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Regulation of Hox target genes by a DNA bound Homothorax/Hox/Extradenticle complex

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SUMMARY

To regulate their target genes, the Hox proteins of *Drosophila* often bind to DNA as heterodimers with the homeodomain protein Extradenticle (EXD). For EXD to bind DNA, it must be in the nucleus, and its nuclear localization requires a third homeodomain protein, Homothorax (HTH). Here we show that a conserved N-terminal domain of HTH directly binds to EXD in vitro, and is sufficient to induce the nuclear localization of EXD in vivo. However, mutating a key DNA binding residue in the HTH homeodomain abolishes many of its in vivo functions. HTH binds to DNA as part of a HTH/Hox/EXD trimeric complex, and we show that this complex is essential for the activation of a natural Hox target enhancer. Using a dominant negative form of HTH we provide evidence that similar complexes are important for several Hox- and *exd*-mediated functions in vivo. These data suggest that Hox proteins often function as part of a multiprotein complex, composed of HTH, Hox, and EXD proteins, bound to DNA.

Key words: *homothorax*, MEIS, Hox, *extradenticle*, *pbx*, *Drosophila melanogaster*

INTRODUCTION

In developmental biology, the term selector gene has been used to describe genes that are able to trigger an entire developmental pathway (Garcia-Bellido, 1975). For example, in *Drosophila melanogaster* the genes *eyeless* and *twin of eyeless* are sufficient, at least in many places in the body, to trigger eye development (Halder et al., 1995; Czerny et al., 1999). Similarly, the genes *Distal-less* and *vestigial* have been suggested to be selector genes for ventral (i.e. legs and antenna) and dorsal (i.e. wings and halteres) appendages, respectively (Cohen et al., 1989; Williams et al., 1991; Kim et al., 1996; Gorfinkiel et al., 1997). The Hox genes are also examples of selector genes and, like many selector genes, they encode transcription factors (Lewis, 1978; McGinnis and Krumlauf, 1992). However, a unique aspect of the Hox genes is that they encode a family of related proteins that execute parallel pathways at different positions along the anterior-posterior axis. Studies in other species, both invertebrate and vertebrate, suggest that Hox genes perform analogous functions in many animals, and that changes in gene regulation by Hox proteins have been important during animal evolution (Lewis, 1978; Kenyon, 1994; Warren et al., 1994; Burke et al., 1995; Weatherbee et al., 1998).

The central role that the Hox genes play in animal development is difficult to reconcile with their molecular biology. One puzzling aspect of these genes is that, unlike many other selector genes, outside of their homeodomains the Hox proteins have been poorly conserved between animal species. Yet, despite this limited sequence conservation, Hox functions have been conserved. For example, when assayed in *Drosophila*, vertebrate Hox proteins elaborate structures that are very similar to those generated by their fly orthologs (Malicki et al., 1990; McGinnis et al., 1990; Zhao et al., 1993; Bachiller et al., 1994). Another puzzling aspect of the Hox proteins is that, in vitro, they bind to DNA with overlapping and low sequence specificities (Ekker et al., 1994; Mann, 1995). The loose DNA recognition properties of the Hox proteins is difficult to understand given the high degree of functional specificity they show in vivo.

To some extent, these paradoxes have been partially resolved by the finding that Hox proteins often bind to DNA as a heterodimer with another homeodomain protein, called Extradenticle (EXD) in flies and PBX in vertebrates (Mann and Chan, 1996). Unlike Hox proteins, EXD and PBX are highly homologous throughout most of their sequences (Rauskolb et al., 1993). EXD and PBX proteins cooperatively bind to DNA with Hox proteins as a tightly spaced heterodimer to a ten basepair sequence, TGATNNATNN (Lu et al., 1995; Phelan et al., 1995; Chan and Mann, 1996; Mann and Chan, 1996; Passner et al., 1999; Piper et al., 1999). There are several examples of Hox target enhancers from both flies and mice that are regulated during development by Hox/EXD or Hox/PBX heterodimers bound to such sequences (Pöpperl et al., 1995; Chan et al., 1997; Grieder et al., 1997; Maconochie et al., 1997; Ryoo and Mann, 1999). When EXD binds together with Hox

proteins, it apparently increases their DNA binding specificity and affinity, and also modifies their transcriptional regulatory properties in vivo (Mann and Chan, 1996; Chan et al., 1997; Grieder et al., 1997; Pinsonneault et al., 1997; Li and McGinnis, 1999; Li et al., 1999; Ryoo and Mann, 1999).

Although Hox proteins gain specificity when binding as heterodimers with EXD, the properties of Hox/EXD heterodimers cannot, on their own, completely account for Hox specificity. For example, although different Hox/EXD binding sites prefer to bind different Hox/EXD heterodimers, the in vitro specificity exhibited by these heterodimers for particular binding sites is not absolute (Shen et al., 1996; Chan et al., 1997; Phelan and Featherstone, 1997). One way to reconcile these disparities is to suggest that there are additional factors that contribute to Hox function, both in vitro and in vivo. Such factors might be expected to interact with and perhaps bind to DNA with Hox/EXD heterodimers.

A good candidate for a factor that might bind to DNA with Hox/EXD heterodimers is the product of the gene *homothorax* (*hth*). *hth* was previously shown to be indirectly required for Hox function because, in *hth* mutant embryos, EXD is found exclusively in the cytoplasm, and therefore cannot act as a Hox cofactor (Rieckhof et al., 1997; Kurant et al., 1998). *hth* encodes a homeodomain protein that has very similar relatives in vertebrates called the MEIS and PREP proteins (Moskow et al., 1995; Nakamura et al., 1996; Steelman et al., 1997; Berthelsen et al., 1998b). Like EXD, HTH is not only conserved in its homeodomain, but also within a large N-terminal domain called HM (Rieckhof et al., 1997; Pai et al., 1998). HTH and EXD proteins directly interact with each other, and the nuclear localization of EXD depends on this protein-protein interaction (Rieckhof et al., 1997; Abu-Shaar et al., 1999; Berthelsen et al., 1999).

The protein-protein interaction between EXD and HTH suggests that these two homeodomain proteins might remain bound to each other in nuclei. In addition, the presence of a highly conserved homeodomain in HTH suggests that HTH is a DNA binding protein. These observations raise the possibility that, for many Hox target genes, Hox proteins bind to DNA as a HTH/Hox/EXD trimeric complex. Although such complexes could be highly relevant to Hox function, HTH's nuclear functions, if any, have been difficult to define because removing *hth* activity also results in the loss of nuclear EXD. In this work, we separated the DNA binding and nuclear import functions of HTH. We find that, in addition to importing EXD into nuclei, HTH is part of an essential DNA-bound HTH/Hox/EXD trimeric complex. Our data suggest that such complexes are required for several Hox- and *exd*-mediated functions in vivo. The ability of Hox proteins to assemble sequence-specific protein complexes may account for how such poorly conserved transcription factors play such central roles in animal development.

MATERIALS AND METHODS

P element constructs and fly stocks

All fly stocks had the w^{1118} mutation to monitor the presence of the mini-white gene in the P elements (Pirrotta, 1988). P-element transformation was carried out using standard procedures. All HTH constructs for analysis in flies contained HTH sequences fused to GFP or MYC and cloned into the pUAST vector (Brand and Perrimon, 1993). GFP-HTH includes amino acids 36 to its C terminus (Casares and Mann, 1998). MYC-HTH includes the complete HTH protein fused to the myc epitope in p131 (Abu-Shaar et al., 1999). GFP-HTH^{51A} has an engineered mutation of Asn51 in the HTH homeodomain into Ala but otherwise is identical to GFP-HTH. GFP-HM includes amino acids 36 to 245; GFP-HM^{A}, amino acids 36 to 125; GFP HM^{B}, amino acids 150 to 245; GFP-HD, amino acids 318 to the C terminus. NLS-EXD has the entire EXD ORF fused to a Myc epitope and the NLS of the SV40 large T antigen (Abu-Shaar et al., 1999). The *lab48/95-lacZ* and *lab550-lacZ* transgenes and expression patterns have been described (Grieder et al., 1997). The *hth* allele used was hth^{C1} (Rieckhof et al., 1997). For all *lacZ* reporter genes, the levels of expression were found to be similar in multiple lines with different P element insertions. The sequences of lab48/95, lab48/95^{exd}, lab48/95^{lab}, and lab48/95^{hth} are shown in Fig. 4A. lab48/$95^{72,73}$ has two point mutations adjacent to the HTH binding site (CCGACTGTCA → CtaACTGTCA) and has wild-type enhancer activity (data not shown). To observe an effect on lab48/95 activity, both maternal and zygotic *exd* functions must be removed as previously described (Chan et al., 1994).

For ectopic expression experiments, *ptc:Gal4*, *Dll:Gal4*, *dpp:Gal4*, *pnr:Gal4*, and *48Y:Gal4* driver lines were used (Brand and Perrimon, 1993; Calleja et al., 1996; Martin-Bermudo et al., 1997). All animals were aged at 25°C with the following exceptions: embryos expressing various HTH constructs with the *48Y:Gal4* driver were collected at 25°C overnight and aged at 29°C for 6 hours before fixation. For expression in the endoderm, we compared lines that expressed similar levels of protein as assayed by GFP staining (not shown). At 29°C, GFP-HM-mediated repression of *lab48/95-lacZ* and *lab550-lacZ* was observed in virtually 100% of the embryos of the correct genotype. Expression of NLS-EXD in imaginal discs was carried out at 29°C. For the analysis of antennae, *Dll:Gal4*; UAS:GFP-HM or UAS:GFP-HTH^{51A} flies were raised in 29°C. A partial transformation of antenna to leg was observed in >90% of the flies of the correct genotype.

Immunostains

Embryos and imaginal discs were stained using standard procedures and previously described antibodies (Grieder et al., 1997; Rieckhof et al., 1997; Abu-Shaar and Mann, 1998; Abu-Shaar et al., 1999; Ryoo and Mann, 1999).

GST pull-downs

GST-HTH was made by subcloning the full length HTH coding sequence into pGEX-5x-3. GST-HM includes amino acids 59 to 245 in pGEX-3x, GST-(HM^{B}+HD) includes amino acids 160 to the C terminus in pGEX-5x-3, and GST-HD includes amino acids 318 to the C terminus in pGEX-3x. Fusion proteins encoded by these constructs were expressed in BL21 pLysS cells (Novagen) according to the manufacturer's protocols. *In vitro* interaction between the GST fusion proteins and ^{35}S-EXD and ^{35}S-EXD(144-376) proteins were carried out as described by Abu-Shaar et al. (1999). Bacterial extracts expressing a GST fusion protein were incubated with ^{35}S-EXD proteins, incubated with GST-agarose beads, and washed three times before eluting with SDS gel loading buffer and resolved on SDS-PAGE gels. The bands were detected by autoradiography.

DNA binding experiments

Electrophoretic mobility shift assays (EMSAs) were carried out as described previously (Chan et al., 1996). His-tagged recombinant proteins were purified for these assays. His-LAB was from amino acid 158 to its C terminus (Chan et al., 1996) and His-EXD from amino acid 1 to 323 (Chan et al., 1997). The His-HTH construct included amino acids 59 to the C terminus subcloned into pET14b (Novagen). His-HTH^{51A} had the same Asn51 to Ala mutation as GFP-HTH^{51A}. The amount of protein added in each binding reaction (25 μl total) shown in Fig. 4 is as follows: in all lanes where they were added, 10

ng of LAB was used and 80 ng of EXD was used. For HTH, 7 ng were used in lanes 8 and 15; 20 ng in lanes 9 and 16; 10 ng in lane 19; 50 ng in lanes 20; and 60 ng in lanes 3, 4, 6, 10, 11, 13, 17. For HTH^{51A}, 10 ng was used in lane 21 and 50 ng was used in lane 22.

RESULTS

The HM domain of HTH directly interacts with EXD

EXD directly binds to HTH (Abu-Shaar et al., 1999) and to the mammalian HTH homolog, MEIS1 (Rieckhof et al., 1997), suggesting that EXD interacts with a domain that is conserved between these two proteins. HTH and MEIS1 have two highly conserved domains, the HM (Homothorax-Meis) domain near the N terminus, and the homeodomain near the C terminus (Fig. 1A,B) (Rieckhof et al., 1997; Pai et al., 1998). In addition, based on sequence comparisons with the related vertebrate protein PREP1, the HM domain can be considered to have two subdomains, HM^A and HM^B, that are more highly conserved (Berthelsen et al., 1998b; Mann and Affolter, 1998) (Fig. 1A). We used a glutathione S-transferase (GST) pull-down assay to determine which part of HTH interacts with EXD. GST-HTH and GST-HM (Fig. 1B), were both able to interact with ^{35}S-labeled EXD protein in vitro (Fig. 1C, lanes 2, 3). In contrast, GST-(HM^B+HD), which begins in the middle of the HM domain and extends to end of the protein, or GST-HD, which spans the homeodomain (Fig. 1B), did not interact with ^{35}S-EXD (Fig. 1C lane 4, 5). As a negative control, we tested whether GST-HTH or GST-HM could bind to an EXD protein lacking the PBC-A domain [EXD(144-376)] (Fig. 1B), which is necessary for the HTH-EXD interaction (Abu-Shaar et al., 1999). As expected, ^{35}S-EXD(144-376) was unable to interact with either GST-HTH or GST-HM in this assay (Fig. 1C, lane 7, 8). These results demonstrate that the HM domain of HTH is necessary and sufficient for the interaction with the PBC-A domain of EXD. Further, these results are consistent with the interaction domains defined in the vertebrate proteins, MEIS1 and PBX1 (Chang et al., 1997; Knoepfler et al., 1997).

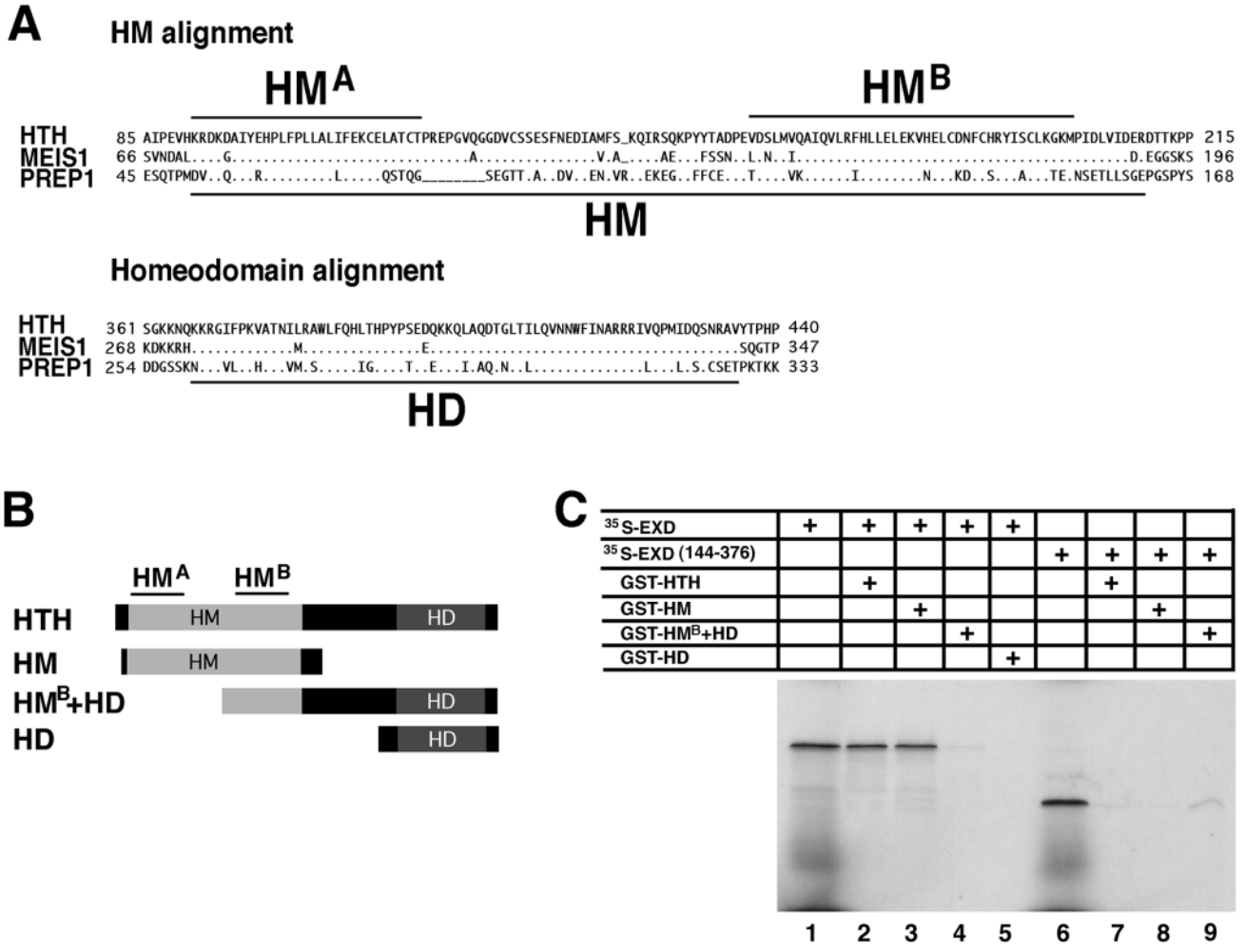

Fig. 1. HTH binds to EXD via its HM domain. (A) Sequence alignments of the HM and homeo domains of HTH, MEIS1 and PREP1. The two subregions of the HM domain that are conserved in PREP1 are referred to here as HM^A and HM^B. (B) Schematic diagram of HTH deletion constructs. All HTH proteins were synthesized in *E. coli* as N-terminal GST fusion proteins (GST sequences are not shown). (C) GST pull down experiment with the fusion proteins depicted in B and ^{35}S-labeled EXD proteins. Lanes 1 and 6 show 25% of the ^{35}S-EXD and ^{35}S-EXD[144-376], respectively, that was used in the binding reactions. GST-HTH (lane 2) and GST-HM (lane 3) bind approximately 25% of the input ^{35}S-EXD, whereas GST-(HM^B+HD) (lane 4) and GST-HD (lane 5) do not bind ^{35}S-EXD in this assay. ^{35}S-EXD[144-376], which lacks the PBC-A domain of EXD, does not bind any of the HTH derivatives.

The HM domain of HTH is sufficient to induce the nuclear localization of EXD

To determine the function of the HM and homeo domains in vivo, we fused mutant and wild-type HTH coding sequences to green fluorescent protein (GFP), and expressed these fusion genes in flies under the control of the yeast transcription factor Gal4 (Brand and Perrimon, 1993) (Fig. 2A). In wild-type *Drosophila* imaginal wing discs, EXD is cytoplasmic in cells that will generate the future wing blade, but is nuclear in cells surrounding the wing blade region (Mann and Abu-Shaar, 1996) (Fig. 2B,C). EXD is usually nuclear only in those cells where HTH is present, but when expressed at high levels or when fused to an additional nuclear localization sequence (NLS-EXD), EXD becomes partially nuclear (Fig. 2D) (Rieckhof et al., 1997; Gonzalez-Crespo et al., 1998; Pai et al., 1998; Abu-Shaar et al., 1999). When GFP-HTH expression was driven in wing discs by the *ptc:Gal4* driver line, which is expressed in a stripe of cells that bisects the wing blade, the endogenous EXD was shifted into the nucleus in GFP-HTH-expressing cells (Fig. 2E). To test if the HTH homeodomain was required for EXD's nuclear localization, two mutant proteins were tested, GFP-HM and GFP-HTH^{51A}, which has Asn 51 of the HTH homeodomain mutated to alanine (Fig. 2A). Asn 51 is conserved in all known homeodomains and

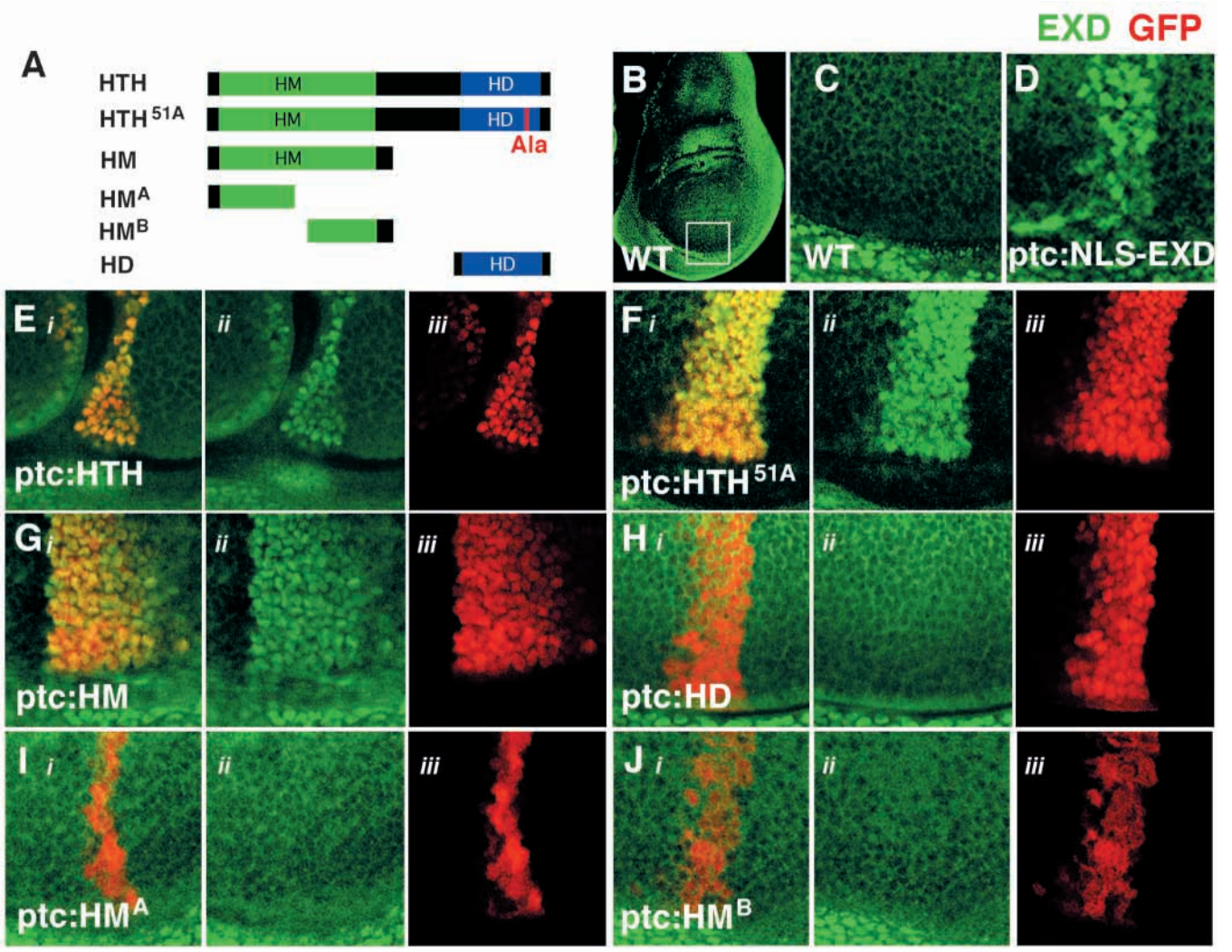

Fig. 2. The HM domain of HTH is necessary and sufficient for the nuclear import of EXD. (A) Schematic diagram of wild-type and mutant HTH proteins, which were expressed in imaginal discs as N-terminal GFP fusion proteins (GFP sequences are not shown). (B,C) A wild-type wing imaginal disc stained for EXD (green) shows cytoplasmic staining in wing pouch cells and nuclear staining in more peripheral regions. The box in B indicates the approximate region shown in C-J . (D) Confocal micrograph showing expression of NLS-EXD using the *ptc-Gal4* driver line and stained for EXD protein (green). NLS-EXD, which has an additional NLS fused to the N terminus, is predominantly nuclear in the absence of HTH. (E-J) Confocal images of wing discs ectopically expressing GFP-HTH variants using the *ptc:Gal4* driver. The discs were stained for EXD (green) and GFP (red) to detect the GFP-HTH variants. The *i* panels show the double images and the *ii* and *iii* panels show individual green or red channels, respectively. Endogenous EXD is imported into nuclei by GFP-HTH (E), GFP-HTH^{51A} (F), and GFP-HM (G), but not by GFP-HD (H), GFP-HM^{A} (I), or GFP-HM^{B} (J). GFP-HM (G*iii*) and GFP-HD (H*iii*) are nuclear, GFP-HM^{A} is present in both the nucleus and cytoplasm (I*iii*) and GFP-HM^{B} is predominantly detected in the cytoplasm (J*iii*). GFP-HM^{A} or GFP-HM^{B} could also be detected with an anti-HTH antibody (not shown), confirming the presence of HTH sequences in these fusion proteins. Note also that *ptc*-driven expression of HTH induces a fold in the wing pouch (E) whereas the other HTH derivatives do not, indicating that HTH must be competent to bind DNA to induce this morphological change.

Table 1. Effects of mis-expressing mutant and wild-type forms of HTH

Phenotype (driver)	HTH	HTH^{51A}	HM	NLS-EXD	EXD
Leg truncations (*Dll:Gal4*)	++++	++	++	Not tested	++*
Repression of *Dll* (*Dll:Gal4*)	++++	No effect	No effect	No effect	No effect*
Split wing (*ptc:Gal4*)	++++	No effect	No effect	No effect	Not tested
Eye suppression (*ptc:Gal4*)	++++	No effect	No effect	No effect	Not tested
Eye suppression (*dpp:Gal4*)	++++	No effect	No effect	No effect	Not tested

*Data not shown and Gonzalez-Crespo and Morata (1996); Gonzalez-Crespo et al. (1998).

makes essential DNA contacts (Gehring et al., 1994). GFP-HTH^{51A} was able to induce the nuclear localization of EXD in wing pouch cells, suggesting that the HTH homeodomain does not need to bind to DNA for this function (Fig. 2F). GFP-HM was also able to induce the nuclear localization of EXD, demonstrating that the HM domain is sufficient for this activity (Fig. 2G). GFP-HD, which lacks the HM domain but contains an intact homeodomain, was unable to induce EXD's nuclear localization (Fig. 2H). These data suggest that *hth* does not induce the nuclear localization of EXD by transcriptionally regulating a third factor. Instead, together with the in vitro interaction data (Fig. 1), they suggest that HTH induces the nuclear localization of EXD via a direct interaction between the HTH HM domain and the EXD PBC-A domain.

We also tested if either of the HM subdomains was sufficient to induce EXD's nuclear translocation. GFP-HM^A, which includes only the amino terminal conserved half of the HM domain, and GFP-HM^B, which includes only the carboxy terminal conserved half of the HM domain, were both unable to induce EXD's nuclear translocation (Fig. 2A,I,J). Thus, an intact HM domain is required for this activity.

GFP-HD, which spans the HTH homeodomain, is localized in nuclei (Fig. 2H*iii*). In contrast, GFP-HM^B is predominantly present in the cytoplasm (Fig. 2J*iii*) and GFP-HM^A is present in both the nucleus and cytoplasm (Fig. 2I*iii*). These data suggest that there is a nuclear localization sequence (NLS) within or close to the HTH homeodomain, no NLS within HM^B, and perhaps a weak NLS within HM^A. GFP-HM is more clearly nuclear than GFP-HM^A, consistent with the idea that the HM domain activates an NLS in EXD (see also Abu-Shaar et al., 1999). In addition, the NLS in HTH probably contributes to the nuclear localization of the HTH-EXD dimer (Abu-Shaar et al., 1999).

Nuclear import of EXD is not the sole function of HTH

The ectopic expression of HTH results in various morphological abnormalities, including the suppression of eye and leg development (Casares and Mann, 1998; Pai et al., 1998). We tested if a wild-type HTH homeodomain is required to generate these phenotypes. When GFP-HTH^{51A} was expressed ectopically, it was unable to generate most the phenotypes generated by GFP-HTH (Table 1). For example, using the *dpp:Gal4* driver line GFP-HTH was able to suppress eye development (Pai et al., 1998) (Fig. 3C). In contrast, expression of GFP-HTH^{51A} (Fig. 3D), GFP-HM, or NLS-EXD (data not shown) did not suppress eye development. Another example is when GFP-HTH was expressed using *ptc:Gal4*, wing cells along the anterior-posterior compartment boundary failed to develop, resulting in a split wing phenotype (Fig. 3A). In contrast, expression of either GFP-HTH^{51A}, GFP-HM, or NLS-EXD did not generate this phenotype (Fig. 3B and Table 1).

During leg development, expression of the homeobox gene *Distal-less*, which is required for ventral limb development (Cohen et al., 1989; Gorfinkiel et al., 1997), is mutually antagonistic with HTH/EXD function: DLL is a repressor of

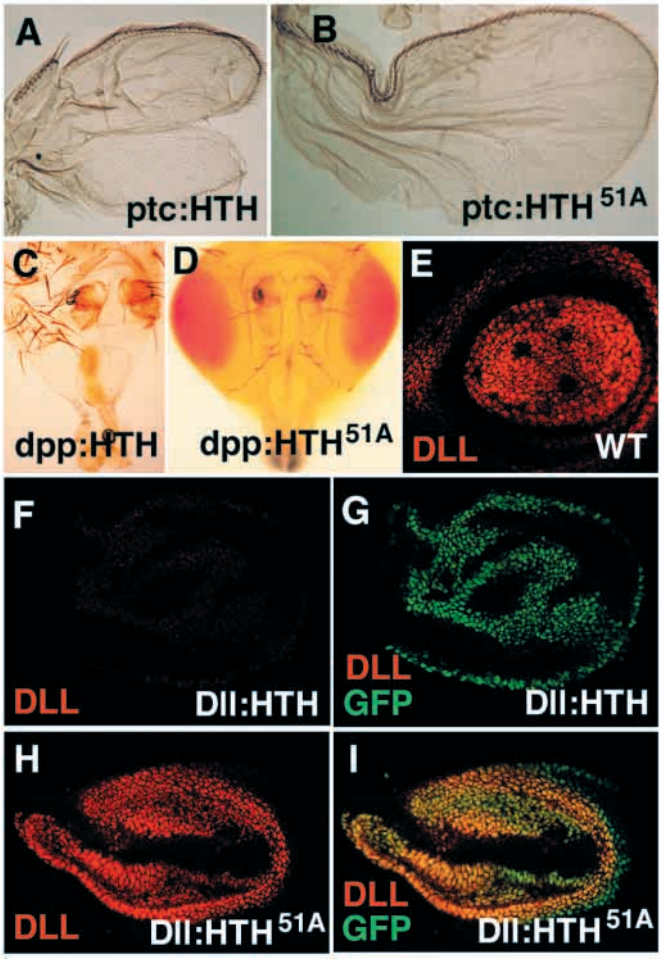

Fig. 3. The HTH homeodomain is required for HTH activity. (A,B) Adult wings resulting from ectopic expression of GFP-HTH (A) or GFP-HTH^{51A} (B) using the *ptc:Gal4* driver line. The wing is split in A. (C,D) Adult heads resulting from ectopic expression of GFP-HTH (C) or GFP-HTH^{51A} (D) using the *dpp:Gal4* driver line. Eyes are completely absent in C. (E-I) Leg imaginal discs in which GFP-HTH (F,G) or GFP-HTH^{51A} (H,I) were ectopically expressed using the *Dll:Gal4* driver line. E shows a wild-type disc for comparison. The discs were stained for DLL (red) and GFP (green); E,F,H show only the red channel and (G,I) show both channels. Expression of GFP-HTH represses *Dll* expression (F,G), but expression of GFP-HTH^{51A} does not repress DLL (H,I).

hth and HTH can also repress *Dll* (Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998; Wu and Cohen, 1999). HTH's ability to repress *Dll* requires its homeodomain: when expressed using the *Dll:Gal4* driver, GFP-HTH repressed *Dll*, but GFP-HTH^{51A} did not (Fig. 3E-I). Similarly, high levels of wild-type EXD or NLS-EXD, which localize to nuclei in the absence of HTH, or expression of GFP-HM, were also unable to repress *Dll* (Abu-Shaar and Mann, 1998) (Table 1).

From these ectopic expression assays, we conclude that although the HTH homeodomain is not required to induce EXD's nuclear localization, it is necessary for many of HTH's functions, including the regulation of specific target genes such as *Dll*. The one known exception is that all forms of HTH, including GFP-HTH^{51A} and GFP-HM, were able to interfere with distal leg development when expressed with the *Dll:Gal4* driver (Table 1). This phenotype, however, is also observed when wild-type EXD is expressed with this driver, and therefore does not require any HTH input (Gonzalez-Crespo and Morata, 1996). The different in vivo activities of HTH and HTH^{51A} indicate that HTH has functions in addition to localizing EXD to nuclei, and that these functions require HTH to bind DNA.

HTH, LAB and EXD form a complex on a Hox target enhancer

The tight interaction between HTH and EXD proteins, together with the requirement for the HTH homeodomain for many of HTH's functions, suggested that HTH might be binding to the same target enhancers as Hox/EXD heterodimers. One well characterized Hox/EXD target is an autoregulatory enhancer from the *labial* (*lab*) gene, called lab550 (Tremml and Bienz, 1992; Grieder et al., 1997). A 48 bp fragment of lab550, lab48/95, is necessary for lab550 activity and, in one copy, is sufficient to direct a *labial*- and *exd*-dependent pattern of expression in endodermal cells (Fig. 4A,C) (Grieder et al., 1997). In lab48/95 there is a single LAB/EXD heterodimer binding site, TGATGGATTG, and this binding site is necessary for the activity of lab550 (Grieder et al., 1997). Also in lab48/95 there is a binding site that resembles a high affinity site for MEIS1, GACTGTCA, a murine HTH homolog (Chang et al., 1997; Knoepfler et al., 1997) (Fig. 4A). To test if this site is a bona fide HTH binding site we performed band shift experiments with LAB, HTH, and EXD proteins on the wild-type lab48/95 oligo, and on an oligo with point mutations in the putative HTH binding site, GACTtatA (lab48/95^{hth}) (Fig. 4A,B). Neither LAB, EXD, nor HTH were able to bind lab48/95 on their own (Fig. 4B, lanes 2,3,5). The combination of EXD plus HTH was able to weakly bind this DNA (Fig. 4B, lane 4). Because binding was diminished on lab48/95^{hth} (Fig. 4B, lane 11), these data suggest that EXD and HTH exhibit weak cooperative binding to lab48/95, consistent with previous studies with MEIS1 and PBX1 (Chang et al., 1997).

LAB cooperatively bound with EXD to lab48/95 (Fig. 4B, lane 7) and the binding of this heterodimer requires both the EXD and LAB half sites (Grieder et al., 1997). In contrast, no complex formation was observed when HTH and LAB were combined (Fig. 4B, lane 6). However, when increasing amounts of HTH were added to a constant amount of LAB plus EXD, the LAB/EXD band disappeared and in its place a HTH/LAB/EXD trimeric complex was observed (Fig. 4B, lane 8-10). The HTH/LAB/EXD band was more intense than the LAB/EXD band (compare lanes 7 and 10), suggesting that HTH contributes to the DNA binding affinity of the trimeric complex.

To test if the formation of the HTH/LAB/EXD complex requires the putative HTH binding site we tested the ability to form these complexes on lab48/95^{hth} (Fig. 4A). On this oligo, LAB/EXD complexes formed as well as on the wild-type oligo (compare lanes 7 and 14). Formation of the trimeric HTH/LAB/EXD complex was observed on this oligo, but compared to lab48/95 it required approximately five-fold more HTH (compare lanes 8-10 with 15-17). In addition, in the absence of a strong HTH binding site, HTH appeared to destabilize the LAB/EXD complex (lanes 14-16). HTH's ability to weakly shift the LAB/EXD complex bound to lab48/95^{hth} is likely due to the protein-protein interaction between HTH and EXD. These results suggest that a HTH binding site is required for optimal trimeric complex formation.

These conclusions were further supported by band shift experiments in which the binding of a mutant HTH protein, HTH^{51A}, was compared to wild-type HTH on lab48/95. In this experiment, the HTH binding site was wild type, but the HTH homeodomain was impaired in its ability to bind DNA due to mutation of Asn 51 to Ala. In contrast to HTH, HTH^{51A} was only able to weakly supershift the LAB/EXD complex (Fig. 4B, lanes 21,22). In fact, HTH^{51A} forms trimeric complexes on lab48/95 more poorly than HTH forms them on lab48/95^{hth} (Fig. 4B and data not shown). Moreover, the HM domain of HTH can also weakly supershift the LAB/EXD complex, but the HTH homeodomain cannot (data not shown). These data suggest that the protein-protein interaction between HTH and EXD is necessary for the formation of the HTH/LAB/EXD complex, but that DNA binding by the HTH homeodomain contributes to the stability of this complex.

The HTH binding site is required for lab48/95 activity in embryos

When a single copy of lab48/95 was cloned upstream of a minimal promoter driving *lacZ*, expression of β-galactosidase (β-gal) was observed in a central domain of the midgut endoderm in embryos (Fig. 4C; Grieder et al., 1997). lab48/95 is part of a larger enhancer, lab550, and previous experiments demonstrated that both the LAB and EXD half sites are required for the full activity of lab550 in vivo (Grieder et al., 1997). lab48/95 activity is completely abolished when either the EXD or LAB half sites are mutated (Fig. 4D,F). Embryos that are mutant for either *hth* or *exd* are also unable to activate *lab48/95-lacZ* (Fig. 4G,H). *hth* is probably required in part because in the absence of *hth* function EXD is cytoplasmic in endodermal cells (Rieckhof et al., 1997). In addition, the requirement for *hth* might also be because HTH must directly bind to lab48/95. To test this possibility, we assessed the ability of lab48/95^{hth} to activate transcription of a *lacZ* reporter gene in vivo. Unlike lab48/95 (Fig. 4C), lab48/95^{hth} had almost no enhancer activity; only a few β-gal^{+} nuclei were observed in some *lab48/95^{hth}-lacZ* embryos (Fig. 4E). In contrast, mutating basepairs adjacent to the HTH binding site did not interfere with enhancer activity (data not shown, see Materials and Methods). These data demonstrate that the HTH binding site is specfically required for lab48/95 activity in vivo. The weak residual activity of lab48/95^{hth} is in concert with the band shift

results in which the intensity of the HTH/LAB/EXD band was diminished, but not abolished, in the absense of an optimal HTH binding site.

Although mutation of the EXD or LAB half sites in lab550 nearly eliminated its activity (Grieder et al., 1997), when we introduced the mutation of the HTH binding site into lab550, and generated *lab550^{hth}-lacZ* transgenic flies, expression of β-gal appeared wild type (data not shown). However, although the HTH binding site is not required for lab550 activity, additional experiments (see below) suggest that HTH must be part of the Hox protein complex for this enhancer to be active.

Expression of the HM domain interferes with HTH function

The above results support the idea that a DNA bound HTH/LAB/EXD triple complex is activating *lab48/95-lacZ* in vivo. If this is the case, then it might be possible to interfere with the stable assembly of this complex by expressing the HM domain, which binds to EXD and therefore might compete with the interaction between EXD and HTH. To test this possibility, we first determined if ectopic expression of GFP-HM affected the activities of *lab48/95-lacZ* and *lab550-lacZ*.

Ectopic expression of LAB, EXD, or GFP-HTH using an endodermal-specific Gal4 driver, *48Y:Gal4*, had no affect on the activities of *lab48/95-lacZ* or *lab550-lacZ* (Fig. 5A,B,E,F and data not shown). In contrast, when we expressed GFP-HM using this driver, *lab48/95-lacZ* expression was nearly eliminated (Fig. 5D) and *lab550-lacZ* expression was undetectable (Fig. 5H). Interestingly, expression of GFP-HTH^{51A} had no affect on the activities of either reporter gene (Fig. 5C,G). These results suggest that GFP-HM can bind to EXD and compete for the interaction with full-length, endogenous HTH. In addition, they suggest that disrupting the interaction between EXD and HTH interferes with their ability to activate both *lab48/95-lacZ* and *lab550-lacZ*.

If GFP-HM is interfering with HTH and EXD function in vivo, its over-expression should be able to phenocopy other *hth* or *exd* mutant phenotypes. One function of *hth* is to direct antennal development; in the absence of either *hth* or *exd* activities antennal structures are autonomously transformed into leg identities (Gonzalez-Crespo and Morata, 1995; Casares and Mann, 1998) (Fig. 5I,L). Consistent with GFP-HM acting as a dominant negative, its expression in the *Dll* domain transformed distal antenna into distal leg (Fig. 5K). The antenna to leg transformations observed in GFP-HM-expressing animals show bristles with bracts, typical of a distal leg identity. In contrast, expression of GFP-HTH^{51A} did not generate this transformation (Fig. 5J). Together with their effect on the reporter genes, these results suggest that GFP-HM, but not GFP-HTH^{51A}, interferes with *hth* function. This would indicate that GFP-HM has dominant negative activity whereas GFP-HTH^{51A} behaves as a hypomorph (see Discussion).

We also tested GFP-HM's ability to alter the segment identity of the adult abdomen which, unlike antennal development, requires input from both *exd* and Hox genes (Lewis, 1978; Sánchez-Herrero et al., 1985; Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995). In wild-type male abdomens, posterior tergites have darker pigmentation and a lower density of small hairs (trichomes) than anterior tergites (Fig. 6A,G). *hth*$^{-}$ clones, like *exd*$^{-}$ clones, in the second or third tergite of a male fly show an increase in pigmentation and a decrease in trichome density, consistent with a transformation into a more posterior abdominal identity (Fig. 6B,C,H). When we expressed GFP-HM using *pnr-Gal4*, we observed an increase in pigmentation in anterior tergites, consistent with an anterior-to-posterior transformation of abdominal segment identity (Fig. 6D). However, no effect on trichome density was observed following GFP-HM expression, suggesting that this transformation is incomplete (not shown). In contrast, expression of wild-type GFP-HTH using *pnr-Gal4* resulted in a decrease in pigmentation and an increase in trichome density in tergites 5 and 6, consistent with a posterior-to-anterior shift in cell fate (Fig. 6E,I). Expression of GFP-HTH^{51A} generated a weak version of this transformation (Fig. 6F). These results suggest that interfering with *hth* function by expressing the HM domain can interfere with a Hox-dependent function, such as tergite identity in the adult abdomen. Moreover, they suggest that different amounts of *hth* activity in the abdomen contribute to differences in tergite identity.

DISCUSSION

Multiprotein Hox complexes in development

We have demonstrated that the assembly of a HTH/Hox/EXD trimeric complex is required for the activity of a natural Hox target enhancer, lab48/95. All three of these proteins contain homeodomains, and the individual protein-DNA interactions made by each of them contribute to the stability of this complex in vitro and to the activity of the enhancer in vivo. Using a dominant negative form of HTH, we provided evidence that the assembly of Hox protein complexes may be important for other *hth*- and Hox-dependent functions in vivo. These data support a model in which Hox proteins function as part of a DNA-bound HTH/Hox/EXD trimer, which we refer to as the Hox protein complex (Fig. 7).

That Hox proteins function as part of a multiprotein complex helps to explain several remarkable attributes of these selector proteins. Despite the central role they play in development, in general Hox proteins from different species are only conserved within their homeodomains and 'YPWM' motifs (Mann, 1995). Yet, despite this limited sequence conservation, the functions of Hox proteins have been conserved between flies and vertebrates (Malicki et al., 1990; McGinnis et al., 1990; Zhao et al., 1993; Bachiller et al., 1994). Another intriguing observation is that Hox proteins, for example Ultrabithorax, retain much of their specific activities even when large portions of these proteins have been deleted (Gibson et al., 1990; Mann and Hogness, 1990). Taken together, these observations suggest that the homeodomain region and 'YPWM' motif may be sufficient for executing most Hox functions in vivo. These Hox domains are also likely to be sufficient for forming Hox/EXD heterodimers and, consequently, HTH/Hox/EXD, trimeric complexes (Passner et al., 1999; Piper et al., 1999; Ryoo and Mann, 1999). An extreme view is that Hox proteins may do little more than help to assemble and influence the sequence recognition properties of these multiprotein complexes. Hox proteins may rely on the other components of the Hox protein complex, which have been highly conserved

evolutionarily, to carry out their essential functions in animal development.

EXD and HTH exhibit different modes of cooperative binding to DNA

As defined here, the Hox protein complex is composed of at least three homeodomain proteins, Hox, EXD and HTH. Although EXD and HTH have both been suggested to be Hox cofactors, the ways in which these proteins bind to lab48/95 are distinct. The EXD and LAB binding sites overlap resulting in a composite, Hox/EXD binding site (Chan and Mann, 1996; Grieder et al., 1997; Passner et al., 1999; Piper et al., 1999). In contrast, LAB, HTH, or LAB/HTH heterodimers do not bind to this DNA. Instead, HTH binds at a distance of approximately 12 basepairs away from the LAB/EXD heterodimer (Fig. 7). Moreover, for HTH to bind this DNA with high affinity, LAB/EXD heterodimers must be bound to their composite site.

Other differences between EXD and HTH are the protein domains that contribute to complex formation. The interaction between HTH and the LAB/EXD heterodimer is mediated primarily by an interaction between the PBC-A domain of EXD and the HM domain of HTH. For both proteins, these domains are located close to their N termini, and far from their DNA binding domains. In contrast, the interaction between EXD and Hox proteins is primarily mediated by the YPWM sequence of the Hox protein directly inserting into a hydrophobic pocket within the EXD homeodomain (Passner et al., 1999; Piper et al., 1999). This pocket in EXD is due in part to the extra long loop between the first and second α-helices of its homeodomain, which is the defining feature of the TALE family of homeoproteins (Bürglin, 1997). Because HTH is also a TALE homeoprotein it may have a similar binding pocket in its homeodomain, raising the possibility that there may be other proteins that associate with the Hox protein complex by interacting with the HTH homeodomain.

The differences between HTH and EXD are also reflected in their different contributions to the activities of lab48/95 and of the larger enhancer, lab550. In lab550, which integrates additional input from the *decapentaplegic* (*dpp*) pathway (Tremml and Bienz, 1992; Grieder et al., 1997), the EXD and LAB binding sites are both required for full activity (Grieder et al., 1997). In contrast, the HTH binding site is not required for the activity of lab550, but all three binding sites are essential for the activity of lab48/95. However, although a high affinity HTH binding site in lab550 is dispensible, the protein-protein interaction between EXD and HTH is required, because interfering with this interaction by expressing GFP-HM decreased the activities of both lab48/95 and lab550. These results suggest that HTH must be part of the Hox protein complex to activate lab550 (and lab48/95), but that the protein-protein interaction between HTH and EXD is more important for the stability of the complex than the interaction between HTH and DNA. Further, these results suggest that in the

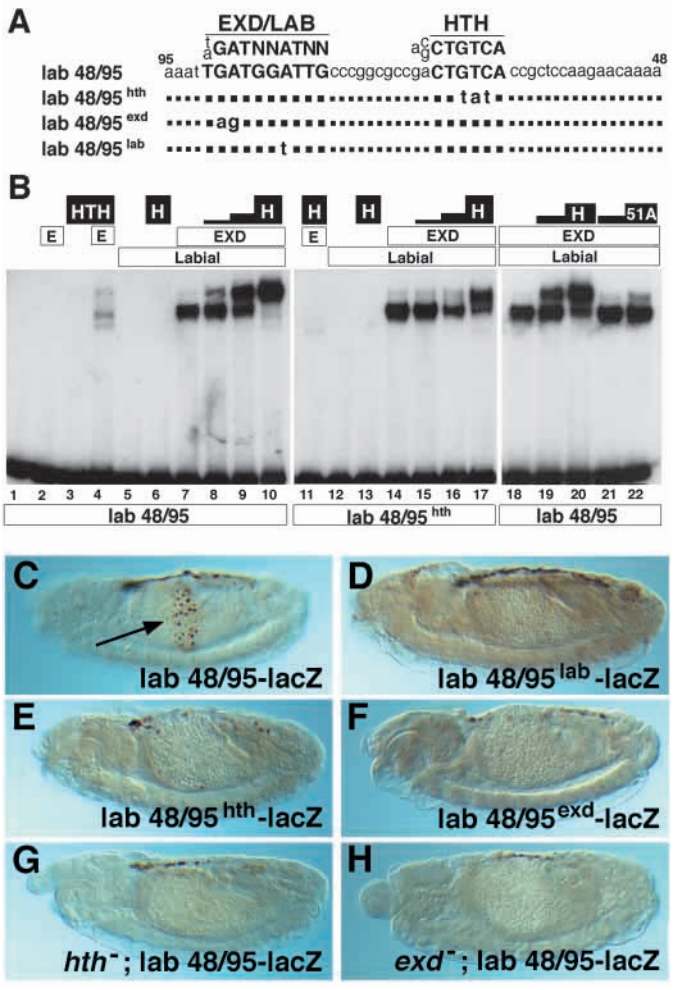

Fig. 4. HTH, LAB and EXD form an essential complex on the Hox target, lab48/95. (A) Sequence of lab48/95 and mutant derivatives. The EXD/LAB and HTH binding sites are indicated in bold; mutations are indicated in small letters. For comparison, the Hox/EXD and MEIS1 consensus binding sites are shown above the lab48/95 sequence (Mann and Chan, 1996; Chang et al., 1997). (B) Band shift experiments using ^{32}P-labelled lab48/95 (lanes 1-10 and 18-22) and lab48/95^{hth} (lanes 11-17) oligos. Neither EXD (lane 2) nor HTH (lane 3) bind this DNA on their own. When EXD and HTH (H) are combined they form a weak complex (lane 4). Neither LAB (lane 5) or a mixture of LAB plus HTH (lane 6) bind this DNA. In contrast, LAB plus EXD bind to this DNA (lane 7) and increasing amounts of HTH shift this band to a slower migrating, HTH/LAB/EXD complex (lane 8-10). On lab48/95^{hth} EXD plus HTH bind very poorly (lane 11), but LAB plus EXD bind as well as on lab48/95 (lanes 7 and 14). Upon addition of increasing amounts of HTH, a HTH/LAB/EXD band forms only at the highest HTH concentration (lane 15-17). In the right-most panel, HTH is able to supershift the LAB/EXD complex (lanes 19-20) whereas the same concentrations of HTH^{51A} (51A) resulted in only a very weak supershift (lanes 21-22). As indicated, the amounts of HTH and HTH^{51A} in lanes 19 and 21 are intermediate to the amounts of HTH in lanes 8 and 9; similarly, the amounts in lanes 20 and 22 are intermediate to the amounts in lanes 9 and 10. (C-H) lab48/95 requires *exd*, *hth* and the LAB, EXD and HTH binding sites. Transgenic embryos were immunostained for β-gal and detected using HRP (brown). In wild-type *lab48/95-lacZ* embryos (C) strong nuclear staining is observed in a central band of endoderm cells (arrow); staining is absent in *hth*$^{-}$ (G) or *exd*$^{-}$ (H) embryos. lab48/95 activity also requires *lab* (Grieder et al., 1997). No endoderm staining is observed when the EXD (F) or LAB (D) binding sites are mutated, and is very weak when the HTH binding site is mutated (E). Dorsal staining, unrelated to *labial* expression, is observed in all of these embryos (Grieder et al., 1997).

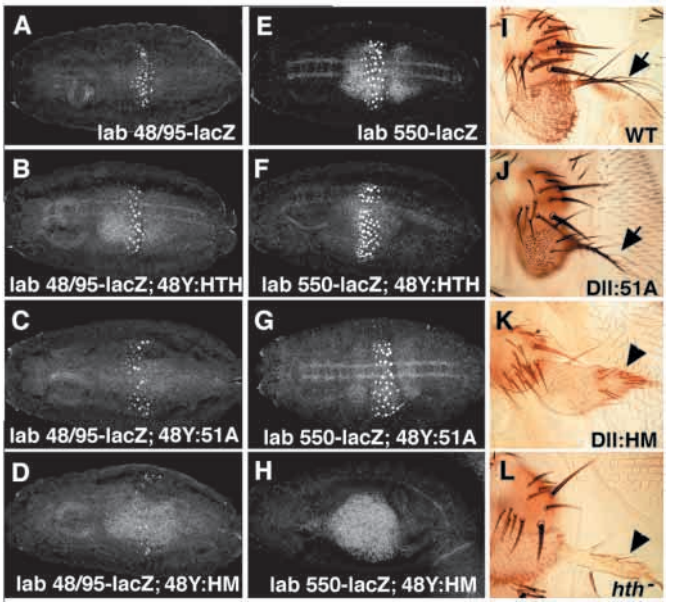

Fig. 5. The HM domain interferes with *hth* function. lab48/95 (A-D) or lab550 (E-H) expression in wild-type embryos (A,E) and embryos expressing GFP-HTH (B,F), GFP-HTH^{51A} (C,G), or GFP-HM (D,H) using the endodermal Gal4 driver line, *48Y:Gal4*. Compared to wild-type levels (A,E), GFP-HTH (B,F) or GFP-HTH^{51A} (C,G) expression had no affect on β-gal levels, whereas GFP-HM expression reduced or eliminated β-gal levels (D,H). In A-H β-gal was detected by immunofluorescence. (I-L) Adult antennas. A wild-type antenna (I), antennas in which GFP-HTH^{51A} (J) or GFP-HM (K) were expressed using the *Dll:Gal4* driver, and an antenna with a distal *hth*$^{-}$ clone, identified by *yellow*$^{-}$ tissue (L). In both (K) and (L) a transformation of distal antenna towards distal leg was observed (arrowheads). The antennae in I and J are wild type (arrows).

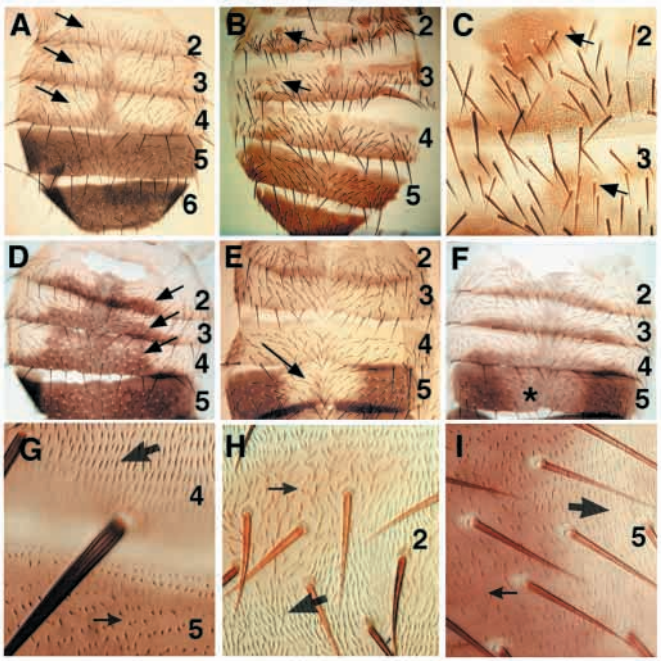

Fig. 6. Altering *hth* activity affects abdominal segment identity. Shown are dorsal views of adult male abdomens; the numbers on the right in each panel refer to the tergite. (A,G) Wild type; (B,C,H) abdomens with *hth*$^{-}$ clones (arrows in B,C) marked with *y*$^{-}$; (D) *pnr:Gal4*; UAS-GFP-HM; (E,I) *pnr:Gal4*; UAS-MYC-HTH; (F) *pnr:Gal4*; UAS:GFP-HTH^{51A}. (A) In wild-type males the anterior two-thirds of tergites 2, 3 and 4 (arrows) are lightly pigmented whereas tergites 5 and 6 are darkly pigmented. Reducing *hth* activity in tergites 2, 3 or 4 by mosaic analysis (B,C) or by expressing GFP-HM (D) resulted in darkly pigmented cuticle, typical of a posterior tergite identity (arrows). Expression of MYC-HTH (E) resulted in a transformation of tergites 5 and 6 into a more anterior, lightly pigmented identity (arrow); GFP-HTH generates an identical transformation (not shown). GFP-HTH^{51A} is also weakly able to produce this transformation (F, asterisk). In D-F the transformations are observed in a dorsal band of cells, which is where the *pnr:Gal4* driver is expressed (Calleja et al., 1996). (G) In addition to differences in pigmentation, anterior tergites (e.g. tergite 4; large arrow) also have a higher density of trichomes, or small hairs, than posterior tergites (e.g. tergite 5, small arrow). (H) *hth*$^{-}$ clones in anterior tergites have a lower density of trichomes (small arrow) than the surrounding wild-type cuticle (large arrow), indicating a transformation to a more posterior identity. (I) A close-up of the border between MYC-HTH-expressing cells (lighter pigmented region on the right) and wild-type cells (darker pigmented region on the left) in tergite 5. In addition to affecting pigmentation, expression of MYC-HTH in posterior segments also resulted a higher density of trichomes (large arrow). Expression of GFP-HM did not affect trichome density, suggesting that the reduction of *hth* activity is incomplete (not shown). In G-I large and small arrows point to regions of higher and lower trichome density, respectively.

context of lab550 (but not lab48/95) there are other factors that stabilize HTH binding, even in the absence of an optimal HTH binding site.

How general is the Hox protein complex? One piece of suggestive evidence is that MEIS and PBX proteins seem to be complexed with each other in mammalian cells (Chang et al., 1997; Knoepfler et al., 1997). However, in most enhancers where Hox/EXD (or PBX) binding sites have been characterized, HTH (or MEIS and PREP) binding sites have not been identified, and it is not known if such binding sites are required (Pöpperl et al., 1995; Chan et al., 1996; Di Rocco et al., 1997; Berthelsen et al., 1998a; Swift et al., 1998; Ryoo and Mann, 1999). Nevertheless, even in the absence of a clear binding site, MEIS or PREP proteins can enhance the transcriptional activity of PBX-containing complexes in cell culture transfection experiments (Berthelsen et al., 1998a; Swift et al., 1998). Thus, as our experiments with lab550 also indicate, the protein-protein interaction between MEIS and PBX may be more important than an interaction between MEIS and DNA. More recent experiments carried out with a HOXB1-activated enhancer from the *Hoxb2* gene demonstrated that a MEIS/HOXB1/PBX trimeric complex is required for enhancer activity (Jacobs et al., 1999). Although this appears to be an analogous complex to the one described here, it is interesting that the arrangement of binding sites in the *Hoxb2* enhancer is different from their arrangement in lab48/95: in lab48/95 HTH binds on the Hox side of the LAB/EXD dimer whereas in the *Hoxb2* enhancer MEIS binds on the PBX side of the HOXB1/PBX dimer. This difference suggests that there may be significant flexibility in the position of the MEIS or HTH binding site relative to the Hox/EXD (or PBX) binding site. This flexibility may be important for the recruitment of additional factors, which may be different in different enhancers.

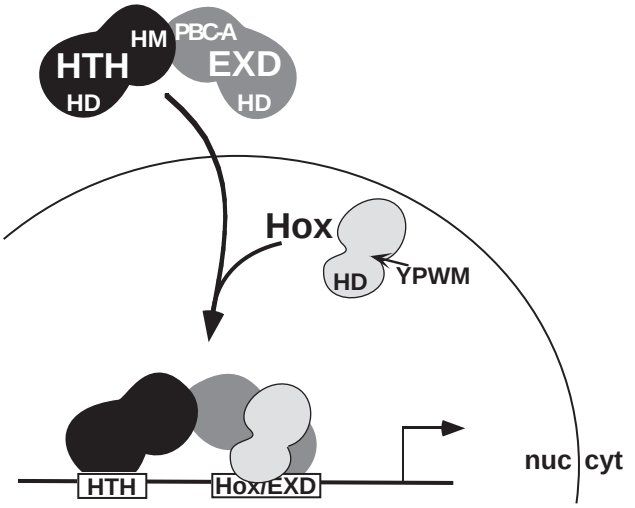

Fig. 7. Formation of a Hox protein complex on a Hox target enhancer. The cytoplasmic to nuclear translocation of EXD requires a direct interaction between the HM domain of HTH and the PBC-A domain of EXD. Once in the nucleus, HTH remains associated with EXD and, together with a Hox protein, can assemble into a Hox protein complex that requires an interaction between the Hox 'YPWM' motif and the EXD homeodomain. The Hox protein complex recognizes DNA via all three homeodomains (HD) with a high degree of sequence specificity. In lab48/95, HTH binds at a distance from and helps to stabilize the Hox/EXD heterodimer. We suggest that the conserved regions of HTH and EXD are likely to recruit additional proteins to this complex to regulate transcription. nuc, nucleus; cyt, cytoplasm.

Both lab48/95 and the *Hoxb2* enhancer are activated by paralog 1 Hox proteins, LAB and HOXB1, respectively. To generalize these findings to other Hox proteins, it will be important to determine if trimeric complexes, analogous to the HTH/LAB/EXD complex defined here, are required for their activity. The experiments presented here suggest this may be the case because expression of GFP-HM was able to alter the apparent activity of the abdominal Hox proteins, which are representatives of paralogs 7 to 13. However, this conclusion should remain tentative until enhancers that are directly activated by these Hox proteins have been characterized at high resolution.

A role for HTH in transcriptional activity

Our experiments suggest that the protein-DNA and protein-protein contacts made by HTH contribute to the overall stability of the DNA-bound Hox protein complex. Unlike EXD, we have not been able to uncover a role for HTH in influencing Hox DNA binding specificity (H. D. R. and R. S. M., unpublished observations). However, like EXD, HTH may also contribute to the transcriptional activation of Hox response elements like lab48/95. This conclusion stems from the different in vivo activities of GFP-HM and GFP-HTH^{51A}. Both of these proteins can induce the nuclear localization of EXD and therefore must be able to compete with the interaction between EXD and endogenous HTH. However, although GFP-HM inhibits *hth* activity, for all of the phenotypes we examined GFP-HTH^{51A} does not block endogenous *hth* activity. One way to reconcile this difference is to suggest that sequences present in GFP-HTH^{51A} and absent from GFP-HM, for example the homeodomain, contribute to transcriptional activation, perhaps by recruiting additional factors to the Hox protein complex. According to this idea, GFP-HTH^{51A} does not have dominant negative activity because, although it still competes for the interaction with HTH, it is able to interact with these additional factors and can therefore, at least partially replace wild-type HTH functions. In contrast, we suggest that GFP-HM behaves like a dominant negative because it is unable to interact with these additional factors.

GFP-HM has other activities in addition to interfering with *hth* activity. Because GFP-HM has the ability to induce EXD's nuclear localization, it also generates gain-of-function phenotypes when expressed in places where there is normally no *hth* expression. An example is in leg development: *hth* is not expressed in the distal portion of the leg, and genetic studies show that *hth* and *exd* have no function there (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995; Rieckhof et al., 1997; Casares and Mann, 1998). Yet expression of either high levels of EXD, which result in its nuclear localization, or GFP-HM, which induces the nuclear localization of endogenous EXD, results in leg truncations (Gonzalez-Crespo and Morata, 1996). Thus, the ability to block leg development is due to the presence of nuclear EXD, and does not require HTH. Similarly, we would predict that any other gain-of-function phenotype that can be induced by nuclear EXD in the absence of HTH would also be generated by GFP-HM.

Conclusions

In summary, we have separated HTH's ability to bind DNA from its ability to induce the nuclear localization of EXD. By doing so, these experiments demonstrated that HTH has functions in addition to inducing the nuclear localization of EXD. Consistent with HTH having a homeodomain that has been highly conserved during evolution, HTH binds to DNA together with Hox/EXD heterodimers to form a HTH/Hox/EXD trimeric complex. Formation of this complex is essential for the activity of a *labial* target enhancer, lab48/95. Further, these experiments suggest that HTH/Hox/EXD complexes may be necessary for other Hox-mediated activities. We suggest that the Hox selector proteins execute their specific functions in vivo largely because they have the ability to direct the assembly of sequence-specific, multiprotein complexes containing highly conserved cofactors such as EXD and HTH.

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