Development 128, 3405-3413 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV8792

Genetic evidence for the transcriptional-activating function of Homothorax during adult fly development

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Accepted 14 June 2001

SUMMARY

Homothorax (HTH) is a homeobox-containing protein, which plays multiple roles in the development of the embryo and the adult fly. HTH binds to the homeotic cofactor Extradenticle (EXD) and translocates it to the nucleus. Its function within the nucleus is less clear. It was shown, mainly by in vitro studies, that HTH can bind DNA as a part of ternary HTH/EXD/HOX complexes, but little is known about the transcription regulating function of HTH-containing complexes in the context of the developing fly. Here we present genetic evidence, from in vivo studies, for the transcriptional-activating function of HTH. The HTH protein was forced to act as a transcriptional repressor by fusing it to the Engrailed (EN) repression domain, or as a transcriptional activator, by fusing it to the VP16 activation domain, without perturbing its ability to translocate EXD to the nucleus. Expression of the repressing form of HTH in otherwise wild-type imaginal discs phenocopied *hth* loss of function. Thus, the repressing form was working as an antimorph, suggesting that normally HTH is required to activate the transcription of downstream target genes. This conclusion was further supported by the observation that the activating form of HTH caused typical *hth* gain-of-function phenotypes and could rescue *hth* loss-of-function phenotypes. Similar results were obtained with XMeis3, the *Xenopus* homologue of HTH, extending the known functional similarity between the two proteins. Competition experiments demonstrated that the repressing forms of HTH or XMeis3 worked as true antimorphs competing with the transcriptional activity of the native form of HTH. We also describe the phenotypic consequences of HTH antimorph activity in derivatives of the wing, labial and genital discs. Some of the described phenotypes, for example, a proboscis-to-leg transformation, were not previously associated with alterations in HTH activity. Observing the ability of HTH antimorphs to interfere with different developmental pathways may direct us to new targets of HTH. The HTH antimorph described in this work presents a new means by which the transcriptional activity of the endogenous HTH protein can be blocked in an inducible fashion in any desired cells or tissues without interfering with nuclear localization of EXD.

Key words: Meis, Transcription, Activation, Homeodomain, Imaginal discs, Homothorax, *Drosophila melanogaster*

INTRODUCTION

The epidermis and cuticle of the cephalic and thoracic segments, the appendages, and the external genitalia of the adult fly, are derived from the imaginal discs. In each type of disc, specific patterns of gene expression determine the identity of the developing organs as well as their anterior-posterior, dorsal-ventral and proximal-distal axes (Cohen, 1993). Homothorax (HTH), a homeobox-containing protein, plays multiple roles in the formation of the adult fly organs, both as a selector of identity and an organizer of proximal-distal axis (Casares and Mann, 1998; Abu-Shaar and Mann, 1998; Wu and Cohen, 1999; Casares and Mann, 2000).

In the head, HTH activity leads to opposite effects on eye and antennal development, functioning as a negative regulator of eye development and a positive regulator of antennal development. In the absence of *hth* expression, ectopic eyes are formed in the ventral head region (Pai et al., 1998). Reciprocally, missexpression of *hth* in the developing eye results in a partial to complete suppression of eye development (Pai et al., 1998). In the antenna, *hth* mutant clones in proximal segments cause an antenna-to-leg transformation (Pai et al., 1998; Casares and Mann, 1998), whereas ectopic expression of *hth* in the distal region leads to a duplication of distal antennal segments (Yao et al., 1999). In addition, ectopic expression of *hth* or its murine homologue, *Meis1*, has been shown to induce the formation of ectopic antennae in different positions, such as the head, the legs, the genitalia and the analia (Casares and Mann, 1998; Dong et al., 2000).

In the leg and wing discs HTH is required for normal formation of the proximodistal axis. In the leg, *hth* mutant clones cause fusion of proximal segments, whereas ectopic

expression of *hth* results in distal truncation of the leg and appearance of proximal structures in more distal locations than normal (Pai et al., 1998; Casares and Mann, 1998; Mercader et al., 1999). In the wing disc, absence of *hth* from the proximal region results in loss of proximal wing structures (Casares and Mann, 2000), whereas ectopic expression of *hth* prevents distal wing development (Mercader et al., 1999; Casares and Mann, 2000; Azpiazu and Morata, 2000).

Great similarity exists between loss-of-function phenotypes of HTH and Extradenticle (EXD), another homeodomain-containing protein. For example, similarly to HTH, absence of EXD from the antennal disc causes a transformation of the antenna towards distal leg identity (González-Crespo and Morata, 1995; Rauskolb et al., 1995). In addition, *exd* mutant clones in the region of the rostral membrane differentiate ectopic eyes (González-Crespo and Morata, 1995). EXD can be found in the cytoplasm or the nucleus, but only the nuclear protein is functional (Mann and Abu-Shaar, 1996; Aspland and White, 1997). When nuclear, EXD can heterodimerize with different HOX proteins, altering their DNA binding specificity (Chan et al., 1994; van Dijk and Murre, 1994; Pöpperl et al., 1995). HTH enables the nuclear localization of EXD and in its absence EXD remains cytoplasmic (Rieckhof et al., 1997; Pai et al., 1998; Kurant et al., 1998). EXD is required to maintain normal levels of the HTH protein (Kurant et al., 1998; Abu-Shaar and Mann, 1998). Although the nuclear localization of EXD seems to be a major role of HTH, recent studies clearly indicate that this is not its only role. The fact that HTH contains a homeodomain suggests that it may bind DNA, as part of a complex, or on its own. In fact, HTH has been shown to participate in a ternary complex with EXD and the HOX protein Labial in vitro (Ryoo et al., 1999). In addition, PREP1 (also known as Pknox1) and Meis1, murine homologues of HTH, have been shown to form ternary complexes with Pbx1, the mouse homologue of EXD, and a HOX protein (Berthelsen et al., 1998; Jacobs et al., 1999). In vivo, when the homeodomain of HTH was missing or mutated, HTH was found to still localize EXD to the nucleus, yet it was unable to induce some of its ectopic expression phenotypes, suggesting that in some contexts it functions as a transcription factor (Ryoo et al., 1999; Jaw et al., 2000; Kurant et al., 2001).

Our aim was to determine whether HTH functions as a transcription factor and if so, whether it is an activator or a repressor of transcription. We generated and expressed in transgenic flies chimeric forms of HTH, fusing it to the Engrailed (EN) repression domain or to the VP16 activation domain, thus forcing HTH to act as a repressor or activator of transcription respectively. Expression of the transcriptional-repressing form of HTH phenocopied *hth* loss of function, whereas the transcriptional-activating form of HTH mimicked the native protein and caused gain-of-function phenotypes. These results are consistent with HTH acting as a transcriptional activator in many developmental processes.

MATERIALS AND METHODS

Generation of DNA constructs and transgenic flies

The *EN-XMeis*$^{1\text{-}333}$ fusion construct was generated by Dibner et al. (Dibner et al., 2001) in the pCS2 vector (Kessler, 1995). The *EN-XMeis*$^{1\text{-}333}$ fusion was then subcloned into the *Eco*RI-*Xba*I sites of the *pUAST* vector (Brand and Perrimon, 1993). To generate the *pUAST-EN-HTH*$^{1\text{-}430}$ construct, amino acids 1-430 of HTH were amplified by PCR from *hth* cDNA. A *Xho*I site was added to the 5′ end of the PCR product and a *Xba*I site to its 3′ end. The PCR product was cloned into the *Xho*I-*Xba*I sites of the *pUAST-EN-XMeis*$^{1\text{-}333}$ plasmid from which the *XMeis* gene was excised. The same strategy was used to generate the *pUAST-VP16-HTH*$^{1\text{-}487}$ construct, but the full-length *hth* ORF was amplified. The constructs were sequenced to verify that no mutations were incorporated during the PCR. Several transgenic strains were generated and analyzed for each construct.

Immunohistochemistry

Staining of whole-mount embryos and imaginal discs was performed using standard techniques (Patel, 1994) with minor modifications. Primary antibodies were, anti-EXD monoclonal antibody (mAb) B11M (Aspland and White, 1997), mouse anti-ELAV (obtained from the Developmental Studies Hybridoma Bank at the university of Iowa) and mAb 22C10 (Fujita et al., 1982). Secondary antibodies were Cy3-conjugated anti-mouse (Jackson), or biotinylated anti-mouse detected with Vecta-Stain Elite ABC-HRP kit (Vector Laboratories). Stained embryos and discs were viewed using bright-field and confocal microscopy (Zeiss Axioskop and Radiance 2000, BioRad).

Cuticle preparations

Anesthetized adult flies were boiled in a 10% NaOH solution for 10 minutes. The cuticles were washed in water, dissected and mounted in Hoyer's. The preparations were examined using a Zeiss Axioskop microscope.

Fly strains

The following strains were used: *dpp-Gal4* (Staehling-Hampton et al., 1994), *arm-Gal4* (Sanson et al., 1996), *Kr-Gal4* (Castelli-Gair et al., 1994; Obtained from Maria Leptin), and *UAS-hth*^{12} (Pai et al., 1998). For the rescue experiments *Kr-Gal4, hth*$^{64\text{-}1}$*/TM6, Tb P[w*$^{+}$*, abdA-lacZ]* and *UAS-VP16-HTH*$^{1\text{-}487(\#24\text{-}3)}$*;hth*$^{64\text{-}1}$*/TM3* strains were generated. Fly strains for competition experiments: The *UAS-hth*^{12}, *UAS-EN-HTH*$^{1\text{-}430(\#51\text{-}1)}$, and *UAS-EN-XMeis*$^{1\text{-}333(\#32\text{-}3)}$ transgenes are all inserted on the third chromosome. *UAS-hth*^{12}, *UAS-EN-HTH*$^{1\text{-}430(\#51\text{-}1)}$ and *UAS-hth*^{12}, *UAS-EN-XMeis*$^{1\text{-}333(\#32\text{-}3)}$ strains were generated by recombination. The presence of both transgenes was verified by PCR.

RESULTS

Generating repressing and activating forms of HTH

The fact that HTH contains a homeodomain suggests that it binds DNA and affects transcription. To establish if HTH is indeed a transcription factor, acting as an activator or repressor of gene expression, we uncoupled the two functions of HTH, namely the nuclear localization of EXD and regulation of transcription. We generated two recombinant forms of HTH that act in opposing manners, as an activator and a repressor of transcription without perturbing the ability of these HTH variants to translocate EXD to the nucleus. The rationale behind this experiment is that if HTH is indeed a transcription factor, one of these chimeric forms should mimic the native protein, whereas the other form should work as an antimorph. If the activating form of HTH leads to a gain-of-function phenotype and the repressing form to a loss-of-function phenotype, then we can conclude that HTH functions normally as an activator of transcription. If, however, the activating form causes a loss-of-function phenotype and the repressing form results in a gain-of-function effect, then the conclusion would be that HTH is a transcriptional repressor.

The transcription-activating form of HTH was created by fusing the viral VP16 activation domain (Sadowski et al., 1988) to the amino terminus of a full-length HTH (VP16-HTH$^{1-487}$). The repressing form of HTH was generated by fusing the *Drosophila* EN repression domain (Han and Manley, 1993) upstream to a truncated form of HTH (amino acids 1-430; EN-HTH$^{1-430}$). The *VP16-HTH$^{1-487}$* and *EN-HTH$^{1-430}$* fusion constructs were cloned into the *pUAST* vector (Brand and Perrimon, 1993) under the regulation of the yeast upstream activating sequence. Several independent transgenic strains containing either *UAS-VP16-HTH$^{1-487}$* or *UAS-EN-HTH$^{1-430}$* were generated.

To establish whether HTH functions normally as an activator or repressor of transcription, we expressed the *UAS-VP16-HTH$^{1-487}$* and *UAS-EN-HTH$^{1-430}$* transgenes in imaginal discs under the regulation of the *dpp-Gal4* driver and examined the phenotypes of the emerging adults.

The repressing form of HTH, EN-HTH$^{1-430}$, induces loss-of-function phenotypes

Driving expression of the *UAS-EN-HTH$^{1-430}$* construct in imaginal discs by the *dpp-Gal4* driver at 29°C resulted in pupal lethality. At 24°C, pupal development and metamorphosis were completed. However, pharate adults were unable to emerge and had to be taken out from their pupal cases for examination. More than 30 *dpp-Gal4/UAS-EN-HTH$^{1-430}$* flies were examined and all of them exhibited some or all of the following phenotypes. The eyes and eye imaginal discs were often enlarged and ectopic eyes appeared occasionally on the ventral head capsule and antennae (Fig. 1A-D). Numerous ectopic bristles were often situated at different locations along the eye margin (Fig. 1E-F). The appearance of ectopic eyes in the ventral head region is typical of *hth* mutant clones (Pai et al., 1998) and thus the EN-HTH$^{1-430}$ protein seems to work as a HTH antimorph in this context.

Notably, in all of the examined flies, we observed a partial antenna-to-leg transformation. The first and second segments of the antenna appeared normal, but while the third segment was unaffected in its proximal part, it was transformed into leg in its distal portion (Fig. 1G). The structure emerging from a3 was rounded and densely covered with bracted bristles, typical of distal leg, with its appearance resembling mostly that of tibia (Fig. 1H). More distal leg structures were not observed in any of the transformed antennae. Antenna-to-leg transformations were previously observed in *hth* mutant clones (Pai et al., 1998; Casares and Mann, 1998), and in cases when HTH expression in the antennal disc was repressed by ectopic expression of different homeotic genes (Casares and Mann, 1998; Yao et al., 1999).

All three legs were also severely malformed and truncated. Proximal leg structures, coxa and trochanter, were reduced in size and fused together. More distal leg structures consisted of a round mass, which appeared to be a shortened, thickened femur fused to an extremely thickened and rounded tibia. In some of the legs small segments with the appearance of distal tibia or pretarsus were present (Fig. 1I,J). However, more distal structures, including the claws, were not evident. The leg discs lost their normal structure of concentric folds and appeared bloated (Fig. 1K,L). Reduction in size and fusion of proximal leg segments were observed in *hth* mutant clones induced in the presumptive proximal region of leg discs (Pai et al., 1998; Wu and Cohen, 1999).

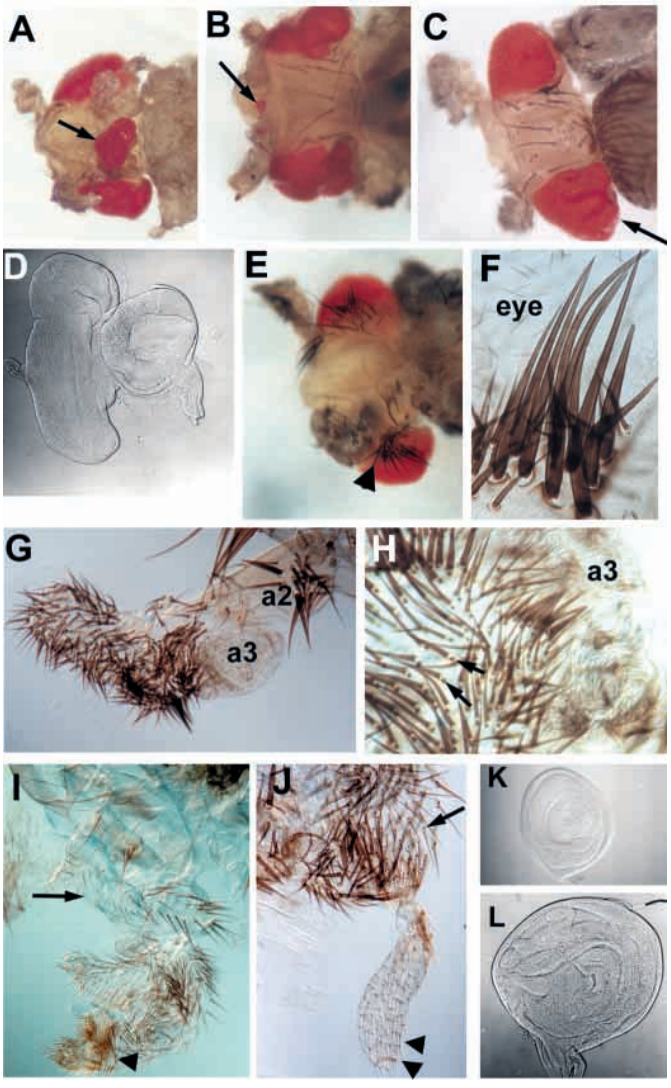

Fig. 1. Phenotypic effects of EN-HTH$^{1-430}$. Phenotypes characteristic of *dpp-Gal4/UAS-EN-HTH$^{1-430}$* flies grown at 24°C. (A-B) Ectopic eyes form in various positions on the ventral head cuticle, including the region of the maxillary palp (A) and the antennal foramen (B). (C,D) Hyperplasia of the eye is evident in adult flies (C) and eye imaginal discs dissected from a third instar larva (D). (E,F) Expression of *EN-HTH$^{1-430}$* in the eye-antenna disc causes tufts of large bristles to appear in ectopic positions along the eye margin. (G,H) Cuticle preparations of antennae from *dpp-Gal4/UAS-EN-HTH$^{1-430}$* flies showing an antenna-to-leg transformation. (G) The a2 antennal segment is relatively normal. The distal portion of the a3 segment is replaced by a leg-like structure. (H) Higher magnification of the transformed antenna reveals the presence of bracted bristles (arrows) typical of distal leg segments. (I,J) Cuticle preparations of a third (I) and second (J) leg of *dpp-Gal4/UAS-EN-HTH$^{1-430}$* flies. The proximal leg segments are reduced in size and fused together (arrow in I). Distal parts of the truncated legs consist of disfigured leg structures with bristle patterns typical of tibia (arrow in J) or pretarsus (arrowheads in I and J). (K-L) Third leg discs from wild-type (K) or *dpp-Gal4/UAS-EN-HTH$^{1-430}$* (L) third instar larvae shown at the same magnification. The *EN-HTH$^{1-430}$* expressing disc is significantly enlarged and exhibits abnormal pattern of epithelial folds.

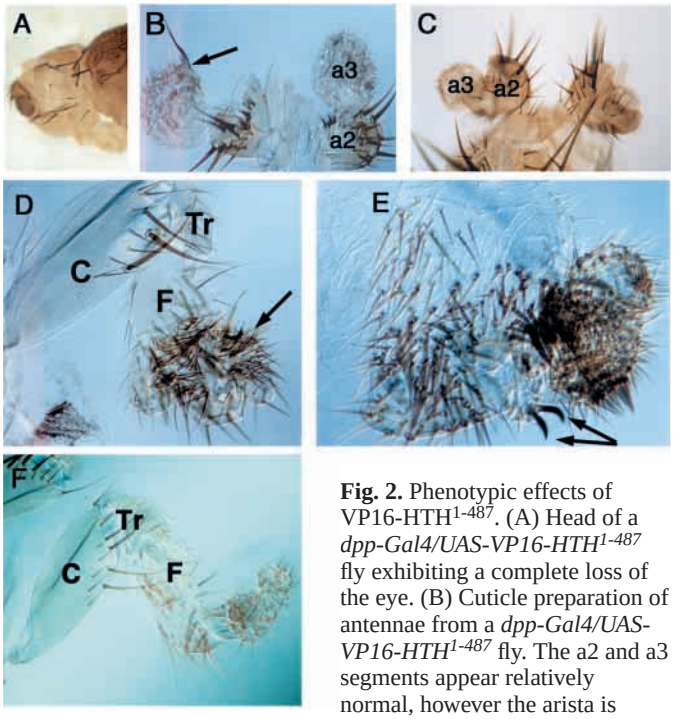

Fig. 2. Phenotypic effects of VP16-HTH$^{1-487}$. (A) Head of a *dpp-Gal4/UAS-VP16-HTH$^{1-487}$* fly exhibiting a complete loss of the eye. (B) Cuticle preparation of antennae from a *dpp-Gal4/UAS-VP16-HTH$^{1-487}$* fly. The a2 and a3 segments appear relatively normal, however the arista is either spineless and fused to the a3 segment (arrow) or completely missing. (C) A typical antenna of a *dpp-Gal4/UAS-hth^{12}* fly. The a3 segment is reduced in size and the arista is missing. (D-E) Cuticle preparations of a first leg (D) and a second leg (E) of a *dpp-Gal4/UAS-VP16-HTH$^{1-487}$* fly. C, coxa; Tr, trochanter; F, femur. The coxa, trochanter and proximal femur are unaffected. Distal leg structures are deformed and condensed together. The arrows indicate the claws. (F) First leg of a *dpp-Gal4/UAS-hth^{12}* fly. Proximal segments are intact, as they are in the leg in D. The leg is truncated and the remaining distal structures are malformed.

Altogether, the described observations demonstrate, that when HTH was forced to act as a repressor of transcription, it caused phenotypes typical of its loss of function. This suggests, that normally, HTH acts as an activator of transcription.

The activating form of HTH induces gain-of-function phenotypes and can substitute for the endogenous protein

To further test the hypothesis that HTH normally functions as an activator of transcription, we examined the effects of the activating form VP16-HTH$^{1-487}$ on adult development. If HTH is indeed a transcriptional activator, it is predicted that expressing its activating form will cause gain-of-function phenotypes.

When the *UAS-VP16-HTH$^{1-487}$* construct was expressed under the regulation of the *dpp-Gal4* driver at 29°C, the affected flies exhibited similar phenotypes to those caused by ectopic expression of the native form of HTH under the same driver. The eyes were missing or extremely reduced in size (Fig. 2A) as observed in flies ectopically expressing native HTH (Pai et al., 1998). Although the antennae appeared normal in their proximal portion, the aristae were abnormal, varying from fusion of the base or loss of the spines, to its complete absence (Fig. 2B). Ectopic expression of normal HTH in the antenna was shown to cause a duplication or reduction in the size of a3 and a loss of the arista (Yao et al., 1999 and Fig. 2C).

Additionally, the effects of VP16-HTH$^{1-487}$ on all three legs were generally similar to those of native HTH. The ectopic expression of either of the transgenes did not affect the proximal segments, coxa, trochanter and proximal femur (Fig. 2D,F) but interfered with the development of the distal region of the leg. In VP16-HTH$^{1-487}$-expressing flies, the leg parts distal to the femur were shortened and distorted. In most cases, all distal elements, including the claws, could be detected (Fig. 2D,E). The sex combs of the male first leg could be seen, but the organization of the teeth was abnormal (not shown). Bifurcations in the distal region were also characteristic of the affected legs. Ectopic expression of native HTH resulted in similar phenotypes. The main difference was that the ectopic expression of native HTH abolished tarsus formation, and distal structures such as the claws were not observed (Fig. 2F).

To further verify that the VP16-HTH$^{1-487}$ chimera functions similarly to the endogenous HTH protein we tested its ability to rescue phenotypes caused by the loss of native HTH. We performed the rescue experiments in the embryonic peripheral nervous system (PNS) where phenotypes associated with loss of HTH activity are well characterized (Kurant et al., 1998; Kurant et al., 2001). Most typically, *hth* null mutants exhibit loss of neurons and dorsal localization of the lateral chordotonal (LCh5) neurons (Fig. 3B,E). The VP16-HTH$^{1-487}$ chimera was expressed in the *hth* null background (*hth$^{64-1}$*) under the regulation of the *Kr-Gal4* driver (Castelli-Gair et al., 1994), which drives expression in embryonic segments T3-A4. The resulting PNS phenotypes were evaluated by anti-Futsch/22C10 (Fujita et al., 1982; Hummel et al., 2000) staining. The anterior abdominal segments (A1-A4) of these embryos presented a dramatic rescue of the localization and number of LCh5 neurons. In contrast, such a rescue did not take place in more posterior segments in which the *VP16-HTH$^{1-487}$* transgene was not expressed (Fig. 3C,F). These results clearly demonstrate that the activating form VP16-HTH$^{1-487}$ can substitute for the loss of the endogenous protein and corroborate our conclusions about the normal function of HTH.

The repressing form of HTH (EN-HTH$^{1-430}$) localizes EXD to the nucleus

The described experiments were designed to uncouple the two functions of HTH: nuclear localization of EXD and regulation of transcription. To verify that the EN-HTH$^{1-430}$ protein indeed causes loss-of-function phenotypes by interfering with the transcriptional activity of the endogenous HTH, rather than with the nuclear localization of EXD, we examined the subcellular localization of EXD in embryos expressing high levels of the *EN-HTH$^{1-430}$* transgene. In late stage 13 wild-type embryos, high levels of nuclear EXD are evident in the thoracic ectoderm and lower levels are observed in the abdominal ectoderm (Kurant et al., 1998; Fig. 4A). In *arm-Gal4/UAS-EN-HTH$^{1-430}$* embryos high levels of nuclear EXD were observed throughout the ectoderm (Fig. 4B), indicating that EN-HTH$^{1-430}$ does not interfere with the nuclear localization of EXD and that it is capable of driving EXD into the nucleus.

The *Xenopus* homologue of HTH, XMeis3, functions similarly to HTH

HTH homologues in vertebrates have been shown to translocate EXD to the nucleus in transgenic flies (Rieckhof et

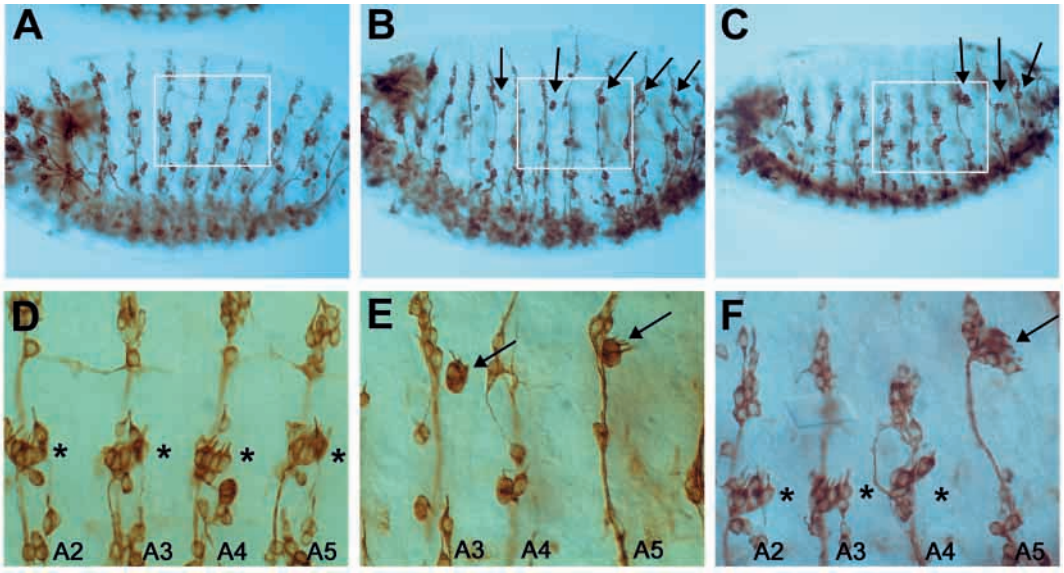

Fig. 3. VP16-HTH$^{1-487}$ can rescue the loss of native HTH. The pattern of PNS neurons in stage 15-16 embryos as revealed by mAb 22C10 staining. In all panels anterior is to the left and dorsal is up. The boxed areas in A-C are shown in higher magnification in D-F; segment identity is indicated. (A,D) A heterozygous *hth*$^{64-1}$ embryo showing a normal pattern of the PNS. The abdominal lateral chordotonal (LCh5) neurons can be clearly seen in the lateral PNS cluster of each abdominal segment A1-A7 (asterisks). (B,E) Similar views of a homozygous *hth*$^{64-1}$ embryo. A loss of neurons and dorsal localization of the LCh5 neurons (indicated by arrows) are evident in all abdominal segments. (C,F) Expression of *UAS-VP16-HTH*$^{1-487}$ was driven by *Kr-Gal4* in a *hth*$^{64-1}$ background. The LCh5 neurons are correctly positioned in abdominal segments A1-A4 (asterisks) where the transgene is expressed. More posterior segments do not exhibit such a rescue. Note the dorsal position of the LCh5 neurons in A5-A7 (arrows in C and F).

al., 1997). In order to check whether this functional homology applies to the transcriptional activity of these proteins as well, we generated transgenic flies carrying chimeric forms of *XMeis3*, the *Xenopus* homologue of *hth* (Salzberg et al., 1999). Inducing the transcription of a *UAS-EN-XMeis3*$^{1-333}$ construct, using the *dpp-Gal4* driver, resulted in phenotypes almost identical to those caused by the *UAS-EN-HTH*$^{1-430}$ transgene. At 29°C the EN-XMeis3$^{1-333}$ caused pupal lethality. At 24°C, all pharate adults taken out of their pupal cases exhibited enlarged eyes, and ectopic eyes were observed in many of them (Fig. 5A-C). The antennae were always partially transformed into legs (Fig. 5D). All three legs were malformed and truncated, with reduced proximal segments, abnormal mid leg, and loss of the most distal structures (Fig. 5E). These observations suggest that HTH and XMeis3 affect gene expression in a similar manner, thus extending the known functional similarity between HTH and its vertebrate counterparts.

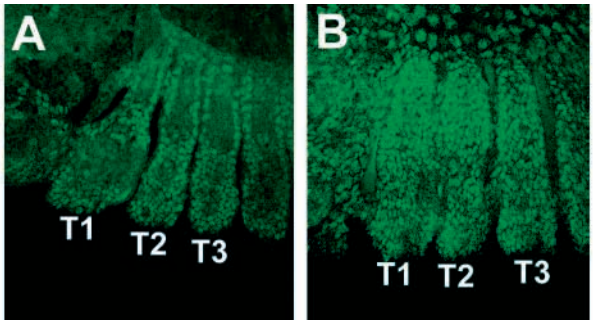

Fig. 4. EN-HTH$^{1-430}$ localizes EXD to the nucleus. Lateral view of stage 13 embryos stained with anti-EXD antibody. The thoracic segments are indicated. (A) A wild-type embryo. (B) An *arm-Gal4/UAS-EN-HTH*$^{1-430}$ embryo. Nuclear localization of EXD is clearly evident in both types of embryos.

EN-HTH$^{1-430}$ and EN-XMeis3$^{1-333}$ cause novel *hth*-related phenotypes

In addition to their effects in the eye, antenna and leg, EN-HTH$^{1-430}$ and EN-XMeis3$^{1-333}$ interfered with normal development of derivatives of the wing, genital and labial discs. Some of the phenotypes observed in these appendages have not been associated previously with *hth* mutations. The wings of the affected flies were severely malformed. Wing hinge structures were replaced by abnormal tissue (Fig. 6A) and bracted bristles were evident in this region (Fig. 6B). The anterior region of the wing blade was distorted, with the same cuticular appearance as the wing hinge. Bristles of the anterior wing margin were condensed into a very small area (Fig. 6A-C). The posterior part of the wing blade differentiated into wing blade tissue; however, it did not posses normal structures such as wing veins (Fig. 6A). In some of the EN-HTH$^{1-430}$ flies, a duplication of the wing was observed (Fig. 6D). The ectopic wing extended from the mesothorax, dorsal to the original wing, and was smaller in size. A similar phenotype consisting of loss of wing hinge structures and formation of ectopic wing tissue was reported when *hth* mutant clones were generated in the developing proximal wing (Casares and Mann, 2000). The wing disc was substantially enlarged. The folds of the blade region did not form properly and large epithelial sacs protruded from the normal plane of the disc (not shown).

The mouthparts of EN-HTH$^{1-430}$- and EN-XMeis3$^{1-333}$-expressing flies were also severely deformed. In the proboscis region, outgrowths were occasionally extending bilaterally from the labellum. Bracted bristles were observed on these outgrowths, suggesting a distal leg identity (Fig. 6F). Such a phenotype has never been described in *hth* mutants, however, a homeotic transformation of the labial palp into leg was observed in *proboscipedia* mutants (Kaufman, 1978)

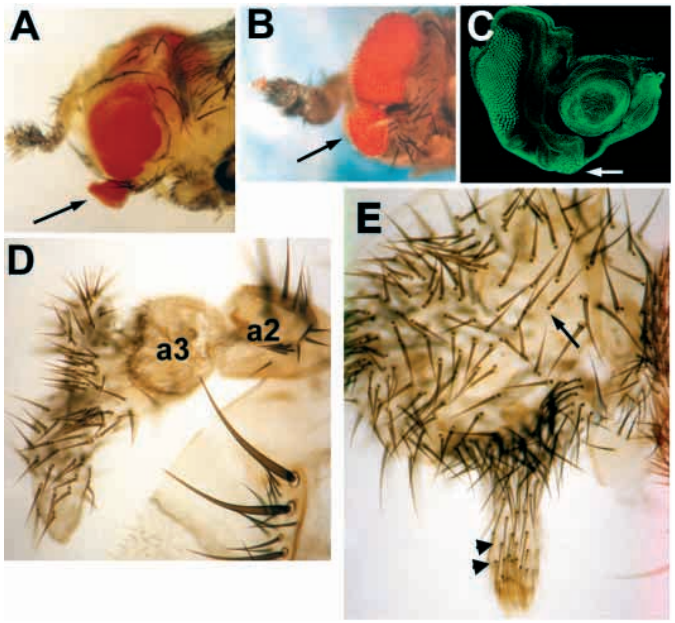

Fig. 5. Phenotypic effects of EN-XMeis3$^{1-333}$. (A,B) Phenotypes characteristic of *dpp-Gal4/UAS-EN-XMeis3*$^{1-333}$ flies grown at 24°C. Ectopic eyes are frequently observed in the ventral head region (arrows). (C) An eye-antenna disc from a third instar larva stained with anti-ELAV antibody. Ectopic photoreceptors are present in the presumptive ventral head region (arrow). (D) A typical antenna-to-leg transformation. The a2 segment appears normal. The a3 segment is transformed distally to distal leg tissue, as suggested by the presence of bracted bristles. (E) The distal region of a deformed and truncated third leg. Bristle patterns suggest that these structures are of tibial (arrow) and pretarsal (arrowheads) identities.

suggesting a possible interaction between *hth* and *proboscipedia*.

One dramatic phenotype, which was observed mainly in EN-XMeis3$^{1-333}$ expressing flies, was the outgrowth of leg-like structures from the anal region. These structures were of distal leg identity, as suggested by the presence of bracted bristles (Fig. 6G). In the genital discs, a substantial enlargement of the presumptive anal region was observed (Fig. 6I). Such enlargements were evident in genital discs from *EN-HTH*$^{1-430}$ flies as well (not shown), and even though the outgrowth of leg-like structures was rarely observed in the adult, the morphology of the genitalia and analia was abnormal.

EN-XMeis3$^{1-333}$ and EN-HTH$^{1-430}$ compete with the transcriptional activity of HTH

As described above, inducing EN-XMeis3$^{1-333}$ or EN-HTH$^{1-430}$ expression by *dpp-Gal4* resulted in dramatic *hth* loss-of-function phenotypes. However, there is a possibility that these constructs affect different downstream targets than the endogenous HTH, and therefore, do not act as true antimorphs of HTH. To verify that the putative antimorphs affect the same target genes as native HTH, we carried out competition experiments. Several strains carrying both the *UAS-hth*^{12} (Pai et al., 1998) and either the *UAS-EN-XMeis3*$^{1-333}$ or the *UAS-EN-HTH*$^{1-430}$ transgenes, were generated. When the concomitant expression of the transgenes was induced using the *dpp-Gal4* driver at 29°C, the increased level of native HTH not only rescued the lethality induced by the expression of the antimorphs, but was also able to generate gain-of-function phenotypes. Though some variability was observed, the legs and antennae of all examined strains showed typical HTH gain-of-function phenotypes. The eye phenotypes were variable, ranging from enlarged eyes in one of the strains to a complete loss of the eyes in another strain. Similar results were obtained when the cross was performed at 24°C, although the HTH gain-of-function phenotypes were generally milder. These results suggest that the repressing forms of HTH or XMeis3 competed with the transcriptional activity of the native form of HTH, thus working as true antimorphs.

DISCUSSION

HTH as a transcriptional activator

The existing data concerning the multiple roles of HTH in *Drosophila* adult development come from studies of HTH expression pattern and from its loss-of-function and gain-of-function phenotypes. Many loss-of-function phenotypes have been studied by generating *hth* mutant clones. In this type of analysis, unless the molecular nature of the mutation and its implication on HTH activity are known, it is impossible to determine which of the missing functions of HTH is responsible for the observed phenotypes. Such interpretation requires the ability to distinguish between the different functions of HTH. One characterized function of HTH is localizing EXD to the nucleus (Rieckhof et al., 1997; Pai et al., 1998; Kurant et al., 1998). Another, less characterized function of HTH, is participating in protein complexes that affect transcription. Several lines of evidence from previous reports suggest that HTH is directly involved in regulating transcription of target genes. In vitro, HTH has been shown to bind DNA as a part of a ternary HTH/EXD/HOX complex (Ryoo et al., 1999). In vivo, ectopic expression of nuclear EXD, or an HTH protein with a defective homeodomain, was unable to generate some of the typical HTH gain of function phenotypes (Ryoo et al., 1999; Jaw et al., 2000). Moreover, mutational and phenotypic analyses of *hth* revealed that when the homeodomain was mutated or absent, EXD remained nuclear, yet the development of the embryonic PNS was perturbed (Kurant et al., 2001). To clarify the role of HTH as a putative transcription factor in the context of the developing fly we manipulated the transcriptional activity of HTH without disturbing its ability to bring EXD to the nucleus. The repressing form of HTH, EN-HTH$^{1-430}$, phenocopied all of the previously reported loss-of-function phenotypes (Pai et al., 1998; Casares and Mann, 1998; Wu and Cohen, 1999; Casares and Mann, 2000). These include the appearance of ectopic eyes in the ventral head region, antenna-to-leg transformation, fusion of proximal leg segments and deformations in proximal wing structures. The activating form of HTH, VP16-HTH$^{1-487}$, was able to induce typical HTH gain-of-function phenotypes, such as loss of eyes, loss of aristae, and abnormal distal leg development. Furthermore, we demonstrated that VP16-HTH$^{1-487}$ could substitute for the loss of native HTH. The ability of the activating chimera to rescue *hth* loss-of-function phenotypes cannot be attributed only to nuclear localization of EXD, since these phenotypes could not be rescued by nuclear EXD alone (E. K. and A. S., unpublished data). Altogether

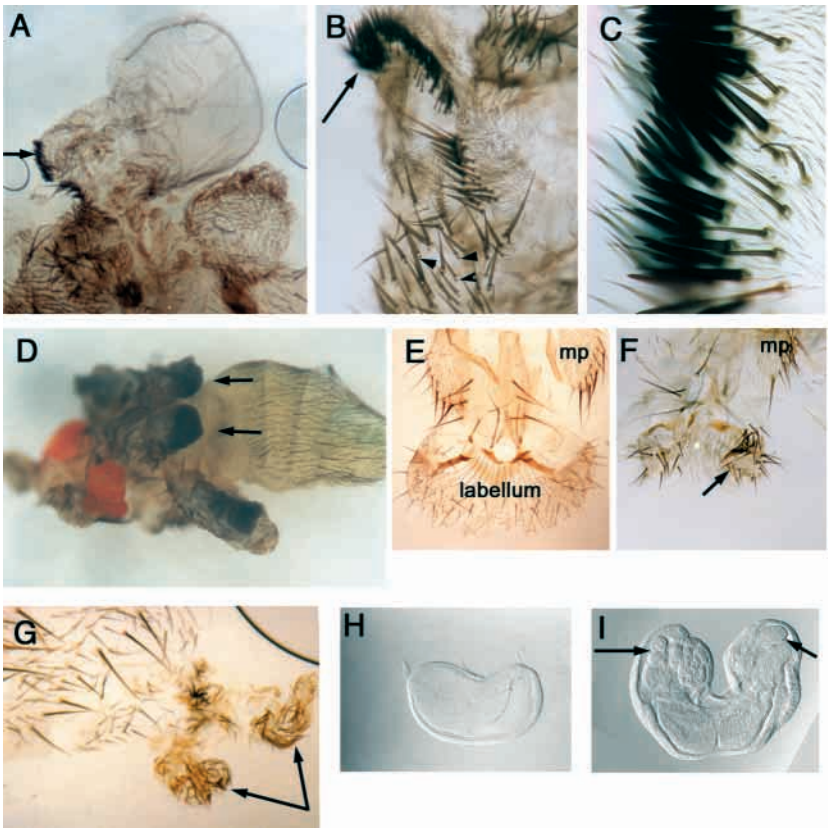

Fig. 6. The effects of EN-HTH$^{1-430}$ and EN-XMeis3$^{1-333}$ on derivatives of the wing, labial and genital discs. (A-C) Cuticle preparations of wings from *dpp-Gal4/UAS-EN-HTH$^{1-430}$* flies. (A) Low magnification showing the general organization of the affected wing. The arrow indicates the bristles of the anterior wing margin. (B) Higher magnification of the proximal region, which is covered by bracted bristles (arrowheads). The arrow indicates the bristles of the anterior wing margin. (C) Higher magnification of the anterior wing margin bristles concentrated in a small region of the proximal portion of the wing. (D) A *dpp-Gal4/UAS-EN-HTH$^{1-430}$* fly exhibiting a duplication of the wings. Extra wings are indicated by arrows. (E-F) Cuticle preparation of mouth parts of a wild-type (E) and a *dpp-Gal4/UAS-EN-HTH$^{1-430}$* fly (F). Note the outgrowths with bracted bristles on the labellum in F (arrow). (G) Cuticle preparation of a *dpp-Gal4/UAS-EN-XMeis3$^{1-333}$* fly showing a typical outgrowth from the anal region (arrows). (H-I) Genital discs of a wild-type (H) and a *dpp-Gal4/UAS-EN-XMeis3$^{1-333}$* (I) larvae. Note the abnormal morphology and hyperplasia of the presumptive anal region in I (arrows).

these data indicate that normally HTH functions in complexes that activate transcription. The exact role of HTH within these transcriptional activating complexes is not clear yet, however in order to simplify this discussion we hereafter refer to HTH as a transcriptional activator. This conclusion is further supported by the finding, that the *Xenopus* homologue of HTH, XMeis3, functions as a transcriptional activator inducing the expression of posterior neural markers. Like HTH, the XMeis3 protein could be turned into an antimorph by fusing it to the EN repression domain and forcing it to act as a transcriptional repressor (Dibner et al., 2001).

The inducible HTH antimorph described in this work enables the separation of the two known functions of HTH, and can therefore be used to answer further questions regarding the way in which HTH affects transcription. For example, can HTH with a defective homeodomain retain some of its transcription-regulating activity by binding through EXD to the DNA-binding complex? Moreover, this antimorph presents a new means by which the activity of the endogenous HTH can be blocked in an inducible fashion in specific cells and tissues in any developmental stage. The use of this tool is not restricted to *Drosophila* research; similar results were obtained recently in frog embryos using the EN-XMeis3 fusion protein (Dibner et al., 2001).

HTH as a transcriptional activator in appendage development

The results described in this work indicate that HTH normally works as a transcriptional activator in all examined tissues. Yet, its activity can have opposite effects on organ development in different contexts. For example, HTH ectopic expression in the developing eye leads to a reduction in size or a complete loss of this organ (Pai et al., 1998; this work), implying that HTH is a negative regulator of eye development. Conversely, in the antenna HTH is required for the formation of the organ, functioning as an antennal determining gene (Casares and Mann, 1998; Dong et al., 2000). It is possible that in the context of the developing eye, HTH induces the transcription of a repressor of eye development. In contrast, in the developing antenna HTH may activate the transcription of gene/s that promote antennal development. For example, *spalt* was suggested by Dong et al. (Dong et al., 2000), to be a downstream target of the combined action of HTH and Distal-less (DLL) in this pathway.

In the leg, HTH is required for proper proximodistal axis formation. Normally, HTH expression is limited to the proximal segments of the developing leg disc and DLL is expressed in the presumptive distal leg. The expression domains of HTH and DLL are mutual exclusive (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). When HTH was ectopically expressed in the DLL domain or along the anteroposterior (AP) border, distal leg structures failed to form, suggesting that HTH interferes with DLL function (Pai et al., 1998; Casares and Mann, 1998; Mercader et al., 1999; Ryoo et al., 1999). When the expression of EN-HTH$^{1-430}$ was induced along the AP border, all leg segments were affected. Proximally, fusion of coxa, trochanter and proximal femur was evident; a phenotype that was also observed when *hth* mutant clones were generated in the developing proximal leg region (Pai et al., 1998; Wu and Cohen, 1999). All tarsal segments were missing, and the remaining structures, which appeared to be mostly of tibial identity, were extremely deformed. The

phenotypes caused by either EN-HTH$^{1-430}$ or *hth* loss of function in the proximal leg are the same, and are therefore in accordance with our assumption that HTH normally induces transcription. Intriguingly however, in the distal region the ectopic expression of either normal HTH or EN-HTH$^{1-430}$ led to a loss of distal leg structures. This result can be interpreted in several ways. First, it is possible that in the specific context of the developing distal leg, ectopic HTH does repress the function of *Dll* and perhaps other genes, and in doing so abolishes distal leg formation. Another possibility is that the main role of HTH in interfering with distal leg development is the nuclear localization of EXD. It has been shown that when the ectopic expression of EXD was driven by *Dll-Gal4*, it was able to induce the same phenotype of leg truncation as ectopic HTH (González-Crespo and Morata, 1996). When EXD was expressed along the AP border it was able to disrupt distal leg formation to a lesser degree (González-Crespo and Morata, 1996; Jaw et al., 2000). Another observation in support of this view is that a defective HTH, in which the homeodomain was inactivated by a mutation (Ryoo et al., 1999) or a deletion (Jaw et al., 2000), was able to interfere with distal leg development when ectopically expressed, driven by *Dll-Gal4*. This effect was probably caused by the ability of the defective HTH to induce ectopic nuclear localization of EXD. Furthermore, in contrast to the effects caused by ectopic HTH, the ectopic expression of EXD or the homeodomain-defective HTH in the developing eye and antenna did not generate abnormal phenotypes (Ryoo et al., 1999; Jaw et al., 2000). This suggests that in the eye and antenna the transcriptional activity of HTH is required, whereas, in the specific context of distal leg, the transcriptional activity of HTH may be less relevant.

Evolutionary conservation of Meis proteins transcriptional activity

Another question we addressed is whether the transcriptional activity of HTH is conserved in its vertebrate homologues. HTH belongs to the Meis family of proteins. These proteins contain both a homeodomain and a Meis-homology domain. The Meis-homology domain is required for their interaction with PBC proteins and the nuclear localization of the latter (Knoepfler et al., 1997; Berthelsen et al., 1998; Ryoo et al., 1999; Jaw et al., 2000). The homeodomain is highly conserved between the different Meis proteins (Bürglin, 1997). However, whether different Meis proteins share a conserved transcriptional activity is not known.

Functional homologies between HTH and other members of the Meis family have been described. For example, ectopic expression of HTH in *Xenopus* embryos caused the same effects as the ectopic expression of XMeis3, the *Xenopus* homologue of HTH (Salzberg et al., 1999). Similarly, ectopic expression of XMeis3 in fly embryos led to the same phenotypes as HTH ectopic expression (A. S., unpublished). The results described here demonstrate that the repressing forms of both HTH and XMeis3 cause almost identical *hth* loss-of-function phenotypes in the adult fly, suggesting that XMeis3 affects transcription in the same way as HTH. Altogether, these results may indicate that Meis family proteins share another conserved function, as transcriptional activators. The ability to block the activity of the endogenous XMeis3 protein in frog embryos by using the EN-XMeis3 chimera further supports this conclusion (Dibner et al., 2001). XMeis3 and HTH act similarly in spite of a substantial size difference between the two proteins; 487 amino acids in HTH and 385 amino acids in XMeis3. The homology between the two proteins consists of two main regions, the MH-box and the homeodomain, and several small stretches of homologous amino acids. The exact contribution of each conserved region to the transcriptional activity and functional homology of the different Meis proteins requires further characterization.

We wish to thank Henry Sun, Robert White, Maria Leptin and the Bloomington Stock Center for antibodies and fly strains, Cai Ayjun for maintaining our fly stocks and Ze'ev Paroush for helpful discussion and critical reading of this manuscript. This work was supported by a Research Career Development Award to A. S. from the Israel Cancer Research Fund and grants (No.60/97 and 219/00) from The Israel Science Foundation founded by the Israel Academy of Sciences and Humanities. D. F. was supported by a grant from the Israel Cancer Research Fund.

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