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Distinct functions of homeodomain-containing and homeodomain-less isoforms encoded by *homothorax*

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The *homothorax* (*hth*) gene of *Drosophila melanogaster* is required for executing Hox functions, for head development, and for forming the proximodistal (PD) axis of the appendages. We show that alternative splicing of *hth* generates two types of protein isoforms, one that contains a DNA-binding homeodomain (HthFL) and one that does not contain a homeodomain (HDless). Both types of Hth isoforms include the evolutionarily conserved HM domain, which mediates a direct interaction with Extradenticle (Exd), another homeodomain protein. We show that although both HthFL and HDless isoforms of Hth can induce the nuclear localization of Exd, they carry out distinct sets of functions during development. Surprisingly, we find that many of *hth*'s functions, including PD patterning and most Hox-related activities, can be executed by the HDless isoforms. In contrast, antennal development shows an absolute dependency on the HthFL isoform. Thus, alternative splicing of *hth* results in the generation of multiple transcription factors that execute unique functions in vivo. We further demonstrate that the mouse ortholog of *hth*, *Meis1*, also encodes a HDless isoform, suggesting that homeodomain-less variants of this gene family are evolutionarily ancient.

[*Keywords*: *homothorax*; homeodomain; Hox; selector gene; *Drosophila melanogaster*]

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Multicellular organisms show an astounding degree of morphological and behavioral complexity, a reflection of their proteome size and the intricacy of interactions between proteome components. The total number of genes in an organism represents only the baseline upon which different mechanisms act to increase coding capacity. Indeed, the number of distinct functional proteins can exceed the number of genes by orders of magnitude, due to biological processes such as DNA recombination, mRNA processing, and post-translational protein modification (Maniatis and Tasic 2002). Alternative pre-mRNA splicing is thought to have one of the most widespread roles in generating protein diversity, in terms of both the number of affected genes and the range of organisms in which it occurs. The diversity that may be produced by alternative splicing ranges from subtle differences in protein activity to the generation of isoforms with antagonistic functions (Lopez 1995; Graveley 2001; Maniatis and Tasic 2002).

Drosophila melanogaster displays complex developmental, morphological, and behavioral traits, despite a genome of only ~14,000 genes (Adams et al. 2000; Celniker and Rubin 2003). Spectacular examples of diversity generated by alternative splicing have been observed in *D. melanogaster*, such as encoded by the gene *Downs syndrome cell adhesion molecule* (*Dscam*). This axon guidance receptor gene can potentially generate up to 38,016 isoforms, two to three times the number of annotated genes in the genome (Schmucker et al. 2000; Wojtowicz et al. 2004).

Alternative splicing also provides additional complexity to genes encoding nuclear transcription factors (Read and Manley 1992; Lopez 1995; Bayer et al. 1996; Goeke et al. 2003). Transcription factors are highly modular in nature, often containing separable domains for DNA binding, dimerization, subcellular localization, and transcriptional activation or repression. Alternative splicing operates on this modularity to expand genomic coding capacity, producing isoforms with potentially subtle or highly diverse functional differences. In principle, transcription factor variants could impact widely on the regulation of transcriptional networks, providing a means for fine-tuning the system as well as creating additional functional diversity. However, the relevance of the majority of alternative splicing events remains elusive, as distinct roles of alternatively spliced isoforms are

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typically not well established but only inferred from variations in predicted protein sequences.

Here we demonstrate that alternative splicing at the *homothorax* (*hth*) locus of *Drosophila* leads to the generation of multiple protein isoforms that carry out distinct functions in vivo. Previous analyses of *hth* demonstrated that it encodes a homeodomain (HD) transcription factor of the three-amino-acid extension loop (TALE) subfamily, homologous to the vertebrate Meis and Prep proteins (Burglin 1997; Rieckhof et al. 1997; Pai et al. 1998). This subfamily of homeodomain proteins displays a high degree of conservation, both within the HD and in an N-terminal Hth/Meis (HM) domain (Rieckhof et al. 1997; Pai et al. 1998). The HD is responsible for DNA binding while the HM domain mediates direct interactions with members of another TALE-homeodomain subfamily, Extradenticle (Exd) in *Drosophila* and Pbx in vertebrates (Knoepfler et al. 1997; Rieckhof et al. 1997; Berthelsen et al. 1998b; Ryoo et al. 1999).

In *Drosophila*, Exd is widely expressed throughout development, but its subcellular localization is tightly regulated by Hth. In the absence of Hth, Exd resides in the cytoplasm, while in the presence of Hth, the proteins interact and translocate into the nucleus to regulate transcription. Importantly for the results described here, the HM domain of Hth is sufficient to translocate Exd from the cytoplasm to the nucleus (Abu-Shaar et al. 1999; Ryoo et al. 1999). The interaction between Exd and Hth has an additional consequence: Just as Exd's nuclear localization depends on Hth, Hth is unstable in the absence of Exd. These observations suggest that Hth and Exd are obligate partners that work together as a single functional unit. Consistent with this notion, identical phenotypes are generated by the lack of *exd* or *hth* function (Gonzalez-Crespo and Morata 1995; Rieckhof et al. 1997; Casares and Mann 1998, 2000; Abu-Shaar et al. 1999; Ryoo et al. 1999).

Hth and Exd are also intimately associated with the Hox proteins, another family of highly conserved homeodomain proteins that instruct many cell fate decisions during animal development (McGinnis and Krumlauf 1992). In the absence of Exd and/or Hth, Hox proteins are unable to regulate many of their target genes (Rauskolb and Wieschaus 1994; Ryoo and Mann 1999; Ryoo et al. 1999; Ebner et al. 2005). One reason for this link between Hth/Exd and Hox is that, for some target genes, Hth/Exd behaves as a Hox cofactor. Several examples of enhancers that bind Hox/Exd/Hth complexes have been described in which Hox DNA-binding selectivity and/or affinity are enhanced by Hth/Exd (Popperl et al. 1995; Maconochie et al. 1997; Jacobs et al. 1999; Ryoo et al. 1999; Gebelein et al. 2002, 2004; Ebner et al. 2005). Structural studies have shown that the Exd TALE peptide, a three-amino-acid insertion between helices 1 and 2 of its HD, mediates direct interactions with a conserved motif called YPWM that is present in most Hox proteins, enabling complex formation and cooperative DNA binding (Passner et al. 1999; Piper et al. 1999; LaRonde-LeBlanc and Wolberger 2003).

In addition to Hox-dependent activities, Hth and Exd have many Hox-independent functions during *Drosophila* development. For instance, they act as master regulators of the antennal developmental program, superimposing antennal identity on an appendage ground-state plan (Casares and Mann 1998, 2001; Dong et al. 2000, 2002). Hth and Exd are also required for leg and wing development, for the correct establishment of the proximal structures of these appendages (Abu-Shaar and Mann 1998; Wu and Cohen 1999, 2000; Azpiazu and Morata 2000, 2002; Casares and Mann 2000; Dong et al. 2001;).

In this study, we show that in addition to the previously characterized full-length protein (HthFL), alternative splicing of *hth* generates two very similar isoforms that lack the HD but contain the HM domain (HDless). Hth HDless variants can interact with Exd and are extensively coexpressed with the HthFL isoform, implying that distinct Hth/Exd complexes are present in the same nuclei. Surprisingly, an analysis of *hth* mutant alleles that are unable to produce any Hth protein or can only express HDless isoforms demonstrate that the HD is dispensable for a substantial number of Hth functions during development, suggesting that the HDless variants are capable of correctly regulating many Hth target genes. The HD is essential, however, for a subset of Hth tasks, such as the instruction of antennal identity. These observations demonstrate that different Hth isoforms have unique transcriptional properties, which depend on the presence or absence of the HD. Thus, *hth* alternative splicing is used as a mechanism to expand the diversity of Hth/Exd complexes, increasing the complexity of the transcriptional networks that use these transcription factors. We also report that HDless isoforms are encoded by the vertebrate *Meis1* gene, suggesting that this division of labor among HD-containing and HDless forms of this gene family may also exist in vertebrates.

Results

homothorax *encodes splice variants that lack a DNA-binding domain*

hth includes 16 annotated exons distributed over >100 kb of genomic DNA (Fig. 1A; FlyBase, http://www.flybase.org). All functionally characterized isoforms of *hth* include both the HM domain, encoded by exons 2–6, and the HD, encoded by exons 11–13 (Fig. 1A). In addition, *hth* encodes at least two additional alternatively spliced variants that have an intact HM domain but no HD. Both alternatively spliced mRNAs code for two almost identical HM-containing proteins that are largely derived from the first six coding exons. Both of these HDless isoforms have an additional 24 amino acids at their C termini encoded by alternate exons (Fig. 1A,C). One of these variants (the 7′ isoform) uses an alternative exon 7 (exon 7′). Sequence comparisons between *D. melanogaster* and *Anopheles gambiae hth* genes enabled us and others (Glazov et al. 2005) to identify a second *hth* splice variant that is also missing the HD (see also FlyBase,

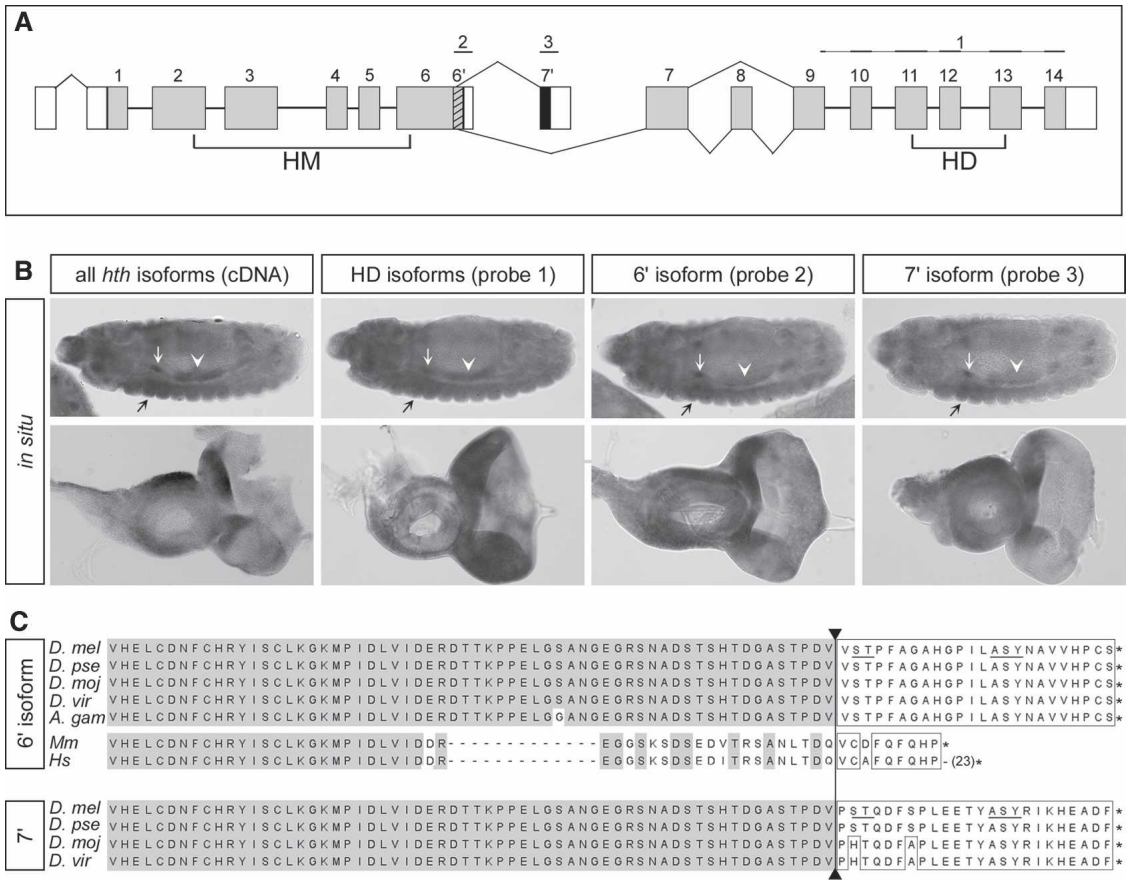


Figure 1. The *hth* locus encodes for evolutionary conserved HDless variants. (*A*) Genomic organization of *hth* (not shown to scale). White and gray boxes denote noncoding and protein-coding exons, respectively, while angled lines represent sites of alternative splicing. The 6′- and 7′-specific ORFs are indicated by the dashed and black boxes, respectively. (*B*) In situ hybridization using probes for specific *hth* isoforms shows very similar expression patterns during both embryonic (whole embryo, *top* panels) and larval (e.g., eye-antennal imaginal disc, *bottom* panels) stages. White and black arrows indicate high levels of *hth* expression in the gastric caeca and the thoracic ectoderm, respectively. White arrow heads point to *hth* staining in the visceral mesoderm. The positions of the probes used are indicated in *A*. See also Supplementary Figure 1. (*C*) Comparison of the predicted protein sequences of the 6′ and 7′ isoforms. The species indicated are *D. melanogaster* (*D. mel*), *D. pseudoobscura* (*D. pse*), *D. mojavensis* (*D. moj*), *D. virilis* (*D. vir*), *A. gambiae* (*A. gam*), *M. musculus* (*Mm*)**, and** *Homo sapiens* (*Hs*). The protein sequences of exon 6 and alternative exon 6 and 7 are compared. Amino acids conserved across all species are outlined by the gray shading. Sequence conservation for the 6′ and 7′ exons within the insect or mammal subgroups are boxed. The two black triangles demarcate the bypassed splice junction at the end of exon 6. Underlined in black are two small motifs common to the 6′ and 7′ isoforms.

http://www.flybase.org). This isoform (the 6′ isoform) is generated when the splice site at the 3′ end of exon 6 is not used, generating an extended ORF (Fig. 1A,C). We confirmed the existence of both 6′ and 7′ isoforms in vivo by sequencing ESTs and performing RT–PCR on mRNA isolated from embryonic and larval tissues (data not shown). The presence of 6′ and 7′ isoforms raised the possibility that HDless variants of Hth might carry out distinct functions, suggesting a functional diversification of the *hth* gene that depends on alternative splicing.

To test if the different isoforms have separate spatial or temporal expression profiles, we examined their distributions by in situ hybridization (Fig. 1B). All the probes revealed very similar expression patterns during both embryonic and larval stages, suggesting the coexpression of HthFL and HDless isoforms in the same tissues (Fig. 1B; data not shown). The in situ hybridization results are supported by immunohistochemical analysis of imaginal discs with antibodies that specifically recognize the HthFL and 6′ isoforms (Supplementary Fig. 1).

6′ and 7′ HDless orthologs

Comparative sequence analysis of the *hth* locus in other *Drosophila* species, including *Drosphila pseudoobscura, Drosophila virilis*, and *Drosophila mojavensis*, revealed a high degree of conservation of the alternative 6′ and 7′ exons, which translate into Hth proteins that are nearly identical to their *D. melanogaster* ortholog (Fig. 1C). The conservation of the 6′ HDless form also extends to the mosquito *A. gambiae*, which diverged from *Drosophila* as long as 250 million years ago (Zdobnov et al. 2002). No analogous 7′ exon could be identified in the *Anopheles* genome using BLAST searches. The high degree of con-

servation observed among the *Drosophila* species, as well as in the mosquito (for the 6′ form), suggests that HDless Hth variants may exert conserved functions in Dipterans.

We further tested the evolutionary conservation of HDless isoforms by analyzing the genomic organization of *Meis1*, a vertebrate ortholog of *hth*. Strikingly, the *Meis1* intronic sequence immediately downstream of exon 6 is unusually conserved between the mouse and human genes. Moreover, if the splice site at the end of exon 6 is bypassed, as it is in Diptera, a HDless variant of Meis1 may be encoded (Fig. 1C). RT–PCR using mRNA from embryonic day 10.5 (E10.5) mouse embryos confirmed the presence of transcripts predicted to encode a HDless isoform that contains the first six exons and nine novel, additional amino acids (Fig. 1C). These additional residues are unrelated to the 24 C-terminal residues found in the *Drosophila* 6′ or 7′ isoforms. The presence of 6′ variants in both Diptera and vertebrates indicates a strong selective pressure to maintain both HD-containing and HDless isoforms of Hth/Meis, underscoring the functional significance of HDless variants in animal development.

The hth$^{100\text{-}1}$ *allele only expresses HDless isoforms that productively interact with Exd*

To begin to address the role of HDless forms of Hth in vivo, we characterized two *hth* alleles in more detail. The first allele, *hth*^{P2}, is a strong hypomorph, generated by a P-element insertion in the *hth* promoter. In homozygous *hth*^{P2} tissue, no Hth protein can be detected as assayed by immunostaining with an anti-Hth antibody that detects both HthFL and HDless isoforms (anti-Hth) (see below and Materials and Methods). The second allele, *hth*$^{100\text{-}1}$, is predicted to encode only HDless isoforms due to an Arg321 to opal mutation in exon 9 (Kurant et al. 2001). We confirmed these predictions by carrying out immunoblot analyses with wild-type and mutant embryonic extracts. When probed with anti-Hth, wild-type extracts show two bands of the predicted sizes for the HthFL and HDless isoforms, respectively (Fig. 2A). Neither band is observed in *hth*^{P2} extracts (Fig. 2A). In *hth*$^{100\text{-}1}$ extracts, no HthFL is observed but the faster migrating species is still present, accompanied by additional *hth*$^{100\text{-}1}$-specific isoforms. These new variants are likely the result of the premature stop codon in exon 9 and are therefore predicted to be HM-containing, HDless forms with additional residues derived from the linker region between the HM and HD. These results suggest that *hth* expresses both HthFL and HDless isoforms. These two alleles provide valuable tools for comparing a strong loss-of-function state (*hth*^{P2}) versus a state in which only HDless forms are present (*hth*$^{100\text{-}1}$).

Because both HDless variants include an intact HM domain, which is necessary and sufficient to mediate an interaction with Exd (Ryoo et al. 1999), we predicted that they should both be able to interact with Exd. To test this prediction, we immunoprecipitated Exd from total embryonic extracts and probed these immunoprecipitates (IPs) with anti-Hth. Two bands having the predicted sizes for the HthFL and HDless isoforms are observed (Fig. 2B). To confirm that the slower, but not the faster, migrating band contains the Hth HD, we also probed these IPs with an antibody that was generated against the C-terminal HD-containing portion of Hth (anti-HthHD) (see Materials and Methods). Consistent with our predictions, this antibody detects the slower, but not the faster, migrating band (Fig. 2B).

We also confirmed that the HDless isoforms encoded by *hth*$^{100\text{-}1}$ are able to translocate Exd into nuclei in vivo. As expected, no Hth or nuclear Exd can be detected in *hth*^{P2} mitotic clones (Fig. 2C). In contrast, *hth*$^{100\text{-}1}$ mutant clones present in proximal domains of the wing or leg imaginal discs have nuclear Exd (Fig. 2C; data not shown). Also as predicted, *hth*^{P2} clones show no immunostaining with the anti-HthHD antibody or with an antibody specifically raised against the unique sequences in exon 6′ (anti-Hth6′) (see Materials and Methods). In contrast, *hth*$^{100\text{-}1}$ clones are stained with the anti-Hth6′ antibody but not with the anti-HthHD antibody (Fig. 2D).

In summary, these data demonstrate that both full-length and HDless isoforms of Hth can interact with and translocate Exd into nuclei. Further, consistent with the immunoblot analyses described above, these immunostaining experiments suggest that *hth*^{P2} makes no detectable Hth protein whereas *hth*$^{100\text{-}1}$ produces only HDless isoforms.

HDless forms of Hth are required for patterning the Drosophila *embryo*

The *hth*$^{100\text{-}1}$ allele allows us to examine a situation in which no full-length, HD-containing Hth isoforms are expressed. In contrast, there are no known *hth* alleles that express only HD-containing isoforms. Multiple attempts to use RNA interference (RNAi) hairpin constructs to specifically eliminate HDless isoforms in vivo failed, perhaps due to the very limited sequences available for targeting (data not shown). Instead, to mimic a HDless-depleted state in vivo, we codepleted the 6′ and 7′ isoforms by injecting small interfering RNAs (siRNAs) specifically directed against these splice variants into preblastoderm embryos. RT–PCR from a population of injected embryos revealed a significant reduction, but not complete loss, of both *hth* HDless transcripts (Fig. 3A; Supplementary Fig. 2A). No decrease was observed for the HthFL transcript, confirming the target specificity of the siRNAs (Fig. 3A). Consistently, siRNA-injected embryos show a selective loss of the HDless isoforms as detected by immunoblot analysis (Supplementary Fig. 2A).

Using these tools, we compared the contributions of HthFL and HDless isoforms to embryonic development. Toward the end of embryogenesis, the embryo secretes a cuticle, whose stereotyped features are a direct readout of patterning events that occurred during embryogenesis. *hth* is required both for proper segmentation and, as a Hox cofactor, to instruct segmental identities along the

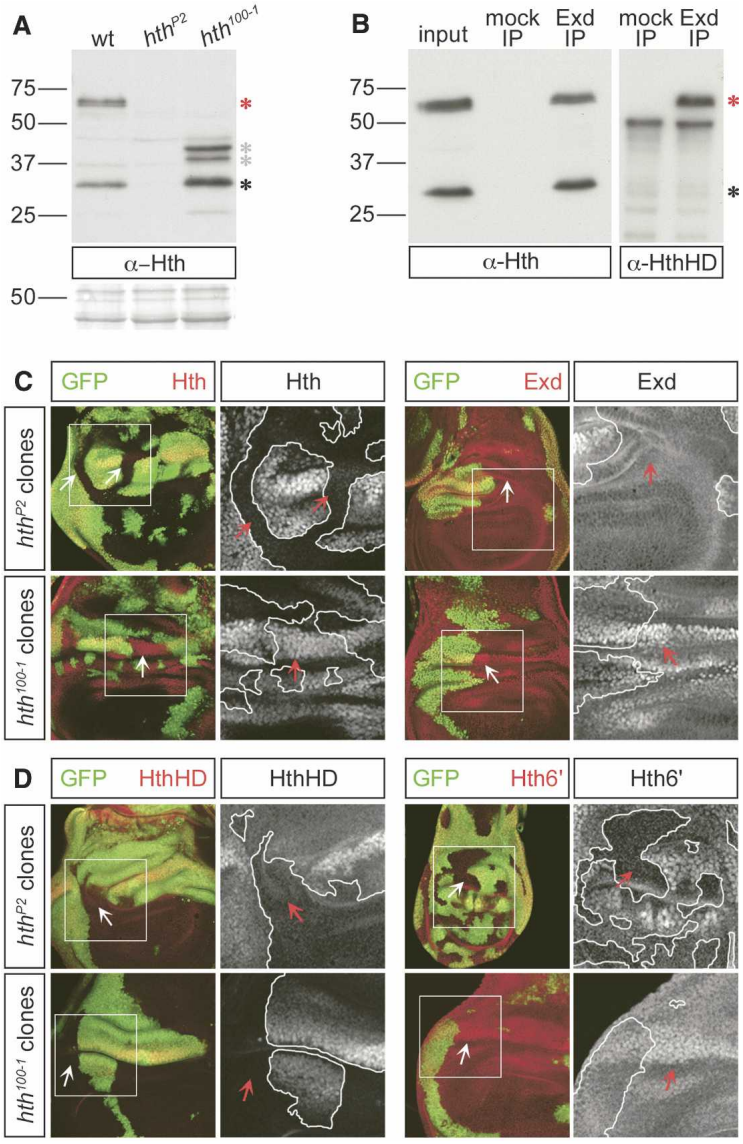


Figure 2. Hth HDless isoforms are capable of interacting with Exd. (*A*) Immunoblot of wild-type (wt), hth^{P2}, and $hth^{100\text{-}1}$ embryos stained with an anti-Hth antibody that detects all HM-containing isoforms (α-Hth). No Hth isoforms are detected in hth^{P2} embryos, while only putative HDless isoforms are present in $hth^{100\text{-}1}$ embryos. (Red asterisk) HthFL; (black asterisk) HDless; (gray asterisks) additional HDless isoforms are observed in $hth^{100\text{-}1}$ extracts, probably as a result of the premature stop codon in exon 9. (*Bottom*) Blue Coomassie staining of a portion of the same membrane showing equivalent loading. (*B*) Immunoprecipitation of total embryonic extracts with either preimmune serum (mock IP) or anti-Exd antibody (Exd IP). The blot was probed with either an α-Hth (*left* panel) or an antibody raised specifically against the HD and C-tail of Hth (α-HthHD; *right* panel). Both full-length and HDless variants of Hth are coimmunoprecipitated with Exd, but only the larger isoform is detected with the anti-HthHD antibody. Equivalent amounts of extracts were loaded (data not shown). The additional band observed in both mock and Exd IPs in the *right* panel is likely due to cross-reactivity with the antibody used for the IP. (*C*) Wing imaginal discs with mitotic clones of hth^{P2} and $hth^{100\text{-}1}$, marked by the absence of GFP (green), stained with anti-Hth and anti-Exd as indicated. hth^{P2} clones have no detectable Hth staining and no nuclear Exd, while Hth and nuclear Exd are still detected in $hth^{100\text{-}1}$ clones. Arrows point to mutant tissue. (*D*) Wing or haltere imaginal discs with mitotic clones of hth^{P2} and $hth^{100\text{-}1}$, marked by the absence of GFP (green), stained with anti-HthHD and anti-Hth6′ as indicated. $hth^{100\text{-}1}$ mutant clones are devoid of the HD-containing isoforms, but still stain for the 6′ HDless variant. Neither form was detected in hth^{P2} mutant tissue. Arrows point to mutant tissue.

anteroposterior (AP) axis (Rieckhof et al. 1997). hth^{P2} cuticles show severe head defects, segmental fusions, and Hox-dependent, posterior-directed transformations of the abdominal segments. In the thorax, an almost completely naked epidermis with some sparse denticles replaces the rows of small denticles that are present in wild type. Transformations toward more posterior fates are evident in the first abdominal segment (A1) that takes on an A3-like identity (Fig. 3B). Denticle belt fusions, especially evident in the abdominal segments, are indicative of segmentation defects arising from the loss of *engrailed* (*en*) and *wingless* (*wg*) expression (Rieckhof et al. 1997).

When otherwise wild-type embryos were coinjected with siRNAs directed against the 6′ and 7′ isoforms, the embryos all died at the end of embryogenesis and their cuticles showed weaker versions of all of the hth^{P2} phenotypes (Supplementary Table 1). Buffer injected embryos were wild type (data not shown). In addition to head involution defects, the 6′ + 7′ siRNA-injected embryos had a partial T1 "beard" in the second (T2) and/or third (T3) thoracic segments. This phenotype, although not observed in the complete absence of *hth* or *exd*, is seen in the absence of zygotic *exd* activity, a partial loss-of-function situation (Peifer and Wieschaus 1990). Keilin's organs (KOs), thoracic-specific sensory structures, were often abnormal. A transformation of segmental identities was apparent in the abdomen, where A1 was partially transformed toward A2, and A2 transformed to A3, on both the ventral and dorsal surfaces (Fig. 3B; data not shown). These embryos also had segmental fusions at a frequency (~40%) comparable to hth^{P2} animals (Fig. 3B), suggesting that HDless isoforms of Hth are required for proper embryonic development. These phenotypes are likely to represent an underestimate of the role these forms play during embryonic development because the siRNA injections do not completely eliminate these mRNAs (Fig. 3A; Supplementary Fig. 2A). Moreover,

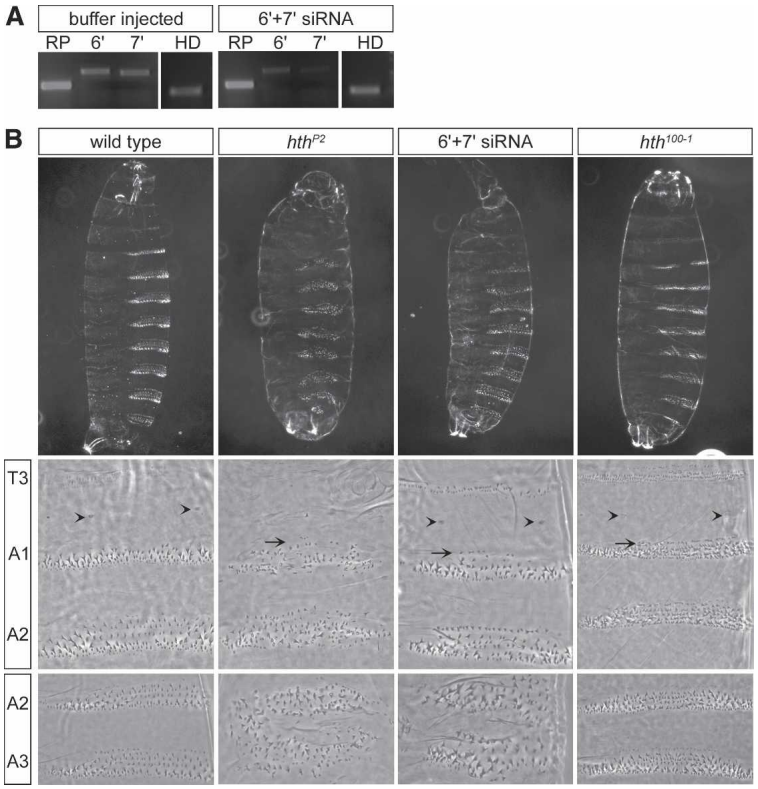


Figure 3. HDless isoforms are necessary for embryonic patterning. (*A*) RT–PCR from embryos injected either with buffer or siRNAs directed against the 6′ and 7′ isoforms. Specific reduction of both the 6′ and 7′ transcripts, but not the HD-containing mRNAs was observed. RT–PCR for *ribosomal protein 49* transcripts (RP) was used as a control. (*B*) Cuticle preparations of wild-type, hth^{P2}, $hth^{100\text{-}1}$, and 6′ + 7′ siRNA-injected embryos. (*Top* panels) Head involution defects can be observed in all mutant and siRNA-injected embryos. Strong posterior-directed transformations of the A1 and A2 segments were observed in hth^{P2} and 6′ + 7′ siRNA-injected embryos and weaker transformations of the A1 segment were observed in $hth^{100\text{-}1}$ embryos (arrows). Arrowheads identify the KOs. (*Bottom* panel) Segmental fusions were observed in hth^{P2} and 6′ + 7′ siRNA-injected embryos but not in $hth^{100\text{-}1}$ cuticles.

these phenotypes are unlikely to be due to a general reduction in Hth levels because they are not observed in $hth^{100\text{-}1}$ homozygous or in $hth^{100\text{-}1}/hth^{P2}$ embryos (see below). Indeed, a 50% reduction in the total amount of Hth, observable in hth^{P2} heterozygous flies (Supplementary Fig. 2B), results in normal animals.

In contrast to the 6′ + 7′ siRNA results, $hth^{100\text{-}1}$ cuticle preparations are remarkably normal (Fig. 3B; Supplementary Table 1). Although head defects were apparent, the thoracic segments were normal except for the rare appearance of a small number of ectopic denticles in T3 (Fig. 3B). A1 was weakly transformed toward A2, but the rest of the abdominal segments had a wild-type pattern of denticle belts (Fig. 3B). These cuticles also lacked segmental fusions, and *en* and *wg* were expressed normally (Fig. 3B; data not shown). Similar phenotypes were observed in $hth^{100\text{-}1}/hth^{P2}$ embryos (data not shown). These results suggest that the Hth HD is not required for proper segmentation and that it plays a relatively small role in the instruction of segmental identities. We note, however, that $hth^{100\text{-}1}$ embryos also express HDless isoforms containing additional residues not present in the 6′ or 7′ isoforms (Fig. 2A). Although these isoforms are also HDless, they may have functions that are not present in the naturally expressed HDless isoforms. In addition, even though Hth HD is largely dispensable for some embryonic functions, HthFL isoforms are required for other Hth-dependent functions, such as peripheral nervous system development, as $hth^{100\text{-}1}$ embryos are defective in this tissue (Kurant et al. 2001).

The Hth HD is not required for the transcriptional regulation of some direct Hth target genes

The dispensability of the HD for a subset of Hth functions during embryonic development suggests that distinct Hth/Exd complexes, HthFL/Exd and HDless/Exd, might control the transcriptional output of different Hth target genes. For some targets, Hth and Exd function as Hox cofactors, enhancing both the DNA-binding affinity and specificity of Hox proteins (Chan et al. 1994, 1996; Ryoo and Mann 1999). To assess the necessity of the Hth HD in the formation of functional complexes with Exd and Hox in vivo, we analyzed the expression of two previously characterized Hox/Exd target elements derived from the genes *forkhead* (*fkh*) and *labial* (*lab*), in $hth^{100\text{-}1}$ and hth^{P2} mutant embryos.

During embryonic development, an autoregulatory enhancer from the *lab* gene (*lab550*) drives expression in a band of endodermal cells (Fig. 4A; Tremml and Bienz 1992; Grieder et al. 1997). A 48-base-pair (bp) fragment of *lab550* (*lab48/95*) also drives expression in the endoderm and cooperatively assembles a Lab/Exd/Hth complex in vitro. Like *lab*, both of these reporter genes require *labial*, *exd*, and *hth* for expression (Ryoo et al. 1999). For *lab48/95* to be active, binding sites for all three HD proteins (Lab, Exd, and Hth) are required (Ryoo et al. 1999). In contrast, *lab550*, which integrates additional inputs from the *decapentaplegic* (*dpp*) pathway (Tremml and Bienz 1992; Grieder et al. 1997), does not require the Hth-binding site for activity, while it still strictly depends on both Lab- and Exd-binding sites (Ryoo et al.

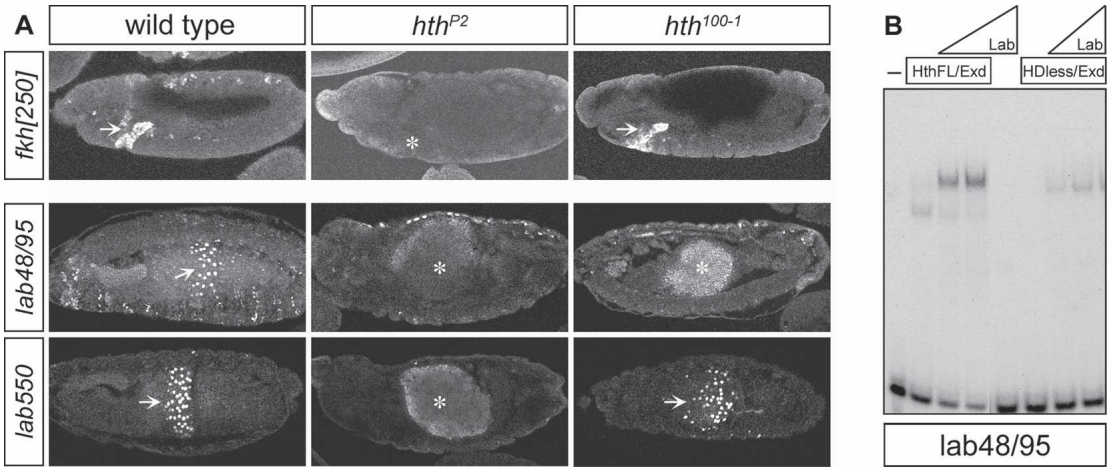


Figure 4. Differential requirement of the Hth HD for the transcriptional regulation of *fkh* and *lab*. (*A*) Embryos carrying *fkh[250]-lacZ* (*top* panels), *lab48/95-lacZ* (*middle* panels), and *lab550-lacZ* (*bottom* panels), stained for β-galactosidase (β-gal). *fkh250-lacZ* is expressed in wild-type and $hth^{100\text{-}1}$ embryos, but not in hth^{P2} embryos. Wild-type embryos carrying the *lab48/95-lacZ* or *lab550-lacZ* transgenes show a very similar pattern of nuclear β-gal in a central band of endodermal cells. In $hth^{100\text{-}1}$ embryos, *lab 48/95-lacZ* expression is lost, while *lab550-lacZ* staining is still present, underlying a differential requirement for the Hth HD by the two enhancers. In hth^{P2} embryos, both *lab 48/95-lacZ* and *lab550-lacZ* are lost. (Arrows) LacZ positive staining; (asterisks) no *lacZ* expression, although some background fluorescence is observed in these midguts. (*B*) HDless/Exd and Lab can form a complex on the *lab48/95* element in vitro, as attested by EMSA analysis. Binding is cooperative, but weaker than that observed for HthFL/Exd/Lab.

1999). These observations suggest that the Hth HD is not necessary for the assembly of a Lab/Exd/Hth complex in the context of the larger enhancer, probably due to the presence of additional factors involved in *lab550* activation. Consistent with these observations, *lab550-lacZ* is expressed normally in $hth^{100\text{-}1}$ embryos, while *lab48/95-lacZ* in not expressed in these embryos (Fig. 4A). These conclusions are further supported by electrophoretic mobility shift assays (EMSAs). To mimic the 6′ and 7′ isoforms found in vivo, we expressed and purified a version of Hth that contains only the HM domain, referred to here as HDless. We find that a HDless/Exd/Lab complex can cooperatively assemble on *lab48/95*, although approximately fourfold more weakly than HthFL/Exd/Lab (Fig. 4B). Thus, the activation of *lab550* does not require a Hth-binding site or the Hth HD, whereas the activation of *lab48/95* requires both.

Another Hox target element, *fkh[250]*, derived from the gene *fkh*, is a natural target of the Hox gene *Sex combs reduced* (*Scr*). Like the endogenous *fkh* gene, *fkh[250]-lacZ* activation requires Scr and Exd, and these two homeoproteins bind cooperatively to this 37-bp element in the absence of Hth (Ryoo and Mann 1999). No Hth-binding site has been found in this element, and consistently, *fkh[250]-lacZ* is expressed normally in $hth^{100\text{-}1}$ embryos (Fig. 4A). In contrast, *fkh[250]-lacZ* is no longer active in hth^{P2} embryos (Fig. 4A; Ryoo and Mann 1999). These results suggest that the HD of Hth is not required to activate the *fkh* element but that the HM domain is necessary. Consistent with our previous observation that Exd/Scr can bind to this element in the absence of Hth (Ryoo and Mann 1999), a HDless/Exd/Scr complex can also cooperatively bind to *fkh[250]* (data not shown). Thus, for both of these direct Hox/Exd targets (*lab550* and *fkh[250]*), the HthFL isoform is not required for activation, suggesting that the HDless isoforms are sufficient to induce the nuclear localization of Exd and activate these elements in vivo. In contrast, a weakened version of *lab550* (*lab48/95*) requires a Hth-binding site and the HthFL isoform for activation, suggesting that in some contexts, the Hth HD plays a role, perhaps by helping to stabilize the DNA-bound Hox/Exd/Hth complex.

The HthFL isoform is essential for antennal identity

The experiments described above suggest, surprisingly, that the Hth HD is dispensable for many of Hth's embryonic functions. Hth and Exd are also required for many adult functions, including the specification of antennal fates and formation of the proximodistal (PD) axis of the legs and wings. Instruction of antennal identity requires the activation of antennal-specific genes, as well as the repression of leg-specific genes in the antennal imaginal disc (Casares and Mann 1998, 2001; Dong et al. 2001). To analyze post-embryonic requirements for the Hth HD, we generated hth^{P2} and $hth^{100\text{-}1}$ clones in imaginal tissues. As previously reported, large hth^{P2} clones in the antenna result in its transformation toward a leg, containing a complete tarsus (including five segments plus the claw) and a single proximal domain (Fig. 5A; Casares and Mann 1998, 2001). The proximal domain derives from the fusion of proximal and medial leg segments, and contains both bracted and unbracted bristles, which are markers for distal and proximal leg identities, respectively. $hth^{100\text{-}1}$ clones result in antenna-to-leg transformations that are nearly indistinguishable from those generated by hth^{P2} clones (Fig. 5A).

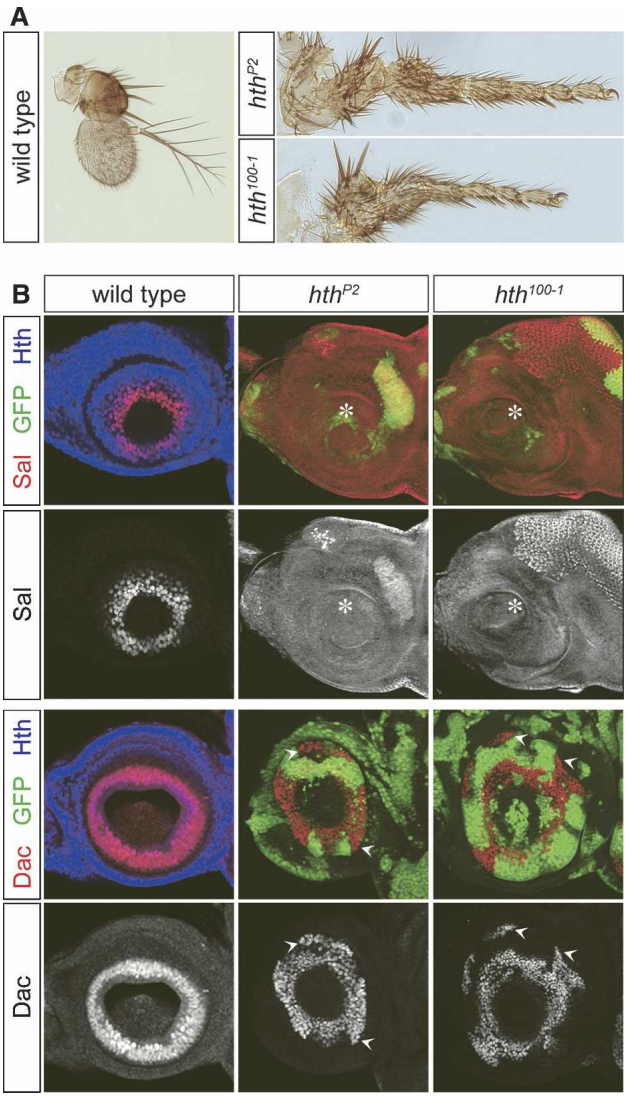


Figure 5. The Hth HD is required for the specification of antennal fates. (*A*) Adult wild-type, *hth*^{P2}, and *hth*$^{100\text{-}1}$ mutant antennae. Transformation of the antenna toward leg is observed in both *hth*^{P2} and *hth*$^{100\text{-}1}$ appendages, suggesting a strict requirement for the HD in the instruction of antennal identity. (*B*) *hth*^{P2} and *hth*$^{100\text{-}1}$ mitotic clones in antennal imaginal discs, marked by the absence of GFP. (*Top* panels) *hth*^{P2} and *hth*$^{100\text{-}1}$ clones are devoid of Sal staining, a marker of antennal fates. Asterisks mark the mutant tissue. (*Bottom* panels) The Hth HD is also necessary for the repression of leg identity in the antenna, as shown by the expansion of *dac* expression into a leg-like pattern in both *hth*^{P2} and *hth*$^{100\text{-}1}$ mutant clones (arrowheads).

Consistent with these adult phenotypes, expression of *spalt* (*sal*), an antennal-specific marker (Dong et al. 2000), is lost in both *hth*^{P2} and *hth*$^{100\text{-}1}$ mutant antennal imaginal discs (Fig. 5B). Conversely, ectopic expression of HthFL, but not a HDless isoform, in the antenna results in ectopic *sal* expression (Supplementary Fig. 3) and ectopic antennae in approximately one-fourth of the flies (see below). Thus, both loss- and gain-of-function experiments suggest an absolute requirement for the Hth HD to generate antennal fates.

To assess if the Hth HD is also required to repress leg identity during antennal development, we monitored the expression of *dachshund* (*dac*). *dac* is expressed in both leg and antenna imaginal discs but with distinct patterns (Dong et al. 2001). In the antennal disc, *dac* is expressed in a narrow domain, whereas in the leg, *dac* expression encompasses a much larger region that will give rise to medial leg segments. Both *hth*^{P2} and *hth*$^{100\text{-}1}$ clones in the antenna result in the derepression of *dac* in a leg-like expression pattern (Fig. 5B). Thus, the Hth HD is not only required to activate antennal-specific genes, such as *sal* but is also required to repress the leg-like expression of *dac*.

The Hth HD is not required for PD axis formation or to pattern the proximal region of the ventral appendages

Large *hth*^{P2} clones in the legs result in the fusion of the proximal segments, the coxa and trochanter, with medial segments and the body wall (Casares and Mann 2001; Azpiazu and Morata 2002). The resulting fused proximal domain has both bracted and unbracted bristles, suggesting it is comprised of both proximal and distal fates (Fig. 6B,D). In leg imaginal discs, *hth*^{P2} clones show two effects, a derepression of *dac* in some clones and a loss of expression of *teashirt* (*tsh*), a proximal domain marker (Fig. 6F). These phenotypes are consistent with the loss of proximal structures and the acquisition of more medial/distal identities (Abu-Shaar and Mann 1998; Wu and Cohen 2000).

In contrast to *hth*^{P2}, *hth*$^{100\text{-}1}$ mutant legs are normal along their entire PD axis: The coxa and trochanter are unaltered, and there are no fusions of these proximal segments with more distal leg segments (Fig. 6C,E). However, in approximately one-third of the *hth*$^{100\text{-}1}$ mutant legs, the tibia and femur were partially fused, suggesting a minor role for HthFL in separating these leg segments (data not shown). No change in *tsh* expression was observed and *dac* expression was usually unaffected in *hth*$^{100\text{-}1}$ clones (Fig. 6G). Consistent with these loss-of-function phenotypes, we also found that both HthFL and HDless isoforms of Hth were able to block distal leg development when ectopically expressed in the distal domain (data not shown). These results suggest that the Hth HD is largely dispensable for PD axis formation and for specifying proximal identities in the leg.

hth is also required for PD axis formation in the wing by limiting the size and position of the distal domain, the presumptive wing blade, by repressing *wingless* along the dorsoventral (DV) boundary (Casares and Mann 2000). As with the ventral appendages, Hth HDless isoforms are sufficient to correctly establish and maintain the PD axis in the wing disc and to limit the growth of the distal portion of the wing (Supplementary Fig. 4). In contrast to the proximal leg, however, the Hth HD is

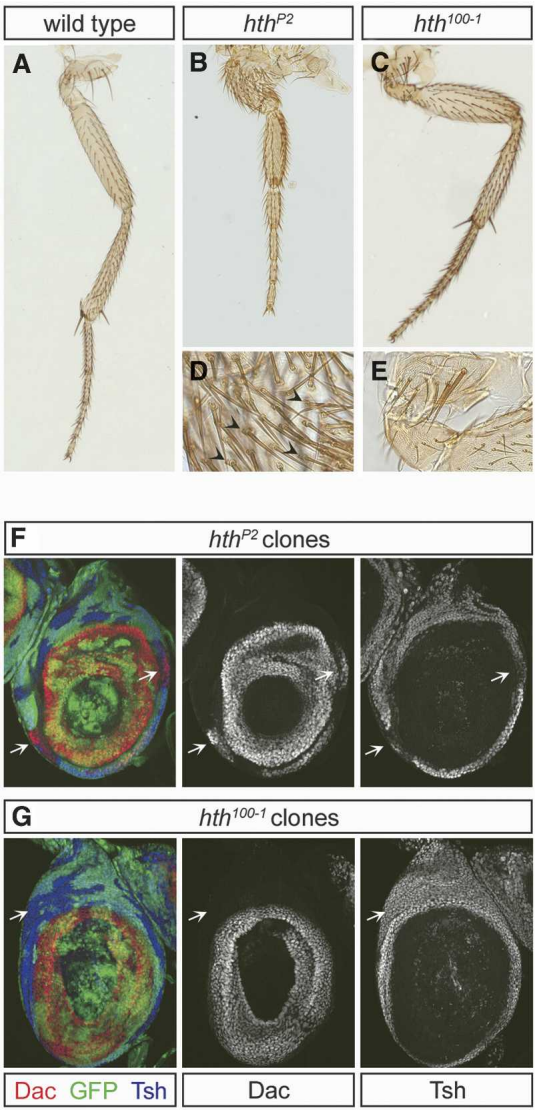
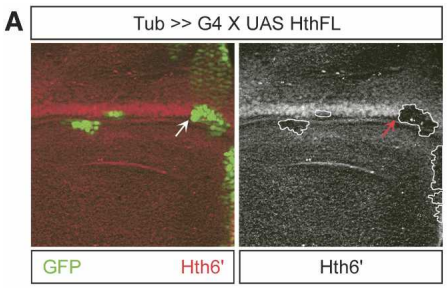


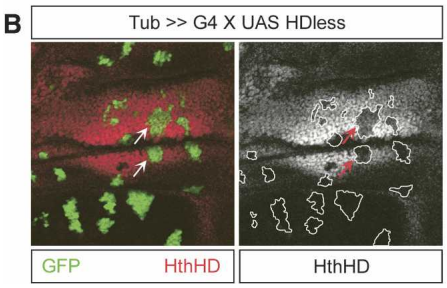
Figure 6. The Hth HD is dispensable for the formation of a correct PD axis in ventral appendages. (*A–C*) Adult structures are shown for wild-type (*A*), hth^{P2} (*B*), and $hth^{100\text{-}1}$ mutant legs (*C*). hth^{P2} mutant legs are characterized by the loss of proximal elements, while $hth^{100\text{-}1}$ mutant legs have a normal PD axis. (*D,E*) High-magnification views of the proximal domains of typical hth^{P2} (*D*) and $hth^{100\text{-}1}$ (*E*) legs. Bracted bristles, indicative of more medial/distal identities can be observed in hth^{P2}, but not $hth^{100\text{-}1}$, proximal domains (arrowheads). (*F,G*) Mitotic clones for hth^{P2} (*F*) and $hth^{100\text{-}1}$ (*G*) in leg imaginal discs. hth^{P2} clones show loss of *tsh*, a proximal fate marker, and the derepression of *dac*, a medial marker (arrows). $hth^{100\text{-}1}$ clones show no loss of *tsh*, or derepression of *dac*. All clones are marked by the absence of GFP (green). Although *dac* expression was usually normal, we observed derepression in some $hth^{100\text{-}1}$ clones located in the dorsal-most region of the disc (data not shown). This rare *dac* derepression might account for single bracted bristles in otherwise normal trochanters of some $hth^{100\text{-}1}$ legs (data not shown).

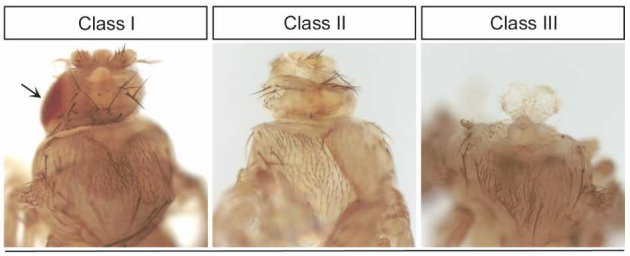
required for the proper patterning of the wing hinge, as its morphology is aberrant in $hth^{100\text{-}1}$ clones (Supplementary Fig. 4).

Headless phenotypes generated by ectopic expression of HthFL but not a HDless isoform of Hth

The above experiments demonstrate that some Hth-dependent functions, for example, antennal specification, strictly require the Hth HD while other functions, such as proximal appendage development, largely do not require HD-containing isoforms. We also find that both HthFL and HDless isoforms are coexpressed in the same cells throughout development. Taken together, these results suggest that within individual cells two sets of *hth* target genes are being regulated, those that depend on the HD for regulation and those that do not require the HD, and that these different sets of target genes have partially nonoverlapping roles in development. An additional test of this idea is to examine the consequences of overexpressing either HthFL or HDless isoforms. In principle, because there is a limiting amount of Exd, overexpression of HthFL or HDless forms should effectively reduce the amount of HDless/Exd or HthFL/Exd complexes, respectively. This prediction was confirmed by immunostaining experiments using isoform-specific antibodies in imaginal discs containing clones that ectopically express either HthFL or HDless isoforms (Fig. 7A,B). Expressing higher levels of one type of isoform (e.g., HthFL) lowers the levels of the other isoforms (e.g., HDless). To analyze these effects in vivo, we used a *hth*-Gal4 driver line that is only active in a subset of cells that normally express *hth* (data not shown). Consistent with the idea that these isoforms carry out distinct functions, *hth*-Gal4; UAS-HDless flies exhibit a range of phenotypes that is distinct from those observed in *hth*-Gal4; UAS-HthFL flies (Fig. 7C,D). In the head, the predominant phenotype resulting from HDless overexpression is an antenna-to-leg transformation (69%; $n = 42$), consistent with a requirement for the HthFL isoform to make this structure. In contrast, HthFL overexpression results in a large reduction in the sizes of the eyes and head (class II + III; ~93%; $n = 63$). Strikingly, a significant fraction of *hth*-Gal4; UAS-HthFL flies have nothing left of the head except a proboscis (class III; ~16%; $n = 63$) (Fig. 7C,D), a phenotype that was never observed in *hth*-Gal4; UAS-HDless flies. Other phenotypes are also consistent with a division of labor between HthFL and HDless isoforms. *hth*-Gal4; UAS-HthFL flies often have no wing blades and no halteres (25% and 79%, respectively) whereas *hth*-Gal4; UAS-HDless flies rarely show these phenotypes (0% and 2.4%, respectively) (Fig. 7D). Consistent with the absolute requirement for the Hth HD in antennal development, *hth*-Gal4; UAS-HthFL flies frequently (24%; $n = 38$) have ectopic antennae in their heads, whereas *hth*-Gal4; UAS-HDless flies did not show this phenotype. Importantly, all of these phenotypes are significantly reverted when both HthFL and HDless forms are coexpressed using the *hth*-Gal4 driver, which effectively restores the availability of both HthFL/Exd and HDless/Exd complexes (Fig. 7D). Thus, altering the relative levels of Hth isoforms results in distinct phenotypes, supporting our conclusion that they execute distinct functions in vivo.







D

	GFP	HDless	HthFL	HDless;HthFL
Head/eye phenotypes (%)				
wild type	100	100	0	11
class I	0	0	7	39
class II	0	0	77	50
class III	0	0	16	0
n	37	42	63	64
Other phenotypes (%)				
antenna to leg	0	69	1.8 (n=53)	0
no wing blade	0	0	25	0
no halteres	0	2.4	79	0
n	37	42	63	64

Figure 7. Altering the balance of HthFL and HDless isoforms produces unique phenotypes. (*A*,*B*) Wing imaginal discs containing flip-out clones that ectopically express HthFL (*A*) or HDless (*B*) Hth isoforms. Clones are marked by the presence of GFP (green). Expression of HthFL leads to a reduction in the amount of endogenous HDless isoforms, as visualized by anti-Hth6′ staining (*A*, arrow), while expression of HDless leads to a reduction in the amount of endogenous HthFL, as visualized by anti-HthHD staining (*B*, arrows). (*C*) The range of head phenotypes observed in *hth-Gal4*; UAS-HthFL adult flies. (Class I) Small eyes (arrow); (class II) no eyes; (class III) no head, only the proboscis remains. (*D*) Frequencies of phenotypes observed in *hth-Gal4*; UAS-GFP, *hth-Gal4*; UAS-HDless, *hth-Gal4*; UAS-HthFL, *hth-Gal4*; UAS-HthFL, UAS-HDless adults. "*n*" refers to the number of flies examined; for class I and II, eyes were scored individually. Because there were 10 class III (headless) flies in the HthFL experiment, only 53 flies were examined for the antenna-to-leg transformation.

Discussion

The discovery that genes are mosaics of expressed sequences (exons) embedded in a matrix of "silent DNA" (introns) raised the question of why genes are constructed in pieces (Gilbert 1978). It was quickly posited that this medley gene structure might enhance the coding capacity of the genome, as well as form an ideal framework for evolution to exploit (Gilbert 1978). Indeed, alternative splicing mechanisms have been shown to use this gene architecture to produce functionally distinct proteins from a single gene, expanding the proteome and its complexity. However, although alternative splicing can in principle lessen the discrepancy between genome coding capacity and organismal complexity, for most genes the functional differences between alternatively spliced variants have remained elusive.

In this study, we address the functional relevance of alternatively spliced isoforms of Hth, a transcription factor involved in a wide variety of developmental programs that are critical for the construction of the *D. melanogaster* body plan. We report that *hth* encodes for both HD-containing and HDless variants, both capable of forming functional Hth/Exd complexes with putative distinct transcriptional properties. Analysis of $hth^{100\text{-}1}$ mutant tissues during both embryonic and larval stages have demonstrated a strict requirement for the HD in a surprisingly small subset of developmental functions, such as the instruction of antennal identity and the correct patterning of the wing hinge. In contrast, partial loss of function of HDless forms, resulting either from siRNA injection against the 6′ and 7′ isoforms or from the ectopic expression of HthFL, suggest that these forms carry out crucial functions in vivo. Intriguingly, our data further suggest that HthFL is apparently unable to substitute for at least a subset of HDless functions. This idea rests primarily on our observation that 6′ + 7′ siRNA-injected embyros exhibit *hth* loss-of-function phenotypes yet still express HthFL. However, we cannot exclude the possibility that the injected siRNAs might have off-target effects, even though the specificity of the observed phenotypes suggests that it is unlikely. In future experiments, it may be possible to more definitively test this idea by generating *hth* mutant alleles that are unable to express the 6′ and 7′ isoforms.

On the other hand, the generally weak phenotypes observed in $hth^{100\text{-}1}$ embryos and adults support the hypothesis that HDless/Exd dimers work as bona fide transcription factors that are essential for the correct regulation of many *hth*-dependent functions. In some respects, these findings are reminiscent of reports showing that an artificially truncated and HDless version of the segmentation protein Fushi tarazu (Ftz) retains many of the activities of full-length Ftz (Fitzpatrick et al. 1992; Copeland et al. 1996). These earlier findings provide addi-

tional support to the idea that HDless forms of some homeoproteins retain biological activity, probably due to their ability to assemble stable protein complexes in vivo. What is unique to our results is that *hth* normally expresses HDless isoforms and that there is a division of labor between HDless and HthFL isoforms. This is best exemplified by our finding that HthFL isoforms are essential for antennal development but largely dispensable for proximal leg development. Based on these observations, we suggest that distinct Hth/Exd dimers may bind to partially overlapping sets of target genes in vivo, and that the presence or absence of the DNA-binding HD expands the range of target genes that Hth/Exd can select and regulate.

Alternative splicing as a means to expand Hth functional diversity

Hth is composed of two conserved modules: the HM domain that mediates an interaction with Exd and the DNA-binding HD. As the 6′ and 7′ isoforms do not have a HD, they are unable to directly interact with DNA. However, the presence of the HM domain allows them to complex with Exd, whose HD can mediate DNA binding, as demonstrated by the formation of cooperative HDless/Exd/Hox complexes on the *fkh[250]* and *lab48/95* elements. Consistent with our results, Meis has also been shown to form trimeric complexes with Pbx and Hox without binding directly to the DNA (Shanmugam et al. 1999).

The absence of the Hth HD has several implications for the transcriptional properties of HDless/Exd complexes. First, it is likely that HDless/Exd and HthFL/Exd complexes have distinct DNA-binding specificities because the latter complex contains two HDs, while the former contacts DNA exclusively through Exd's HD. We imagine that the two types of complexes regulate partly overlapping sets of target genes by decoding different *cis*-regulatory architectures, possibly in the same cells. For example, the HthFL isoform appears to be unable to carry out some *hth* functions since the 6′ + 7′ siRNA-injected embryos exhibit *hth* loss-of-function phenotypes. This observation suggests that the presence of the HD might be incompatible with a subset of the *cis*-regulatory architectures that bind HDless/Exd. Second, HDs can also be protein interactions motifs, raising the possibility that the absence of the Hth HD from HDless/Exd could influence its ability to contact other transcription factors, coactivators, and/or corepressors. The Exd and Pbx TALE HD mediate direct interactions with Hox factors (Passner et al. 1999; Piper et al. 1999; LaRonde-LeBlanc and Wolberger 2003), and with the HD-containing transcription factor Engrailed (En) (Peltenburg and Murre 1996; Gebelein et al. 2004). The HD of Hth, which is also of the TALE family, is also likely to interact with other transcription factors, including Hox proteins (Gebelein et al. 2004). Thus, through alternative splicing, the modular architecture of Hth is exploited to produce unique transcription factor complexes that are likely to have distinct protein and DNA-binding properties.

The HD is required for Hth to function as a selector gene

Given that HthFL and HDless isoforms have some unique functions during development, it is tempting to suggest some generalizations about which functions require the Hth HD and which do not require this domain. Insect body plans are made up of repeated units that develop into diverse body parts in the adult due to the activity of selector genes, transcription factors that instruct morphological identities by regulating unique sets of target genes (Mann and Carroll 2002). Legs and antennae in *Drosophila* represent an example of serially homologous appendages that develop from a leg-like ground-state in response to different selector activities: Hox factors select for legs while Hth/Exd select for antenna (Struhl 1981, 1982; Casares and Mann 1998, 2001). Our demonstration that a $hth^{100\text{-}1}$ mutant antenna is completely transformed toward a ground-state leg-like appendage demonstrates that the antennal selector function of Hth is absolutely dependent on its HD.

In contrast to its antennal selector role, our data suggest that the Hth HD is largely dispensable for at least some of the Hox-cofactor functions of Hth/Exd. This surprising conclusion is based in part on the cuticle phenotypes of $hth^{100\text{-}1}$ and 6′ + 7′ siRNA-injected larvae. Specifically, $hth^{100\text{-}1}$ larvae show no or very weak transformations of segmental identity, whereas 6′ + 7′ siRNA-injected larvae show clear posterior-directed transformations. Consistently, $hth^{100\text{-}1}$ mutant embryos still express two directly activated Hox/Exd/Hth targets, *fkh[250]* and *lab550*. Repression of *Distalless* (*Dll*), which also requires direct Hox/Exd/Hth input (Gebelein et al. 2004), also occurs normally in $hth^{100\text{-}1}$ mutant embryos (data not shown). Thus, from these diverse observations we conclude that the Hth HD is largely dispensable for the Hox-cofactor function of Hth/Exd. However, we note that there are exceptions to this generalization. Although activation of *lab550* does not require the Hth HD, activation of a weakened derivative of this enhancer, *lab48/95*, does require the Hth HD. Similarly, mutation of the Hth-binding site in the *Dll* repressor element, DllR, results in weak abdominal derepression (Gebelein et al. 2004). Taken together, these data suggest that the transcription factor complexes binding to the *lab* and *Dll* regulatory elements contain the Hth HD, but that its presence is only required when the activity of these elements is compromised or weakened.

A third well-characterized function of Hth/Exd is its role in the establishment of the PD axis in both ventral (legs) and dorsal (wings and halteres) appendages. Our experiments suggest that the Hth HD is not required for PD axis formation or for specifying proximal identities in the legs. In the wing, the Hth HD is also apparently dispensable for forming a correct PD axis (in particular, repression of *wg* at the DV boundary) but is partially required for specifying proximal (hinge) fates. Notably, both functions in which the Hth HD is largely dispensable (PD axis formation and Hox cofactor activity) appear to be evolutionarily ancient. Like Hth/Exd, Meis/Pbx are

Hox cofactors and are also instrumental for establishing the PD axis of the vertebrate limb (Phelan et al. 1995; Berthelsen et al. 1998a,b; Jacobs et al. 1999; Mercader et al. 1999; Piper et al. 1999; Ferretti et al. 2000; LaRonde-LeBlanc and Wolberger 2003). In contrast, the antennal-specifying activity of Hth/Exd, which requires the Hth HD, is not known to have a vertebrate correlate. Thus, it is tempting to speculate that, in *Drosophila*, the Hth HD is more essential for executing evolutionarily recent, invertebrate-specific Hth functions and plays a less crucial, supplemental role in evolutionarily ancient Hth/Meis activities. Consistent with the idea that the HDless activities of Hth are ancient is our identification of an analogous HDless isoform made by *Meis1* in *Mus musculus,* which underscores the functional relevance of HDless isoforms for the fulfillment of Hth/Meis-dependent functions during both invertebrate and vertebrate development. Interestingly, *Prep2,* another vertebrate gene related to *hth,* also appears to encode both HD-containing and HDless isoforms (Haller et al. 2004). Although the functions of these isoforms are not known, our results suggest that there may be a similar division of labor of HD-containing and HDless isoforms encoded by the *Meis1* and *Prep2* genes of vertebrates.

In summary, our results strongly support the idea that alternative splicing of Hth and its vertebrate orthologs is an evolutionarily conserved mechanism to expand the architectural diversity of Hth/Exd and Meis/Pbx transcriptional complexes. We propose that by excluding or including the HD of Hth, Hth/Exd complexes acquire distinct DNA-binding and protein interaction properties, which allow them to regulate different sets of target genes and execute unique developmental programs in vivo.

Materials and methods

Genotypes and genetic manipulations

Loss-of-function experiments. Mutant clones of *hth* were generated by flp-mediated recombination. Males *yw; FRT82B* hth^{P2}*/TM6B or yw; FRT82B* $hth^{100\text{-}1}$*/TM6B,* y^+ were crossed to females *yw, hs-flp; FRT82B ubi-GFP, M/TM6B* to analyze mutant tissue during imaginal disc development. Mutant tissue was marked by the absence of GFP. The same males were crossed to females *yw, hs-flp*; *FRT82B hs-CD2,* y^+ *M/TM2* for the analysis of adult structures, with homozygous mutant tissue marked by *y* bristles. The progeny were heat-shocked for 15 min to 1 h at 37°C, at 24–48, 48–72, 72–96, and 96–120 h after egg laying (AEL).

fkh[250]-lacZ expression was analyzed in *fkh[250]-lacZ; FRT82B* hth^{P2} or *FRT82B* $hth^{100\text{-}1}$*/Cyo-TM3 GFP* embryos, with mutant embryos marked by the absence of GFP. To define the requirement of the Hth HD for *lab* expression, *FRT82B* $hth^{100\text{-}1}$*/Cyo-TM3 GFP* flies were crossed to *lab550-lacZ; FRT82B* hth^{P2}*/cyo-TM6B* or *lab 48/95-lacZ,* hth^{P2}*/TM2*. No *lab48/95-lacZ* staining was observed in any of the GFP-negative embryos, while *lab550-lacZ* staining was detected in half of the GFP-negative embryos analyzed. *FRT82B* hth^{P2}*/TM6B* flies were crossed to *lab550-lacZ; FRT82B* hth^{P2}*/Cyo-TM6B* or *lab 48/95-Z,* hth^{P2}*/TM2*. hth^{P2} embryos were identified by the loss of anti-Hth staining.

Gain-of-function experiments. The Gal4/UAS system (Brand and Perrimon 1993) was used to ectopically express GFP and HthFL (Casares and Mann 1998) and/or HDless, previously denoted as Hth-HM (Ryoo et al. 1999), using the *hth-Gal4* (GETDB-Gal4 Enhancer Trap Insertion database) driver line. Both HthFL and HDless are GFP tagged. Crosses were grown at 25°C for 12 h and then transferred at 29°C for the reminder of development.

yw hs-flp; tub > hs-CD2>Gal4 was used to drive expression of HthFL (Casares and Mann 1998) and/or HDless (Ryoo et al. 1999) in clones. Larvae were heat shocked for 10–15 min at 37°C and then returned to 25°C until dissection at mid to late third instar.

The *ptc-Gal4* driver line was used to misexpress GFP, HthFL, and HDless in eye-antenna imaginal discs. All the experiments were performed at 25°C.

Whole-mount in situ hybridizations and cuticle preparations

The probe for the detection of all *hth* isoforms was prepared by using a full-length *hth* cDNA as template, while the probe specific for the HD-containing variants includes the *hth* cDNA sequence starting from part of exon 9 to the end of the protein. The 6′ and 7′ variant probes include the 6′ and 7′ exons, respectively, as well as part of the following exclusive 3′UTRs. The probes were labeled with digoxigenin-UTP as instructed by the manufacturer (Roche), and in situ hybridizations were performed as previously described (Tautz and Pfeifle 1989). Embryonic cuticle preparations were performed as described in Wieschaus and Nusslein-Volhard (1986).

RNAi

RNAi was performed by microinjecting preblastoderm embryos as described (Kennerdell and Carthew 1998). Twenty-one-nucleotide double-stranded RNA (dsRNA) (Dharmacon, A4 option) against the 6′ and 7′ isoforms was coinjected at a final concentration of 2.5 and 5mg/mL. Buffer was injected as control.

Targets sequences used were as follows: *exon6′* isoform, 5′-GG AGCACATGGTCCTATAT-3′; *exon7′* isoform, 5′-GCAGTTC TCTATGAATATA-3′.

RT–PCR

Total RNA was isolated from populations of buffer or 6′ and 7′ siRNA-injected *Drosophila* embryos, or E10.5 mouse embryos, using standard procedures (Trizol). Synthesis of first-strand cDNA was primed by oligo (dT) and followed by RT–PCR. Oligonucleotides sequences are available upon request. The putative mouse 6′ homolog was sequenced revealing a bona fide 6′ variant.

Immunohistochemistry

Antibodies used were as follows: rabbit anti-β-galactosidase (Cappel), mouse anti-Dac (Iowa University Hybridoma bank; Mardon et al. 1994), rabbit anti-Exd (Mann and Abu-Shaar 1996), rat anti-Sal (Barrio et al. 1999), guinea pig anti-Tsh (from G. Struhl), guinea pig anti-Vg (from G. Struhl), and mouse anti-Wg (4D4, University Hybridoma bank; Neumann and Cohen 1997). The guinea pig (Ryoo and Mann 1999) and the rabbit anti-Hth were raised against the full-length protein. The rabbit anti-Hth6′ was produced against the 6′exon sequence N′-VSTPFAGAHGPILASYNAVVHPCS-C′ and used at 1:500 dilution. The guinea pig anti-HthHD was raised against a Hth fragment containing the HD and the C-terminal tail (from K363 to the end of the protein) and used at 1:500 dilution.

Immunoblot analysis and coimmunoprecipitations

Twenty wild-type, hth^{P2}, or $hth^{100\text{-}1}$ embryos were directly lysed in SDS loading buffer and boiled before PAGE. Proteins were transferred to PVDF membranes and probed with the guinea pig anti-Hth (1:2000). As a loading control, total proteins transferred to the membrane were stained with GelCode blue (Pierce).

Immunoprecipitations were performed using equivalent amounts of 0–12 h OregonR embryonic lysate. Embryo lysates were incubated with a rabbit anti-Exd (1:100) or a preimmune serum (1:100) and protein A/G-conjugated beads overnight at 4°C. Beads were washed three times with RIPA buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 0.5% NP-40) prior to SDS-PAGE and immunoblot analysis, using a guinea pig anti-Hth (1:2000) or a guinea pig anti-HthHD (1:500) antibody.

Protein purification and EMSA

EMSAs were carried out as described (Gebelein et al. 2002). The HDless construct includes the first 247 amino acids and was subcloned into pET14B (Novagen). Hox, Exd and Hth proteins used were purified as His-tagged fusions using Ni-chromatography from BL21 bacteria (Gebelein et al. 2002). Protein concentrations were determined by the Bradford assay and then confirmed by SDS-PAGE and Blue Coomassie analysis (GelCode Blue, Pierce). The amounts of Hth/Exd (50 ng/lane) and HDless/Exd dimers used were equimolar. The amounts of Lab used in Figure 4B were 80 and 160 ng. The lab48/95 probe has been previously described (Ryoo and Mann 1999; Ryoo et al. 1999).

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