The Abd-B-like Hox Homeodomain Proteins Can Be Subdivided by the Ability to Form Complexes with Pbx1a on a Novel DNA Target

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Previous studies showed that the Hox homeodomain proteins from paralog groups 1–8 display cooperative DNA binding with the non-Hox homeodomain protein Pbx, mediated by a canonical YPWM. Although the Abd-B-like Hox proteins in paralogs 9–13 lack this sequence, Hoxb-9 and Hoxa-10 were reported to bind with Pbx1a to DNA. We show that these interactions require a tryptophan 6 amino acids N-terminal to the homeodomain. Binding site selection for Hoxb-9 with Pbx1a yielded ATGATTTACGAC, containing a novel TTAC Hox-bind- phan 6 amino acids N-terminal to the homeodomain. DNA. We show that these interactions require a tryptophan residues N-terminal to the homeodomain. These data extend previous findings that interactions with Pbx define a Hox protein binding code for different DNA sequences across paralog groups 1 through 10. Members of the 11, 12, and 13 paralogs do not cooperatively bind DNA with Pbx1a, despite the presence of tryptophan residues N-terminal to the homeodomain in Hoxd-12 and Hoxd-13. Hoxa-11, Hoxd-12, or Hoxd-13, in the presence of Pbx1a, selected a TTAC Hox site but lacking a Pbx1a site. These data suggest that Abd-B-like Hox proteins bind to a novel TTAC site and can be divided by their cooperative binding to DNA with Pbx1a.

The Drosophila HOM-C genes are master developmental regulatory genes which share a conserved 183-nucleotide homeobox sequence. The 39-vertebrate Hox homeobox genes are arranged in four parallel loci (A, B, C, and D) such that the genes in each cluster can be aligned on the basis of homology within the homeobox to form so-called paralog groups (2). Although the Hox genes from paralogs 1 through 8 can be related to specific HOM-C genes on the basis of sequence homology within the homeobox, paralogs 9 through 13 appear to be equally related to the Drosophila Abd-B gene (3). Thus the homeobox sequences of human, murine, or chicken Hox genes from paralogs 9–13 are equally similar to the Abd-B homeobox. Hox-d cluster genes from paralogs 9 to 13 are expressed in spatially and temporally distinct patterns in the developing limb (4), suggesting that the Abd-B-like gene products play specific developmental regulatory roles.

The Hox homeodomain proteins are thought to function as transcription factors (5). X-ray crystal structure analysis has shown that the most conserved portion of the homeodomain, helix three, forms a portion of the DNA recognition surface (6, 7). This conservation is reflected by the fact that the homeodomains of many Hom-c and Hox proteins appear to bind preferentially to DNA oligomers containing a TAAT core recognition sequence. This observation of a shared DNA consensus binding site has been puzzling, since different homeodomain proteins have distinct biologic functions, as judged by the observed phenotypic differences caused by over-expression or targeted disruption of specific homeobox genes (1).

One mechanism for increasing functional specificity would be the interaction of Hox proteins with protein partners which might provide enhanced DNA specificity or differential binding affinity. We and others have reported that Hox and Hom-c proteins cooperatively bind to DNA with Pbx and Exd, respectively (8–12). These interactions appeared to be mediated by a conserved N-terminal YPWM sequence in Hox proteins from paralogs 1 to 8 (10, 13–16). We demonstrated that the Hox-4 protein requires at least the tryptophan and methionine residues from this tetrapeptide for complex formation with Pbx1a and DNA (13). These studies also revealed that complex formation occurred with representative proteins from paralogs 1 to 8, even though the YPWM motif is variably spaced, occurring 5 to 53 residues N-terminal to the homeodomain. We and others initially reported that Abd-B and the Abd-B-like Hox proteins did not cooperatively bind DNA with Exd or Pbx (8, 9, 11). However, we have recently shown that the lack of cooperative binding originally observed for Hoxa-10 was due to an inappropriate target DNA. Hoxa-10, which lacks a YPWM motif, formed a strong DNA binding complex with Pbx1a, mediated by an N-terminal ANW motif, on an ATGATTTATG1 target (16). In addition, Peltenburg and Murre (17) have recently demonstrated that the engrailed homeodomain protein interacts with Pbx or Exd via tryptophan residues located N-terminal to the homeodomain. The current study was initiated to determine whether members of the other Abd-B-like Hox paralogs (9, 11, 12, and 13), three of which contain tryptophan residues located N-terminal to the homeodomain, are capable of cooperative binding with Pbx1a to an appropriate DNA target, and to determine whether Pbx1a also provides DNA selectivity to these homeodomain proteins.

EXPERIMENTAL PROCEDURES

Protein Expression—Since in previous studies, proteins from the same paralog appeared to have similar DNA binding preferences (13), cDNAs encoding representative full-length Hox proteins from each paralog and Pbx1 were subcloned into either an sp65 vector containing an SP6 promoter (Promega, Madison, WI) engineered to express proteins containing an N-terminal FLAG epitope tag (MDYKDDDDK) (Pbx1a, Hoxb-7, and Hoxa-10); or into a pET vector (Novagen, Madison, Wisconsin).

1 Throughout the text the core sequence within the putative Hox homeodomain protein recognition site is underlined.
containing a T7 promoter, which produces proteins with an N-terminal T7 epitope tag (Pbx1a, Hoxb-8, Hoxb-9, Hoxa-11, Hoxd-12, and Hoxd-13). The identity of each Hox protein was confirmed by Western blot analysis of bacterially expressed proteins using specific polyclonal antisera. For gel shift and DNA target selection assays, proteins were synthesized containing the full-length homeodomain protein fused to the respective epitope tag using the TNT-coupled in vitro transcription-translation system (Promega), in parallel reactions in the presence and absence of [35S]methionine. Electrophoresis of the labeled proteins demonstrated synthesis of the appropriate full-length products (data not shown).

**FIG. 1.** Hoxb-9 and Hoxa-10 cooperate with Pbx1a to bind DNA. A, EMSA were performed with epitope-tagged Hox and Pbx1a proteins produced by in vitro transcription-translation, on a DNA target (ATGATTATGAC) containing the consensus Pbx1a/Hoxa-10 site previously identified by site selection (see text). Each lane contains an equivalent amount of the reticulocyte lysate used to synthesize the proteins to control for the contribution of a variable nonspecific gel shift band (lanes marked L) which migrates just below the bands observed for the Hox-Pbx-DNA complexes. Strong cooperative binding for Hoxb-8, Hoxb-9, and Hoxa-10 with Pbx1a and weak cooperative binding for Hoxb-7 with Pbx1a is observed by comparing the lack of specific gel shifts for Pbx1a alone (lanes 2, 8, 14, and 20) or the weak binding of the Hox proteins alone (lanes 3, 9, 15, and 21) with the strong bands observed in the presence of both Hox and Pbx1a proteins (lanes 4, 10, 16, and 22). Specific antisera to either the epitope tag fused to the Pbx1a protein (lanes 5, 11, 17, and 23) or a different tag fused to the Hox protein (lanes 6, 12, 18, and 24) were used to supershift each of the Hox-Pbx-DNA complexes. Although each of the other three Abd-B-like paralog members, Hoxa-11, Hoxd-12, or Hoxd-13 was capable of binding this oligonucleotide probe (lanes 25, 29, and 33), no cooperative gel shift bands were seen for these proteins in the presence of Pbx1a (lanes 26, 30, and 34). B, EMSA assays were performed with epitope-tagged or non-tagged Hox or Pbx1a proteins to demonstrate the lack of influence of the epitope tag on complex formation. Both Hoxb-9 (lanes 8–10) and Hoxa-10 (lanes 17–19) reacted with epitope-tagged Pbx1a to form complexes which were indistinguishable from those formed between the epitope-tagged Hoxb-9 (lanes 4–7) or Hoxa-10 (lanes 11–14) and tagged Pbx1a. In addition, native Pbx1a formed a complex with epitope-tagged Hoxa-10 which migrated with the same mobility as the complex formed with tagged Pbx1a (compare lanes 12 and 16).

**TABLE I**

| Protein      | N-terminal sequence | Homeodomain |
|--------------|---------------------|-------------|
| Abd-B        | LHEETGQVS           | 12345678    |
| Hoxa-9       | NPAANNLHARS         |            |
| Hoxb-9       | NPSANNLHARS         |            |
| Hoxc-9       | NPVANNLHARS         |            |
| Hoxd-9       | NPEANNLHARS         |            |
| Hoxa-10      | ENAAANNLHARS        |            |
| Hoxd-10      | ENAAANNLHARS        |            |
| Hoxb-10      | TPTSNKLHARS         |            |
| Hoxc-10      | TPTSNKLHARS         |            |
| Hoxd-12      | KGGLPGAAAPGRA       | A           |
| Hoxa-11      | RRPPESSPESSSHEDRAGSSGQR | T.C...T.I |
| Hoxd-13      | QQPHLWCKSSL.PDVVWFDASNYR | G.V...V.L |
| Hoxb-8       | NGPGFRAAFAESPVSQHPFPDGCAFRR | G.I...G.L |
| Hoxd-15      | QSSHFPKSSFPGDVALNGQFPNCTYYRR | G.V...V.L |

* The tryptophan residue thought to be important for potential interaction with Pbx1a is underlined.

WI) containing a T7 promoter, which produces proteins with an N-terminal T7 epitope tag (Pbx1a, Hoxb-8, Hoxb-9, Hoxa-11, Hoxd-12, and Hoxd-13). The identity of each Hox protein was confirmed by Western blot analysis of bacterially expressed proteins using specific polyclonal antisera. For gel shift and DNA target selection assays, proteins were synthesized containing the full-length homeodomain protein fused to the respective epitope tag using the TNT-coupled in vitro transcription-translation system (Promega), in parallel reactions in the presence and absence of [35S]methionine. Electrophoresis of the labeled proteins demonstrated synthesis of the appropriate full-length products (data not
shown). Using autoradiography and densitometry of the $^{35}$S-labeled proteins, and calculating the incorporation of labeled methionine of known specific activity into each protein, we estimated that the relative protein concentrations used were within a 2-fold range. Hoxb-9, Hoxa-10, and Pbx1a were cloned in Bluescript (Stratagene, La Jolla, CA), for synthesis of non-epitope-tagged proteins, in parallel reactions with and without $^{35}$S)methionine to check protein size and to estimate relative concentrations. Each of the epitope-tagged Abd-B-like proteins was also shown to be functional in DNA site selection assays (see "Results").

Human Hoxb-7 and Hoxa-10 were cloned previously (18, 19). A full-length Hoxd-12 cDNA was cloned from 12-day mouse embryo RNA by standard reverse transcriptase-polymerase chain reaction using primers from the published sequence (3), and checked by DNA sequencing. Other full-length cDNA clones were: murine Hoxb-9 (20), murine Hoxa-11 (21), murine Hoxb-8 (22), and chicken Hoxd-13 (23). A full-length cDNA encoding human Pbx1a was kindly by Dr. Michael Cleary (24). The codon encoding the tryptophan residue located 6 amino acids N-terminal to the homeodomain in the Hoxb-9 cDNA was changed to encode glutamine using a Muta-Gene M13 in vitro mutagenesis kit (Bio-Rad).

Electrophoretic Mobility Shift Assays—Complementary oligonucleotides containing consensus binding sites determined by site selection for Hoxb-9 with Pbx1a (CTGCAGATGATTTACGACCGC) and Hoxa-10 with Pbx1a (CTGCAGATGATTTATGACCGC) were synthesized (Operon Technologies, Alameda, CA). Standard conditions used were similar to those previously described (16). Double-stranded, end-labeled DNA (50,000 cpm/binding reaction, 10 nM) was incubated with 2 µl of reticulocyte lysate mixture containing the Hox protein (1 nM) either in the presence of 2 µl of reticulocyte lysate mixture containing Pbx1a (1 nM) or with 2 µl of the lysate control, in 75 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris-HCl (pH 7.5), 6% glycerol, 2 µg of bovine serum albumin, and 2 µg of poly(dI-dC) as nonspecific competitor, in a final reaction volume of 15 µl. Experiments designed to detect complex formation (Figs. 1–3) were performed with a 30-min incubation at 4 °C. Reaction mixtures were run on a 6% polyacrylamide gel to visualize complex formation by retardation of the $^{32}$P-labeled target DNA. In some experiments, polyclonal antisera to the appropriate epitope tag was incubated with aliquots of the reaction mixture for an additional 30 min. The Hox protein was fused to one epitope tag while the Pbx1a molecule was fused to a different epitope tag, such that it was possible to use specific antisera to identify the presence of the Hox protein or the Pbx1a protein in the complex by supershifting the retarded complex band. In experiments designed to measure dissociation rate constants, reaction mixtures were incubated at 30 °C for 30 min and either applied directly to a 6% polyacrylamide gel (zero time sample) or mixed with a 100-fold excess of unlabeled oligonucleotide followed by incubation for fixed times (1–30 min) prior to application to the running polyacrylamide gel.

**Table II**

| Position | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|----------|---|---|---|---|---|---|---|---|---|----|----|----|
| Consensus| A | T | G | A | T | T | A | C | G | A | C | T |
| Frequency| 100| 100| 100| 100| 100| 100| 100| 100| 91| 100| 100| 100|

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**A**

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Consensus| ATCGATGATTTACGACTA | ATCGATGATTTACGAC | ATCGATGATTTACGAC | ATCGATGATTTACGAC | ATCGATGATTTACGAC | ATCGATGATTTACGAC | ATCGATGATTTACGAC |
| Frequency| 100 | 100 | 100 | 100 | 100 | 100 | 100 |

**B**

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Consensus| GCGGGTTTACGAC | GCCAATTTAACGAC | ATGTTTTACGACCTG | CTTCTTTACGACTTAA | ATTAACTTTTACGATC | TACATTTATGACCCAT | GACCGGATTAACGA |
| Frequency| 90 | 100 | 60/40 | 100 | 80 | 90 | 90 |

**FIG. 2.** A tryptophan residue is required for cooperative DNA binding by Hoxb-9 and Pbx1a. Site-directed mutagenesis was used to change the tryptophan six residues N-terminal to the homeodomain of Hoxb-9 to glutamine. The mutant protein still possessed the capacity to bind to DNA in the absence of Pbx1a (compare lanes 2 and 3). However, the mutant protein was incapable of cooperative interactions with Pbx1a and DNA (compare lanes 4 and 5).
amide gel. Gel electrophoresis was performed in 0.25 × TBE buffer as described previously. For each gel shift reaction, a control containing the reticulocyte lysate and appropriate viral polymerase was used to detect possible DNA binding by endogenous factors. Lysate controls showed variable intensity gel shift bands with the DNA target. These bands varied with both the lysate batch and the batch of poly(dI-dC) used as non-competitive inhibitor.

Calculation of Complex Half-lives—Electrophoretic mobility shift assay gels were autoradiographed for densitometric quantitation of complex band using a MacIntosh 8500 Power PC computer and the NIH-Image software program. Each gel was autoradiographed for various times to ensure that the densities measured were within the linear range of the scanner and software program. A dissociation rate was calculated for each Hox-Pbx1a-DNA complex from the slope of the regression line generated by plotting the log of the complex band intensities versus time (Fig. 4C). For each dissociation experiment, the correlation coefficient for the line was >0.96. For each complex, the half-life was calculated using the equation, $T_{1/2} = \frac{-\ln(0.5)}{k_d}$.

DNA Site Selection Protocol—Site selection was performed following the basic protocol described by Blackwell (25). The T7-epitope tag Hox protein of interest and native Pbx1a were synthesized in vitro and incubated at 4 °C for 30 min with a 59-mer containing a random 18-mer core flanked by arms which contained cloning sites (GCTCGAATTCAAGCTTCTN18CATGGATCCTGCAGAATTTCAGT). Bound DNA was immunoprecipitated using an antiserum to the T7 tag sequence. After six selection cycles, the amplified DNA was subcloned into M13mp19 (New England BioLabs, Beverly MA) and sequenced using the dideoxy method with 35S-labeled adenosine triphosphate. Consensus sequences were determined by visual alignment of sequences from unique clones. The number of independent clones used to define each consensus are given in parentheses: Hoxb-9 plus Pbx1a (11); Hoxa-11 alone (10); Hoxa-11 (34); Hoxd-12 (17); and Hoxd-13 (18).

RESULTS

Hoxb-9 and Hoxa-10 but Not Hoxa-11, Hoxd-12, or Hoxd-13 Cooperatively Bind with Pbx1a to a TTAT-containing Target—Previous studies presented conflicting data concerning the capability of Abd-B-like Hox proteins to cooperatively bind DNA with Pbx1a. We initially used an oligonucleotide (ATGATT-TATGA), which was identified in a DNA site selection protocol using Hoxa-10 with Pbx1a (16), to examine the ability of representative Abd-B-like Hox proteins to cooperatively bind to DNA with Pbx1a. The first five nucleotides, ATGAT, comprise the Pbx consensus binding site (26–28). As described below, the Hox site overlaps the Pbx site and consists of the TTATGTA
sequence. Electrophoretic mobility shift assays (EMSA)\(^2\) were used to detect complex formation between the labeled oligonucleotide and Hox and Pbx1a proteins. Since we have previously observed that full-length Hox proteins behave differently from the truncated homeodomain fragments used in many experiments (13), all of these studies have been performed using full-length proteins. In most experiments the Hox and Pbx1a proteins were synthesized as fusion molecules containing short N-terminal T7 or FLAG epitope sequences to permit identification using epitope-specific antisera.

Hoxb-9 and Hoxa-10 formed strong cooperative complexes with Pbx1a on this target DNA, under conditions in which the Hox proteins alone bound very weakly and Pbx1a binding alone was undetectable (Fig. 1A). For comparison, the neighboring paralog proteins, Hoxb-8 and Hoxb-7, formed weak complexes with Pbx1a and this oligonucleotide. Since uncharacterized DNA-binding proteins present in the reticulocyte produced a variable gel shift band in the same position as some of the specific complex bands, supershift experiments using antibodies to the epitope tags were used to show that retarded bands ascribed to the Hox-Pbx1a-DNA complex contained both Pbx1a and Hox protein. Although each of the other three Abd-B-like paralog members, Hoxa-11, Hoxd-12, or Hoxd-13, were capable of binding this oligonucleotide probe, none was able to form a detectable cooperative complex with Pbx1a and this DNA target.

To demonstrate that the epitope tag does not alter complex formation, DNA binding reactions were performed using one tagged protein with the other protein being synthesized without an epitope tag. Both native Hoxb-9 and Hoxa-10 behaved similarly to the epitope-tagged proteins in DNA binding reactions with epitope-tagged Pbx1a (Fig. 1B). Native Pbx1a formed a complex with epitope-tagged Hoxa-10 which migrated with the same mobility as the complex formed with tagged Pbx1a.

A Conserved Tryptophan in Hoxb-9 Confers Complex Forming Capability—Although we reported that the interaction of Hoxa-10 with Pbx1a is mediated by a conserved ANW sequence located N-terminal to the homeodomain in Hoxb-9 and Hoxa-10 (Table I) (16), the importance of individual amino acids for complex formation was not defined. Since we and others had previously shown that Pbx1a interaction with other Hox proteins required a tryptophan residue, we focused our studies on the invariant tryptophan within this amino acid triplet. A mutant Hoxb-9 protein containing a glutamine in place of this tryptophan was unable to form a complex with Pbx1a on the Hoxa-10 DNA target, under conditions in which the wild type Hoxb-9 formed a very strong complex (Fig. 2, compare lanes 4 and 5). This mutation did not prevent DNA binding since the mutant protein was still capable of shifting DNA in the absence of Pbx1a (Fig. 2, compare lanes 2 and 3).

Hoxb-9 Selectively Binds to an Oligonucleotide Containing a TTAC Core Site—Since Hoxa-10 complex formation with Pbx1a is highly dependent on the DNA target sequence (16), we performed DNA site selection experiments to determine the pre-

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\(^2\) The abbreviation used is: EMSA, electrophoretic mobility shift assays.

| Position | Consensus | Frequency |
|----------|-----------|-----------|
|          | G G G G C C A G G T T T T A C G T A C C | 35 35 58 59 97 100 65/35 97 97 79 75 72 |

**TABLE III**

Consensus binding site for Hoxa-11 in the presence of PBX1a

Underlined nucleotides in selected sequences are from the univarient linker regions of the target oligonucleotide and were not scored in frequency calculations.
ferred binding sites of each of the other Abd-B-like Hox proteins in the presence of Pbx1α (see also below). We initially used an epitope-tagged Hoxb-9 in the presence of Pbx1α to select a very highly conserved 12-nucleotide sequence: AT-GATTTAATGAC (Table II, part A). This sequence was identical to that previously selected for Hoxa-10 with the important exception of the occurrence of a C in place of a T at position 9 in the putative Hox homeodomain core recognition site (underlined region), as well as being extended by one extra 3′-nucleotide (see Table IV). The first five nucleotides of this sequence (ATGAT) correspond to that obtained previously for Pbx1α (see Table IV). The first five nucleotides of this sequence (ATGAT) correspond to that obtained previously for Pbx1α (see Table IV). The first five nucleotides of this sequence (ATGAT) correspond to that obtained previously for Pbx1α (see Table IV). The first five nucleotides of this sequence (ATGAT) correspond to that obtained previously for Pbx1α (see Table IV). The first five nucleotides of this sequence (ATGAT) correspond to that obtained previously for Pbx1α (see Table IV). The first five nucleotides of this sequence (ATGAT) correspond to that obtained previously for Pbx1α (see Table IV). The first five nucleotides of this sequence (ATGAT) correspond to that obtained previously for Pbx1α (see Table IV). The high specificity obtained in the presence of Pbx1α-binding site was relaxed to some degree for Hoxb-9 alone. In contrast, Hoxb-7 and Hoxb-8 appeared to bind to the TAAT containing sequence somewhat more strongly, reflecting an apparent greater preference for this core recognition sequence by the Hox proteins in the middle of the locus (see below).

**Hox Proteins from Paralog Groups 11, 12, and 13 do Not Cooperatively Bind DNA with Pbx1α—**Proteins representing the three paralog groups located at the extreme 5′ end of the loci, Hoxa-11, Hoxd-12, and Hoxd-13, did not form detectable complexes with Pbx1α on DNA targets containing either the core consensus sequence for the Hoxb-9 protein (TTAC), the core consensus for Hoxa-10 (TTAT), or an oligonucleotide containing a TAAT core sequence (Figs. 1 and 3). The fact that each of these proteins bound DNA in the absence of Pbx1α suggested that the lack of cooperativity was not due to denatured proteins. Since Hoxa-11 does not contain a tryptophan residue within the 50 amino acids N-terminal to the homeodomain (21), it seemed likely that this protein might not cooperatively bind DNA with Pbx1α. However, Hox proteins from both the 12 and 13 paralogs contain tryptophan residues which are 9 and 21 residues N-terminal to the homeodomain, respectively (Table I), suggesting that given a different DNA binding target they might form complexes with Pbx1α. In this regard, it should be noted that restrictions on the distance between the tryptophan residue which mediates cooperative binding and the homeodomain appear to be relatively modest for the Hox proteins since linker arms from 5 to 53 amino acids are tolerated (13).

To search for putative DNA targets on which Hoxa-11, Hoxd-12, and Hoxd-13 might form cooperative complexes with Pbx1α, each protein was used for site selection in the presence of Pbx1α. After six rounds of selection, there was a clear consensus Hoxa-11 binding site consisting of TTTATACGGC (Table III and IV), but there was no apparent binding site for Pbx1α. In a similar manner, site selection experiments using Hoxd-12 and Hoxd-13 yielded clear consensus sequences containing Hox but not Pbx1α-binding sites (Table IV). Thus there do not appear to be unique DNA sequences on which these Hox proteins will cooperatively bind with Pbx1α. These data also confirmed that each of these Abd-B-like proteins was capable of binding DNA. Taken together with the lack of gel shifting seen
with an oligonucleotide target which is extremely similar to that selected by the Hoxa-11, Hoxd-12, and Hoxd-13 proteins (Fig. 3A), these data demonstrate that these three Abd-B-like Hox proteins do not cooperatively bind DNA with Pbx1a, under conditions where members of the other Hox paralogs all cooperatively interact with Pbx1a to bind DNA.

The Abd-B-like Hox Proteins Favor a TTAC(C/T) Core Sequence—It is of interest that both Hoxa-11 and Hoxd-12 showed a DNA binding consensus of TTT/AACGAC, which was identical to that found for Hoxb-9 in the absence of Pbx1a (Table IV). The core region (TTAC) within the consensus sequence for Hoxd-13 was the same as that found in the other three consensus sequences which we determined. However, the Hoxd-13 consensus was unique in having a specificity for G at position 12, while the other Abd-B-like proteins appeared to prefer a C in this position. Hoxd-13 also had a higher selectivity for a T at position 4 than was observed for the other proteins. These results differ from those previously obtained for the Hoxa-10 and Abd-B proteins, both of which appear to prefer a TTAT core sequence (16, 29). However, as shown in Fig. 3A, Hoxa-10 formed a strong complex with the sequence containing a TTAC core (see also below). In addition, the site selection protocol which identified a TTAT core recognition site for Hoxa-10 with Pbx1a (16), also yielded a significant number of sequences containing a TTAC core site.

To further investigate the DNA-binding site selectivity of the Hoxb-9 and Hoxa-10 proteins in the presence of Pbx1a, we performed dissociation rate determinations using these proteins, along with Hoxb-7 and Hoxb-8 for comparison to the neighboring non-Abd-B-like proteins in the Hox loci. In these studies, complexes were formed at 30 °C between each of the respective epitope-tagged Hox and Pbx1a proteins with the DNA targets. Following removal of a time 0 sample, a 100-fold excess of cold-competitor DNA was added to the pre-formed complex, and at specified times aliquots were loaded onto the running gel. Fig. 4 shows a representative experiment for the dissociation of Hoxb-9 and Hoxa-10 from complexes with Pbx1a on a probe consisting of either the Hoxa-10 consensus site containing a TTAT core or the Hoxb-9 site containing a TTAC core. As seen in Table V, the dissociation rates for complexes formed by either Hoxb-9 or Hoxa-10 with Pbx1a on an oligonucleotide containing a TTAC core were lower than those observed for the dissociation of these proteins from an oligonucleotide containing a TTAT core site. In contrast, Hoxb-7 and Hoxb-8 exhibited higher dissociation rates for the oligonucleotide with the TTAC core sequence. The differences in stability of complexes formed between either Hoxb-9 or Hoxa-10 with Pbx1a on both targets were relatively modest, reinforcing the gel shift results which suggested that Hoxb-9 and Hoxa-10 form strong cooperative complexes with Pbx1a on both TTAC and TTAT containing targets. Table V also shows that a DNA target containing a TTAT core showed the highest dissociation rate with all four proteins. Assuming that the association rate constants are the same, the observed high dissociation rates are in agreement with the site selection and gel shift results showing that, in the presence of Pbx1a, the conventional TAAT core sequence was not preferred by either the Abd-B-like proteins or the neighboring proteins from the 5′ side of the Hox-b locus.

DISCUSSION

We show that the Abd-B-like Hox homeodomain proteins can be divided into those from the 9 and 10 paralog groups which cooperatively interact with Pbx1a to bind DNA and the 11 to 13 paralog proteins which do not bind cooperatively to DNA with Pbx1a. A number of studies have shown that an N-terminal tryptophan appears to mediate the interaction of the Hox homeodomain proteins with the Pbx/Ekd homeodomain proteins (13–17). However, the structural context for the tryptophan residue within the Hox proteins is not clear. In Hoxb-1 through Hoxb-8, the tryptophan resides within a relatively conserved, but variably spaced hexapeptide motif (30). In the 9 and 10 paralog proteins the tryptophan is located 6 residues N-terminal to the homeodomain within a conserved ANW. Further...

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3 C.-P. Chang, personal communication.

4 We have preliminary data showing that other members of the 9 and 10 paralog groups, Hoxa-9, Hoxd-9, and Hoxd-10, also bind cooperatively with Pbx1a to DNA targets.
sequence, appears to be unique to the vertebrate Abd-B-like Hox proteins, since neither the Drosophila Abd-B protein (29) or other Hox homeodomain proteins (31) have been shown to bind to DNA targets containing a TTAC core. Current x-ray crystallographic studies do not provide an explanation for the preference for a C at position 9 of the recognition sequence (6, 7).

**Pbx Confers Selective DNA Binding to Hox Proteins Across the Loci**—It has been difficult to explain Hox protein function given the apparent lack of DNA binding specificity across the Hox loci. Chang et al. (16) initially proposed that cooperative binding with Pbx conferred a differential DNA binding selectivity to Hox proteins across paralog groups 1 to 10, based on the nucleotide at position 7 of the consensus sequence ATGATTGAT (16). As shown in Fig. 5, these studies demonstrated that, in the presence of Pbx, Hox proteins from paralogs 1–5 preferentially bound oligonucleotides containing a TGAT core sequence, while proteins from paralogs 6–10 appeared to prefer a TTAT core. Proteins from the middle of the locus (paralog groups 3 to 8) also tolerated a TTAT core sequence, although this sequence was not observed during site selection using Hoxb-4 or Hoxb-6 with Pbx. We have now extended these observations to show that the proteins from paralog groups 9 and 10 bind a TTAT core recognition sequence in the presence of Pbx1a. We propose that the Abd-B-like Hox proteins can be subdivided by their ability to bind to DNA cooperatively with Pbx, such that proteins from groups 9 and 10 will form much stronger complexes than the proteins from groups 11, 12, and 13. In addition, the 9 and 10 paralog proteins can, through interactions with Pbx1a, exhibit increased selectivity for DNA targets due to the longer recognition site bound by the combination of Hox and Pbx proteins. Although the studies of Chang et al. (16) focused on the role of the second nucleotide in the core sequence, selection preferences were also noted for individual Hox proteins in positions 10, 11, and 12. The fact that Hoxd-13 selects a G at position 13, while Hoxa-11 and Hoxd-12 select a C, suggests that this difference may provide in vivo binding selectivity between proteins from these paralogs.

The scheme shown in Fig. 5 provides a possible rationale for DNA binding specificity for Hox proteins across the vertebrate loci. It is encouraging that one of the first observations to show that the proteins from paralog groups 9 and 10 bind a TTAT core recognition sequence are insufficient to specify the Pbx1a-mediated Hox homedomain protein binding to DNA. Thus we have recently shown that a DNA target containing a TGAT core, which was preferred over other DNA targets by Hoxb-1 through Hoxb-3 in gel shift assays with Pbx1a (16), actually exhibited a 100-fold lower dissociation rate for Hoxb-6 and Hoxb-5 compared with Hoxb-2 or Hoxb-3 (13). While recogniz-
ing that dissociation rate data provide only one component of the equilibrium binding constant, it seems likely that the in vitro experimental systems employed in these and other studies provide only partial insights as to the in vivo binding specificity of these transcription factors. A greater understanding of the physiological interactions of Hox and Pbx homeodomain proteins with DNA targets will require identification of the natural regulatory targets of these putative transcription factors.

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