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Nuclear Translocation of Extradenticle Requires *homothorax*, which Encodes an Extradenticle-Related Homeodomain Protein

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Summary

We show that *homothorax* (*hth*) is required for the *Hox* genes to pattern the body of the fruit fly, Drosophila melanogaster. *hth* is necessary for the nuclear localization of an essential HOX cofactor, Extradenticle (EXD), and encodes a homeodomain protein that shares extensive identity with the product of *Meis1*, a murine proto-oncogene. MEIS1 is able to rescue *hth* mutant phenotypes and can induce the cytoplasmic-to-nuclear translocation of EXD in cell culture and Drosophila embryos. Thus, *Meis1* is a murine homolog of *hth*. MEIS1/HTH also specifically binds to EXD with high affinity in vitro. These data suggest a novel and evolutionarily conserved mechanism for regulating HOX activity in which a direct protein–protein interaction between EXD and HTH results in EXD's nuclear translocation.

Introduction

Throughout the animal kingdom, the *Hox* genes are important regulators of developmental pathways (reviewed by McGinnis and Krumlauf, 1992; Krumlauf, 1994). Because of the central role these genes play in development, the *Hox* genes are subject to several levels of regulation. In Drosophila melanogaster, *Hox* gene transcription is governed by a complex set of factors that control where and when the *Hox* genes are initially transcribed (Qian et al., 1991; Muller and Bienz, 1992; Zhang and Bienz, 1992; Shimell et al., 1994; Pirrotta et al., 1995), as well as by the Polycomb and Trithorax groups of proteins that epigenetically maintain these expression patterns over many cell divisions (reviewed by Paro and Harte, 1996; Pirrotta, 1997). A second level of *Hox* regulation is achieved by modulating the activity of the protein products of the *Hox* genes. For example, the phenomenon termed phenotypic suppression suggests that the activities of some HOX proteins are suppressed by other HOX proteins (Gibson and Gehring, 1988; Gonzalez-Reyes and Morata, 1990; Gonzalez-Reyes et al., 1990; Mann and Hogness, 1990; Duboule, 1991; Chisaka et al., 1992; Lufkin et al., 1992). Phosphorylation appears to modulate HOX activity and might contribute in part to phenotypic suppression (Jaffe et al., 1997).

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The Drosophila *extradenticle* (*exd*) and vertebrate *Pbx* genes provide a third mechanism for regulating *Hox* functions. All *Hox* genes encode homeodomain proteins that control cell fates by regulating the transcription of target genes (reviewed by Graba et al., 1997). HOX proteins bind DNA without a high degree of specificity; however, their DNA binding specificity can be increased by interactions with the homeodomain proteins encoded by the *exd* and *Pbx* genes. These homeodomain proteins, collectively referred to as the PBC family (Burglin, 1994), bind to DNA cooperatively (reviewed by Mann and Chan, 1996) and noncooperatively (Pinsonneault et al., 1997) with Hox proteins and are required for the HOX proteins to execute their functions in vivo (Peifer and Wieschaus, 1990; Chan et al., 1994; Rauskolb and Wieschaus, 1994; Gonzalez-Crespo and Morata, 1995; Pöpperl et al., 1995; Rauskolb et al., 1995; Chan et al., 1996, 1997; Pinsonneault et al., 1997). Surprisingly, unlike other homeodomain proteins, EXD is often found in the cytoplasm during development (Mann and Abu-Shaar, 1996; Aspland and White, 1997). EXD protein is exclusively cytoplasmic in blastoderm embryos and is translocated into specific nuclei at precise times during development. In at least one case, the endoderm cells of the embryonic midgut, EXD's nuclear translocation is triggered by the secreted signaling molecules Wingless (WG) and Decapentaplegic (DPP) (Mann and Abu-Shaar, 1996). Thus, EXD's ability to control target gene transcription, either on its own or as a HOX cofactor, is regulated. It follows that for HOX functions that require EXD as a cofactor, the control of EXD's nuclear translocation is also a mechanism to control HOX activity.

Although *exd* clearly contributes to *Hox* specificity, EXD–HOX interactions are not sufficient to explain the precise functions that the HOX proteins execute in vivo (Chan et al., 1997; Pinsonneault et al., 1997). To identify additional factors that contribute to HOX specificity, we examined other Drosophila mutations that affect embryonic pattern (Jürgens et al., 1984). We looked for mutations that, like *exd* (Peifer and Wieschaus, 1990), alter antero-posterior pattern without affecting the expression of the trunk *Hox* genes. We found that mutations in the gene *homothorax* (*hth*) appear very similar to *exd* mutations. Genetic epistasis experiments suggest that in the absence of *hth*, EXD protein is nonfunctional. Consistent with this observation, in *hth* mutant embryos EXD is found predominantly in the cytoplasm. Surprisingly, *hth* encodes a homeodomain protein with extensive amino acid identity to the murine proto-oncogene *Meis1* (Moskow et al., 1995), and the HTH homeodomain is approximately 45% identical to the EXD homeodomain. EXD is nuclear where *hth* is transcribed and EXD directly interacts with MEIS1 and HTH in vitro. Further, MEIS1/HTH can induce the cytoplasmic to nuclear translocation of EXD in cell culture and in Drosophila embryos. These results suggest a novel mechanism in which EXD protein translocates into nuclei due to a direct interaction with another homeodomain protein encoded by *hth*.

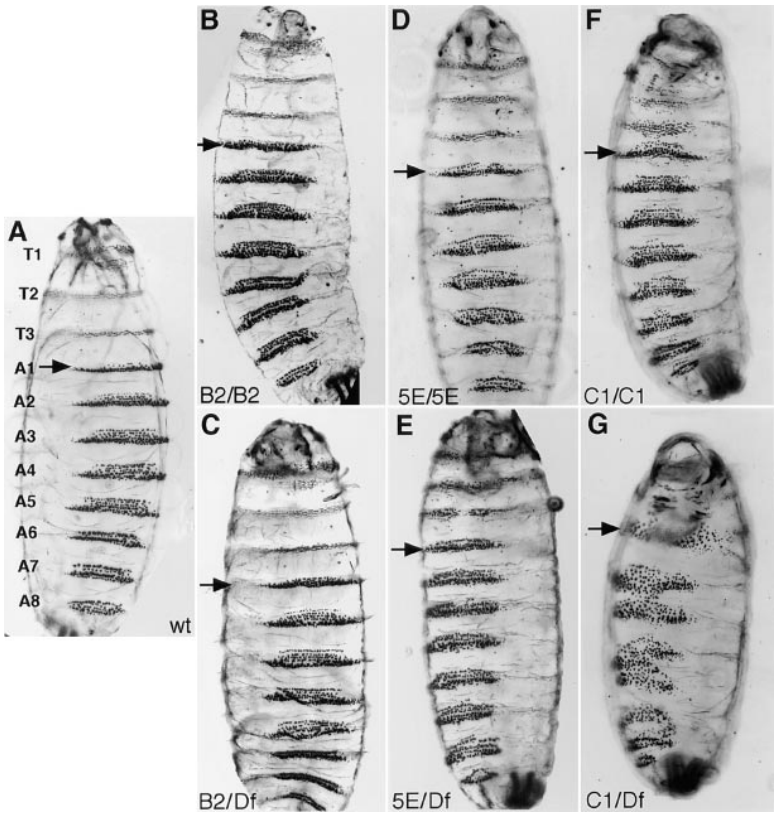

Figure 1. *hth* Phenotypes

Ventral surfaces of the cuticles secreted by wild type (A), hth^{B2}/hth^{B2} (B), $hth^{B2}/Dfhth$ (C), hth^{5E}/hth^{5E} (D), $hth^{5E}/Dfhth$ (E), hth^{C1}/hth^{C1} (F), and $hth^{C1}/Dfhth$ (G) embryos. The arrows point to the position of A1, which for hth^{5E} (D) and hth^{C1} (F) resembles A3 and A5, respectively.

Results

hth Is Required for Patterning the Drosophila Embryo

At the end of embryogenesis, the Drosophila embryo secretes a cuticle in which each thoracic and abdominal segment can be identified by morphological differences. The main determinants of these differences are the *Hox* genes. *hth* is also required for the normal pattern of the cuticle. Three EMS-induced alleles, hth^{B2}, hth^{5E}, and hth^{C1}, disrupt the normal pattern to differing degrees, in the order B2 < 5E < C1 (Figures 1B, 1D, and 1F). When in *trans* to an X-ray-induced *hth* allele (*Dfhth*, an RNA null allele; see below), all three EMS alleles generate stronger alterations of the cuticle than when they are homozygous (Figures 1C, 1E, and 1G). These results suggest that none of the three EMS alleles are complete loss-of-function mutations. However, the phenotype of $hth^{C1}/Dfhth$ suggests that this genotype is close to a complete loss of *hth* function (see below).

In general, loss of *hth* function results in severe head defects, including a failure of head involution, and in the transformation of the thoracic and abdominal segments into a more posterior identity. For example, in hth^{C1} homozygotes, the denticle belts present in the thoracic segments have an abdominal-like morphology, and the first abdominal segment (A1) is transformed into an identity that resembles the fifth abdominal segment (A5-like) (Figure 1F). Similar posterior-directed transformations are seen in other combinations of *hth* alleles. In the strongest allelic combination, $hth^{C1}/Dfhth$, segmental fusions are observed in addition to these transformations (Figure 1G). These fusions are a consequence, at least in part, of the loss of *engrailed* (*en*) expression (see below).

hth Is Required for the *Hox* Genes to Function Normally

The posterior-directed transformations observed in *hth* embryos could be due to the ectopic expression of the abdominal *Hox* genes, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), or *Abdominal-B* (*Abd-B*). To address this possibility, we examined the expression of these *Hox* genes in *hth* embryos. We also examined the expression of *Sex combs reduced* (*Scr*) and *Antennapedia* (*Antp*), which are required for thoracic development. All five of these *Hox* genes appear to be expressed normally in the ectoderm of *hth* embryos, including $hth^{C1}/Dfhth$ embryos (Figure 2). Therefore, the pattern alterations observed in *hth* mutants cannot be explained by obvious alterations in the expression of these *Hox* genes.

Although *Hox* gene expression appears normal in *hth* embryos, a low level of ectopic expression might be difficult to detect. To address this possibility, we determined if *hth* mutations cause pattern alterations when four of the five trunk *Hox* genes are eliminated by mutation. Embryos of this genotype, *Scr Antp abd-A Abd-B*, in which the only functional trunk *Hox* gene is *Ubx*, secrete cuticles that have a reiteration of the A1 segment throughout the abdomen (Figure 3A). When *hth* is also mutant in this background (*Scr Antp* hth^{5E} *abd-A Abd-B*), the abdominal segments all resemble the third abdominal segment (A3-like) (Figure 3B). Thus, the hth^{5E} mutation alters the pattern generated by *Ubx* even when the other four trunk *Hox* genes are nonfunctional.

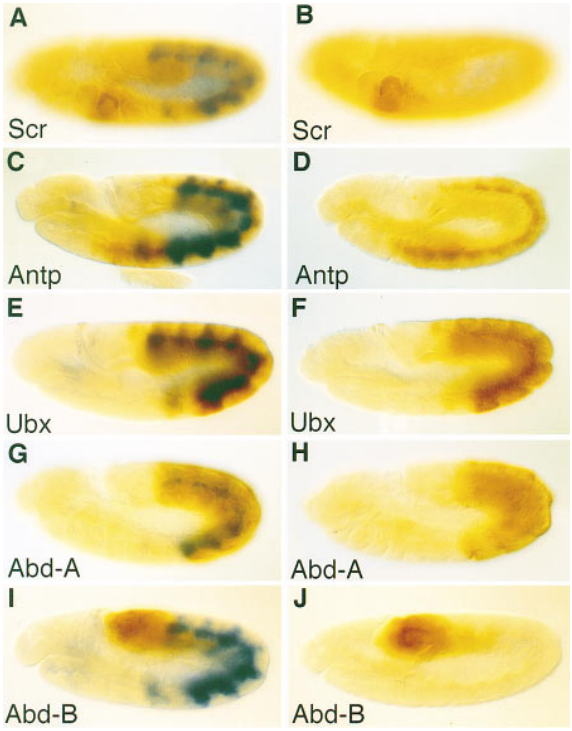

Figure 2. *hth* Does Not Affect Most *Hox* Gene Expression

Stage 11 *hth*/+ (A, C, E, G, and I) or hth^{C1}/*Dfhth* (B, D, F, H, and J) embryos stained for β-gal (all panels) and the HOX proteins SCR (A and B), ANTP (C and D), UBX (E and F), ABD-A (G and H), or ABD-B (I and J). The HOX proteins are detected with HRP (brown), and the absence of the β-gal staining (blue; derived from the *Ubx-lacZ* reporter gene present on the balancer) allowed the identification of the mutant embryos.

Although this experiment rules out the possibility that ectopic expression of *abd-A* or *Abd-B* are responsible for the transformations observed in *hth* embryos, it is still possible that effects on *Ubx* expression contribute to the *hth* phenotype. To address this possibility, we examined the segmental pattern generated by the ubiquitous expression of *Ubx*, in either the presence or absence of *hth* function. In the presence of hth^{+}, uniform *Ubx* expression transforms all three thoracic segments and 2–3 head segments into A1-like segments (Figure 3C) (Gonzalez-Reyes and Morata, 1990; Mann and Hogness, 1990). In contrast, in hth^{C1}/hth^{C1} embryos, ubiquitous *Ubx* expression transforms these segments into A5-like segments (Figure 3D). Thus, *hth* is required for *Ubx* to generate an A1 identity even when *Ubx* is expressed from a heterologous and ubiquitously active promoter. Using a similar approach, we also observe that *hth* is required for *Antp* to generate its normal output, a second thoracic (T2) segment (Figures 3E and 3F). These results suggest that *hth* does not function by modulating *Hox* gene expression.

Loss of *hth* Function Resembles the Complete Absence of *exd* Function

Like *hth*, embryos that are mutant for *exd* have posterior-directed segmental transformations but normal expression of the trunk *Hox* genes (Peifer and Wieschaus, 1990). *exd* is expressed both maternally and zygotically, and both sources of *exd* transcription contribute to embryogenesis (Peifer and Wieschaus, 1990; Rauskolb et al., 1993). In the absence of zygotically expressed *exd* (exd^{zyg^-}), weak posterior-directed transformations are observed in all three thoracic segments and in the anterior segments of the abdomen. When maternal and zygotic *exd* functions are eliminated (exd^{mat^-,zyg^-}), the transformations of segment identity are more extreme and segmental fusions are apparent (Peifer and Wieschaus, 1990). Strikingly, many of the characteristics of exd^{mat^-,zyg^-} embryos are very similar to those of hth^{C1}/*Dfhth* embryos (Figures 1G and 4H). In addition to the similar transformations observed in the embryonic cuticle, both hth^{C1}/*Dfhth* and exd^{mat^-,zyg^-} embryos show a loss of *en* expression in the ectoderm of stage 12 and older embryos (Figures 4A and 4B) (Peifer and Wieschaus, 1990). As with exd^{mat^-,zyg^-} embryos, younger hth^{C1}/*Dfhth* embryos show normal *en* expression (not shown). In the embryonic midguts of both hth^{C1}/*Dfhth* and exd^{mat^-,zyg^-} embryos, *teashirt* (*tsh*) expression is reduced and *decapentaplegic* (*dpp*) is ectopically expressed anterior to its normal expression domain (Figures 4C and 4D and data not shown) (Rauskolb and Wieschaus, 1994).

Unlike *exd*, *hth* functions do not appear to be maternally provided. When crossed to hth^{C1}/balancer males, females with hth^{C1}/hth^{C1} germlines generate embryos that appear indistinguishable from hth^{C1}/hth^{C1} embryos derived from females with hth^{C1}/hth^{+} germlines (data not shown). Thus, reducing *hth* function from the maternal germline does not increase the severity of the *hth* mutant phenotype.

Because the zygotic loss of *hth* appears to phenocopy the exd^{mat^-,zyg^-} phenotype, we wondered if *exd* and *hth* acted independently or within the same genetic pathway. If they act in the same pathway, then one gene might be epistatic to the other. In fact, when *exd* maternal and zygotic functions are eliminated, the *hth* genotype appears irrelevant: the cuticles secreted by exd^{mat^-,zyg^-}; hth^{C1}/*Dfhth* embryos appear indistinguishable from those secreted by exd^{mat^-,zyg^-}; hth^{+} embryos (data not shown). These data suggest that for many of its functions, hth^{+} requires exd^{+}. We also observe a genetic interaction between *hth* and *exd*: exd^{zyg^-}; hth^{C1}/hth^{C1} embryos are more strongly transformed than exd^{zyg^-} embryos or hth^{C1}/hth^{C1} embryos (Figures 4E–4G). Moreover, the phenotype of exd^{zyg^-}; hth^{C1}/hth^{C1} embryos resembles the exd^{mat^-,zyg^-} phenotype (Figures 4G and 4H).

In the Absence of hth^{+}, EXD Protein Is in the Cytoplasm

The subcellular localization of EXD protein is regulated during Drosophila development (Mann and Abu-Shaar, 1996; Aspland and White, 1997). Furthermore, most places where there is a genetic requirement for *exd*, EXD protein is localized to nuclei (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995). Therefore, the genetic data described above could be explained if hth^{+} is required for EXD's nuclear localization. To test this possibility, we immunostained *hth* embryos with an antibody to detect EXD protein. Throughout embryogenesis, EXD is observed primarily in the cytoplasm in hth^{C1}/

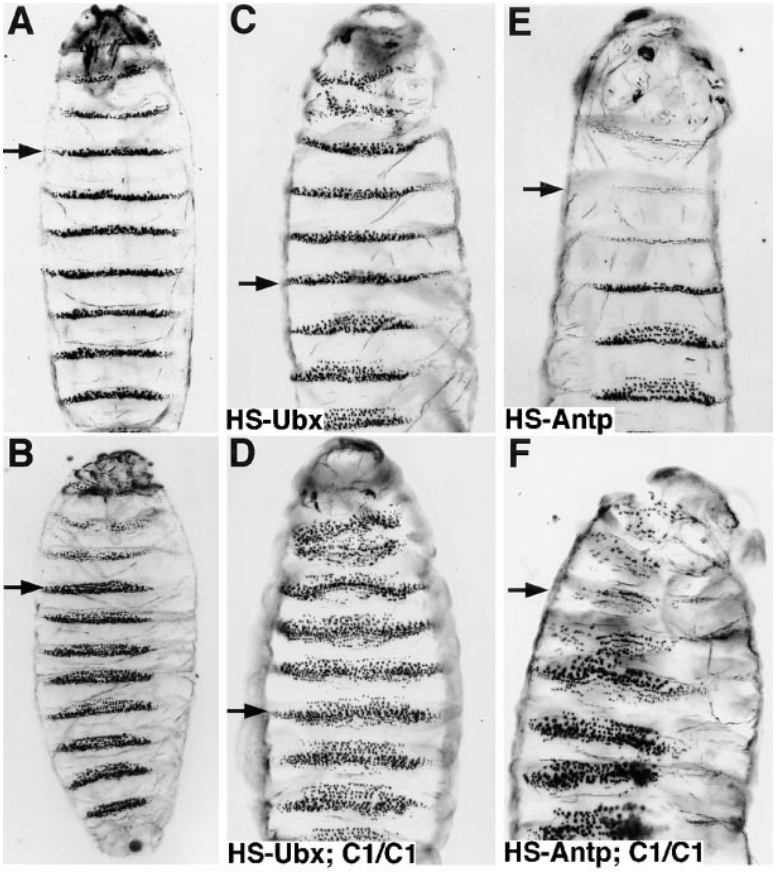

Figure 3. *hth* Is Required for the *Hox* Genes to Execute Their Normal Functions

The ventral surfaces of the cuticles secreted by *Scr Antp abd-A Abd-B* (A), *Scr Antp hth*^{5E} *abd-A AbdB* (B), *HS:Ubx* (C), *HS:Ubx*; *hth*^{C1}/*hth*^{C1} (D), *HS:Antp* (E), and *HS:Antp*; *hth*^{C1}/*hth*^{C1} (F) embryos. The arrows point to the position of A1 (A–D) or T2 (E and F).

Dfhth embryos (Figures 5B, 5D, and 5F). Double stains with other nuclear proteins, for example TSH, UBX, or EN, illustrate that these proteins are localized to nuclei normally in these embryos (Figure 5). Moreover, in embryos that are homozygous for *Dfhth* (an RNA null allele), EXD is only observed in the cytoplasm (data not shown). The requirement for *hth*$^{+}$ for EXD's nuclear localization is apparent in many embryonic tissues, including the ectoderm, visceral mesoderm, and endoderm (Figure 5). This requirement is observed in cells where the signaling molecules WG and DPP contribute to EXD's nuclear translocation (e.g., the endoderm) (Mann and Abu-Shaar, 1996), and in tissues where no signaling molecules are known to be required for EXD's nuclear localization (e.g., the visceral mesoderm).

hth Encodes a Homeodomain Protein with Similarity to Murine MEIS1

We identified two *lacZ*-encoding P element insertions generated by the Drosophila genome project that are allelic to *hth* (*hth*^{P5} and *hth*^{P6}). Excision of these P elements using P transposase reverts the *hth* mutation present on these chromosomes. These P elements map very closely to the Drosophila homolog of a murine homeobox gene called myeloid ecotropic insertion site 1 (*Meis1*), at 86C1 (Moskow et al., 1995; Sun et al., 1995; H. Sun, personal communication). The homeodomain encoded by *Meis1* is approximately 50% identical to the EXD homeodomain (Moskow et al., 1995). Because of the similarity between the *hth* and *exd* mutant phenotypes, as well as between the MEIS1 and EXD homeodomains, we tested the possibility that *hth* is a Drosophila homolog of *Meis1* by attempting to rescue the *hth* mutant phenotype using a *Meis1* cDNA. When expressed via a heat shock promoter during embryogenesis, the *Meis1* cDNA can partially rescue the *hth*^{C1}/*hth*^{C1} embryonic phenotype (data not shown). Together with the evidence presented below, we conclude that *hth* is a Drosophila homolog of *Meis1*.

Using the *hth*^{P5} and *hth*^{P6} P elements, we rescued genomic DNA fragments from the *hth* locus and used these to identify genomic phage DNA clones derived from this region. We also isolated several Drosophila cDNAs that hybridized under low stringency to *Meis1*. One of these, clone #7, hybridizes to genomic phage that are derived from the *hth* locus. When used in in situ hybridization experiments, clone #7 sequences identify an expression pattern, described below, that is absent in *Dfhth* embryos. The clone #7 cDNA is 3.5 kb and contains a 458 amino acid open reading frame with two regions that are very similar to MEIS1 (Figure 6). The first is a region in the N-terminal third of the protein, termed the Homothorax-MEIS (HM) domain, that is identical to MEIS1 in 105/119 amino acids. The second region includes the homeodomain and is identical to MEIS1 in 69/73 amino acids. The HTH homeodomain belongs to an atypical class that is characterized by an extra three amino acids between helices 1 and 2 (Burglin, 1994). EXD's homeodomain is also in this class, and the

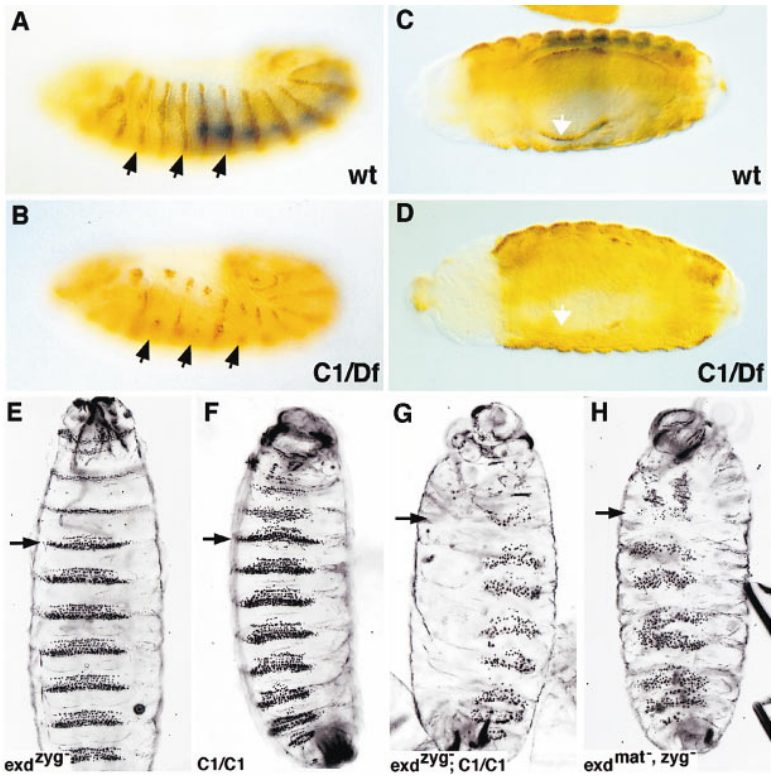

Figure 4. Loss of *hth* Function Is Similar to the Complete Loss of *exd*

(A–D) Wild-type (A and C) or $hth^{C1}/Dfhth$ (B and D) embryos stained for β-gal (all panels), and EN (A and B) or TSH (C and D). EN and TSH were detected by HRP staining (brown), and the absence of β-gal (blue) identified the mutant embryos. The arrows in (A) and (B) point to even-numbered EN stripes that are more strongly affected than odd-numbered stripes in the mutant. The arrows in (C) and (D) point to TSH expression in the anterior visceral mesoderm, which is absent in the mutant.

(E)–(H) Cuticles secreted by exd^{zyg^-} (E), hth^{C1}/hth^{C1} (F), exd^{zyg^-}; hth^{C1}/hth^{C1} (G), and exd^{mat^-, zyg^-} (H) embryos.

HTH and EXD homeodomains are identical (similar) in 27 (37)/63 amino acids.

hth Is Expressed Where EXD Is Localized to Nuclei

We characterized the *hth* expression pattern by in situ hybridization with cDNA probes and by monitoring *lacZ* expression from the *hth* P element insertions P5 and P6. With the exception that P5 is expressed more weakly than P6, all three methods of detecting *hth* expression produce very similar results. Consistent with a lack of a maternal function, *hth* transcripts are first detected at approximately 3 hr of embryogenesis in a broad domain in the central portion of the blastoderm embryo, from approximately 15% to 85% egg length. In addition to a lack of expression at both poles, the ventral-most cells of the embryo, corresponding to the mesoderm primordium, are unstained (Figure 7A). As embryogenesis proceeds, the expression pattern becomes very dynamic. Expression is strongest in the trunk and in isolated regions of the head (Figures 7B and 7D). Beginning at ~stage 9, the thoracic segments stain more strongly than the abdominal segments, and this difference increases as embryogenesis proceeds. By stage 14, expression in the thorax, including the central nervous system, remains strong but has been down-regulated in the abdomen. In the midgut, expression is strongest in the gastric caeca primordia and in a central, broad domain in the endoderm. Expression is absent in the most anterior and posterior regions of the midgut endoderm (Figure 7D). By stage 16, strong expression is observed in the malphigian tubules, and expression in the endoderm begins posterior to the first midgut constriction and ends just anterior to the third midgut constriction, with a peak of expression at the second midgut constriction.

Because *hth* is important for EXD's nuclear localization, we determined to what extent *hth* expression correlates with nuclear-localized EXD. To address this, we compared the pattern of nuclear-localized EXD detected with the anti-EXD antibody with the *hth* expression pattern detected by in situ hybridization. In addition, we doubly stained embryos and imaginal discs from the P6 *hth* enhancer trap line with antibodies to EXD and β-galactosidase (β-gal). Strikingly, in all cells where *hth* or the P6 element are expressed, EXD is localized to nuclei. Conversely, in most, but not all, cells where EXD is nuclear, *hth* is expressed. For example, during embryogenesis, EXD is cytoplasmic in the labial segment and in the limb primordia cells that express the gene *Distal-less* (*Dll*) (Figures 7C and 7H) (Mann and Abu-Shaar, 1996). For both of these cell types, both enhancer traps (P5 and P6) and *hth* are not expressed (Figures 7B and 7G and data not shown). Conversely, *hth* is expressed in neighboring cells where EXD is present in nuclei (Figures 7B and 7G).

The correlation between *hth* expression and nuclear-localized EXD can also be observed in leg and antennal imaginal discs: EXD is nuclear in only the peripheral cells of leg and antennal discs and is cytoplasmic in the central portion of these discs (Figures 7E, 7F, 7I, and 7J). Like nuclear EXD, *hth* is expressed only in the peripheral cells of these discs, and expression of β-gal from the P6 element colocalizes with nuclear EXD (Figures 7I and 7K). We note, however, that in a few places in the

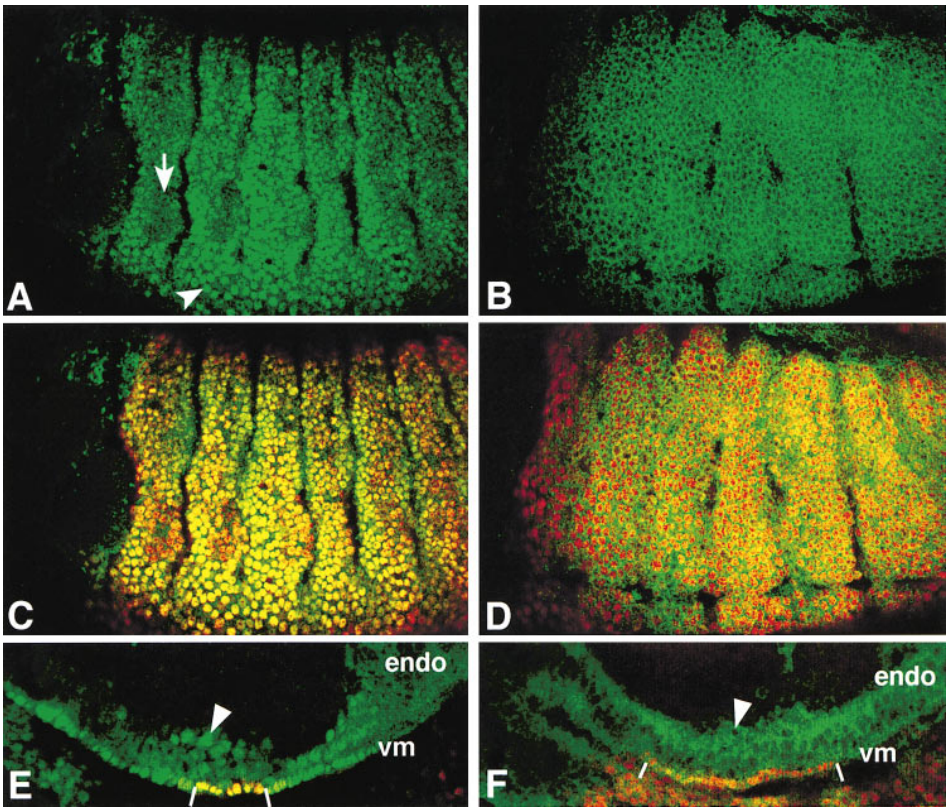

Figure 5. *hth* Is Required for EXD's Nuclear Localization

Confocal images of wild-type (A, C, and E) or hth^{C1}/*Dfhth* (B, D, and F) stage 14 embryos stained for EXD (in green; all panels), TSH (in red; [C] and [D]), or UBX (in red; [E] and [F]) proteins. The embryos in (B) and (D) are the same as those in (A) and (C), respectively; in (A) and (C), only the EXD signal is shown. The focal plane in (A)–(D) shows the ectoderm nuclei of the thoracic and anterior abdominal segments. The normal pattern of EXD (A) includes cytoplasmic (arrow) and nuclear (arrowhead) localizations, whereas TSH (C) is exclusively nuclear. In (E) and (F), the focal plane shows the visceral mesoderm (vm) and endoderm (endo) of the midgut. The small open bars indicate the extent of UBX expression in the (vm); note that in the mutant (F), UBX expression is broader than in the wild type (E). Whereas TSH and UBX are localized to nuclei in wild-type and hth^{C1}/*Dfhth* embryos, EXD is nuclear only in wild type.

imaginal discs, for example, the region of the antennal disc that gives rise to the maxillary palps, EXD is localized to nuclei without detectable expression of *hth* (Figures 7E and 7F). Thus, *hth* expression correlates with nuclear-localized EXD in most, but not all, imaginal disc cells.

MEIS1/HTH Can Induce the Nuclear Translocation of EXD

If *hth* is responsible for localizing EXD to nuclei, it might be possible to induce EXD's translocation in a cell culture system by expressing *hth* or *Meis1*. To test this, we constructed a version of the MEIS1 protein with a MYC epitope at its N terminus (MYC-MEIS1) and performed transfection experiments in Schneider Line 2 (S2) cells. In untransfected S2 cells, *exd* is expressed and EXD protein can be detected only in the cytoplasm (Figures 8A–8C). Even when expressed at high levels by transient transfection, EXD is predominantly found in the cytoplasm; however, under these conditions, some EXD is observed in nuclei (data not shown). In contrast, MYC-MEIS1 is predominantly found in nuclei following its expression in S2 cells. Moreover, virtually all of the cells expressing MYC-MEIS1 have EXD present in nuclei, not in the cytoplasm (Figures 8B and 8C). The shift in EXD's subcellular localization can be observed in less than one hour after induction of MYC-MEIS1 expression. The nuclear translocation of EXD is also induced by HTH in S2 cells (data not shown). Further, HTH can induce EXD's nuclear transport even when Asn-51 of the HTH homeodomain, which in other homeodomains provides important contacts with DNA, has been mutated to Ala ($HTH^{[N51A]}$) (data not shown). In contrast, expression of the Labial homeodomain protein, which interacts with EXD in vitro (Chan et al., 1996), does not alter the subcellular localization of EXD in S2 cells (Figure 8A).

We also generated transformed fly lines that express the MYC-MEIS1 fusion protein via a heat shock promoter (*HS:Myc-Meis1*). In wild-type blastoderm embryos, EXD is found exclusively in the cytoplasm (Figure 7D). In contrast, when MYC-MEIS1 is expressed uniformly in blastoderm embryos, EXD is observed in all nuclei (Figure 8E). MYC-MEIS1 is also only observed in nuclei in these embryos (not shown). Thus, in either S2 cells or when ectopically expressed in a Drosophila embryo, a mouse homolog of *hth* can induce the cytoplasmic to nuclear translocation of EXD.

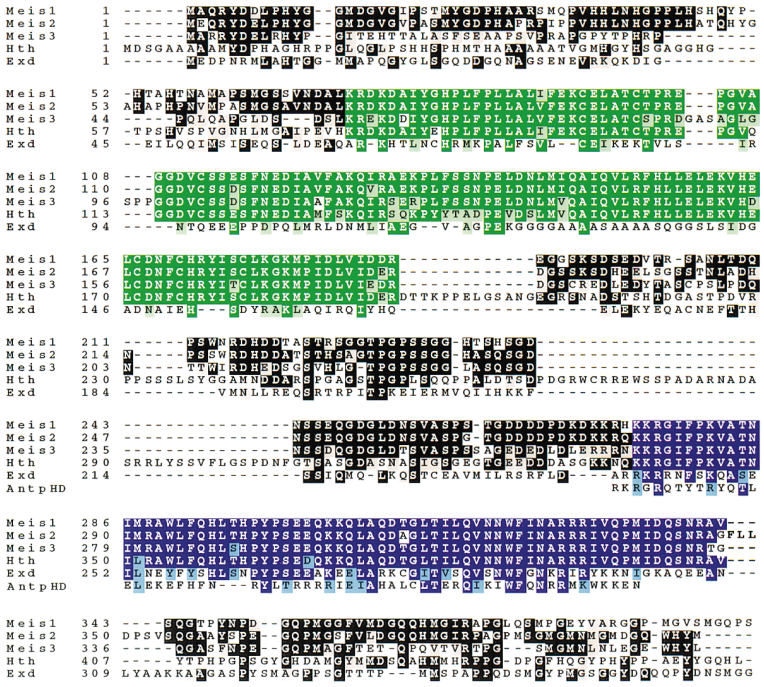

Figure 6. The Sequence of the Predicted HTH ORF

The HTH ORF is compared to MEIS1b (Moskow et al., 1995), MEIS2 and MEIS3 (Nakamura et al., 1996a), and EXD (Rauskolb et al., 1993). Green and blue highlighted regions indicate the HM and homeodomains, respectively; darker and lighter colors indicate identities and similarities, respectively. Amino acids that are highlighted in black/gray indicate additional homology outside these domains. For reference, the 60 amino acid ANTP homeodomain (ANTP-HD) is shown; the gap in the ANTP-HD sequence is in the loop between helices 1 and 2, where three additional amino acids are present in the HTH and MEIS homeodomains. Of the three MEIS proteins, HTH has the best match to MEIS1. Although EXD is most similar to the MEIS/HTH proteins in the homeodomain, EXD appears weakly related throughout its ORF.

MEIS1/HTH Interacts with EXD In Vitro

MEIS1/HTH could induce the nuclear translocation of EXD by a direct interaction between these two proteins. Alternatively, MEIS1/HTH could act indirectly, by inducing the expression of another gene whose product is directly responsible for EXD's nuclear translocation. The first possibility predicts that EXD and MEIS1/HTH might directly interact in vitro. To address this, we tested if a fusion protein between glutathione-S-transferase and MEIS1 (GST-MEIS1) is able to "pull-down" EXD from a Drosophila embryo extract. Crude E. coli extracts containing GST or GST-MEIS1 were incubated with a crude Drosophila embryo extract and complexes were purified using glutathione-agarose (Figure 8F). Complexes were washed, resolved by SDS–PAGE, transferred to nitrocellulose, and probed with the anti-EXD antibody. A band was detected in the GST-MEIS1 lane (lane 4), but not in the GST lane (lane 3), that comigrates with full-length, purified histidine-tagged EXD (His-EXD) synthesized in E. coli (lane 1) and with a band detected in the input embryo extract (lane 2). Thus, MEIS1 is able to specifically bind to EXD present in an embryo extract.

It is possible that the MEIS1–EXD interaction identified in the pull-down experiments is mediated by another protein present in the Drosophila extract. To determine if EXD and MEIS1 can directly interact with each other, we mixed crude E. coli extracts containing GST-MEIS1 or GST with purified His-EXD, and GST-containing complexes were purified using glutathione-agarose. SDS–PAGE and Western blotting with the anti-EXD antibody demonstrate that GST-MEIS1, but not GST, is able to directly bind EXD in vitro (Figure 8G). In addition, other GST fusion proteins (for example, GST-RHR, containing the rel homology region of p65) do not interact with EXD in this assay (not shown). From these experiments, we estimate that EXD has an affinity for MEIS1 that is greater than 10^{-9} M. Like MEIS1, we have confirmed that HTH has a similar affinity for EXD in vitro (data not shown).

Discussion

A Conserved Pathway Integrating HOX Specificity with the Control of HOX Activity

A central problem in understanding how the HOX proteins control developmental pathways has been to reconcile their highly specific functions in vivo with their promiscuous in vitro DNA binding properties. DNA binding cofactors, such as EXD and PBX (the PBC family), have provided a partial explanation to this paradox. PBC proteins can bind to DNA cooperatively with HOX proteins, and PBC-HOX heterodimers bind DNA with greater sequence specificity than HOX monomers (reviewed in Mann and Chan, 1996). Moreover, consistent with EXD's ability to interact with HOX proteins, *exd* is required for the HOX proteins to execute their normal functions in vivo (Peifer and Wieschaus, 1990; Chan et al., 1994, 1996, 1997; Rauskolb and Wieschaus, 1994; Gonzalez-Crespo and Morata, 1995; Pöpperl et al., 1995; Rauskolb et al., 1995; Pinsonneault et al., 1997). The discovery that the subcellular distribution of EXD protein is regulated during development (Mann and Abu-Shaar, 1996; Aspland and White, 1997) suggests an additional function for EXD: for those HOX functions that require EXD as a cofactor, the regulation of EXD's subcellular localization is also a way to control HOX activity.

Here we demonstrate that *hth* plays an important part in the control of EXD's nuclear localization. We show that, like *exd*, *hth* alters the functional output of the HOX proteins. Further, at least some of *hth*'s functions are mediated by *exd* because *hth* is required for EXD's transport into nuclei in many places during development. Our results are consistent with the idea that the expression

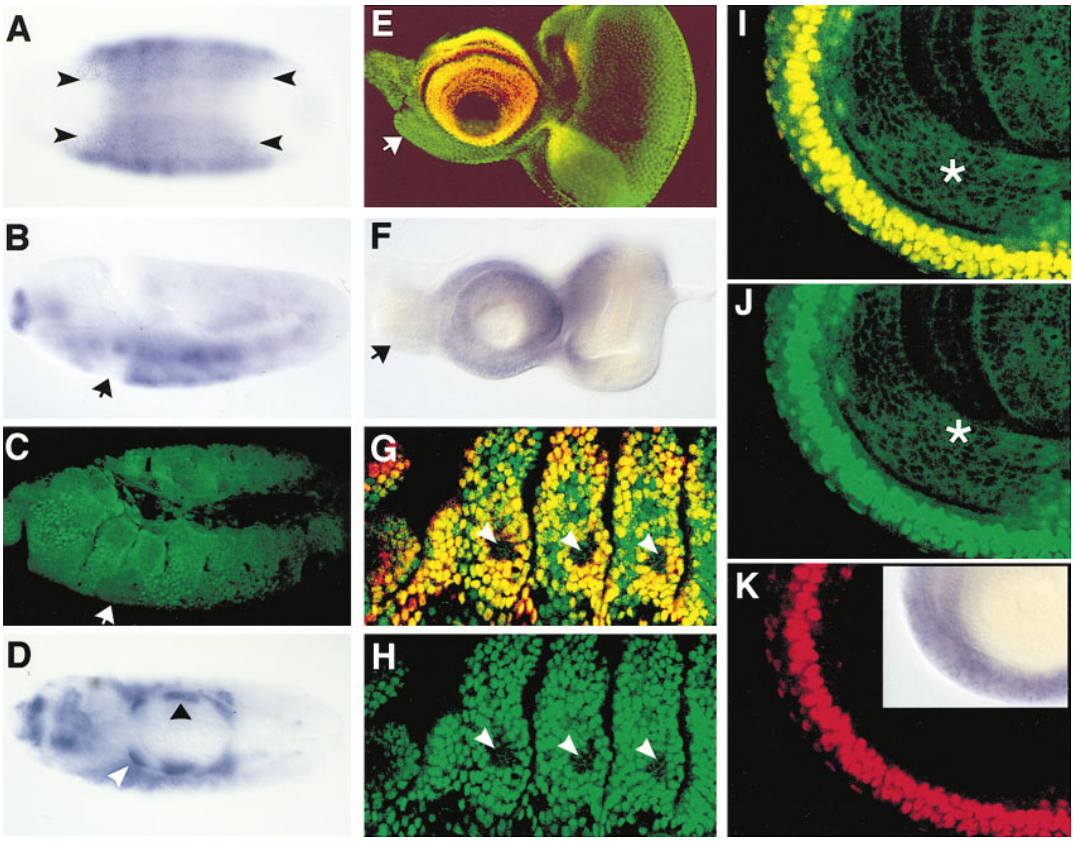

Figure 7. *hth* Expression Correlates with Nuclear-Localized EXD

(A) Blastoderm embryo stained for *hth* RNA. No *hth* expression is seen at the poles or close to the ventral midline; the arrowheads point to sharp ventral expression boundaries.

(B and C) Stage 11 embryos, stained for *hth* RNA (B) or EXD protein (C). The arrows point to cytoplasmic EXD (C) and lack of *hth* expression (B) in the labial segment.

(D) Stage 14 embryo stained for *hth* RNA showing strong expression in the head, gastric caeca primordia (open arrowhead), and central portion of the midgut endoderm (closed arrowhead).

(E and F) Eye-antennal imaginal discs. (E) is stained for EXD protein (green) and β-gal from the hth^{P6} enhancer trap (red), and (F) is stained for *hth* RNA. Although most of the nuclear-localized EXD correlates with *hth* expression, the maxillary palp primordia (arrows) has nuclear EXD and no detectable *hth* expression.

(G and H) The leg primordia region of a stage 14 embryo stained for EXD protein (green) and β-gal from the hth^{P6} enhancer trap (red). EXD is cytoplasmic in the leg primordia (arrowheads) but nuclear in the surrounding cells, where *hth* is also expressed.

(I–K) A sector of a leg imaginal disc stained for EXD protein (green) and β-gal from the hth^{P6} enhancer trap (red). Cytoplasmic EXD is visible in the central region of the disc (asterisk), whereas nuclear EXD and *hth* expression are visible at the periphery of the disc. The inset in (K) shows a leg disc stained for *hth* RNA, confirming expression only at the periphery of the disc.

of *hth* is sufficient to induce the nuclear localization of EXD. We note, however, that while *hth* expression is required for localizing EXD to most nuclei, some imaginal disc cells can localize EXD to the nucleus in a *hth*-independent manner. For these cells, EXD's nuclear transport may require another as yet unidentified protein. Although HTH is most similar to MEIS1, in the mouse there are at least three *Meis1*-related genes (*Meis1*, *Meis2*, and *Meis3*) (Nakamura et al., 1996a), suggesting the possibility that there may be additional members of this gene family in Drosophila.

Because HTH is responsible for localizing EXD to many nuclei, it follows that the control of *hth* expression, which appears to be complex, indirectly controls the ability to form EXD-HOX heterodimers and, consequently, affects HOX functions that require EXD as a cofactor. Such a mechanism provides an additional level of complexity that integrates HOX specificity with the control of HOX activity. A knowledge, therefore, of how *hth* expression is regulated will be important for understanding this mechanism of HOX regulation. For example, in the endoderm cells of the embryonic midgut, where EXD's nuclear localization is triggered by DPP and WG (Mann and Abu-Shaar, 1996), *hth* is strongly expressed (Figure 7), and strong expression at this stage requires these signaling molecules (our unpublished data). Thus, the induction of EXD's nuclear localization by DPP and WG in these cells is probably a consequence of activating *hth* transcription. Similarly, other controls of *hth* expression, which may not depend on DPP or WG, regulate EXD's localization and, consequently, HOX activity.

This mechanism for controlling HOX activity is likely to be evolutionarily conserved. All of the relevant components identified in Drosophila (HOX, EXD, and HTH) are also present in vertebrates (HOX, PBX, and MEIS).

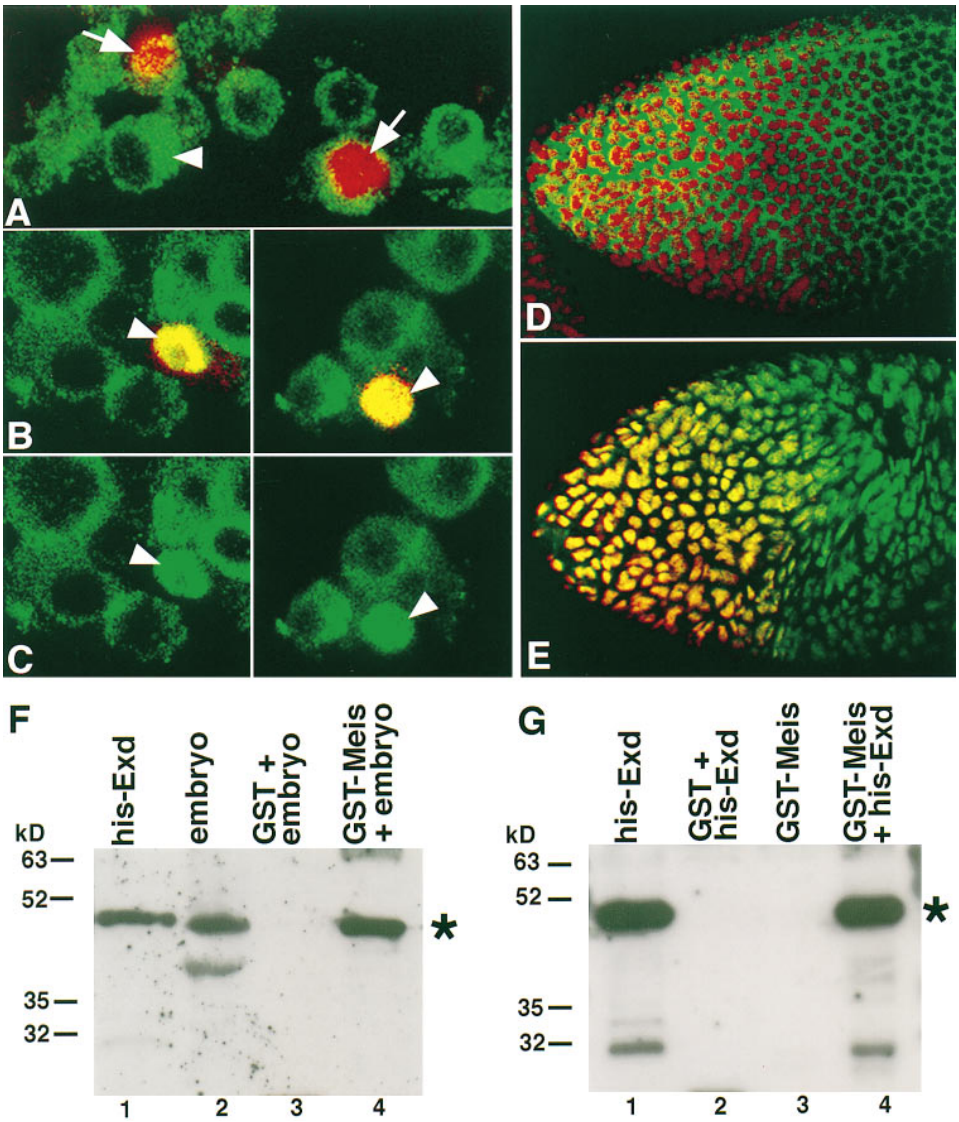

Figure 8. MEIS1 Induces the Cytoplasm to Nuclear Translocation in S2 Cells and in Blastoderm Embryos and Directly Interacts with EXD In Vitro

(A–E) Confocal images of S2 cells (A–C) or blastoderm embryos (D and E) stained for EXD (in green; all panels), Labial (in red; [A]), MYC-MEIS1 (in red; [B]), or Hunchback (in red; [D] and [E]). The cells in (A) were transfected with a *labial* expression construct, and two *labial*-expressing cells can be seen (arrows). EXD is cytoplasmic in the transfected and untransfected cells (arrowhead).

(B and C) Two examples of S2 cells transfected with a *Myc-Meis1* expression construct and imaged for EXD and MYC-MEIS1 (B) or only EXD (C). EXD is nuclear only in the cells in which MYC-MEIS1 is present (arrowheads).

(D and E) The anterior halves of heat-shocked wild-type (D) or *HS:Myc-Meis1* (E) precellular blastoderm embryos stained for Hunchback (in red; a marker for anterior nuclei) and EXD (in green). Although EXD is cytoplasmic in the heat-shocked wild-type embryo (D), it is nuclear after ubiquitous expression of MYC-MEIS1 (E). The nuclei appear slightly disordered due to the very early heat shocks.

(F and G) Western blots of SDS–PAGE gels probed with the anti-EXD antibody. (F) 100 ng His-EXD (lane 1); embryo extract (lane 2); embryo extract pulled-down with GST (lane 3); embryo extract pulled down with GST-MEIS1 (lane 4). (G) 100 ng His-EXD (lane 1); 100 ng His-EXD pulled down with GST (lane 2); GST-MEIS1 (lane 3); 100 ng His-EXD pulled down with GST-MEIS1 (lane 4). The asterisks indicate the position of EXD.

Further, HOX-EXD and HOX-PBX heterodimers, together with their binding sites, appear to be functionally and structurally analogous (reviewed by Mann and Chan, 1996; Chan et al., 1997). Finally, the data presented here demonstrate that the murine MEIS1 protein can compensate for the absence of HTH in Drosophila embryos. Thus, it is likely that the vertebrate HOX proteins are being similarly regulated by the PBX and MEIS proteins.

Does MEIS1/HTH Form Heterodimers with HOX Proteins?

The similarity between the MEIS/HTH and EXD homeodomains raises the possibility that, like EXD, MEIS/HTH binds to DNA cooperatively with HOX proteins. Although MEIS/HTH may interact with HOX proteins on some binding sites, we have observed that HTH and EXD have different DNA binding properties, and that HTH-HOX heterodimers do not form on previously characterized

EXD-HOX binding sites (H. D. R. and R. S. M., unpublished data).

However, there is some evidence that MEIS1 collaborates with HOX proteins in vivo. *Meis1* was identified because retroviral insertions into the *Meis1* gene of BXH-2 mice result in myeloid leukemias (Moskow et al., 1995). Interestingly, most (19/20) of the leukemias that have an insertion at *Meis1* also have a second retrovirus inserted at *Hox-a7* or *Hox-a9* (Nakamura et al., 1996b). This finding implies that these leukemias are caused by the coactivation of *Meis1* and either *Hox-a7* or *Hox-a9*. This coactivation could suggest that MEIS1 and HOX proteins cooperatively interact in vivo, in a manner that is analogous to EXD–HOX interactions (Nakamura et al., 1996b). Although this may be the case, our results suggest an additional possibility: that the activation of *Meis1* results in the inappropriate nuclear localization of one or more of the murine homologs of EXD, the PBX proteins. Consistent with this possibility, although human chromosomal translocations that activate *Pbx1* (by fusing it to the *E2A* gene) cause pre-B-cell leukemias, in mice the *E2A-Pbx1* oncogene can cause myeloid leukemias similar to those induced by activation of *Meis1* (Dedera et al., 1993; Kamps and Baltimore, 1993). Thus, it remains an open question if the leukemias induced by *Meis1* activation are caused by a direct interaction between MEIS1 and HOX proteins, by a mechanism that acts indirectly via the PBX proteins, or by a combination of these two mechanisms.

Homeodomain Proteins as Nuclear Escorts

Homeodomain proteins are important for many aspects of development and are generally thought to function in the nucleus by binding to DNA and regulating the transcription of specific target genes (Gehring et al., 1994). One exception is the ability of the Bicoid homeodomain protein to specifically bind the *caudal* mRNA and repress its translation (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996; Chan and Struhl, 1997). Here, we provide evidence that homeodomain proteins also control the subcellular localization of other proteins.

We envision three models for how HTH controls EXD's subcellular localization. The first is an indirect mechanism whereby HTH acts as a transcription factor that regulates the expression of another gene whose product directly controls EXD's localization. The second and third models both involve a direct interaction between the HTH and EXD proteins. In the first of these direct interaction models, HTH acts in the nucleus as an "EXD trap" by binding to EXD and preventing its export to the cytoplasm. In the second of these models, HTH binds to EXD in the cytoplasm and acts as its nuclear escort.

Although we cannot exclude the indirect model, our data suggest that HTH controls EXD's nuclear translocation by a direct protein–protein interaction, and not by regulating the expression of another gene product. This suggestion is based on the findings that MEIS1/HTH can directly interact with EXD in vitro and that MEIS1/HTH can induce the translocation of EXD from the cytoplasm to the nucleus in S2 cells. The speed in which MEIS1 can induce EXD's nuclear translocation (less than 1 hr) makes it less likely that MEIS1 is acting via an intermediate gene. Further, EXD's nuclear transport in S2 cells can be induced by HTH$^{[N51A]}$, in which a key DNA binding residue in the HTH homeodomain has been mutated to Ala. Although we favor a mechanism that depends on a direct interaction between HTH and EXD, we cannot at this time discriminate between the trapping and escort models.

Multiple Partners for EXD

Previous work has suggested that when present in nuclei, EXD functions, at least in part, by forming heterodimers with HOX proteins on specific DNA sequences (reviewed by Mann and Chan, 1996). The data presented here suggest that EXD also binds to HTH and that the HTH–EXD interaction is important for EXD's nuclear localization. The HTH–EXD interaction, which is at least in the nanomolar range, may be stronger than most PBC–HOX interactions (Chan et al., 1996; Lu and Kamps, 1996). Moreover, like EXD and HOX proteins, HTH contains a homeodomain that is likely to bind DNA. These findings raise the following questions: how does EXD interact with both HOX and HTH proteins and what is the role of the HTH homeodomain? We suggest that for some binding sites, HTH is displaced from the HTH–EXD complex upon the formation of an EXD–HOX–DNA complex, whereas for other binding sites, the HTH–EXD interaction is maintained, resulting in a HTH–EXD–HOX–DNA complex. The HTH homeodomain and the sequence of the binding site may determine if HTH is displaced or not. Consistent with this idea, we have observed that in the presence of EXD and the appropriate HOX protein, HTH can bind some EXD–HOX binding sites, but not others (our unpublished data). Thus, some, but not all, EXD–HOX binding sites also contain a HTH binding site. In the future, it will be important to determine the function of HTH binding sites in vivo, especially those located close to EXD–HOX binding sites.

Finally, we note that an interesting implication of these findings is that the control of HOX activity by HTH may, in turn, provide additional specificity to the HOX proteins. The presence of a HTH binding site might increase specificity by providing additional protein–protein and protein–DNA contacts. HTH might also modify how EXD or HOX proteins bind DNA. Our findings suggest that the control of HOX activity by EXD and HTH is intimately linked to the control of HOX specificity.

Experimental Procedures

Fly Stocks

hth^{5E} (Jürgens et al., 1984) has a partially penetrant dominant phenotype: extra pigment in the fourth abdominal tergite of adult males (first recognized by S. Tiong and R. Whittle). This phenotype is easier to score when in *trans* to *Abd-B*$^{Mcp\text{-}1}$, which also affects the pigmentation of this tergite (Karch et al., 1985). Additional *hth* alleles were generated by mutagenizing males with EMS and mating them to *Abd-B*$^{Mcp\text{-}1}$/+ females. Of ~300,000 F1 males, ~50 showed the adult phenotype, and 2 (*hth*^{B2} and *hth*^{C1}) did not complement *hth*^{5E}. *Dfhth* is an X-ray-induced excision of P[w^+ (l(3)j9A5)] at 85F15–16. *Dfhth* shows the tergite phenotype, fails to complement other *hth* alleles, has a rearrangement at 86B-C (by polytene chromosome squashes), and is an RNA null allele of *hth* (by in situ hybridization). *Dfhth* homozygotes do not secrete a cuticle. Because *hth*^{C1}/*Dfhth* embryos are similar to *exd*$^{mat^-,zyg^-}$ embryos and have a strong effect

on EXD's localization, this genotype may be close to a complete loss of *hth* function. *hth*^{C1}/*Dfhth* embryos were identified by the lack of β-gal expression from the TM6B-22UZ balancer. *hth*^{P5} and *hth*^{P6}, generated by the BDGP (l(3)05745^{05745} and l(3)05745^{06762}, respectively) (Karpen and Spradling, 1992), fail to complement other *hth* alleles and generate weak transformations of the cuticle. In addition, *hth* alleles fail to complement P{lacW}MEIS1^{P2} (Sun et al., 1995). The *Scr*^{C1} *Antp*$^{Ns+RC3}$ *hth*^{5E} *abd-A*^{M1} *Abd-B*^{M8}/TM6B, *hth*^{C1}/TM6B; *HS:Ubx*/CYO, and *hth*^{C1}/TM6B; *HS:Antp*/CYO stocks were generated using previously described stocks (Feinstein et al., 1995). To test if *hth* is required maternally, *FRT82 hth*^{C1}/*FRT82 ovo*^{D}; *heat-shock-flp*/+ larvae were heat-shocked and resulting females were crossed to *hth*^{C1}/MKRS males (Chou et al., 1993). Of ~100 cuticles, none were more severe than the *hth*^{C1} zygotic phenotype. To generate *exd*$^{mat^-,zyg^-}$ embryos, we used flp-mediated recombination, an X-linked *ovo*^{D} mutation, and the *exd* null allele *exd*^{XP11}.

hth Cloning and Rescue by *Meis1*

Genomic sequences next to the P5 and P6 P elements were isolated (Hamilton et al., 1991) and used to probe a λ-DashII genomic library (Stratagene). A 1.1 kb SmaI fragment from the *Meis1* cDNA (Moskow et al., 1995) was ^{32}P-labeled and used to probe an antennal cDNA library under low stringency (from H. Amrein, L. Vosshall, and R. Axel). The longest clone (#7) was sequenced. Blast searches (NCBI) identified MEIS1b (Moskow et al., 1995) as the best match to clone #7. Rescue by MEIS1 was carried out by heat shocking 3–6 hr *hth*^{C1}/TM6B-22UZ; *HS:Myc-Meis1*/CYO larvae for 30 min at 37°C. In ~66% of the *hth*^{C1}/*hth*^{C1} larvae, the thoracic and A1 segments were partially rescued. In a wild-type background, MEIS1 expression results in nuclear EXD and produces cuticle defects that may account for the incomplete rescue.

Expression Constructs

The entire MEIS1b ORF was fused in-frame to a tag containing six MYC epitopes (D. Turner and H. Weintraub) that had been optimized for translation in Drosophila, and the MYC-MEIS1b fusion ORF was inserted into pRM-HA-3 (Fortini and Artavanis-Tsakonas, 1994). The complete *labial* ORF was also cloned into this vector. Transfection, Cu^{++} induction, and antibody staining of S2 cells were described (Fortini and Artavanis-Tsakonas, 1994) except that 0.1% Triton X-100 was used in the incubations. The MYC-MEIS1b ORF was cloned under the control of a heat shock promoter in a P element vector, and Drosophila transformants were generated. The nuclear translocation of EXD by MYC-MEIS1 in embryos was induced by heat shocking 0–3 hr *HS:Myc-Meis1* embryos for 30 min and allowing them to recover for 1 hr before fixation and antibody staining.

Antibody, In Situ, and Cuticle Analysis

The EXD (Mann and Abu-Shaar, 1996), Labial (Chouinard and Kaufman, 1991), UBX (White and Wilcox, 1985), TSH (Roder et al., 1992), EN (Patel et al., 1989), SCR (Pattatucci and Kaufman, 1991), ABD-A (Karch et al., 1990), ABD-B (Celniker et al., 1990), β-gal (Promega), and MYC (Calbiochem) antibodies have been described. Embryos and imaginal discs were prepared for immunofluorescence using FITC- and rhodamine-conjugated 2° antibodies and analyzed with a Biorad confocal microscope. The stains in Figures 2 and 4 were detected using HRP- and AP-conjugated 2° antibodies. Clone #7 was labeled with digoxygenin-conjugated dUTP and used as a probe in in situ hybridization experiments (Tautz and Pfeifle, 1989), and embryonic cuticle preparations were prepared as described (Wieschaus and Nusslein-Volhard, 1986).

Protein Interaction Experiments

A fusion protein between GST and MEIS1b (amino acid 59 to its C terminus) was generated in the pGEX-5X-3 vector (Pharmacia). GST-MEIS1 or GST were expressed in E. coli according to Pharmacia. Crude Drosophila embryo extracts were generated by homogenizing 0.3 ml of dechorionated embryos in 0.6 ml L buffer (1× PBS [150 mM NaCl {pH 7.4}], 1% NP-40, 1 mM PMSF, and 20 μg/ml each of aprotinin, pepstatin, leupeptin, 1 mM sodium vanadate, and 1 mM sodium fluoride). The embryo homogenates were centrifuged, and the aqueous phase mixed with 0.5 ml of crude bacterial extract. The extracts were incubated at 4°C for 2 hr, and the complexes were purified using glutathione-agarose, washed four times in L buffer, eluted by boiling in 20 μl of loading buffer, and resolved by SDS–PAGE. By Coomassie staining, no proteins derived from the Drosophila extract were visible. After blotting to nitrocellulose, the filters were probed with the EXD antibody and detected using the ECL system (Amersham). His-tagged, full-length EXD purified from E. coli was prepared by inducing pET14b-EXD(fl) (constructed by inserting a PCR product containing the entire EXD ORF, with NdeI and BamHI sites at the 5′ and 3′ ends, respectively, into pET14b). In the direct interaction experiment, 500 μl of GST, GST-RHR (from D. Thanos), or GST-MEIS1 bacterial extracts was incubated with 100 ng of His-EXD and complexes purified using glutathione-agarose. Detection of the complexes was the same as for the embryo pull-down experiments.

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