential for cooperative DNA binding.

The second amino acid of this triplet, leucine, is present at that position in all Pbx proteins as well as in each of the bE proteins analyzed to date. We mutated this hydrophobic residue to a bulkier phenylalanine and to an aspartic acid (Fig. 6A, HFS and HDS, respectively). Both substitutions are present with high frequencies in loop structures and the overall structure is predicted to be similar when examined by Chou-Fasman analysis (unpublished data). Exchanging the hydrophobic leucine for a bulkier phenylalanine does not affect cooperativity with either the Hox or En-2 proteins (Fig. 6C, lanes 4; Fig. 6D, lane 5), but mutation to an aspartic acid decreases the Pbx/Hox and Pbx/En-2 cooperativity significantly (Fig. 6C, lanes 5; Fig. 6D, lane 6). Thus, within the triplet, specific residues are required for cooperativity of Pbx1 with Hox and En-2 gene products.

The data shown above suggest a role for the triplet in mediating cooperative DNA binding involving both Hox and Engrailed gene products. In the three amino acid insertion, which is characteristic of many atypical homeodomain proteins, only the leucine residue is conserved in other atypical homeodomain proteins (Table 1). For example, compare HLS in Pbx gene products to TLD in bE6 (Table 1). To determine whether the triplet present in *Ustilago maydis* bE1, bE2, bE5, bE6 and bE7 is capable of replacing the Pbx residues HLS, we exchanged the Pbx amino acids for the triplet TLD (Fig. 6A). Interestingly, the Pbx mutant protein in which the HLS triplet had been substituted into the TLD triplet found in bE proteins still fully supports cooperative DNA binding with Hox proteins (Fig. 6C, lanes 6). However, when tested in conjunction with En-2, no significant cooperative DNA binding can be detected (Fig. 6D, lane 7), indicating differential requirements for interactions of Pbx1 with Engrailed and Hox proteins. Taken together, these data indicate that a three amino acid insertion located in atypical homeodomain proteins is important for cooperativity with both Hox and Engrailed proteins. Furthermore, the triplet derived from the fungus *Ustilago maydis* can functionally replace the Pbx1 residues for cooperative DNA binding in conjunction Hox gene products.

Table 1. Alignment of the regions spanning helix 1 and helix 2 of atypical homeodomain proteins with a three amino acid insertion

Protein	Helix 1	Helix 2
homeodomain	10	37
consensus	RYQLLELEKEFHF	...NRYLT RRRRIELAHS
Pbx1	KQATEILNEYFYS	HLSNPYPS EEAKEELAKK
Pbx2	KQATEVLNEYFYS	HLSNPYPS EEAKEELAKK
Pbx3	KQATEILNEYFYS	HLSNPYPS EEAKEELAKK
exd	KQASEILNEYFYS	HLSNPYPS EEAKEELARK
ceh-20	KQATEVLNEYFYG	HLSNPYPS EEAKEDLARQ
Um bE1	DLPAYHMRKHFLL	TLDNPYPT QEEKETLVRL
Um bE2	DLPAYHMRKHFLH	TLDNPYPT QEEKEGLVRL
Um bE3	DLPAYHMRNHFLH	TLENPYPT QEEKEGLVRL
Um bE4	DLPAYHMRKHFLH	TLENPYPT QEEKETLVRL
Um bE5	DLPAYHMRKHFLL	TLDNPYPT QEEKQNLVRL
Um bE6	DLPAYHMRKHFLH	TLDSPYPT QEEKETLVRL
Um bE7	DLPAYHMRKHFLL	TLDSPYPT QEEKEGLVRL
Kn1	KEARQQLLSWWDQ	HYKWPYPS ETQKVALAES
ZMH1	GDTTSILKQWWQE	HSKWPYPT EDDKAKLVEE
ZMH2	GDTASTLKAWWQA	HSKWPYPT EEDKARLVQE
MATα2	KENVRILESWFAK	NIENPYLD TKGLENLMKN
Sp MAT1Pi	KCTKPHLMRWLLL	HYDNPYPS NSEFYDLSAA
Cc Aβ1-1	PAYVEPCARWLKD	NWYNPYPS GEVRTQIARQ
Cc Aβ4-1	PAYIEPSCRWLKD	NWYNPYPS PQVRSSIAKQ
Sc AαZ3	PPFIGACYEWLLQ	HLHNPYPS KSEKQIILQL
Sc AαZ4	PPYIEPCYRWLVN	HLDNPYPT KAIKEELLDQ

References for sequences Pbx1, Pbx2 and Pbx3 (Kamps et al., 1990; Nourse et al., 1990; Monica et al., 1991), *D. melanogaster* exd (Flegel et al., 1993), *C. elegans* ceh-20 (Bürglin and Ruvkun, 1992), *Ustilago maydis* bE (Kronstad and Leong, 1990; Schulz et al., 1990), Kn1, ZMH1 and ZMH2 (Vollbrecht et al., 1991), *S. cerevisiae* MATα2 (Astell et al., 1981), *Schizosaccharomyces pombe* MAT1Pi (Kelly et al., 1988), *Coprinus cinereus* Aβ1-1 and Aβ4-1 (Kües et al., 1992; Bürglin, 1994), *Schizophyllum commune* AαZ3 and Aα4 (Stankis et al., 1992; Bürglin, 1994).

DISCUSSION

A large variety of homeodomain-containing proteins bind to DNA in a cooperative manner. Among the best characterized examples are two proteins that control cell fate in *Saccharomyces cerevisiae*, MATa1 and MATα2 (Johnson, 1992). Recently, a new family of proteins has been identified that include *Drosophila* gene product extradenticle, and Pbx1, Pbx2 and Pbx3 (Monica et al., 1991). The Pbx family of proteins allows other classes of homeodomain proteins to bind to DNA with higher affinity and specificity (Chan et al., 1994; van Dijk and Murre, 1994; Chang et al., 1995; Lu et al., 1995; Phelan et al., 1995; van Dijk et al., 1995). Among these other classes belong the Hox proteins, members of the Engrailed family and orphan homeodomain proteins, for example STF-1 (Peers et al., 1995). The domains that are required for Hox and Engrailed cooperativity have been well defined (Chang et al., 1995; Knoepfler and Kamps, 1995; Neuteboom et al., 1995; Phelan et al., 1995; Peltenburg and Murre, 1996). They involve two motifs: the hexapeptide in Hox proteins and the EH2 domain in Engrailed. These motifs contain a number of conserved residues and share a highly conserved tryptophan residue. One question that arises is whether the Hox and Engrailed proteins require identical structural determinants for cooperativity. The EH2 domain is quite divergent from the hexapeptide and it is conceivable that the EH2 domain interacts with different residues located in the Pbx protein as compared to the hexapeptide. In previous studies, we showed that synthetic peptides containing either the Hox or Engrailed Pbx interaction motifs can destabilize both Hox/Pbx and Engrailed/Pbx multimeric complexes (Peltenburg and Murre, 1996). However, it was also clear from those experiments that peptides could destablize cooperativity of homologous proteins more efficiently than heterologous proteins. Thus, while there are clearly overlapping residues that allow cooperativity to occur between Pbx and either Hox or Engrailed, there seemed to be also clear differences. We now have extended these studies. The experiments described above indicate that indeed certain residues present in the Pbx homeodomain are required for cooperativity with both Hox and Engrailed, whereas others are required only for Hox or En-2 cooperativity. For example, within the N-terminal portion of the Pbx homeodomain, we identified a residue that is required for cooperative DNA binding involving Hoxb-7, Hoxb-8 and, to a lesser extent, Hoxc-6, but not for cooperative DNA binding involving En-2. Vice versa, a specific amino acid substitution between helix 1 and helix 2 of the Pbx homeodomain abolished cooperativity involving Engrailed-2 but did not reduce cooperativity with either Hoxb-7, Hoxb-8 or Hoxc-6. Thus, while a number of structural determinants are required for cooperativity involving both Hox and Engrailed, others are clearly more specific.

Previous studies have indicated that there are many similarities between the mechanism of cooperative DNA binding involving a1 and α2 and the exd/Pbx members interacting with the Hox proteins (Johnson, 1992; van Dijk and Murre, 1994). Here we show that residues in the a1 homeodomain that mediate interaction with α2 are also required for cooperative DNA binding involving Pbx and Hox gene products. However, we note that there are also significant differences. For example, specific residues in the a1 homeodomain located in the turn separating helix 2 from helix 3 are required for cooperativity in conjunction with α2 (Stark and Johnson, 1994). By analogy, we have mutated similar residues in Pbx1, but did not detect significant reductions in cooperative DNA binding involving either Hox or Engrailed.

There are also strong similaties between the Pbx/Hox interaction and two classes of homeodomain proteins controlling cell determination in three different fungi, *Ustilago maydis*, *Coprinus cinereus* and *Schizophylum commune.* For all of these fungi, the fusion of two haplotypes brings together two homeodomain proteins encoded by two different gene families, the bE and bW genes (Banuett, 1992). Both the bE and bW loci encode homeodomain proteins (Gillissen et al., 1992). Although more complicated, there are similarities between the bE and bW members, and a1 and α2. Both systems bring together two homeodomain proteins that induce the cell to differentiate. However, while in *S. cerevisiae* only one combination of homeodomain proteins occurs, the number of bE and bW proteins is estimated to be at least 25, which in principle can result in at least 625 combinations of homeodomain proteins (Banuett, 1992). Interestingly, the bE homeodomains, like the members of the Pbx family, belong to the group of atypical homeodomain proteins (Bürglin, 1994). Many atypical homeodomain proteins contain three additional residues in the turn between helix 1 and helix 2 (Table 1). We demonstrate here that deletion of these three residues abolishes cooperativity with both Hox and Engrailed, but replacement of three Pbx residues in the loop

between helix 1 and helix 2 with amino acids derived from bE allows Pbx/Hox but does not allow Pbx/Engrailed cooperativity to occur at wild-type levels. While the molecular mechanism of how bE and bW proteins induce differentiation in *U. maydis* has not yet been resolved, it may very well be that the interactions involving bE and bW proteins are similar, as described here for Pbx/Hox and Pbx/Engrailed combinations.

We would like to thank Drs Boncinelli, Saskia Neuteboom and Johan van Es for the generous gift of several plasmids. This work was sponsored by the National Institutes of Health, the Council for Tabacco Research and the Edward Mallinckrodt Jr. Foundation. L. T. C. P. was supported by a fellowship of the Dutch Cancer Society (KWF).

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(Accepted 23 December 1996)