Function and regulation of homothorax in the wing imaginal disc of Drosophila

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SUMMARY

The gene homothorax (hth) is originally expressed uniformly in the wing imaginal disc but, during development, its activity is restricted to the cells that form the thorax and the hinge, where the wing blade attaches to the thorax, and eliminated in the wing pouch, which forms the wing blade. We show that hth repression in the wing pouch is a prerequisite for wing development; forcing hth expression prevents growth of the wing blade. Both the Dpp and the Wg pathways are involved in hth repression. Cells unable to process the Dpp (lacking thick veins or Mothers against Dpp activity) or the Wg (lacking dishevelled function) signal express hth in the wing pouch. We have identified vestigial (vg) as a Wg and Dpp response factor that is involved in hth control. In contrast to its repressing role in the wing pouch, wg upregulates hth expression in the hinge. We have also identified the gene teashirt (tsh) as a positive regulator of hth in the hinge. tsh plays a role specifying hinge structures, possibly in co-operation with hth.

Key words: Wing disc, homothorax, wingless, decapentaplegic, vestigial, Drosophila

INTRODUCTION

In the body of Drosophila, there is a clear distinction between the main body trunk and the cephalic and thoracic appendages. These are formed by the derivatives of the imaginal discs that also contribute to the body trunk (see Morata and Sanchez-Herrero, 1999, for a review). Some developmental genes appear to play a role in establishing the differences between trunk and appendage. The case best characterised so far is that of extradenticle (exd) and homothorax (hth), two related homeobox genes. exd acts as a cofactor of Hox function (Peifer and Wieschaus, 1990; see review by Mann, 1995), but it also has other developmental roles (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995). A characteristic feature of exd is that it is regulated at the subcellular level; in the imaginal discs, exd is transcribed uniformly (Flegel et al., 1993; Rauskolb et al., 1995), but the Exd protein accumulates only in the nuclei of cells that give rise to the proximal region of the discs (Mann and Abu-Shaar, 1996; Aspland and White, 1997). The nuclear transport of Exd depends on the activity of hth, which thus acts as a positive regulator of exd (Rieckhoff et al., 1997). It is not yet clear whether hth has an independent role of its own. In this work, we assume exd and hth encode equivalent developmental functions.

In the leg disc, the distinction between body trunk and appendage is based genetically on the activity of the exd/hth genes in the proximal region and of the Hh/Wg/Dpp signalling pathway in the distal part. The growth of the appendage requires full activity of the Hh/Wg/Dpp pathway (Diaz-Benjumea et al., 1994; Gonzalez-Crespo and Morata, 1996; Gonzalez-Crespo et al., 1998), which activates the response genes Distal-less (Dll), dachshund (dac) (Lecuit and Cohen, 1997) and possibly others, which determine the characteristic development of the appendage. Dll in particular is a principal determinant of ventral appendage development: in absence of its function ventral appendages do not form (Cohen et al., 1989) and ectopic Dll expression induces leg or antennal development, depending on the local genetic context (Gorfinkel et al., 1997). The functions of exd/hth and of the Hh/Wg/Dpp pathway are mutually antagonistic (Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998; Wu and Cohen, 1999): exd activity in the proximal region blocks the response of Hh/Wg/Dpp target genes such optomotor-blind (omb) and dac, whereas high levels of Wg and Dpp signalling in the distal region eliminate exd function by repressing hth. This repression is mediated by the activity of Dll and dac (Abu-Shaar and Mann, 1998).

From the above it can be concluded that one prerequisite for appendage development is the inactivation of the exd/hth genes. This allows full activity of the Hh signalling pathway, which, in turn, induces the necessary proliferation in the cells fated to form the appendage. When exd or hth function is forced in the domain of Hh signalling in the leg (Gonzalez-Crespo and Morata, 1996; Mercader et al., 1999), the appendage does not develop and only the proximal structures differentiate.

The interactions between exd/hth and the signalling pathways in the wing disc have not been studied. This disc forms a part of the trunk, the mesothorax and an appendage, the wing blade, which attaches to the mesothorax through a
proximal region, called the wing hinge. Functional and expression studies indicate that exd is expressed in the nucleus and required in the mesothorax, but is largely dispensable in the wing, except in the hinge region (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995). The various signalling pathways are very well characterised (Affolter et al., 1994; Neumann and Cohen, 1996) and are similar to those in the leg disc. A significant difference is that, in the wing, the Dpp and Wg signals emanate from the anteroposterior (AP) and dorsoventral (DV) compartment borders, respectively, whereas they both originate in the AP border in the leg disc (Affolter et al., 1994; Diaz-Benjumea et al., 1994; Campbell and Tomlinson, 1995). This indicates that, in the wing disc, the two signals are established independently. Other differences concern the response genes; in the wing disc, spalt (sal), omb and vestigial (vg) are activated by appropriate concentrations of Dpp (Lecuit et al., 1996; Nellen et al., 1996; Kim et al., 1997) and, of these, only omb is expressed in a thin dorsal stripe in the leg disc (Gonzalez-Crespo and Morata, 1996). Conversely, Dll and dac are activated by the Wg and Dpp signals in the leg but, in the wing, dac is not expressed and Dll is activated only by wg in late development. In this report, we describe the regulatory interactions between the Dpp and Wg pathways and hth in the wing disc and also explore the developmental function of hth in the wing hinge. We show that the two signalling pathways repress hth activity in the wing pouch. This results in the functional elimination of the Exd protein, which accumulates in the cytoplasm. We have identified vg, a target gene common to both pathways (Kim et al., 1996, 1997), as one of the effectors involved in the control mechanism. In the wing hinge, Wg signalling and the gene teashirt (tsh) upregulate hth expression.

MATERIALS AND METHODS

Drosophila mutations

The following mutant strains were used in this work: vg8327R (Williams et al., 1991), dshf26 (Perrimon and Mahowald, 1987), MadB1 (Wiersdorff et al., 1996), tkv12 (Nellen et al., 1996), tsh12.5 (Fasano et al., 1991) and Df(2L)305, which lacks the tsh gene (Roder et al., 1992).

Generation of mutant clones

The clones were generated by the FRT/FLP method (Chou and Perrimon, 1992). The following stocks were used that had the FRT sequences in the appropriate locations: FRT42 vg8327R, FRT40 MadB1, FRT42 tkv12 and FRT101 dshf26. Recombination was induced in larvae carrying two copies of the FRT insertion and a marker for discs (either arm-lacZ or Myc) in trans to the chromosome carrying the mutation. The FLP recombinase was provided by a 1 hour heat-shock treatment at different developmental stages depending on the experiment. The clones were visualized in discs by staining with either anti-β-galactosidase or anti-Myc antibodies. For staining of discs with anti-Myc antibody a 1 hour heat shock was given to the larvae to induce the Myc expression, followed by 1 hour recovery at 25°C, before the larvae were dissected.

Gal4/UAS experiments

The Gal4/UAS system (Brand and Perrimon, 1993) was used to induce ectopic function of the different gene products. The following Gal4 lines were used: ap-Gal4, omb-Gal4 and nub-Gal4 (Calleja et al., 1996; M. C. and G. M., unpublished data) confer the expression corresponding to the apterous, optomotor-blind and nubbin genes, respectively. The line MS1096 has been described (Guillen et al., 1995); it induces expression mainly restricted to the dorsal wing compartment. The MD735 line found in our laboratory confers expression in most of the prospective wing region. The UAS-hth stock was obtained from A. Salzberg (Pai et al., 1998), the UAS-vg is described (Kim et al., 1996) and the UAS-tsh was constructed in our laboratory by Sergio Gonzalez-Crespo and will be published elsewhere.

To generate marked clones of cells containing ectopic expression of any of the UAS chromosomes, we used the stock ywFLP10; UAS-y abx>Y >Gal4-lacZ. The abx promoter is an Ubx regulatory element that confers high expression levels in the thoracic discs. Clones were heat-shock induced at 37°C at convenient times during larval development. To examine clones in the discs, these were dissected from late third instar larvae, fixed and stained as described below. The clones of cells arising from FLP-induced recombination in the FRT sequences gain Gal4 and lacZ activity and lose the wild-type allele of forked, a bristle and trichoma marker. In the discs, the clones can be scored with anti-β-gal or with antibodies that recognize the protein encoded by the corresponding UAS (hth, vg, tsh) gene. In the adult cuticle, the clones can be scored as they are mutant for the F26 allele and are also y+ because the Gal4 activity triggers the UAS-y construct present in the chromosome.

Immunostaining of discs

Wing discs were dissected in PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. They were blocked in PBS, 1% BSA, 0.3% Triton for 1 hour, incubated with the primary antibody (anti-β-gal diluted 1:2000, anti-Hth 1:2000, anti-Wg 1:10, anti-Tsh 1:200, anti-Myc 1:1000) overnight at 4°C, washed 4x in blocking buffer and incubated with the appropriate fluorescent secondary antibody for 1 hour at room temperature in the dark. They were then washed and mounted in Vectashield. Antibodies were kindly provided by A. Salzberg and H. Sun (anti-Hth), S. Kerridge (anti-Tsh), Sean Carroll (anti-Vg) and the Hybridoma Center (anti-Wg). β-Gal antibody (rabbit) was purchased from Cappel and Myc antibody (mouse) from BABCO. Images were taken in a laser scan Zeiss microscope and subsequently processed using Adobe Photoshop.

Preparation of adult cuticle

The adult flies were dissected in water and cut into pieces. They were subsequently treated with 10% KOH at 95°C for 3-5 minutes to digest the internal tissues, washed with water, rinsed in ethanol and mounted in Euparal. The preparations were photographed on a Nikon AFK-II microscope using Fujichrome 64T film.

RESULTS

Wild-type hth expression in the wing imaginal disc

We have analysed in detail hth expression in the wing disc using a polyclonal antibody (Pai et al., 1998). Protein distribution in second and early third instar discs is uniform in most or all the disc (Fig. 1A,B). In late third instar, hth expression is modulated in the thoracic as well as in the appendage part. There is no expression in the central part of the disc, which corresponds to the wing appendage, but the Hth protein accumulates in two concentric peripheral rings, in the region that corresponds to the wing hinge (Fig. 1C). The domain of hth expression has been mapped by double-label experiments in relation to the domains of wg, nubbin (nub) and vg (Fig. 1D-F). hth and vg are co-expressed in the two rings, whereas hth and vg are expressed in near
complementary domains that only overlap in the anterior and posterior sides of the disc. The expression of nub covers the wing pouch but also extends to the inner ring of hth and wg expression. The rings of wg and hth expression may be used to discriminate between wing hinge and wing pouch; hereafter we will refer to prospective wing pouch as the region that does not express hth, and prospective hinge as the region with hth (and wg) expression.

**Negative regulation of hth in the wing pouch**

(1) vg represses hth expression

The vg gene is a key element in the development of the wing pouch (Williams et al., 1991). Mutant flies lacking vg activity show loss of most of the wing, but the hinge region remains (Williams et al., 1991; Lindsley and Zimm, 1992). It is specifically expressed in the wing pouch cells (Williams et al., 1993) (Fig. 1F) and its ectopic expression in other imaginal discs induces wing tissue (Kim et al., 1996). Because of the near reciprocity of the vg and hth/exd domains and the parallel with Dll function in the leg (Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998), we tested the possibility that vg is involved in the repression of hth in the wing pouch.

We induced clones of cells mutant for the vg83h27R (see Methods for details) and examined hth expression. In the wing pouch, these clones are infrequent and round, suggesting that they sort out from the surrounding wild-type cells. However, those that can be detected show ectopic hth expression (Fig. 2A-C). We note that, although all the clones examined show some ectopic hth activity, it does not affect all the cells. This observation suggests that, although vg is involved in hth repression in the wing pouch, there probably are other genes implicated.

To analyse further the ability of vg to repress hth, we generated clones of vg-expressing cells. As expected, these clones have no effect in the wing pouch but, in the hinge, they repress hth activity (Fig. 2D-F). In contrast to the loss-of-function clones, all the cells expressing vg show repression of hth.

(2) The activities of the Dpp and Wg signalling pathways repress hth

The preceding experiment indicates that vg is part of the mechanism controlling hth expression in the wing. The transcription of vg in the wing is under the control of two
different enhancers, the boundary and the quadrant enhancers (Kim et al., 1996). The activity of the boundary enhancer depends on Notch signalling arising from the DV axis (Kim et al., 1996; Klein and Martinez-Arias, 1999); Notch induces wg, whose product diffuses from the border and activates Dll and vg in third instar wing discs (Diaz-Benjumea and Cohen, 1995; Kim et al., 1996). The quadrant enhancer is responsible for vg expression in the wing pouch away from the DV border and is activated by Dpp (Lecuit et al., 1996; Kim et al., 1997). Thus both the Dpp and the Wg signals activate vg and are likely to be involved in the control of hth expression in the wing pouch.

The role of Dpp was tested by inducing clones of cells that were either mutant for thick veins (tkv) or for Mothers against Dpp (Mad). In the first case, the cells are unable to respond to the Dpp gradient and, in the second, do not transduce the Dpp signal (Newfeld et al., 1996; Wiersdorff et al., 1996). Both experiments gave the same result: either tkv or Mad mutant cells showed ectopic hth expression in the wing pouch (Fig. 3). A significant difference with vg mutant clones is that, in tkv and Mad clones, all the cells show hth derepression, whereas vg mutant clones only activate hth in some cells of the clone. We searched for tkv mutant clones in the adult cuticle as they were marked with yellow, but the great majority of them fail to develop in the distal and medial wing blade. Sometimes round vesicles of invaginated tissue, which are possibly formed by mutant cells, are present. In contrast, tkv clones are readily found in the Costa and alula region, where their differentiation is normal (data not shown; Burke and Basler, 1996), suggesting that the Dpp gradient does not pattern this region.

The involvement of Wg signalling was tested by inducing clones of cells mutant for dsh (dsh), one of the intracellular transducers of the pathway. The loss of dsh activity affects hth expression differently, depending on their position with respect to the AP axis. As observed in Fig. 3C, hth is ectopically activated only in dsh clones localised far from the AP border, but inside the prospective wing pouch region. dsh cells at the AP axis, or close to it, do not show hth expression (Fig. 3B).

Taken together, these observations suggest that the Dpp and Wg signalling pathways repress hth in the prospective wing pouch, probably by activating vg and maybe other response gene(s).

**Positive regulation of hth in the wing hinge**

As described above (Fig. 1), hth expression is uniform in the early wing disc, and it is also known (Rieckhof et al., 1997) that it is uniformly expressed at high levels in the thoracic segments of the late embryo. Thus it is likely that hth expression in the hinge is not activated de novo but inherited from embryonic cells. Nevertheless, we have studied the role of some of the genes co-expressed with hth in the maintenance and regulation of the high expression levels observed in late third instar discs. In particular, we considered wg and tsh. The expression of wg in the wing hinge may be regulated differently from the expression in the DV border and is likely to perform a different developmental role. The zinc-finger gene tsh is co-expressed with hth in the outer ring (Fig. 4A-C) and it is also known that, in the leg disc, tsh is entirely coincident with nuclear extradenticle expression (Gonzalez-Crespo and Morata, 1996), suggesting it may have some functional interaction with hth.

The role of wg was studied as in the previous experiments by inducing clones of cells mutant for dsh allele. The mutant clones generated in the prospective hinge region show reduction, but not elimination, of hth expression (Fig. 4D-F). This observation suggests that wg is necessary for the high levels of hth expression in the hinge, but is not the only factor necessary for hth activity.

We have also examined the possible role of tsh in regulating hth. The effect of the loss of tsh function was not studied because the necessary FRT tsh recombinant chromosome could not be constructed (due to the very proximal location of tsh). The effect of ectopic expression was studied using the Gal4/UAS method (Brand and Perrimon, 1993). Driving tsh activity in the wing disc with the ap-Gal4, MS1096, omb-Gal4 and nub-Gal4 lines (Calleja et al., 1996; Guillen et al., 1995) results in ectopic hth expression in the pouch (Fig. 4J-L), and a reinforcement of its expression in the wing hinge (Fig. 4J-L, and data not shown). We also induced marked clones of tsh-expressing cells. These clones tend to be round suggesting that they sort out from surrounding cells; those in the wing hinge are of normal size considering the time of induction and show increased levels of hth expression (Fig. 4G-I). In the wing pouch, they are more heterogenous; those near the DV border are small and show no hth activity, whereas those closer to the hinge are larger and have ectopic hth expression.

**Developmental effects of ectopic expression of hth and of tsh**

Both hth and tsh are strongly expressed at high levels in the wing hinge region but their respective developmental roles are not clarified. There is good evidence for the requirement for hth/ed function in the hinge; clones of hth or exd mutant cells develop aberrant structures in the hinge and produce outgrowths (Gonzalez-Crespo and Morata, 1995; Casares and Mann, 2000). In contrast, tsh mutant phenotype in the wing is...
not known, although its strong expression in the hinge suggests a function. To study the developmental roles of hth and tsh, we have induced their ectopic activity in the wing pouch. A number of Gal4 lines were used to force expression in various regions of the wing (Brand and Perrimon, 1993). In other experiments, marked clones of hth-expressing and of tsh-expressing cells were induced (see Methods).

The principal result concerning ectopic hth expression is that it prevents growth; in lines conferring high and overall hth activity (nub-Gal4/UAS-hth, 1096/UAS-hth or C765/UAS-hth genotypes), the wing pouch does not develop, but proximal structures like sclerites or costa are normal (Fig. 5). In lines inducing locally restricted expression, the effect on growth is mainly limited to the region containing Gal4 activity. For example, the line omb-Gal4, confers Gal4 activity only to the central region of the wing pouch. In omb-Gal4/UAS-hth flies, the central region of the wing pouch grows very little and differentiates only proximal pattern elements (Fig. 5D). In contrast, the anteriormost and posteriormost wing regions are much less affected.

In addition, some of these genotypes present an excess of proximal pattern elements (also reported in Mercader et al., 1999), probably at the expense of the more distal ones, suggesting that the presence of the Hth product is influencing the cellular response to the proximodistal signals. For example, in the genotype MS1096/UAS-hth the number of alula (a proximal posterior wing structure) bristles is 29.5 (n=14), which is higher than in the wild type (23.5, n=10). We have studied this “proximalizing” effect in some detail using the Gal4 line C735, that confers a general expression in most or all the wing pouch and whose activity responds readily to temperature changes between 18°C and 29°C. The phenotype of C735/UAS-hth wings shows a distal-proximal gradation depending on the temperature; weak hth activity (at low temperature) only affects the distal wing, but stronger activity affects the distal and medial regions (Fig. 5F-H). In the strongest phenotypes only proximal wing elements remain.

Few clones of hth-expressing cells can be recovered in the differentiated cuticle, the majority of them probably sort out and are eliminated. Many of those that can be scored are in the proximal wing. In the margin they are associated with loss of marginal elements (Fig. 5L), while those located closer to the AP border are associated with the loss of large portions of the middle of wing blade (Fig. 5K), suggesting an effect on growth in the proximodistal axis. A few cases were found near the medial DV wing margin; they only differentiate wing trichomes, but appear to induce local duplications of wing margin elements (Fig. 5L, see below).

Since both the effects on growth and on the proximodistal pattern may be due to an interference of the Hth product with the production or diffusion of the morphogenetic signals Wg and Dpp, we examined wg and dpp expressions in the genotypes above. The overall result is that wg expression is altered but that dpp expression is not. As shown in Fig. 6A-C, ap-Gal4/UAS-hth wing discs show loss of wg expression along the DV border. This is accompanied by an enhancement of wg expression in the inner ring in the hinge. This distinct effect of Hth on wg expression in the hinge and in the wing

![Fig. 4.](image-url)
Fig. 5. Effects of hth expression in the adult wing. (A) Wild-type wing showing the normal costa and hinge structures. (B) Wing rudiment of an ap-Gal4/UAS-hth fly. The high and uniform expression of ap in the dorsal wing compartment prevents growth of the wing. (C) Wing of the genotype MS1096/UAS-hth. The wing is reduced in size predominantly in the distal region, but no reduction is seen in the proximal region. (D) Wing of genotype omb-Gal4/UAS-hth showing lack of the distal medial region. The deleted part corresponds largely to the area of expression of omb, which includes the central and distal part of the wing blade. (E-H) A series of wings of genotype C765/UAS-hth grown at 18° (E), 22° (F) 25° (G) and 29°C (H) to illustrate the response to different levels of hth activity. Note the gradual “proximalization” of the wing; the effect of hth is stronger in the distal than in the proximal structures. (I-K) Three cases of hth-expressing clones (marked with y+ and f36a). The clones are localized (arrows) in the proximal regions but appear to reduce or eliminate growth of the distal part of the wing. (L) A small hth-expressing clone that appears to induce the formation of a duplication of the wing margin in the vicinity.

Fig. 6. Effect of ectopic expression of hth on wg and dpp. (A-C) Third instar wing disc of the genotype ap-Gal4/UAS-hth wg-lacZ stained for hth (red) and β-gal (green). Ectopic expression of hth in the wing pouch represses wg expression in the DV border. In the hinge, gain-of-function hth reinforces wg expression in the inner ring, but not in the outer one. (D-F) A clone (arrowhead) of hth-expressing cells (red) in the wing pouch represses local wg expression (green) and induces ectopic wg activity adjacent cells. (G,H) The arrowhead points to a clone of hth-expressing cells (red) in the wing pouch. This clone exhibits normal vg expression (green). (J-L) Third instar wing disc of the genotype omb-Gal4/UAS-hth; dpp-lacZ stained for hth (red) and β-gal (green). Ectopic expression of hth in the omb domain does not alter dpp expression in the AP border.
Clones of hth-expressing cells (see Methods) also have a similar effect on wg activity. As shown in Fig. 6D-F, hth-expressing clones in the DV border repress local wg expression, but, surprisingly, they cause ectopic wg activity in cells located in the vicinity. This is consistent with the observation above (Fig. 5L) that hth-expressing cells are associated with wing margin duplications. Despite the effect on wg, hth-expressing clones do not repress vg (Fig. 6G-I).

The effect on dpp expression was studied in the Gal4 lines described above and is illustrated in the genotype omb-Gal4/UAS-hth (Fig. 6J-L). Wing discs of this genotype show normal dpp expression. This result was anticipated as, in omb-Gal4/UAS-hth flies, the anterior and posterior patterns are near normal (Fig. 5D) and this requires normal dpp activity. The lack of effect on dpp provides an explanation for the lack of effect of ectopic hth on vg expression, as the latter is also activated by Dpp signalling (Kim et al., 1997).

Ectopic expression of tsh strongly modifies the adult wing pattern. In genotypes ap-Gal4/UAS-tsh or 1096/UAS-tsh, the dorsal wing compartment differentiates hinge structures (Fig. 7A,B, data not shown), especially sclerites and proximal costal elements, that derive from a region of the disc with high levels of hth and tsh. In wing discs of these genotypes, the expression of wg in the DV border disappears, although it is likely due to the activation of hth described above (data not shown). The behaviour of clones of tsh-expressing cells also point to a similar conclusion; although most of the clones fail to differentiate properly, we frequently observe vesicles of differentiated tissue bearing the genetic markers of the clone. In favorable cases (Fig. 7C), the pattern differentiated by these clones can be identified as proximal hinge structures.

**DISCUSSION**

**Negative regulation of homothorax in the wing pouch**

In the second instar wing disc, the Hh product accumulates uniformly in the thoracic and appendage regions of the disc (Fig. 1A), but throughout the third larval period hth expression is downregulated and, by the late third instar, Hth only appears in the presumptive regions of the thorax and the wing hinge. The central part of the disc, which gives rise to the wing pouch, shows no hth expression (Fig. 1C). The repression of hth function is important for wing development since, if hth activity is forced in the wing pouch, the wing does not form (Fig. 5B). A similar observation has been made in the leg disc; hth or exd expression in the distal part results in truncated appendage in which all the distal components are missing (Gonzalez-Crespo and Morata, 1996). In the leg, the subdivision between distal and proximal regions results from the antagonism between Hh signalling and exd/hth function (Gonzalez-Crespo et al., 1998). Hh response genes such as Dll and dac are instrumental in repressing hth (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999).

Our results indicate that the downregulation of hth in the wing pouch is a consequence of the activity of the Dpp and the Wg signaling pathways. Cells in which the response to the Dpp signal is prevented, as in tkv or Mad mutant cells, hth is expressed at high levels (Fig. 3A). Similarly, dsh– cells, in which the transduction of Wg is blocked, show ectopic hth activity and consequently nuclear exd expression. These results also indicate that hth is latently active in the wing cells and has to be repressed by the continuous activity of the Dpp and Wg signals. The inability of cell clones, in which the Dpp or the Wg pathways have been totally eliminated, to proliferate may be due to high levels of hth expression. Our results also indicate that the Dpp and Wg pathways repress hth expression independently. This is illustrated by the experiments inducing dsh mutant clones: ectopic hth expression is only observed in clones located away from the AP border. Our interpretation is that the high levels of Dpp expression near the AP border are sufficient to impede hth expression despite the removal of the control by Wg.
We have identified vg as one of the factors involved in the downregulation of hth: the elimination of vg activity in the wing pouch (Fig. 2) results in hth activation and its ectopic expression in the hinge region represses the normal activity of hth. As a principal role of vg is to specify wing development (Williams et al., 1991), it appears that a component of this function is to eliminate hth activity and therefore exd function. As vg is a target gene of both the Hh and the Wg pathways in the wing, it seems that the downregulation of hth by both pathways is mediated by vg. One question that is not fully understood about the role of vg is that, although it is able to repress hth, there is some vg activity normally in the wing hinge that coincides with that of hth. The levels of the Vg protein appear to be similar in the pouch and the hinge regions so that different levels of product do not seem to be a likely reason. We believe that there may be other factors in the hinge; tsh is a likely candidate that counteracts the repression by Vg.

Although vg clearly has a regulatory role, we note that, in contrast with the observed in tkv and Mad clones, not all the cells lacking vg activity in the wing pouch express hth, suggesting that there probably are other response genes involved in the repression of hth. We have not checked other Dpp response genes, such as omb or spalt (sal) (Nellen et al., 1996; Lecuit et al., 1996) that may mediate this control. Dll is a Wg target gene in the wing blade but, unlike in the leg disc (Abu-Shaar and Mann, 1998), Dll mutant clones do not affect hth expression (R. S. Mann, personal communication).

Maintenance of hth expression in the wing disc

The negative regulation of hth discussed above is a modification of the original uniform expression found in the early wing disc. As the wing disc derives from the second thoracic embryonic segment, which shows high and uniform levels of hth expression (Rieckhof et al., 1997), the initial levels of hth in the thoracic region and the wing hinge are likely to be inherited from the progenitor cells. The mechanism of hth activation during embryogenesis is not known, although its expression is modulated by the activity of the BX-C genes (Azpiazu and Morata, 1998).

Our results suggest that, at least during the larval period, hth expression in the wing disc is positively regulated by tsh. This is based on two findings. (1) An increase of Tsh levels in the hth domain results in increased levels of Hth product; possibly, one of the normal functions of tsh in the wing hinge is to maintain high hth levels. (2) Ectopic tsh expression in the wing pouch causes ectopic hth activity, at least in the clones located close to the hinge (or far from the DV border) and in the Gal4 lines. The fact that clones located close to the DV border are not able to induce hth activity, whereas the Gal4 lines do, could be due to a timing effect. However, our results also indicate that tsh is not the only factor involved: hth and tsh are normally co-expressed only in the proximal ring of hth expression, therefore there should be other factor(s) maintaining hth expression in the distal ring. Since dsh mutant clones show a reduction in hth expression in the hinge, a wg response gene is likely to be involved. In addition, the fact that tsh cannot activate hth near the DV border in the wing pouch may suggest that other factors are required.

The developmental role of hth in the wing disc

The main role of hth is to regulate exd function (Rieckhof et al., 1997). The loss of hth activity during adult patterning results in changes in segmental identity and morphogenetic alterations that appear to be similar or identical to those produced by eliminating exd (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995). Thus hth and exd can be considered to perform the same developmental function.

In the wing disc, hth and exd are only required in the wing hinge region and, in their absence, the cells proliferate but form aberrant patterns (Gonzalez-Crespo and Morata, 1995; Casares and Mann, 2000) indicating that hth/exd function is involved in specifying the wing hinge region. The experiments inducing ectopic hth expression suggest that it has a role in controlling growth, for hth is able to prevent the formation of the wing pouch. It is also consistent with the observation (Casares and Mann, 2000) that hth mutant clones in the hinge may reach very large size. The finding that hth suppresses wg activity in the DV border may be related with the repression of growth, a process with which wg has been shown to be involved (Zecca et al., 1996). The lack of effect of hth on dpp expression emphasizes the independence of the AP wing axis from hth/exd function.

One aspect that we do not fully understand is the effect of hth on the proximodistal pattern, which has also been observed on the leg disc and on the chicken limb (Mecardor et al., 1999).

In the experiments, the presence of the Hh product influences the reading of proximodistal signals by the cells towards differentiating more proximal patterns. We do not know which factors are responsible for the proximodistal pattern in the wing, but as hth prevents wg response to Notch, it is possible that a Wg response element or some other Notch response gene may be involved in patterning.

It is also not clear what is the role of hth in the specification of the wing hinge, where it is expressed at high levels. Its ectopic expression in the pouch does not produce any specific transformation towards hinge structures, but rather a general proximalization of the whole pattern. This is in contrast with the effect of ectopic tsh that induces sclerites and very proximal hinge structures. Since tsh activates hth in the hinge (Fig. 4G-I), it suggests that the formation of proximal hinge requires the activity of the two gene products.

Altogether, the results presented here suggest the subdivision of the non-thoracic part of the wing disc into two major domains: the wing hinge, where hth is expressed and Exd is functional (nuclear), and the wing pouch where hth is not expressed, Exd is cytoplasmic and therefore inactive. By homology with the leg disc (Gonzalez-Crespo and Morata, 1996; Gonzalez-Crespo et al., 1998), the latter would be the genuine appendage part of the disc. These two regions are formed by two antagonistic genetic systems: in the hinge, the high levels of hth, inherited from the embryo and probably maintained by wg, tsh and may be other regulators, prevent wg response to Notch signalling, which is necessary for the development of the wing pouch. In the wing pouch, the activities of the Wg and Dpp pathways suppress hth so that Notch may induce wg activity and the appendage is formed (see also Casares and Mann, 2000).

In addition to its role in preventing excessive proliferation, hth may also contribute, together with tsh, wg and nub, to the partition of the wing hinge into two regions that correspond to the outer and inner rings of hth expression. The outer ring domain expresses tsh, wg and hth, has nuclear Exd and does not express vg and nub. The inner domain expresses wg, nub...
and hth, has nuclear Exd and does not express tsh. The individual role of these genes is not yet established, but it is possible that they function in some combinatorial manner.

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