Patterning function of *homothorax/extradenticle* in the thorax of *Drosophila*

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Summary

In *Drosophila*, the morphological diversity is generated by the activation of different sets of active developmental regulatory genes in the different body subdomains. Here, we have investigated the role of the *homothorax/extradenticle* (*hth/exd*) gene pair in the elaboration of the pattern of the anterior mesothorax (notum). These two genes are active in the same regions and behave as a single functional unit. We find that their original uniform expression in the notum is downregulated during development and becomes restricted to two distinct, α and β subdomains. This modulation appears to be important for the formation of distinct patterns in the two subdomains. The regulation of *hth/exd* expression is achieved by the combined repressing functions of the Pax gene *eyegone* (*eyg*) and of the Dpp pathway. *hth/exd* is repressed in the body regions where *eyg* is active and that also contain high levels of Dpp activity. We also present evidence for a molecular interaction between the Hth and the Eyg proteins that may be important for the patterning of the α subdomain.

Key words: Notum patterning, *hth, exd, eyg*

Introduction

One major issue in developmental biology is the manner in which the different body parts are genetically subdivided. In *Drosophila*, several major subdivisions have been identified and characterised: notably, the appearance of anterior and posterior compartments in all body segments and the various domains defined by the Hox genes along the anteroposterior body axis (Lawrence and Morata, 1994; Lawrence and Struhl, 1996; Mann and Morata, 2000). More recently, the gene *pnr* has been demonstrated to subdivide all or most body segments in the dorsoventral axis (Calleja et al., 2000; Herranz and Morata, 2001). All these are general subdivisions that affect several body segments, and therefore the specific development of each body part is not determined by any of the individual genes involved but more likely by a combination of active gene in each part – a genetic address (Garcia-Bellido et al., 1979).

The dorsal mesothoracic structures are formed by the wing imaginal disc and include the mesothorax, the wing hinge and the wing blade. The mesothorax is part of the trunk and is subdivided into an anterior compartment, the notum, and a posterior one, the postnotum. The notum contains approximately 15,000 cells and is the biggest part of the thorax. It is subdivided by the *pannier* (*pnr*) gene (Calleja et al., 2000) into medial and lateral regions, which are in turn further subdivided by the Pax gene *eyegone* (*eyg*) (Aldaz et al., 2003).

All the genes involved in these subdivisions encode transcription factors, and their activities trigger the establishment in the cells of specific developmental programmes, i.e. states of determination. Experiments in which these factors are expressed ectopically, which amount to transplantation experiments, demonstrate that these products are able to induce the formation of the corresponding pattern out of the normal context (Aldaz et al., 2003; Calleja et al., 2000; Diez del Corral et al., 1999).

Among the genes involved in body patterns, a special case is the pair *homothorax* (*hth*) and *extradenticle* (*exd*), which encode related homeodomain proteins (Kurant et al., 1998; Pai et al., 1998; Rauskolb et al., 1993; Rieckhof et al., 1997). *exd* is expressed ubiquitously, but it is only functional when the Exd product is transported to the cell nuclei (Aspland and White, 1997; Pai et al., 1998; Rieckhof et al., 1997). *hth* is required for the nuclear transport of Exd, and thus acts as a positive regulator of *exd*. In turn the Exd nuclear activity is necessary to prevent the degradation of the Hth product (Abu-Shaar and Mann, 1998; Aspland and White, 1997; Mann and Abu-Shaar, 1996; Pai et al., 1998). Thus, the two gene products are mutually necessary. Besides, the phenotype of *hth* and *exd* mutations is similar or identical (Abu-Shaar and Mann, 1998; Azpiazu and Morata, 2002; Kurant et al., 1998; Rieckhof et al., 1997). Altogether these observations suggest that the two genes are involved in the same function to which we refer as *hth/exd*.

A principal role described for *hth/exd* is its function as cofactor of Hox products (Peifer and Wieschaus, 1990; Rieckhof et al., 1997; Ryoo et al., 1999). The Hth and Exd proteins contribute to the specificity of the Hox products by forming complexes with the different Hox products that recognise Hox-binding sites with high affinity and specificity (Chan et al., 1994; Ryoo and Mann, 1999; Ryoo et al., 1999; Van Dijk and Murre, 1994). This raises the possibility that Hth and Exd may also interact with other transcription factors.

In addition to its role as a Hox co-factor, there are *hth/exd*

functions that seem to be independent of Hox activity. It is involved in the subdivision of wings and legs into proximal and distal domains (Abu-Shaar and Mann, 1998; Azpiazu and Morata, 2000; Azpiazu and Morata, 2002; Casares and Mann, 2000; Gonzalez-Crespo et al., 1998), and also has been shown to act as a selector gene in antennal development (Casares and Mann, 1998; Dong et al., 2002). That *hth/exd* may have this kind of function is not totally unexpected as *hth* and *exd* encode transcription factors that may regulate the transcriptional activity of specific target genes.

The notum of the fly is a region where there is no known Hox gene activity and is therefore a convenient place to examine possible *hth/exd* roles that are not dependent on interactions with Hox genes. Previous results have indicated that in the absence of *exd* activity the notum pattern is abnormal (Gonzalez-Crespo and Morata, 1995), and we have also noticed that the expression of *hth* and *exd* in the notum is not uniform, but shows a regional modulation. These two observations suggested a Hox-independent *hth/exd* function in the notum, which we explore in this report. We find that *hth/exd* is necessary to discriminate between two major parts of the notum, scutum and scutellum, and also contributes to scutellum identity. We also provide evidence for a molecular interaction between the Hth and Eyg products.

Materials and methods

Drosophila strains and clonal analysis

The following *Drosophila* strains were used to analyse mutant phenotypes or generate loss of function clones: exd^{Y012}, eyg^{SA2}, *FRT40A* Mad^{B1}*/FRT40AGFP* (Wiersdorff et al., 1996), *FRT2A* eyg^{SA2}*/FRT2A* GFP and *FRT82B* hth^{P2}*/FRT82BGFP*. The FLP/FRT technique (Xu and Rubin, 1993), or X-rays, were used to generate loss-of-function clones. For the first, larvae of the appropriate genotype were heat shocked for 1 hour at 37°C at different larval stages. The clones were visualised in discs by loss of GFP expression. For the second, the exd^{Y012} null allele and the Minute technique were used. Larvae of the genotype *y* exd^{Y012} f^{36}*/M(1)*o^{Sp} were irradiated at 24 and 48 hours after egg laying (AEL) with X-rays (1000 Rad). Mitotic recombination proximal to the *forked* (*f*) locus resulted in a clone of cells homozygous for exd^{Y012}, *y* and *f* that can be easily scored.

For gain-of-function experiments, the following Gal4 lines were used: *ywflp122; act-FRT* y^+ *FRT Gal4 UAS-GFP/SM5 Tb* (Ito et al., 1997), *ap-Gal4* (Rincon-Limas et al., 2000), *248-Gal4* (Sanchez et al., 1997) *eyg-Gal4* (Aldaz et al., 2003) and *hth-Gal4* (GETDB – Gal4 Enhancer Trap Insertion Data Base). The gain-of-function clones were generated by recombination at the FRT sequences. The clones were labelled with *Gal4* and *GFP* activity. In the adult cuticle the clones can be scored because they are mutant for f^{36a} and contain y^+ activity. The *UAS-eyg, UAS-hth* and *UAS-*tkv^{QD} (Hoodless et al., 1996) lines used were described previously. For the apoptosis experiments we used an *UAS-P35* line (Hay et al., 1994). The *lacZ* reporter line used was the DC-*LacZ* (Culi and Modolell, 1998).

Immunostaining of embryos and discs

Discs were dissected in PBS and fixed in 4% paraformaldehyde for 20 minutes at room temperature. They were subsequently washed in PBS, blocked in blocking buffer (PBS, 0.3% Triton, 1% BSA) and incubated overnight with the primary antibody: anti-β-Gal 1:2000 (rabbit), anti-Eyg 1:200 (guinea pig), anti-En 1:10 (mouse), anti-Exd 1:200 (rat), anti-Hth 1:500 (rabbit), anti-Ara 1:200 (rat) and anti-pMad 1:2000 (rabbit) diluted in blocking buffer at 4°C. Washes were performed in blocking buffer, and the appropriate fluorescent secondary antibody was added for 1 hour at room temperature. Following further washes in blocking buffer, the discs were mounted in Vectashield.

Anti-Ara antibody was kindly provided by S. Campuzano, anti pMad by T. Tabata, anti-β-Gal (rabbit) and anti-En were purchased from Cappel and from the Hybridoma Bank respectively. Images were taken in a laser MicroRadiance microscope (BioRad) and subsequently processed using Adobe Photoshop.

Protein interaction experiments

Crude *Drosophila* discs extracts were prepared by homogenizing 0.2 ml of third instar larvae in 0.4 ml of lysis buffer (1×PBS, 1% NP-40, 1 mM PMSF and 20 μg/ml each of peptastin and leupeptin). The homogenates were centrifuged, and the aqueous phase was mixed with the His-tagged, full-length Hth protein extracted under native conditions. The complexes formed were purified using the Ni-NTA Agarose (Quiagen), washed five times in 1×PBS, 0.5 M NaCl and one final time in 50 mM Tris (pH 6.8). They were boiled in loading buffer and resolved by SDS-PAGE. After blotting to nitrocellulose, the filter was incubated with the anti-Eyg antibody and the signal was detected using the Amersham ECL western blotting analysis system.

Preparation of larval and adult cuticles

For good X-gal staining of adult patterns, pharates were removed from the puparium and treated as described by Calleja et al. (Calleja et al., 1996). To examine the cuticular patterns of the different genetic combinations adult flies were prepared by the standard methods for microscopic inspection. Soft parts were digested with 10% KOH, washed with alcohol and mounted in Euparal. Embryos were collected overnight and aged an additional 12 hours. First instar larvae were dechorionated in commercial bleach for 3 minutes and the vitelline membrane removed using heptano-methanol 1:1. Then, after washing with methanol and 0.1% Triton X-100, larvae were mounted in Hoyer's lactic acid 1:1 and allowed to clear at 65°C for at least 24 hours.

Results

Expression of *hth*/*exd*

We have made use of the anti-Hth and anti-Exd polyclonal antibodies (Gonzalez-Crespo and Morata, 1995; Azpiazu and Morata, 2000), to study the expression patterns of *hth* and of *exd* in different stages of the wing disc. As *hth* activity is necessary for the nuclear translocation of the Exd protein, it was expected that there should be a correlation of *hth* expression and nuclear localisation of Exd. We have checked this by double staining mature third instar discs for *hth* and *exd* expression. The result is illustrated in Fig. 1D, which shows a near perfect co-extension of Hth with nuclear Exd. Therefore, for subsequent studies we have used the anti-Hth antibody as indicative of the expression of the two genes.

The expression of *hth* in the notum evolves during imaginal development. At the second instar all or nearly all the prospective notum cells express *hth* (Azpiazu and Morata, 2000) but by the beginning of the third instar period some modulation is already visible (Fig. 1A): *hth* expression is reduced in the central domain, whereas it remains at high levels in a posterior and an anterior region. Mature discs show two well-defined subdomains of expression (Fig. 1B), one located in the anterior region (subdomain α), and the other located more posteriorly (subdomain β).

We have further delimited the expression of *hth* with respect to that of other developmental genes that are active in the notum. Double staining with antibodies directed against the En,

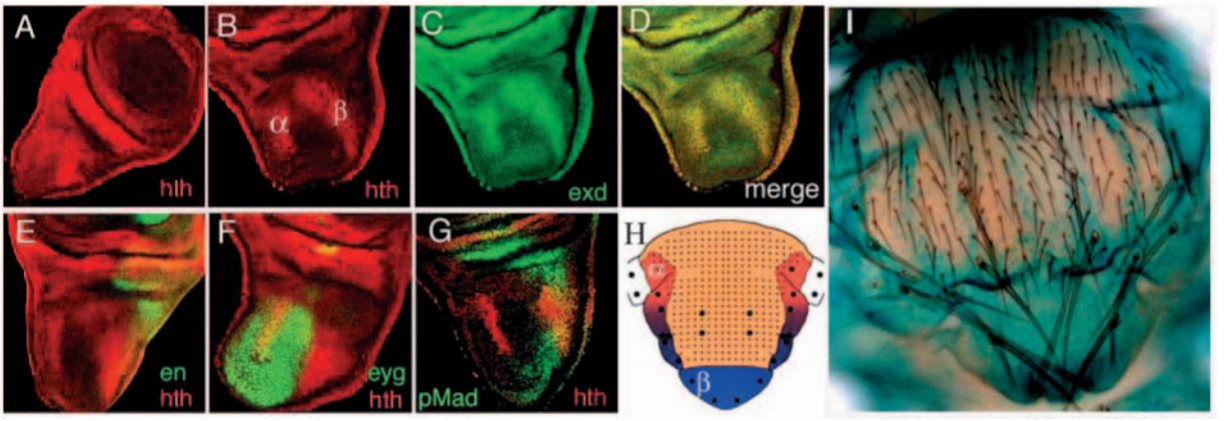

Fig. 1. Expression of *hth/exd* in the *Drosophila* notum. (A) Single staining for *hth* expression in a middle third instar disc. *hth* expression (red) covers most of the notum but some modulation is already visible. (B-D) Notum region of a mature disc doubly stained for *hth* (red) and *exd* (green). The two regions of high *hth* expression are the α and the β subdomains. The image in C shows only *exd* staining and in D the red and green channels are merged. The *hth* and *exd* expression patterns are almost identical. (E) Third instar disc doubly stained for *hth* (red) and *en* (green). Part of the β subdomain is included in the postnotum. (F) Notum part of a mature disc stained for *hth* (red) and *eyg* (green). The β subdomain and the *eyg* expression domain abut and form a sharp border, but the α subdomain is included within the *eyg* domain. (G) Double staining with anti-Hth (red) and anti-pMad (green). The β subdomain contains high levels of Dpp signalling. (H) A schematic of *hth* expression in the adult thorax. (I) Thorax of an *hth-Gal4 > UAS-lacZ* stained with X-gal to reveal *hth* expression in adult structures.

Eyg and pMad (Tanimoto et al., 2000) products is shown in Fig. 1E-G. Of special interest is the comparison of the *eyg* and *hth* domains (Fig. 1F): the α subdomain of *hth* is included within the *eyg* domain, but the β subdomain abuts in its anterior border with the posterior border of *eyg*. This subdomain coincides with high levels of *pMad* (Fig. 1G), indicating that the Dpp signalling pathway is active in this domain. X-gal staining of *hth-Gal4/UAS*lacZ flies (Fig. 1I) shows that the *hth* α subdomain differentiates the anterior lateral part of the adult notum and the β subdomain the scutellar region. It is also of interest to point out that the onset of *hth* modulation at early third instar (Fig. 1A) correlates with the initiation of *eyg* activity, which occurs approximately at that time (Aldaz et al., 2003).

Developmental function of *hth/exd* in the notum

We have studied the developmental role of *hth/exd* in the notum by examining the consequences of the loss of function and of modifying its normal spatial expression.

Notum development in the absence of *hth/exd* function

Null *hth* and *exd* mutants are zygotic lethal, therefore we have induced marked clones to study the behaviour of mutant cells in imaginal discs and the adult patterns they form. As their phenotype is the same, we have used indistinctly *exd* or *hth* mutant clones as convenient. For the adult cuticle we have used *exd* clones as they are better marked for the adult notum structures (see Materials and methods). We made use of the Minute technique (Morata and Ripoll, 1975) to induce large clones. Some of those that initiated early in development occupy a large part of the notum thus allowing the visualisation of the pattern produced in absence of *hth/exd* function (Fig. 2). The results obtained after having examined more than hundred clones can be summarised by saying that the loss of *hth/exd* does not produce a change of identity of the notum cells but gives rise to an abnormal notum pattern. It is illustrated in the cartoon of Fig. 2C, where we compare a normal notum pattern with that formed by the composite of a number of *exd* mutant clones. There are supernumerary macrochaetes on the presutural area (Fig. 2A), the infrascutellar suture disappears and the postalar bristles are not formed. The suture between scutum and scutellum also disappears (Fig. 2B), and the more posterior bristles tend to align along with the dorsocentral ones.

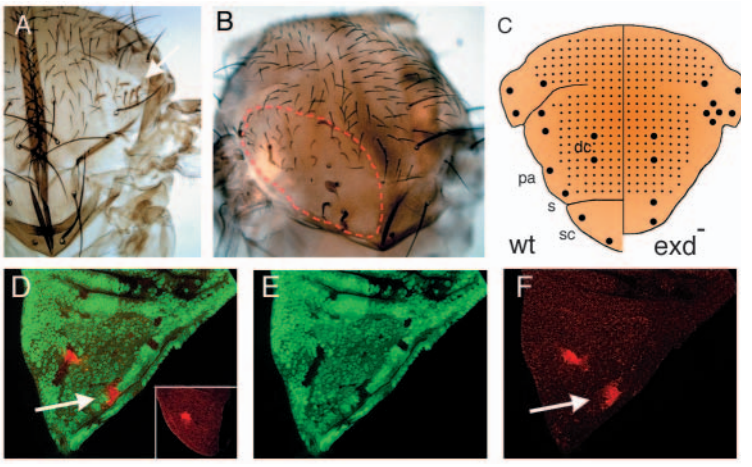

Fig. 2. Developmental effects of the loss of *hth/exd* function. (A) Adult notum containing a exd^- clone (arrow) in the lateral region. The clone differentiates several macrochaetes that are not normally present in that zone. (B) A large M^+ exd^- clone showing alterations of the bristle pattern in the scutellum region and the disappearance of the suture between the scutum and the scutellum. (C) Idealised image of the phenotype of an entire exd^- heminotum (right) compared with a normal heminotum (left). The two sutures are lost and the pattern of macrochaetes is different from the normal one. dc, dorsocentral; pa, postalar; s, suture; sc, scutellum. (D-F) Notum region of the disc containing several hth^- clones, marked black. The red staining marks the activity of the DC enhancer (see main text for details), which is expressed in the precursors of the dorsocentral bristles. The normal activity of this line is indicated in the inset in D. The clone in the posterior region (arrows in D,F) shows ectopic activity of the DC enhancer, indicating a change of the identity towards scutum.

Moreover, these clones often differentiate microchaetes in the scutellum region.

All together, these observations suggest that the loss of *hth/exd* function results in the formation of an abnormal notum with some scutum pattern elements, microchaetes, although not a perfect scutum pattern. This interpretation is further supported by the behaviour of *hth*$^{-}$ clones in the scutellar region of the disc. These clones often show expression of the specific DC enhancer of the *scute* gene (Culi and Modolell, 1998), which is normally active only in the corresponding region of the scutum (Fig. 2D-F).

Notum development with uniform *hth/exd* function

The previous experiment tested the functional role of *hth/exd* in the notum pattern. We then tried to assess the functional significance of the modulation of *hth/exd* activity. We have used the Gal4/UAS method to replace the normal expression pattern by a uniform pattern as driven by the Gal4 lines *ap-Gal4* and *248-Gal4*. As shown in Fig. 3A, the notum region of an *ap-Gal4>UAS-hth* wing disc presents an uniform *hth* activity, very different from the normal pattern – compare with Fig. 1B.

The developmental consequences of the change of the expression pattern are illustrated in Fig. 3B: the anterior part or the thorax, the scutum, is much reduced in size, and lacks most pattern elements like bristles. The effect is more pronounced in the central region, the lateral part of the notum and the scutellum are relatively less affected.

The size reduction observed in these flies seems not to be a consequence of massive apoptosis. We have checked for levels of active caspase in discs of genotype *ap-Gal>UAS-hth* or *248-Gal>/UAS-hth* and did not find a significant increase compared with wild-type discs. Moreover, we were not able to restore the normal size of the notum in flies of those genotypes by co-expressing the caspase inhibitor P35 (not shown).

The conclusion from the previous experiments is that *hth/exd* is not involved in fate specification of the notum cells: in the absence of *hth/exd* function the cells still differentiate notum pattern elements. However, the local modulation of *hth/exd* expression appears to be an important factor in the patterning process.

Regulation of *hth* expression

It follows from the results above that the understanding of the spatial regulation of *hth/exd* may provide insights into the notum patterning. As the *hth* expression in the notum evolves from ubiquitous to spatially restricted during the larval period, one of our aims was to identify the factors responsible for this regulation.

eyg is a negative regulator of *hth*

We suspected that *eyg* might regulate *hth/exd* because, as shown in Fig. 1F, *hth* and *eyg* expressions abut in some places as if they were mutually exclusive. In particular, the anterior border of the *hth* β subdomain abuts with the posterior border of the *eyg* domain.

We first examined *hth* expression in *eyg*^{SA2} homozygous discs. As shown in Fig. 4A it becomes uniform and covers most of the notum, suggesting that in absence of Eyg there is an expansion of the *hth* β subdomain. This result indicates that *eyg* can function as a negative regulator of *hth/exd.* However,

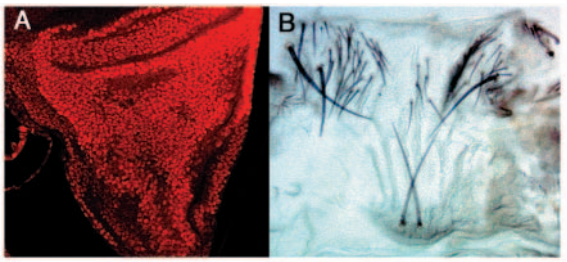

Fig. 3. Effect of uniform *hth* expression in the notum pattern. (A) Uniform expression of *hth* in an *ap-Gal4>UAS-hth* notum. (B) Notum structures differentiated by the same genotype. Some scutellum structures remain but most of the scutum is lacking. There is a general lack of microchaetes and the few that remain are in the lateral region.

in normal development the *hth* α subdomain is included within the *eyg* domain (Fig. 1F), suggesting that the repressive role of Eyg is effective only in part of the domain. To explore this possibility further, we induced clones of mutant *eyg* cells all over the notum and examined their local effect on *hth/exd* expression. We obtained two types of *eyg*$^{-}$ clones in respect of their effect on *hth* expression. Those in the α subdomain, where *hth* and *eyg* are normally co-extensive, have no effect on *hth* expression (not shown). However, the clones in the posterior region of the *eyg* domain, located between the α and β *hth* subdomains (Fig. 4B,C) show ectopic *hth* activity. This result indicates that *eyg* acts as a negative regulator of *hth* in the posterior region of its domain; the sharp border of *hth* in the posterior region probably reflects the repression by *eyg*. We have examined *eyg* expression in *hth*$^{-}$ clones (not shown) and found that it is not modified.

To confirm that *eyg* is indeed capable of repressing *hth* and also that it has distinct local effects, we conducted gain-of-function experiments. We generated clones of *eyg*-expressing cells all over the notum, and checked for *hth* activity in the clones (Fig. 4D-F). As expected from the loss-of-function experiments, *eyg*-expressing cells were only able to repress *hth* in the β subdomain. Clones generated in the α subdomain had no effect. Furthermore, we also ectopically expressed *eyg* in the majority of the notum cells using the *248-Gal4* line. In *248-Gal4>UAS-eyg* discs, the β subdomain is eliminated but the α subdomain remains unaltered (Fig. 4G-I). This distinct behaviour of *eyg* in different parts of the notum suggests the existence of some other local factor contributing to *hth* regulation.

The Dpp pathway is a negative regulator of *hth*

Because high levels of Dpp signalling co-express with the β *hth* subdomain, we wanted to determine whether the Dpp pathway could be involved in *hth* regulation. We generated clones of cells mutant for *Mothers against dpp* (*Mad*), which are unable to transduce the Dpp signal (Newfeld et al., 1996), and examined them for *hth* expression.

According to their position *Mad*$^{-}$ clones have different effects on *hth* expression (Fig. 5): (1) those located in the α subdomain do not affect *hth*; (2) those located between the α and β subdomains exhibit gain of *hth*; and (3) the clones in the β subdomain lose *hth* expression. The loss of *hth* by the *Mad*$^{-}$ clones in the β subdomain appears to be an indirect consequence of the up regulation of *eyg* in absence of activity

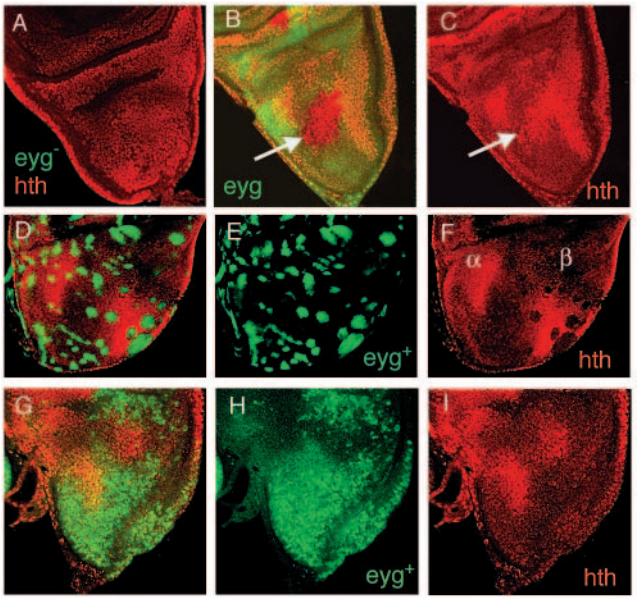

Fig. 4. Regulation of *hth* expression by *eyg*. (A) Mutant *eyg* disc stained with an anti-Hth antibody showing generalised *hth* expression. (B,C) A mutant *eyg*⁻ clone (arrows, labelled by the loss of GFP) in the inter-subdomains zone, exhibiting ectopic *hth* expression. (D-F) Notum region with many clones of *eyg*-expressing cells, marked with GFP. The clones in the α subdomain do not affect *hth* expression, in contrast to those in the β subdomain where *hth* is lost. (G-I) Disc of genotype *248-Gal4>UAS-eyg* doubly labelled with anti-Eyg (green) and anti-Hth (red), showing loss of *hth* in the β but not in the α subdomain.

of the Dpp pathway (Aldaz et al., 2003). As we show above, the Eyg product represses *hth/exd* in this region.

The lack of effect of *Mad*⁻ clones in the α subdomain and the ectopic activation of *hth* in the inter-subdomains region can be explained assuming that Dpp acts as a concentration-dependent negative regulator of *hth*. Because the inter-subdomain region is closer to the Dpp origin than the α subdomain, the level of Dpp activity is stronger and is sufficient to repress *hth*. The α subdomain is located further from the source of Dpp so that the activity levels are too low to be effective.

To confirm the repressor role of Dpp, we generated clones of cells that contain Tkv^{QD} a constitutive form of the Dpp receptor, which produces a hyperactivation of the Dpp pathway (Nellen et al., 1996). The results are illustrated in Fig. 5D-F: Tkv^{QD} clones repress *hth* when they are generated in the α subdomain, but have no effect in the β subdomain.

In summary, from the preceding experiments, both Eyg and the Dpp pathway act as repressors of *hth* in the inter-subdomains region. However, in both cases their activity domains are co-extensive in part with the *hth* domain; the *hth* α subdomain for Eyg and the *hth* β subdomain for the Dpp pathway. This indicates that Eyg and the Dpp pathway require additional factors to fulfil its repressing role (see Discussion).

Interactions between the Hth and the Eyg proteins

The α and the β subdomains differentiate distinct patterns in the adult notum (Fig. 1). The α subdomain, where *hth/exd* and *eyg* are co-extensive, gives rise to a part of the scutum and contains microchaetes as a distinctive feature. The β subdomain, which does not contain *eyg* activity, differentiates the scutellum and part of the lateral region, which do not contain microchaetes. This suggests that the differential expression of *hth/exd* and *eyg* may contribute to the pattern differences. To check on this possibility, we have expressed the Hth and Eyg products under the control of the same driver (*248-Gal4*), both separately and together, and compared the notum patterns obtained. In *248-Gal4>UAS-hth*, we observe (Fig. 6A) a reduction of the notum, which affects mostly the scutum territory; as a result, it differentiates into very few microchaetes and the dorsocentral macrochaetes are missing. We have checked in discs of this genotype the expression of the specific dorsocentral enhancer of *scute* (Culí and Modolell, 1998) and found it is not expressed. In *248-Gal4>UAS-eyg* (Fig. 6B), there is a mirror image duplication of the scutum pattern in the scutellum, as reported previously (Aldaz et al., 2003). This duplication includes macrochaetes, which are part of the *eyg* domain (Aldaz et al., 2003). By comparison, the duplicated notum structures in the *248-Gal4>UAS-hth UAS-eyg* genotype do not contain macrochaetes, suggesting that the duplicated pattern corresponds to the α subdomain. This interpretation is supported by the observation that in discs of this genotype the DC enhancer of *scute* is not expressed (not shown).

The preceding results suggested a genetic interaction between *hth/exd* and *eyg* that may be responsible for the specification of the α subdomain pattern. As Hth/Exd act as co-factors of the Hox genes to confer in vivo specificity to the Hox products (Rauskolb et al., 1993; Rieckhof et al., 1997), there was the possibility that they may act in a similar manner acting as a co-factor of Eyg. Therefore, we attempted to find

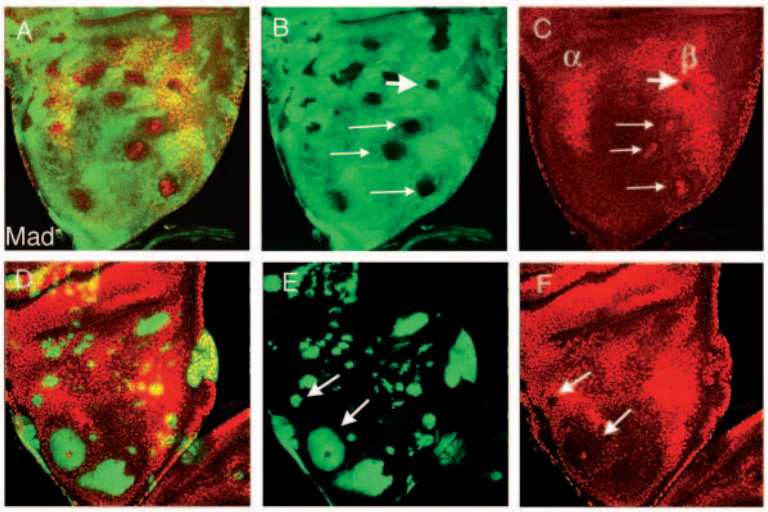

Fig. 5. Regulation of *hth* by the Dpp pathway. (A-C) Disc containing several *Mad*⁻ clones marked by the loss of GFP (green). In the α subdomain, they do not affect *hth* (red) but those in the inter-subdomains zone (small arrows) show gain of *hth*, although not in all the cells. The clone inside the β subdomain (large arrows) shows loss of *hth*. (D-F) Several Tkv^{QD}-containing clones marked by the gain of GFP (E). The clones in the α subdomain (arrows) show loss of *hth* (red) but the clones in the β subdomain do not affect *hth*.

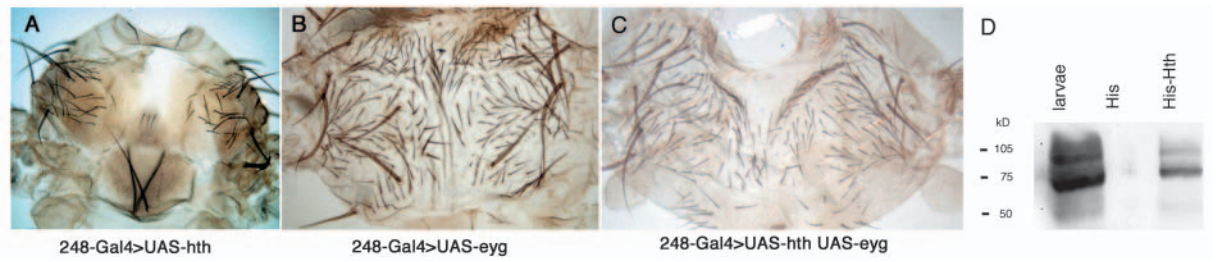

Fig. 6. (A) Adult thorax of genotype *248-Gal4>UAS-hth*. Although the scutellum remains approximately normal, most of the scutum is lacking. Many microchaetes are missing. (B) Thorax of a *248-Gal4>UAS-eyg* fly. As described previously (Aldaz et al., 2003), the scutellum is replaced by a scutum-like pattern in mirror-image orientation. Macrochaetes are present in the duplicated structure. (C) *248-Gal4>UAS-hth UAS-eyg* thorax. The only macrochaetes that remain are those in the lateral region (where the Gal4 line is not expressed), while the central region only differentiates microchaetes. (D) Pull-down assay. All three lanes are tested with the Eyg antibody for the presence of Eyg product (80 kDa band). The first lane corresponds to crude extracts. The second lane to the extract incubated with His-agarose, and in the third lane the extract was incubated with His-Hth protein. Eyg is detected in the larval extract and in a complex formed with Hth.

out if there is a protein-protein interaction between Hth/Exd and Eyg. We used a Ni-NTA pull-down assay to determine if Hth could interact with Eyg (for details, see Materials and methods). Discs from third instar larvae were homogenized and incubated with native extracted His-tagged-Hth protein. The protein complexes formed were resolved by SDS-PAGE and tested with the anti-Eyg antibody for the presence of the Eyg product. As shown in Fig. 6, the anti-Eyg antibody recognizes a 80 kDa protein in the complex that is also present in crude extracts and which corresponds to the Eyg protein. From these results, we conclude that His-tagged-Hth is able to interact with the Eyg protein in vitro (Fig. 6D).

Discussion

Our experiments bear on the problem of the spatial organization and pattern of the *Drosophila* notum, and in particular on the role of the homeobox-containing genes *hth* and *exd*. The function of *hth/exd* on segmental identity is well known; they mediate much of the in vivo specificity of the Hox genes (Chan et al., 1994; Chan et al., 1996; Rauskolb and Wieschaus, 1994; Ryoo et al., 1999) by forming Hth/Exd/Hox complexes that bind DNA. The vertebrate homologues of *hth* and *exd* perform a similar Hox-related function (Chang et al., 1996; Chang et al., 1994).

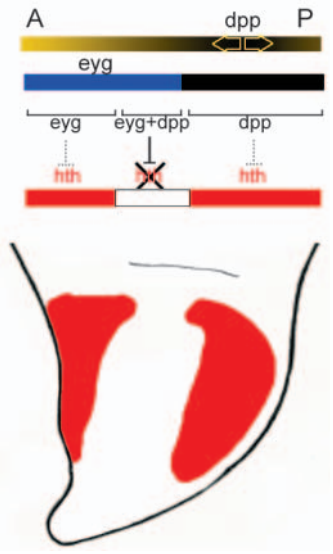

Fig. 7. Model for *hth* regulation. The two horizontal bars indicate expression of the two regulators: the Dpp signalling pathway and *eyg*. In the anterior (A) part of the disc, *eyg* is expressed but the levels of Dpp signalling are too low to repress *hth*. In the inter-subdomain region, *eyg* is present and the levels of Dpp signalling are sufficient to repress *hth*. Close to the posterior (P) border of the disc, high levels of Dpp signalling are present, but there is no *eyg* activity and hence *hth* is not repressed.

Additionally, *hth/exd* has some other roles that are not directly related to Hox gene activity. During limb development, it acts to repress the response of the proximal region to the influence of Hh/Wg/Dpp pathways, thus contributing to the subdivision of appendages along the proximodistal axis (Abu-Shaar and Mann, 1998; Azpiazu and Morata, 2000; Azpiazu and Morata, 2002; Gonzalez-Crespo et al., 1998). This function is also conserved in vertebrate development (Mercader et al., 1999).

Here, we deal with a novel *hth/exd* function: its patterning role in the notum. It is not related to the specification of notum identity because it is not affected by alterations of *hth/exd* activity. For example, in the absence of *hth/exd* the cells still differentiate as notum, if an abnormal one (Fig. 2). Conversely, high and uniform Hth levels also produce notum tissue but with abnormal pattern (Fig. 3). This function is only required in part of the notum and is therefore linked to the modulation of *hth* expression during the development of the disc. The final result of this modulation is the appearance of the α and β subdomains of *hth* that we report here. These two subdomains differentiate distinct notum patterns, suggesting that Hth/Exd interact with other localised products to generate these patterns.

Thus, there are two principal aspects in the patterning function of *hth/exd*: (1) the spatial regulation, that eventually results in the restriction of its expression to the α and β subdomains; and (2) the local interactions of Hth/Exd with other products in either of the subdomains.

Regulation of *hth/exd* in the notum

Although *hth* and *exd* form a single functional unit, their mode of regulation is different: *exd* is expressed ubiquitously but is regulated at the subcellular level by *hth*, which promotes Exd nuclear transport. Therefore, the key element of *hth/exd* regulation is the transcriptional control of *hth*.

Originally, *hth* is expressed in all the notum cells (Azpiazu and Morata, 2002) (this report) and later becomes restricted to the α and β subdomains. Consequently, the principal aspect of *hth* regulation is the mechanism(s) leading to its repression in the regions outside the α and β subdomains. We have identified two negative regulators: the *eyg* gene and the Dpp pathway, which probably acts through some unidentified downstream gene. In the notum *hth* behaves as a downstream target of both the Dpp pathway and *eyg*.

The role of Eyg as a negative regulator of *hth* is based on the following observations: (1) the beginning of the modulation of

hth expression in the notum at the early third instar coincides with the initiation of *eyg* expression (Aldaz et al., 2003); (2) in *eyg* mutants the *hth* domain is expanded, extending to most of the notum (Fig. 4A); (3) mutant *eyg* clones show *hth* derepression in the inter-subdomains region (Fig. 4B,C), and conversely, ectopic *eyg* activity in the β subdomain represses *hth* (Fig. 4). The fact that it fails to affect *hth* in the α subdomain was expected as *eyg* and *hth* are normally co-expressed in this subdomain (Fig. 1F). In conclusion, *eyg* suppresses *hth* in the inter-subdomains region and also acts as a barrier for *hth* in the *eyg*/β-*hth* border.

The role of the Dpp pathway as a negative regulator of *hth* is based on results illustrated in Fig. 5A-C, showing that *Mad*$^{-}$ mutant clones in the inter-subdomains region show activation of *hth*. This is in contrast to the behaviour of those clones in the α subdomain, where they have no effect, or in the β subdomain, where they show suppression of *hth*. We believe that the reason for the latter effect is that *eyg* is up regulated in those clones, as described previously (Aldaz et al., 2003), and in turn Eyg suppresses *hth*, as we discuss above. The lack of effect of *Mad*$^{-}$ clones in the α subdomain is probably due to the low activity of Dpp in that region (see below). The observation (Fig. 5D-F) that the high activity levels generated in the Tkv^{QD} clones suppress *hth* in this subdomain in principle supports this view. Expectedly, Tkv^{QD} clones do not affect *hth* expression in the β subdomain, because it normally possesses high Dpp activity levels.

Taking all the results together, we propose the following model of *hth* regulation (Fig. 7). As *hth* is originally expressed in all trunk embryonic cells (Azpiazu and Morata, 1998) and in all the notum cells in the early disc (Azpiazu and Morata, 2002), the regulation of *hth* during wing disc development essentially reflects local repression in specific parts of the disc. The basic idea is that *hth* is repressed by the joint contribution of *eyg* and high/moderate levels of the Dpp pathway. Neither of these elements can repress *hth* individually. Although *eyg* appears to act uniformly in its domain, the repressing activity of Dpp is concentration dependent. Within the *eyg* domain, the *hth* α subdomain is located in the anterior region, in which the Dpp levels are too low to be effective and Eyg alone cannot repress *hth/exd*. In the inter-subdomains region the Dpp levels are high enough to repress *hth* as here it acts together with Eyg. The β subdomain is outside the *eyg* domain and therefore in the absence of Eyg even the high Dpp levels are not capable of repressing *hth/exd*. The model is also supported by the experiments of overexpressing *eyg*. The *eyg*-expressing clones in the β subdomain suppress *hth* because the two repressors are active in the clones, while they have no effect in the α subdomain because it normally contains high *eyg* levels. In principle the experiments overexpressing the Dpp pathway (Tkv^{QD} clones) appear to support the model. These clones have no effect in the β subdomain, which normally possesses high Dpp activity levels, but they suppress *hth* in the α subdomain. However, these clones are known to suppress *eyg* (Aldaz et al., 2003) and therefore *hth* should not be repressed according to our model. It is possible that in certain circumstances the very high Dpp activity levels induced by these clones may be sufficient to down regulate *hth*, even in the absence of *eyg*.

The presence of two distinct repressors may suggest that the *hth* promoter region contains binding sites for Eyg and for Mad/Medea (Raftery and Sutherland, 1999) that would be responsible for the transcriptional repression. The ubiquitous expression in the absence of these two repressors may be due to a constitutive promoter.

Interactions of Hth/Exd with other products

The second aspect of the late patterning function of *hth/exd* arises from the observation that the α and β subdomains form different patterns with similar levels of *hth*. This suggests the existence of interactions between Hth/Exd and products specifically localised to the different subdomains. In the case of the α subdomain, the obvious candidate for the interaction is Eyg. We show that the joint activity of *hth/exd* and *eyg* specifies a notum pattern that is different from those specified by each of these genes alone (Fig. 6A-C).

Our finding that the Eyg and Hth proteins associate to form a complex in vitro suggest a mechanism to achieve the pattern difference between the α and the β subdomains. As it has been shown to be the case for the in vivo specificity of the Hox genes, the association of Hth/Exd with the different Hox products results in higher affinity and specificity for target sites (Ryoo et al., 1999). Here, the formation of a Eyg/Hth/Exd complex in the α subdomain may result in a constellation of gene activity different from that in the β subdomain where Eyg is not present. In the latter subdomain *hth/exd* may act alone, for after all the two genes encode transcription factors. Alternatively, the Hth/Exd products may interact with some other yet unidentified co-factor.

Genetic subdivisions of the notum

As pointed out in the Introduction, the morphological diversity of the body is achieved by regulating the spatial expression of developmental regulatory genes. For example, the spatial deployment of the various Hox genes along the anteroposterior body axis establishes the identity of the different segments (reviewed by Lawrence and Morata, 1994; Mann and Morata, 2000). Within each segment, this process is reiterated to elaborate the identity of the anterior or posterior compartments, which requires the deployment of *engrailed* (Morata and Lawrence, 1975). Further genetic subdivisions establish the identity of even more discrete regions. In the notum, previous work has identified two subdivisions that appear during the development of the disc. One is established by *pnr*, which subdivides the notum into a medial and a lateral region (Calleja et al., 2000). The second results from the activation of the *eyg* gene and straddles the *pnr* domain, thus originating four genetically distinct regions (Aldaz et al., 2003). Here, we report a new element involved in the notum subdivision: the appearance of *hth* expression in two distinct subdomains. These two *hth* subdomains are superimposed with the genetic subdivisions established by *en*, *pnr* and *eyg*, and contribute to the genetic diversification of the notum and hence to the morphological diversity within it. It is part of a genetic address (Garcia-Bellido et al., 1979) that specifies the final pattern. As shown in Fig. 6C, the combination of Hth with Eyg produces a notum pattern different from that produced by Hth alone (or in combination with some unknown factor). Our demonstration that the Hth and the Eyg products form a protein complex suggests that protein-protein interactions are part of the patterning process.

One interesting aspect of the interaction of *hth/exd* and *eyg* is that it acts in two different ways. At the gene regulation level, *eyg* participates in the spatial control of *hth/exd* activity, but where the two genes are co-expressed their proteins interact,

presumably to contribute to the in vivo affinity and specificity for target genes.

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