MOLECULAR AND CELLULAR BIOLOGY, Sept. 1997, p. 5369–5376 Vol. 17, No. 9
0270-7306/97/$04.00+0

Conformational Changes Induced in Hoxb-8/Pbx-1 Heterodimers in Solution and upon Interaction with Specific DNA

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Received 7 February 1997/Returned for modification 5 March 1997/Accepted 2 June 1997

Two classes of homeodomain proteins, Hox and Pbx gene products, have the ability to bind cooperatively to DNA. In Hox proteins, the homeodomain and a highly conserved hexapeptide are required for cooperative DNA binding. In Pbx, the homeodomain and a region immediately C terminal of the homeodomain are essential for cooperativity. Using fluorescence and circular dichroism spectroscopy, we demonstrated that Hox and Pbx proteins interact in the absence of DNA. The interaction in solution is accompanied by conformational changes. Furthermore, upon interaction with specific DNA, additional conformational changes are induced in the Pbx-1/Hoxb-8 heterodimer. These data indicate that prior to DNA binding, Hox-Pbx interaction in solution is accompanied by structural alterations. We propose that these conformational changes modulate the DNA binding properties of these proteins, ultimately resulting in cooperative DNA binding.

Homeodomain proteins, like many other transcriptional regulators, act in concert to regulate common downstream target genes (12). The combinatorial control exerted by homeodomain proteins has been best described with regard to the control of mating-type-specific gene expression in *Saccharomyces cerevisiae*. During the diploid phase, two homeodomain proteins, a1 and α2, form heterodimers to repress the transcription of a large number of haploid-specific genes (9). a1 and α2 readily form heterodimers in the absence and presence of DNA (7, 17). This interaction requires a short unstructured peptide located immediately C terminal of the α2 homeodomain which, upon interacting with residues located in the a1 homeodomain, is converted into an α-helix (14).

Recently, homeodomain protein interactions have also been described for *Drosophila* and vertebrate Hox gene products (1, 34). The *Drosophila* gene product Ubx binds cooperatively with Extradenticle (Exd), a protein containing an atypical homeodomain, to recognition sites present in the decapentaplegic enhancer (1). Similarly, Ubx and abd-A have the ability to bind cooperatively with Exd to synthetic Hox and Pbx binding sites (34). In vertebrates, three proteins that are closely related to Exd have been identified and are designated Pbx-1, Pbx-2, and Pbx-3 (5, 18, 29). The Pbx members have the ability to modulate the DNA binding properties of the Hox proteins, increasing their DNA binding affinity and specificity (3, 11, 15, 27, 28, 35). Exd and Pbx also have the ability to modulate the DNA binding activity of *Drosophila* and murine Engrailed. The binding specificity of these heterodimers is distinct from that of Hox/Pbx heterodimers (34, 35). These data indicated that the DNA binding properties of these heterodimers are different from those of either protein alone (4, 15, 35).

The structural determinants required for Hox/Pbx cooperativity have been well characterized. A highly conserved hexapeptide motif, located immediately N terminal of the Hox homeodomain, is required for cooperative DNA binding (3, 10, 11, 19, 23, 27). Cooperative DNA binding involving Pbx and Engrailed also requires the presence of a highly conserved peptide, located immediately N terminal of the Engrailed homeodomain (24). The Hox and Engrailed homeodomains are also required for cooperativity, since shuffling of these homeodomains abolishes their ability to bind cooperatively to DNA with the Pbx gene products (19, 23, 24). The Pbx domains required for cooperative DNA binding with Hox proteins have been mapped to the homeodomain and a region immediately C terminal of the homeodomain (3, 16, 25).

To determine whether Hox and Pbx form heterodimers prior to DNA binding, we have analyzed Hox-Pbx interactions in the absence and presence of DNA using fluorescence and circular dichroism (CD) spectroscopy. Both techniques have been used extensively to examine protein-protein and protein-DNA interactions (13, 21, 26, 33). Our data indicate that Hox and Pbx proteins interact in the absence of DNA. CD analysis revealed the induction of conformational changes upon interaction in the absence of DNA. Further conformational changes are induced in the presence of specific DNA but not in the presence of nonspecific DNA. We propose that the structural changes that are induced in solution upon interaction allow Pbx and Hox proteins to bind cooperatively to DNA.

MATERIALS AND METHODS

Plasmid DNA isolation and cloning procedures. Plasmid DNA isolation, cloning techniques, and transformation procedures were done as described by Sambrook et al. (32). Enzymes were obtained from Promega and New England Biolabs and used according to the manufacturers' instructions. A Qiagen II kit was used for DNA fragment purification. The high-pressure liquid chromatography-purified 22-mer oligonucleotides 5′-GAGATGATTTATGACTCTAG TC-3′ and 5′-GCGCGCGCGCGCGCGCGCGC-3′ were obtained from Genset.

Bacterial strains and plasmids. *Escherichia coli* DH101 was used routinely for primary transformation. The new plasmids were transferred into BL21(DE3)Δ*lon* strain for protein overproduction. Bacteria were grown in Luria-Bertani broth, supplemented with antibiotics when required (25 μg of kanamycin per ml), at 37°C. Plasmid pET30b(+) (Novagen) was used for all of the N-terminal histidine tag fusion proteins. Different truncated forms of Hoxb-8 (19) and Pbx-1 (25), as previously described, were cloned in frame into pET30b(+). In addition, Hoxb-8(125-220), the mutant forms, and Hoxb-8(146-220) have a C-terminal His tag fusion produced by cloning into pET30b(+). Pbx-1(233-320), however, contains a proline tag in the C-terminal domain. The changes do not affect functionality, as assayed by gel mobility shift. All the constructs were checked by DNA sequencing.

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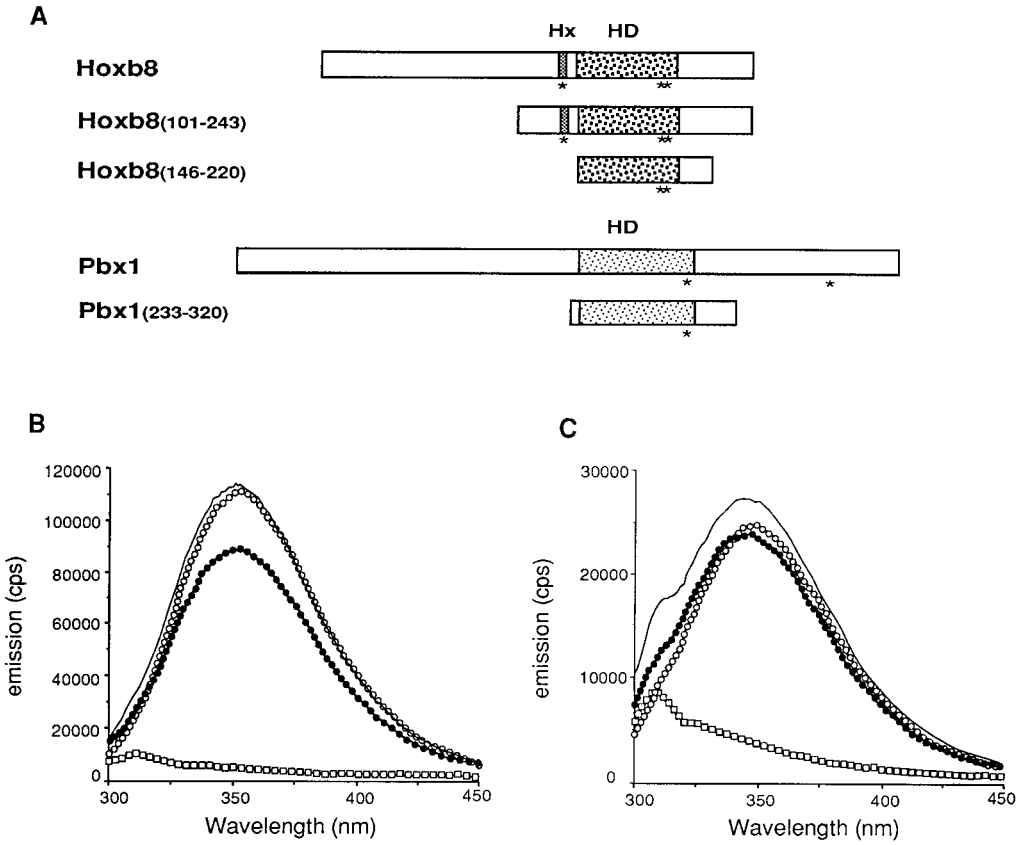

FIG. 1. The homeodomain-containing proteins Hoxb-8 and Pbx-1 interact in the absence of DNA. (A) Schematic representation of the proteins used in this study. HD, homeodomain present in both proteins; Hx, hexapeptide located in Hoxb-8. *, location of tryptophan residue. (B) Fluorescence spectra of Hoxb-8(101-243) (○) (emission λ_{max} = 352 nm), Pbx-1(233-320) (□) (emission λ_{max} = 310 nm), and both proteins together (●). The solid line indicates the theoretical spectrum produced by the combination of the individual spectra. The final concentration of each protein was 3 μM. (C) Fluorescence spectra of Hoxb-8(146-220) (○) (emission λ_{max} = 348 nm), Pbx-1(233-320) (□), and both proteins together (●). The solid line shows the theoretical spectrum produced by the combination of the individual spectra. The final concentration of each protein was 3 μM. The reduction in emission of Hoxb-8(146-220) lacking the hexapeptide compared with the emission of Hoxb-8(101-243) is consistent with the removal of one tryptophan residue located within the hexapeptide.

Protein purification. Bacterial cultures were grown to an optical density at 600 nm of 0.5 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Thirty minutes postinduction, rifampin was added to a final concentration of 25 μg/ml, and the cultures were incubated for an additional 2.5 h at 37°C. Harvesting, cell lysis, and purification by nickel chelate affinity chromatography were performed as described by the manufacturer (Novagen). Purified proteins were incubated with DEAE-Sepharose to remove DNA contamination. The preparations of the various fusion proteins were >90% pure as estimated by Coomassie blue staining of samples run on sodium dodecyl sulfate-polyacrylamide gels. Proteins were dialyzed extensively against 10 mM phosphate buffer, pH 7.8, containing 137 mM NaF and 1 mM EDTA for posterior spectroscopy studies. Samples were centrifuged for removal of precipitates. Protein concentration was determined by measuring the absorbance at 280 nm (6) and with the Bradford assay kit from Bio-Rad. All proteins were tested by electrophoretic mobility shift assays for functionality (8).

Spectroscopy. Fluorescence analysis was performed with a Fluoromax II fluorescence spectrophotometer. Fluorescence spectra were recorded at 25°C with a 1-nm step size and a 0.5-s time constant. Unless otherwise indicated, all the spectra were recorded with a protein and DNA concentration of 3 μM. The sample was incubated for 5 min prior to recording. The excitation wavelength was 280 nm, and emission wavelengths were scanned between 300 and 450 nm. All experiments were duplicated at least once. Quenching studies using potassium iodide were performed as described before (13). CD spectra were generated in a Cary 61 spectropolarimeter as previously described (31) and recorded at 18°C with a 0.5-nm bandwidth, a 0.1-nm step size, and a 0.3-s time constant. The path length was 0.05 cm. The samples were incubated for 5 min prior to recording. All the CD spectra were recorded with a protein and DNA concentration of 10 μM. The mean residue ellipticity, $[\theta]_{MRW}$, is given in 10^3 degrees times square centimeters per decimole. For derivation of spectra in the absence of DNA, we subtracted the free-DNA spectra (not shown) from the protein-DNA spectra (26).

In vitro translation and sucrose gradients. Wild-type and mutant forms of Hoxb-8 (19) were produced with the Promega SP6 TNT rabbit reticulocyte lysate-coupled transcription-translation system according to the manufacturer's protocol. Proteins were labeled with [^{35}S]methionine. Sucrose gradients were performed as previously described (30). Step gradients were formed with 0.25 ml each of 10, 15, 20, 25, and 30% sucrose in 10 mM Tris (pH 7.9), 50 mM NaCl, and 1 mM dithiothreitol buffer. Ten microliters of the in vitro reaction mixture was incubated for 10 min with bovine serum albumin (BSA) (Sigma) or with purified Pbx-1. Samples were layered on top of gradients in centrifuge tubes (Beckman) and centrifuged for 50 min at 54,000 rpm in a TLS-55 rotor at 20°C. Gradients were fractionated and analyzed by polyacrylamide gel electrophoresis. The gels were scanned and analyzed with NIH-Image software.

RESULTS

Hoxb-8 and Pbx-1 interact with high affinity in the absence of DNA. To determine whether Pbx-1 and Hoxb-8 have the ability to form heterodimers in the absence of DNA, Hox-Pbx interactions were analyzed by fluorescence spectroscopy. Fluorescence spectroscopy can be used to detect changes in solvent exposure of tryptophan and tyrosine residues, as well as changes in global stability and compactness. In addition, this technique can be used to study protein-protein interactions at relatively low concentrations (13, 23, 33). In Hoxb-8, one tryptophan residue is located within the hexapeptide and two tryptophan residues are localized in the homeodomain. In Pbx-1, two tryptophan residues are present, one at the C terminus of

the homeodomain and one at the extreme C terminus (Fig. 1A).

Since full-length Pbx-1 and Hoxb-8 are insoluble when synthesized in *E. coli*, the products were produced as truncated forms. Both proteins were fused to a histidine stretch located at the N terminus. The truncated Hoxb-8(101-243) polypeptide lacks a portion of the N-terminal domain but contains the hexapeptide, the homeodomain, and a region C terminal of the homeodomain (Fig. 1A). Pbx-1(233-320) contains the homeodomain and a region C terminal of the homeodomain (Fig. 1A). Both truncated forms have the ability to bind cooperatively to DNA (16). The products were purified to homogeneity by nickel chelate chromatography and analyzed by fluorescence spectroscopy. The fluorescence emission of truncated Hoxb-8 is dominated by the tryptophan residues, as indicated by a maximum at 352 nm (Fig. 1B). The fluorescence emission of truncated Pbx-1 is significantly less and exhibits a maximum at 310 nm (Fig. 1B). Upon denaturation with 6 M guanidinium hydrochloride, the fluorescence emission at 352 nm by Pbx-1 is greatly enhanced, indicating that the emission from the tryptophan residue in native Pbx-1 is quenched (data not shown) and that native Pbx-1 fluorescence is due mainly to its five tyrosine residues. We next analyzed the fluorescence spectra for Hoxb-8 in the presence of Pbx-1. A significant reduction in fluorescence emission can be detected at 352 nm when the proteins are mixed, compared to the theoretical fluorescence spectrum produced by the combination of the individual spectra (Fig. 1B). The quenching of Hoxb-8 fluorescence emission by Pbx-1 indicates an interaction of Pbx and Hox in the absence of DNA (13).

To determine whether the Hoxb-8 homeodomain is sufficient for Pbx-1-induced quenching, a truncated form (residues 146 to 220) containing the homeodomain was purified to homogeneity and analyzed by fluorescence spectroscopy in the absence or presence of Pbx-1 (Fig. 1C). As expected, fluorescence emission of the Hoxb-8 homeodomain exhibits a fluorescence emission which is reduced compared to that of Hoxb-8 containing the hexapeptide, consistent with the deletion of one tryptophan residue located within the hexapeptide (Fig. 1C). Pbx-1 does have the ability to slightly quench fluorescence emitted by the Hoxb-8 homeodomain (Fig. 1C). Quenching of the fluorescence of Hoxb-8 with potassium iodide showed a change in the environment of the tryptophans in the presence of Pbx1 compared to that in Hoxb-8 alone (data not shown). Collectively, these data indicate that Hoxb-8 and Pbx-1, at equimolar concentrations, have the ability to interact in the absence of DNA.

Hoxb-8–Pbx-1 interaction in solution is accompanied by conformational changes. The observations described above indicate that Pbx-1 and Hoxb-8 interact in solution. To analyze whether structural changes are induced upon interaction, we analyzed the secondary structure of truncated Hoxb-8 and Pbx-1 by themselves and together (10 μM), in aqueous solution and at a neutral pH, using CD spectroscopy (Fig. 2). The CD spectra of both proteins display a negative absorption at 208 and 220 nm, indicating significant α-helical content in aqueous solution (Fig. 2A). When Hoxb-8 and Pbx-1 are mixed, a significant change of ellipticity can be detected between 208 and 230 nm, compared to the combined spectra of Hoxb-8 and Pbx-1 by themselves (Fig. 2B). In contrast, when Hoxb-8 lacking the hexapeptide is incubated in the presence of Pbx-1, no significant change in ellipticity is detectable (Fig. 2C). Although aromatic residues may contribute to the loss in ellipticity, the data suggest that the formation of Hoxb-8/Pbx-1 heterodimers in solution is accompanied by a loss of α-helical content. The data also indicate that the hexapeptide is required for the induction of structural changes in the Hox/Pbx heterodimer.

Conserved Hox residues located within the hexapeptide are involved in Hoxb–8-Pbx-1 interactions in the absence of DNA. In previous studies, we and others demonstrated that a conserved hexapeptide located immediately N terminal of the homeodomain is required for cooperative DNA binding (3, 10, 11, 19, 27). As described above, fluorescence emitted by Hoxb-8 lacking the hexapeptide is not significantly quenched by Pbx-1. These data could be interpreted as showing an interaction between the two proteins that is mediated in part by the tryptophan residue present in the Hoxb-8 hexapeptide. To determine whether single substitutions of residues in the hexapeptide affect quenching, we designed three mutations that were previously shown to abolish cooperative DNA binding (19). A conserved phenylalanine was changed to an aspartic acid, a conserved methionine was replaced by a glutamate, and the conserved tryptophan residue was replaced by a tyrosine (Fig. 3A). Fluorescence emission by the F-D mutant is similar to that of the wild-type protein (Fig. 3B and C). Fluorescence emission of the M-E mutant, however, is slightly different from that of the wild-type Hox peptide, possibly due to changes in the polarity surrounding the conserved tryptophan. With the W-Y mutant Hox protein, a dramatic decrease in fluorescence is observed compared to the wild-type protein, confirming that most of the fluorescence is emitted by the tryptophan residue present in the hexapeptide (Fig. 3E). The mutant proteins were incubated with Pbx-1 and analyzed by fluorescence spectroscopy. The fluorescence of the F-D mutant was slightly quenched in the presence of Pbx-1, although significantly less so than that of wild-type Hoxb-8 (Fig. 3B and C). Both other mutants also showed significant but reduced quenching upon incubation with Pbx-1, indicating that single substitutions within the hexapeptide do not abolish Pbx-1–Hoxb-8 interaction in the absence of DNA (Fig. 3B through E).

To further examine Pbx-1–Hoxb-8 interactions in the absence of DNA, we employed sucrose gradient sedimentation. ^{35}S-labeled Hoxb-8 forms were synthesized in vitro, incubated with an excess of either BSA or Pbx-1, and loaded on a sucrose gradient. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. When incubated with BSA, Hoxb-8 wild-type and mutant forms were found essentially in the upper fractions of the gradient (Fig. 3F). In the presence of Pbx-1, wild-type Hoxb-8 migrates to fractions with higher densities upon centrifugation, indicating the formation of the Hoxb-8/Pbx-1 heterodimer. Interestingly, mutant forms of Hoxb-8 incubated with Pbx migrated with intermediate mobility (Fig. 3F). Similar results were obtained with the Hoxb-8 homeodomain (data not shown). Taken together, these data suggest that specific residues within the hexapeptide are involved in Hox-Pbx interaction in solution, although the hexapeptide by itself is not solely responsible for the formation of the Hoxb-8/Pbx-1 hetero-oligomer.

Specific DNA binding of Hoxb-8/Pbx-1 heterodimers induces conformational changes. To determine whether DNA binding induces additional conformational changes in the Hoxb-8/Pbx-1 heterodimer, we examined the fluorescence emission and CD spectra of these proteins in the absence or presence of DNA. We first examined the interaction involving truncated Hoxb-8 and Pbx-1 in the presence of specific DNA. For specific DNA, we used an optimal recognition site for Hox/Pbx heterodimers, previously identified by binding site selection (5′-GAGATGATTTATGACTCTAGTC-3′) (19). Neither Hoxb-8 nor Pbx-1, at a concentration of 3 μM by

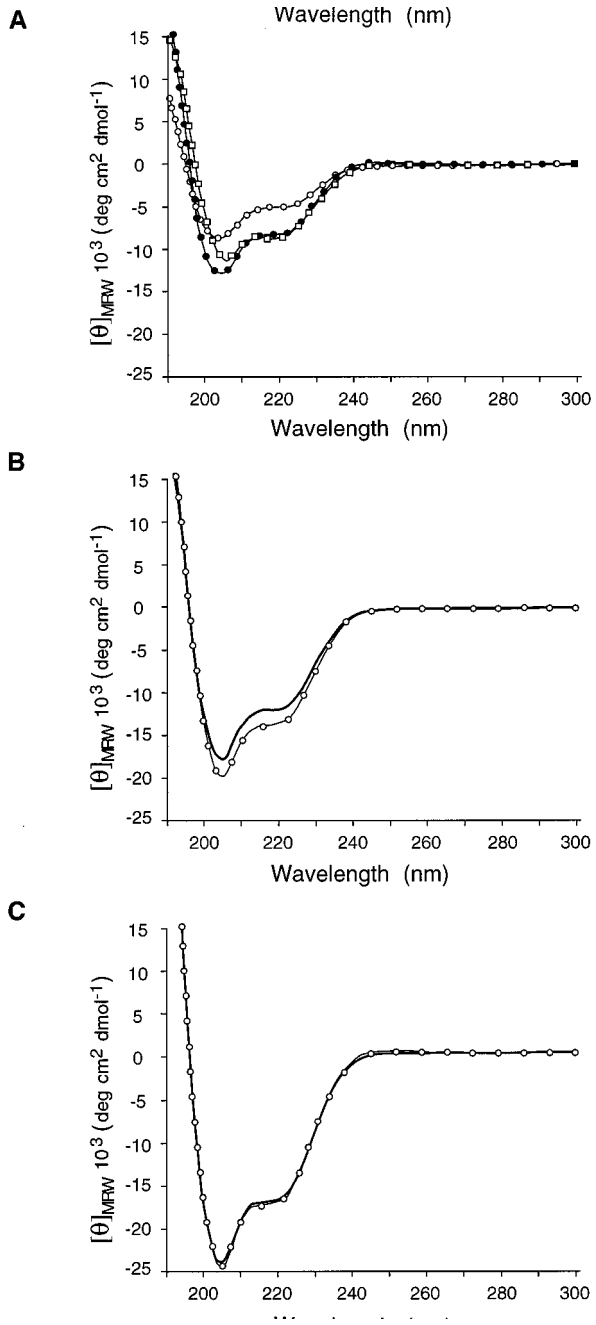

FIG. 2. Hoxb-8–Pbx-1 interaction in the absence of DNA is accompanied by conformational changes. (A) CD spectra of 10 μM Hoxb-8(101-243) (○), Hoxb-8(146-220) (●), and Pbx-1(233-320) (□). (B) Comparison of the theoretical spectrum produced by the combination of the spectra measured for Hoxb-8(101-243) and Pbx-1(233-320) (○) and the spectrum obtained when the proteins are mixed together (-) at a concentration of 10 μM. (C) Comparison of the theoretical spectrum produced by the combination of the individual spectra from Hoxb-8(146-220) and Pbx-1(233-320) (○) and the spectrum obtained when the proteins are mixed together (-) at 10 μM. Each spectrum has been corrected by the subtraction of data for buffer alone.

themselves, shows significant changes in the maximum wavelength of fluorescence emission in the presence of specific DNA (data not shown). In contrast, when both Hoxb-8 and Pbx-1 are present and incubated with specific DNA, a shift in the maximum wavelength (from 352 to 342 nm) of fluorescence emission is induced (Fig. 4A). In the presence of nonspecific DNA (5′-GCGCGCGCGCGCGCGCGCGC-3′), the maximum wavelength of fluorescence emission remains identical (352 nm) (Fig. 4A). This result was also obtained with poly(dA · dT) and poly(dI · dC) (unpublished observations). The same results were also observed with the shorter wild-type form Hoxb-8(125-220) (data not shown). In the presence of both specific and nonspecific DNA, significant quenching of fluorescence is obtained (Fig. 4A).

To assess whether the shift in the maximum wavelength is concentration dependent, constant concentrations of Hoxb-8 and Pbx-1 (3 μM) were mixed with increasing amounts of specific DNA (Fig. 4B). The maximum wavelength of fluorescence emission was monitored for each concentration of specific DNA and was plotted as a function of the concentration of DNA. The data show that the shift in maximum wavelength induced by DNA binding is a saturable response (Fig. 4B). The observation that saturation is reached at a concentration of DNA lower than the concentration of protein indicates that not all of the protein is present in an active form. To rule out the possibility that the shift is due to nonspecific interactions, Hoxb-8/Pbx-1 heterodimers were incubated for 15 min with nonspecific DNA. Subsequently, specific DNA was added and analyzed for fluorescence emission (Fig. 4C). Similarly, Hoxb-8/Pbx-1 heterodimers, preincubated with specific recognition sites, were challenged with nonspecific DNA as described above and analyzed by fluorescence spectroscopy (Fig. 4D). While increasing the concentration of specific DNA causes a shift to UV in the spectrum of the complex preincubated with nonspecific DNA, nonspecific DNA does not alter the maximum wavelength of fluorescence emission of Hoxb-8/Pbx-1 heterodimers (Fig. 4C and D). Taken together, these data indicate that the solvent accessibility of tryptophan residues present in Hoxb-8/Pbx-1 heterodimers changes in the presence of specific recognition sites. Furthermore, fluorescence quenching experiments with potassium iodide indicated that the accessibility of the tryptophans changes in the presence of specific DNA but not in the presence of nonspecific DNA, if we compare to the complex Hox/Pbx alone (data not shown).

To further examine Hox/Pbx binding to DNA, the CD spectra of Hoxb-8/Pbx-1 heterodimers were measured in the presence of 10 μM specific and nonspecific DNA. The CD spectra of Hoxb-8/Pbx-1 obtained in the presence of nonspecific DNA and in the absence of DNA did not show significant differences (Fig. 5A). In contrast, when Hoxb-8/Pbx-1 was incubated in the presence of specific DNA, a slight but significant increase in ellipticity was observed, suggesting a loss of α-helical structure upon binding to specific recognition sites. A change in ellipticity was measured between 230 and 300 nm, suggesting that an aromatic residue(s) is affected upon binding to specific DNA (Fig. 5B), consistent with the shift observed in the fluorescence spectrum. We cannot rule out the possibility that changes in the DNA structure contribute to the changes detected in the CD spectra. In summary, these data indicate that upon binding of DNA, structural changes are induced in the Hoxb-8/Pbx heterodimer.

DISCUSSION

It is well established that Hox and Pbx proteins interact to bind cooperatively to DNA (1, 3, 11, 15, 27, 34, 35). Hox proteins have the ability to bind, albeit weakly, to DNA by themselves. In contrast, Pbx proteins exhibit almost no intrinsic DNA binding activity. However, when incubated with DNA containing a specific recognition site, Hox and Pbx bind cooperatively to DNA with high affinity and specificity (3, 10, 11, 19,

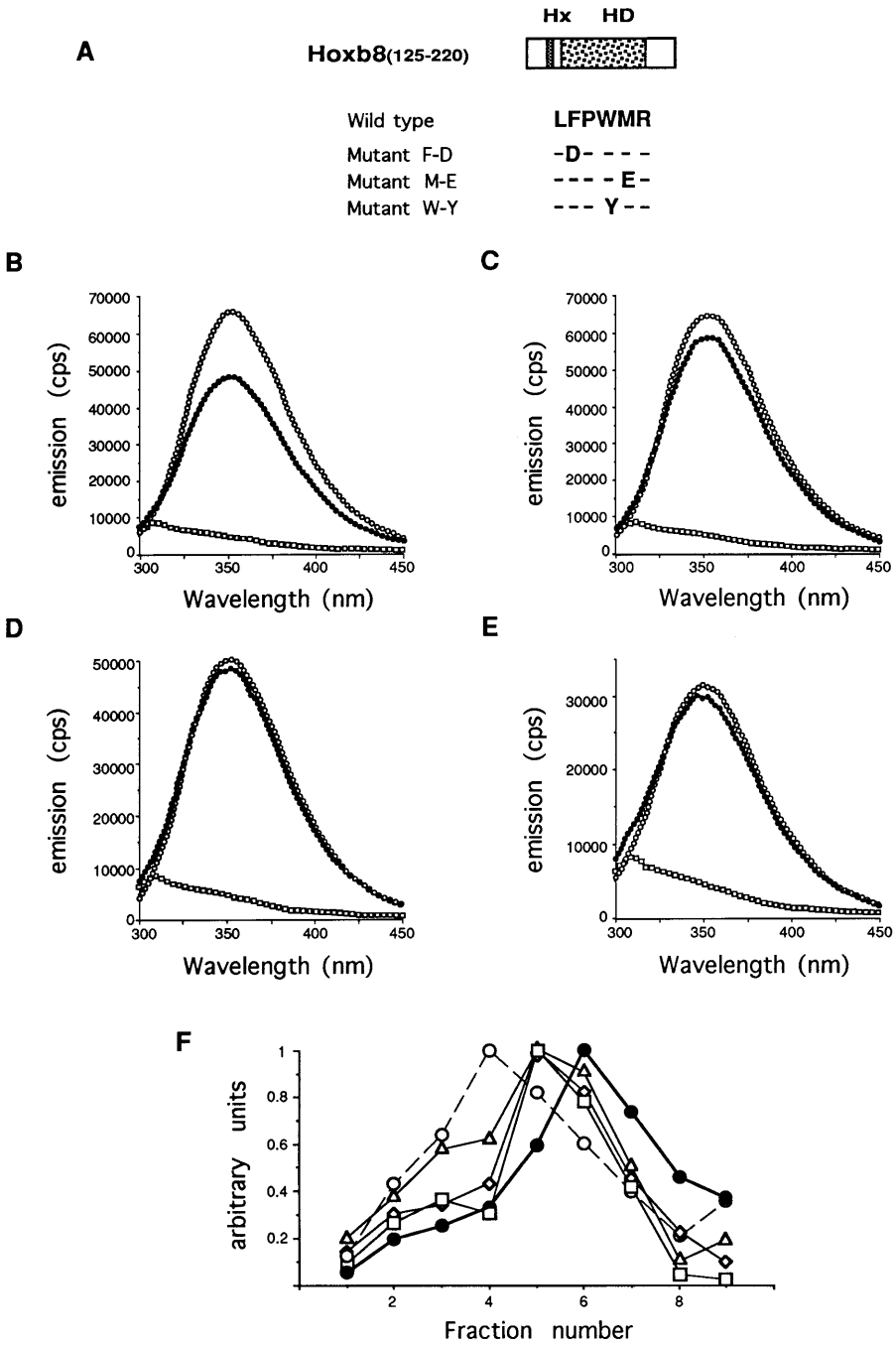

FIG. 3. The Hoxb-8 hexapeptide is essential for interaction with Pbx-1. Fluorescence spectra of Pbx-1(233-320) (□), wild-type and mutant Hoxb-8(125-220) proteins (○), and the Hoxb-8 proteins mixed with Pbx-1(233-320) (●) are shown. (A) Schematic representation of the forms of Hoxb-8 that were incubated with Pbx-1(233-320). The hexapeptide and mutations are indicated. (B) Fluorescence spectra of Hoxb-8(125-220) containing the wild-type hexapeptide (emission λ_{max} = 352 nm) in the presence and absence of Pbx-1. (C) Fluorescence spectra of Hoxb-8 containing a replacement of a phenylalanine by an aspartic acid residue in the hexapeptide (emission λ_{max} = 352 nm) in the presence and absence of Pbx-1. (D) Fluorescence spectra of Hoxb-8 containing a replacement of a methionine by a glutamic acid residue in the hexapeptide (emission λ_{max} = 351 nm) in the presence and absence of Pbx-1. (E) Fluorescence spectra of Hoxb-8 in which the tryptophan residue is replaced by a tyrosine (emission λ_{max} = 350 nm) in the presence and absence of Pbx-1. The concentration of each of the proteins analyzed was 3 μM. (F) Sucrose gradient profile of ^{35}S-labeled Hoxb-8 incubated with BSA (○) or with Pbx-1(233-320) (●). The profiles of the different Hoxb-8 hexapeptide mutants incubated with Pbx-1(233-320) are shown as well: ◇, F-D Hoxb-8; □, M-E Hoxb-8; △, W-Y Hoxb-8. Arbitrary units represent the relative abundance of the proteins. Fractions were collected from the top. Profiles of Hoxb-8 mutant forms in the presence of BSA are very similar to that of the wild-type protein.

27). These data suggested that upon interaction, Hox and Pbx proteins produce a DNA binding activity that is not a simple hybrid of the biochemical properties of each protein alone. We now show that Pbx-1 and Hoxb-8 form heterodimers in the absence of DNA and that these interactions are accompanied by conformational changes. Quenching of fluorescence due to protein-protein interaction has been described before (21, 33). The results obtained in quenching experiments and sucrose

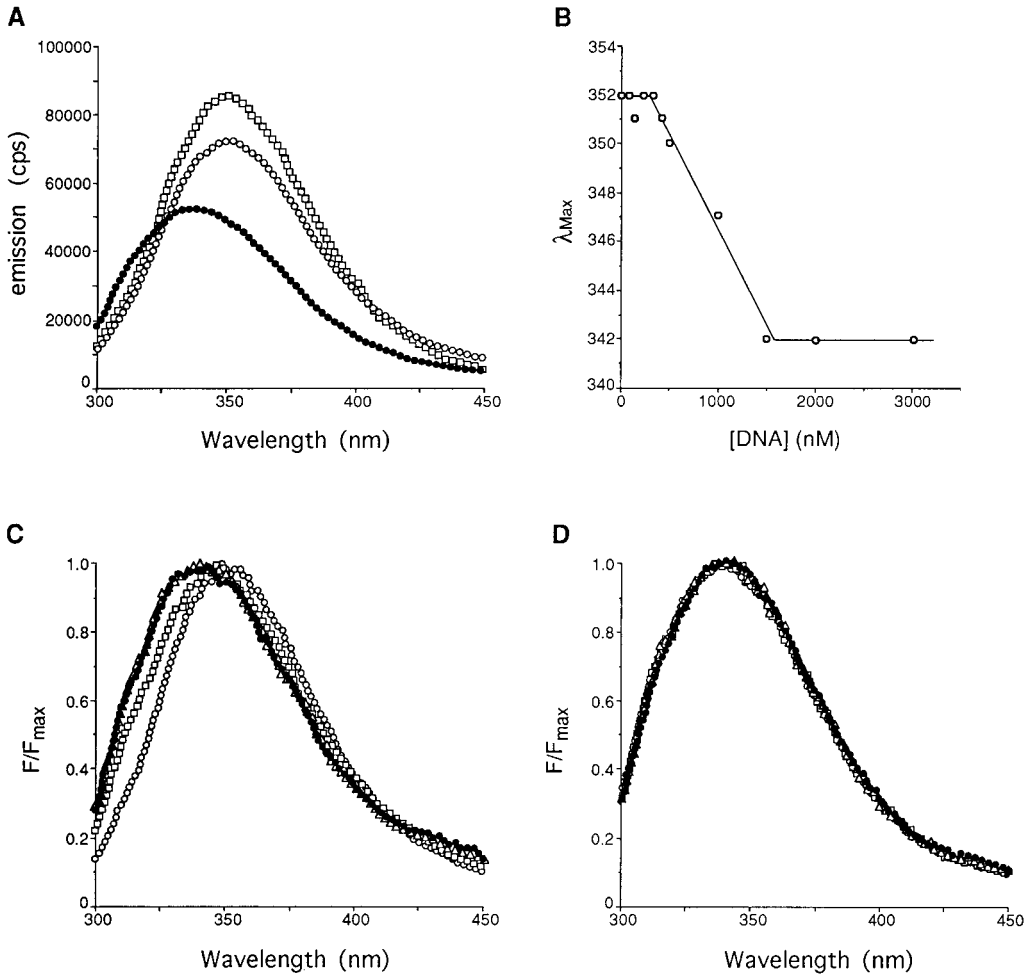

FIG. 4. Specific DNA induces alterations in the fluorescence spectra emitted by a Hoxb-8/Pbx-1 heterodimer. (A) Fluorescence spectra of Hoxb-8(101-243)/Pbx-1(233-320) alone (□) and in the presence of nonspecific (○) and specific (●) DNA. (B) Plot of the maximum wavelength of Hoxb-8/Pbx-1 versus the concentration of specific DNA. (C) Normalized fluorescence spectra of Hoxb-8/Pbx-1 preincubated with nonspecific DNA and challenged with increasing amounts of specific DNA (0 [○], 1 [□], 2 [△], and 3 [●] μM). (D) Normalized fluorescence spectra of Hoxb-8/Pbx-1 preincubated with specific DNA and then challenged with increasing amounts of nonspecific DNA (0 [○], 1 [□], 2 [△], and 3 [●] μM). (C and D) Spectra were normalized to the maximum to avoid the effect of the nonspecific quenching by the DNA. In all experiments the protein and DNA concentration was 3 μM. The specific DNA was the 22-mer 5′-GAGATGATTTATGACTCTAGTC-3′. The nonspecific DNA was the 22-mer 5′-GCGCGCGCGCGCGCGCGCGCGC-3′.

gradients using different mutant forms of Hoxb-8 indicate that Hox and Pbx interact in the absence of DNA in vitro. Moreover, the hexapeptide domain is required to induce structural changes upon interaction of Hoxb-8 and Pbx-1 in solution.

Our observations also showed that conformational changes of the Hox/Pbx heterodimer are induced upon binding to DNA. Conformational changes in DNA binding proteins upon interacting with DNA have been noted before. For example, the CD spectra of two leucine zipper proteins, C/EBP and GCN4, change significantly upon interaction with specific recognition sites (22, 36). It is interesting that upon interaction with DNA, unfolding of a secondary structure in the Hox/Pbx heterodimer is induced. Two precedent examples of unfolding upon interaction with DNA have recently been reported. In *Bam*HI, an α-helix is unfolded upon interaction with DNA (20). In Ets-1, DNA allosterically induces the unfolding of an α-helix that releases an inhibitory domain from the DNA binding domain, and it has been suggested that the unfolded domain may allow interaction of Ets-1 with cofactors upon binding to DNA (26). It is conceivable that structural changes in the Hox/Pbx heterodimer, upon recognition of specific binding sites, similarly allow interaction with other transcriptional regulators. Alternatively, the unfolding may release a cryptic transactivation domain in the Hox/Pbx heterodimers only upon binding to specific DNA. Both mechanisms may further enhance the functional specificity of these highly specific developmental regulators.

Previous studies have suggested that there are many parallels between the mechanisms of cooperative DNA binding involving Pbx/Hox and a1/α2 heterodimers (15, 19, 34). The DNA binding properties, including affinity and specificity, of a1/α2 heterodimers are clearly distinct from those of a1 and α2 alone (7). Similarly, Pbx/Hox heterodimers have biochemical characteristics and DNA binding affinity and specificity that are not simple hybrids of the properties of the two subunits. Interactions between a1 and α2 involve an unstructured peptide, located immediately N terminal of the homeodomain of α2. Hox/Pbx cooperativity also requires the presence of a conserved hexapeptide (3, 11, 19, 27). Previous studies have proposed different roles for the hexapeptide. It has been proposed

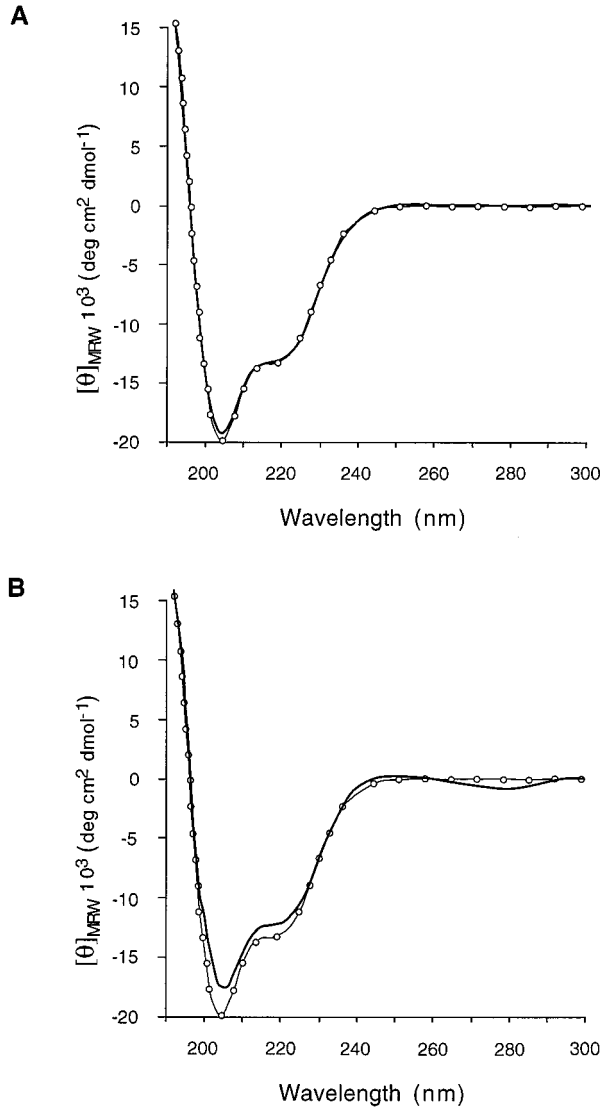

FIG. 5. Hoxb-8/Pbx-1 interaction with specific DNA is accompanied by conformational changes. (A) CD spectra produced by the complex Hoxb-8(101-243)/Pbx-1(233-320) alone (○) and in the presence of nonspecific DNA (▬). (B) CD spectra produced by the complex Hoxb-8(101-243)/Pbx-1(233-320) alone (○) and in the presence of specific DNA (▬). The protein and DNA concentrations were 10 μM for each of the components. The CD spectrum of protein complexed with DNA was corrected by subtraction of the spectrum derived from DNA by itself.

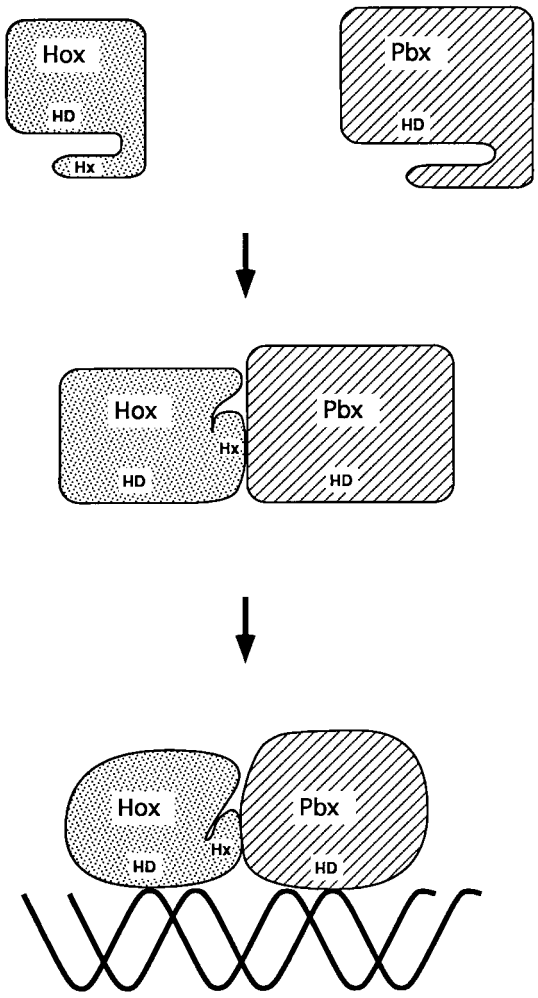

FIG. 6. Model showing interactions involving Hox and Pbx in the absence and presence of DNA. Separately, both proteins are in a conformation exhibiting low intrinsic DNA binding affinity and specificity. In Hoxb-8, the homeodomain (HD) is blocked by an N-terminal hexapeptide (Hx). In Pbx-1, an inhibitory domain located at the N terminus prevents intrinsic DNA binding. The interaction in solution involving the hexapeptide and the Pbx homeodomain induces a conformational change, allowing high-affinity DNA binding. Upon interaction with a specific DNA, further conformational changes are induced.

that the hexapeptide acts to enhance the DNA binding affinity of the heterodimer Hox/Pbx compared to that of either protein alone (19). Second, the hexapeptide has been suggested to function as an inhibitory domain, preventing intrinsic DNA binding activity, which is relieved upon interaction with Pbx-1 (2). Here, we show that the hexapeptide is involved in heterodimerization of Hoxb-8 and Pbx-1 in the absence of DNA. Our data also suggest that the hexapeptide-mediated dimerization of Hox and Pbx proteins can produce structural changes leading to cooperative DNA binding. Based on these findings, we propose a model in which Pbx and Hox proteins interact in solution (Fig. 6). In the absence of a Hox protein, Pbx DNA binding is blocked by the presence of an inhibitory domain, located upstream of the homeodomain (Fig. 6). The presence of such an inhibitory domain has been suggested by studies in which deletion of a domain located at the N terminus of Pbx-1 was shown to allow high-affinity DNA binding by itself (16, 25). The hexapeptide may also function as an inhibitory domain as described above. Upon interaction in the absence of DNA, conformational changes which require the hexapeptide are induced, allowing high affinity DNA binding accompanied by further structural changes (Fig. 6). Such a mechanism is consistent with the findings described here and also with previous observations demonstrating that peptides containing the hexapeptide motif are sufficient to enhance intrinsic Pbx-1 DNA binding activity (11, 24).

ACKNOWLEDGMENTS

We thank Joseph Taulane for the use of the Cary 61 equipment and assistance, Christine Mullen and John Finke for excellent technical assistance, Saskia Neuteboom and Lucy Peltenburg for providing plasmids, Antonio Ferrer-Montiel and Jaime Merino for helpful discussion, and Antonio Ferrer-Montiel for reviewing of the manuscript.

This work was supported by funds from the NIH, the Mallinckrodt Foundation and the Council for Tobacco Research to C.M. M.S. is

supported by a Fulbright fellowship from the Spanish government. P.A.J. is supported by the NIH.

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