Establishing primordia in the Drosophila eye-antennal imaginal disc: the roles of decapentaplegic, wingless and hedgehog

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SUMMARY

The eye-antennal imaginal discs of Drosophila melanogaster form the head capsule of the adult fly. Unlike the limb primordia, each eye-antennal disc gives rise to morphologically and functionally distinct structures. As a result, these discs provide an excellent model system for determining how the fates of primordia are specified during development.

In this study, we investigated how the adjacent primordia of the compound eye and dorsal head vertex are specified. We show that the genes wingless (wg) and orthodenticle (otd) are expressed throughout the entire second instar eye-antennal disc, conferring a default fate of dorsal vertex cuticle. Activation of decapentaplegic (dpp) expression in the posterior eye disc eliminates wg and otd expression, thereby permitting eye differentiation. We also demonstrate that otd is activated by wg in the vertex primordium. Finally, we show that early activation of dpp depends on hedgehog (hh) expression in the eye anlage prior to morphogenetic furrow formation.

Key words: decapentaplegic, wingless, hedgehog, orthodenticle, Drosophila, imaginal disc

INTRODUCTION

A critical question in development is how regional identity is established. The limb imaginal discs of Drosophila melanogaster have provided an important model for studies of regional specification (reviewed in Cohen, 1993). The best understood mechanism for patterning the limb primordia involves compartment formation mediated by selector genes. In this process, cells acquire compartment specific identity by expressing genes in a cell-lineage-restricted manner (Garcia-Bellido et al., 1973). Within the wing and leg discs, for example, engrailed and invected are expressed in all the cells of the posterior compartment where they are required to specify posterior cells fate (Sanicola et al., 1995; Zecca et al., 1995; Tabata et al., 1995; Morata and Lawrence, 1975; Kornberg et al., 1985), while the LIM/homeobox gene apterous specifies dorsoventral identity (Diaz-Benjumea and Cohen, 1993; Blair et al., 1994). A different mechanism governs dorsoventral subdivision of the leg discs. In each leg primordium, dorsoventral territories are established by mutual repression mediated by the secreted DPP and WG proteins (Brook and Cohen, 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Morimura et al., 1996; Penton and Hoffman, 1996). This repression, which does not require compartmentalization, results in dorsal dpp expression and ventral wg expression. If each gene product specifies a distinct differentiation program, the result is regional specification in the absence of selector gene activity.

The mechanisms described above specify subdomains of individual structures, the appendages of the adult fly. To understand how the primordia of different domains are established, we have focused on the patterning of the eye-antennal imaginal discs. These discs are particularly interesting because, unlike the limb discs, they each produce different adult structures (Haynie and Bryant, 1986; reviewed in Jurgens and Hartenstein, 1993). Each disc is subdivided into primordia which give rise to the antennae, compound eyes and specific regions of the head capsule.

How are the adjacent primordia within a single eye-antennal disc distinguished? Clonal analysis indicates that, apart from late anteroposterior subdivision of the antennal anlage, compartments do not form within the eye-antennal discs (Morata and Lawrence, 1979). Therefore, selector gene-mediated compartmentalization is not likely to underlie regionalization outside of the antennal primordium. A second possible explanation involves the embryonic origin of these discs. Unlike the limb discs, which derive from single trunk segments, each eye-antennal disc arises from multiple embryonic head segments (Younossi-Hartenstein et al., 1993). Divisions between segment primordia within the disc, which have not been clearly defined, could contribute to certain aspects of regional specification.

Finally, the early roles of the dpp, wg and hh gene products in patterning the early eye-antennal disc are not well understood.

Most molecular studies of the eye-antennal disc have focused not on early patterning, but on later events in disc differentiation. Of particular interest has been the progression of the morphogenetic furrow, which traverses the disc epithelium beginning in the third instar larval stage and leaves differentiated retinal cells in its wake (Ready et al., 1976; Tomlinson and Ready, 1987). dpp, wg and hh are involved in regulating furrow progression. Mutant analysis and ectopic expression experiments suggest that hh expression in differentiating photoreceptor cells induces both
dpp expression in anteriorly adjacent cells and progression of the furrow (Heberlein, 1993; Ma et al., 1993). Although the role of dpp in furrow progression may be relatively minor (Burke and Basler, 1996), its early expression along the posterior and lateral margins of the eye-antennal disc is necessary for furrow initiation (Blackman et al., 1991; Treisman and Rubin, 1995; Burke and Basler, 1996). dpp-mediated furrow formation is antagonized by wg, which is expressed along the ventral and dorsal margins of the eye primordium (Ma and Moses, 1995; Treisman and Rubin, 1995; Wiersdorff et al., 1996).

Here, we investigated how, before morphogenetic furrow formation, the early eye primordium is distinguished from the adjacent primordium of the dorsal head capsule (also called the head vertex). We show that, in addition to its later role in furrow formation, dpp prevents dorsal head fate in the eye primordium. This early function of dpp is mediated by its repression of both wg and the homeobox gene orthodenticle (otd), which normally collaborate to specify vertex identity. We also show that otd is a wg target gene in the vertex primordium. Finally, we present evidence that this early role of dpp in regional specification depends upon hh expression in the early eye primordium.

MATERIALS AND METHODS

**Fly strains and clonal analysis**

The wild-type strains used in this study were Oregon-R or yw. Reporter genes used were dpp-lac Z BS3.0 (Blackman et al., 1991) and hh330, lacZ (Lee et al., 1992). The dppΔdk allele (Spencer et al., 1982) is a 5 kb deletion of sequences 3' to the dpp transcribed region (Blackman et al., 1991). The mutations In(2L) dpp12 and Df(2L) dpp114 do not affect dpp embryonic function, but disrupt enhancers required for imaginal expression (St. Johnston et al., 1990; Blackman et al., 1991). The Mad1-2 allele is described in Wiersdorff et al. (1996) and the so, dsh and zw3 alleles used were so1, dsh1V153 (a null allele; Flybase) and zw330127 (a null allele; Ruel et al., 1993). Balancer chromosomes and other mutations are described in Lindsley and Zimm (1992).

Clonal analysis was performed using the FLP/FRT system (Xu and Rubin, 1993), using P[winiw+; armadillo-lacZ] as a clonal marker (Vincent et al., 1994).

zw3 and dsh mutant clones were induced in larvae of the genotype w, zw330127 FRT18A/armadillo-lacZ FRT18A; hsP70-flp38/+ and dsh1V153 FRT18A/armadillo-lacZ FRT18; hsP70-flp38+; respectively. Clones were generated by a 1 hour heat shock at 37°C during the first or second larval instar, and dissected and stained in late third instar discs.

Flies homozygous for the temperature-sensitive allele hh152 were raised at 17°C until the end of the first instar stage, then shifted to 29°C. After 24-36 hours, larvae were dissected and eye-antennal discs labeled.

**Histochemistry, immunohistochemistry and analysis of head morphology**

Larvae were grown in uncrowded conditions to ensure optimal disc morphology. Discs were dissected (attached to the larval mouthhooks) in PBS and fixed for 20 minutes at room temperature in 4% paraformaldehyde/PBS saturated with heptane. They were washed briefly in methanol once, 3× 5 minutes in PBT (PBS + 0.1% Tween-20) and incubated overnight at 4°C with the indicated primary antibodies preabsorbed against fixed embryos. Antisera used were rat polyclonal OTD antisera (Wieschaus et al., 1992) used at a 1:500 dilution, rabbit polyclonal WG antisera (van den Heuvel et al., 1993) used at a 1:100 dilution, and a mouse monoclonal antibody to β-galactosidase (Cappel) used at a 1:500 dilution. Following incubation in primary antibody, discs were washed 3× 1 hour in PBT and incubated for 3 hours at room temperature with biotinylated or fluorescein labeled secondary antibodies (Cappel). For immunocytochemistry, after 3× 1 hour washes in PBT, discs were treated for 1 hour with biotinylated horseradish peroxidase-avidin solution (Vector laboratories, Elite ABC Kit) diluted 1:50 in PBT, and washed again for 3× 45 minutes in PBT. Staining was visualized by incubating discs in 0.5 mg/ml diaminobenzidine in PBT in the presence of 0.04% H2O2. Discs were mounted in 80% glycerol in PBS and viewed under Nomarski optics using a Zeiss Axioskop microscope. For immunofluorescence, discs were washed 3× 1 hour at room temperature, and then mounted in 75% glycerol with 50 mg/ml n-propyl gallate and observed with a scanning confocal microscope (BioRad).

For X-gal staining, discs were dissected in cold PBS, fixed in 1% glutaraldehyde for 20 minutes, and washed 3× 10 minutes in PBT. Discs were then incubated in prewarmed staining solution plus 0.2% X-gal.

To analyze dorsal head structures, heads were severed with a razor blade and mounted in 30% glycerol in ethanol.

**RESULTS**

On the *Drosophila* head capsule, the compound eyes are separated dorsally by the head vertex, laterally by the shingle cuticle and ventrally by the gena (Fig. 1A; Haynie and Bryant, 1986). The vertex includes three morphologically distinct domains: (1) ocellar cuticle, containing the three ocelli and surrounding sensory bristles, (2) frons cuticle, consisting of a series of closely spaced parallel ridges flanking the ocellar region, and (3) orbital cuticle, which resembles ocellar cuticle and is also marked by a precise pattern of macrochaetes (Fig. 1A,B). In the vicinity of the ocelli, the compound eye is adjacent to the orbital cuticle, while closer to the antennae, the eye lies immediately next to the frons.

**dpp and wg /otd are expressed in adjacent domains of the early third instar eye-antennal disc**

Mutations that decrease dpp expression in the eye primordia lead to the formation of severely reduced eyes (Masucci et al., 1990; St. Johnston et al., 1990; Blackman et al., 1991). Similarly, the loss of otd or wg function in the vertex primordia causes the elimination of dorsal head structures (Wieschaus et al., 1992; Royet and Finkelstein, 1996). To understand the respective roles of these genes in regional specification, we first compared their expression patterns in the developing eye-antennal disc.

Through disc transplantation experiments, the anlagen of the primordia of adult head structures have been mapped on the third instar eye-antennal disc (Fig. 2A; Haynie and Bryant, 1986). The eye primordium occupies most of the posterior half of the disc (the ‘eye disc’). The head vertex forms from the dorsomedial region of the disc, while the antenna develops from the anterior half of the disc (the ‘antennal disc’).

During the early third instar stage (70-80 hours after egg laying [AEL]), dpp is expressed in a horseshoe-shaped domain along the ventral, posterior and dorsal periphery of the eye disc (Fig. 2B; Masucci et al., 1990). Dorsal dpp expression does not extend as far anteriorly as ventral expression, but instead ends at the vertex primordium. At this stage, otd expression covers the vertex primordium and extends along the edge of the antennal disc (Fig. 2C; Wieschaus et al., 1992; Royet and Finkelstein, 1995). The posterior boundary of otd expression in the vertex anlage approximately coincides with the anterior boundary of the dpp domain (Fig. 2D).
At the same stage of disc development, wg is expressed in two regions of the eye disc (Fig. 2A,E). One corresponds to the future gena and the other to the head vertex. In the early vertex primordium, wg and otd expression approximately colocalize (not shown), with the posterior boundaries of both expression domains lying immediately adjacent to the dpp domain.

**dpp prevents dorsal head development in the eye primordium**

The double-labeling described above showed that wg and otd are coexpressed in the vertex primordium, while dpp is expressed in the adjacent primordium of the compound eye. This juxtaposition of expression suggested that these genes could interact to establish the boundary between the two primordia. To test this hypothesis, we examined the phenotype of flies homozygous for the dpp^d-blk allele. This mutation reduces dpp activity in the eye primordium and permits morphogenetic furrow movement only in the central region of the eye disc (Masucci et al., 1990). The result is a greatly reduced compound eye composed of only a few residual ommatidia (compare Fig. 1E and F).

Closer examination of dpp^d-blk flies revealed that the eyes are replaced largely by frons cuticle, which normally appears only on the dorsal head (compare Fig. 1C,F with B,E). This ectopic frons lies between the orbital cuticle and the remaining ommatidia as well as more anteriorly, between the shingle cuticle and the ommatidia. To determine whether the loss of
Fig. 2. Expression domains of dpp, otd and wg. (A) Schematic of a third instar eye-antennal disc, showing the primordia of the antenna, vertex and compound eye (see Haynie and Bryant, 1986 for a more detailed fate map). In the eye primordium, dorsal is to the right and posterior towards the bottom. (B-D) Double staining of a dpp-lacZ early third instar disc with antibodies against β-galactosidase (red) and OTD (green). D is an overlay of B and C. (B) dpp is expressed in a horseshoe-shaped region along the margins of the eye primordium as well as in a sector of the antennal anlage. (C,D) otd is expressed in the vertex primordium (arrow) and along the margin of the antennal primordium (arrowhead). The posterior limit of otd expression coincides with the anterior limit of the dpp domain (arrowhead). Staining in the posterior region of the eye disc is in the optic stalk and is not detected by in situ hybridization with an otd probe (J. Royet, unpublished results). (E) wg expression in the early third instar disc. wg is expressed in the antennal anlage and in the primordia of the vertex (arrowhead) and gena (arrow). Scale bars: B-E (shown in D), 30 μm. In all panels, anterior is up and dorsal is right.

Ommatidia is always associated with ectopic dorsal head cuticle, we examined flies carrying other mutations that prevent eye formation. In sine oculis (Fig. 1D) or eyes absent (not shown) flies, the eyes are completely lost but are not replaced by ectopic frons. This suggests that dorsal head cuticle does not result simply from loss of the eyes, but is caused instead by the loss of dpp function.

To determine whether other mutations affecting the dpp pathway cause a similar change in regional identity, we examined the effect of Mothers against dpp (Mad) mutant clones on eye development. The Mad gene product is required for the reception of the dpp signal (Raftery et al., 1995; Sekelsky et al., 1995) and Mad clones in the eye field have been reported to give rise to head cuticle (Wiersdorff et al., 1996). We found that Mad clones induced in first instar larvae cause a transformation of ommatidia into frons (Fig. 1G,H).

dpp prevents wg and otd expression in the eye primordium

On the head vertex, the formation of frons cuticle requires both otd and wg function (Wieschaus et al., 1992; Royet and Finkelstein, 1995). To determine whether the ectopic frons seen in dppΔ-bk flies is associated with ectopic otd and wg expression, we examined their expression in dppΔ-bk eye-antennal discs. In mid-third instar (80-90 AEL) dppΔ-bk discs, OTD protein is not limited to the vertex anlage but instead is expressed in a band extending across the entire eye disc along the anterior margin of the eye primordium (compare Fig. 3A and B). In late third instar discs, the otd expression domain expands posteriorly into the eye anlage (compare Fig. 3D and E). Since existing fate maps of the eye-antenal disc are not precise, ectopic otd expression cannot be mapped precisely with respect to the primordia of head structures. However, the otd domain expands towards the anlagen of the shingle cuticle and the compound eye, consistent with the location of ectopic frons cuticle on dppΔ-bk mutant heads. No
such ectopic otd expression can be detected in an so disc (Fig. 3F). It has previously been reported that wg expression also expands in late third instar dpp^{d-blk} mutant discs (Wiersдорff et al., 1996; Chanut and Heberlein, 1997). In the early third instar disc, we found that wg expression in both the head vertex and gena primordia expands significantly towards the posterior edge of the eye disc (compare Figs 3C and 2E).

Although the dpp^{d-blk} mutation severely reduces eye development, several observations suggest that it does not totally eliminate dpp function in the eye primordium (Treisman and Rubin, 1995). dpp^{d-blk} eye-antennal discs are indistinguishable from wild-type discs until the third instar larval stage, suggesting that the mutation does not affect early development of this primordium. To determine the effect of a more severe reduction in dpp expression, we used a transheterozygous combination of alleles (In(2L)dppe^{12}/Df(2L)dppe^{11}) that almost completely eliminates dpp imaginal function and fails to produce adult flies (St. Johnston et al., 1990; Blackman et al., 1991; Diaz-Benjumea et al., 1994). In these mutant discs, we observed a dramatic expansion of the otd expression domain, such that almost all cells of the eye and antennal primordium express otd (Fig. 3G). In the eye primordium, approximately three times as many cells express OTD protein as in wild-type discs at an equivalent stage. We observed a similar expansion of the wg domain (Fig. 3H). This indicates that, in both these primordia, dpp acts to establish domains free from otd and wg expression.

**Ectopic activation of the wg pathway in the eye primordium induces otd expression and vertex formation**

Ectopic wg and otd expression caused by the loss of dpp can be explained in two ways. First, wg and otd could simply be markers of dorsal head cuticle and their expanded expression domains a secondary consequence of the enlarged head vertex of dpp^{d-blk} mutant flies. Alternatively, as suggested by the important roles of wg and otd in vertex formation, their ectopic expression could instruct the formation of dorsal head structures in the eye primordium.

To distinguish between these possibilities, we determined the phenotypic consequence of activating the wg pathway in the eye anlage. To do so, we generated clones mutant for zeste-white 3 (zw3, also known as shaggy) in first or second instar larvae and observed their effect on eye differentiation. Loss of zw3 function results in constitutively activated wg signaling (Siegfried et al., 1992; Siegfried and Perrimon, 1994). A previous study showed that in zw3 clones, ommatidia are replaced by dorsal head cuticle (Heslip et al., 1997). Closer examination revealed that these clones, like Mad clones, contain frons cuticle (Fig. 4A,B), which is normally wg-dependent on the dorsal head. This demonstrates that wg activation is sufficient to respecify cell fate in the eye primordium.

In the vertex primordium, both wg and otd are necessary for frons formation (Royet and Finkelstein, 1996). We therefore tested whether otd is ectopically expressed in zw3 mutant clones. We found that, in the third instar eye disc, such clones express OTD protein in a cell autonomous fashion (Fig. 4C-E). This suggests that otd expression in the vertex primordium is normally activated or maintained by wg. To test this, we made clones in this primordium homozygous mutant for the dishevelled (dsh) gene, which is required for the reception of the wg signal (Klingensmith et al., 1994; Theisen et al., 1994). In these clones, endogenous otd expression was lost (Fig. 4F-I). Combined, these results strongly suggest that otd is a wg target gene in vertex formation.

**wg and otd are expressed throughout the eye primordium of the second instar eye-antennal disc**

The results described above show that dpp acts to exclude wg (and consequently otd) expression from the eye primordium. They raise the question of whether wg and otd are initially expressed throughout this primordium and only later excluded by the activation of dpp. Alternatively, the two genes might never be expressed in the eye anlage during normal development.

To address this issue, we examined wg and otd expression in second instar eye-antennal discs (Fig. 5). We found that, at a stage when the disc consists of fewer than 50 cells (50 hours AEL; Fig. 5A), WG protein expression is present in almost all the cells of the eye primordium (Fig. 5B,C). OTD protein is also evident in virtually all the cells of the eye disc, as well as in the antennal primordium (Fig. 5D).

**Early dpp expression in the eye primordium is hh-dependent**

How is dpp expression initiated in the early eye primordium?
**Early dpp expression creates the eye primordium by eliminating wg expression**

The role of dpp in the patterning and differentiation of the eye primordium has previously been described (see Introduction). Here, we have shown that dpp is also required to specify the fate of this primordium. Early in disc development, both wg and otd are expressed throughout the eye anlage. We propose that, by repressing wg expression, dpp permits the development of the compound eye. In the absence of dpp, the eye is transformed into its default fate, dorsal head cuticle (frons).

After complementary domains of dpp (eye) and wg (vertex) expression are established, they may be maintained by mutual repression. A recent study showed that dsh mutant clones in the vertex primordium develop into ectopic ommatidia on the dorsal head (Heslip et al., 1997). This led these authors to propose dpp/wg mutual repression similar to that which subdivides the leg disc. In addition, Ma and colleagues used a temperature-sensitive wg allele to show that the elimination of wg function causes expansion of the eyes at the expense of dorsal head cuticle (Ma and Moses, 1995). It has not yet been demonstrated, however, that loss of wg activity in the vertex primordium leads to ectopic dpp expression. In addition, our results suggest that wg expression in the vertex primordium does not depend on hh, which differs from the situation in the leg primordia where wg and dpp expression require constant hh signalling.

In the eye-antennal disc, dpp/wg interactions, rather than subdividing a single appendage, distinguish the primordia of two very different structures, the compound eye and dorsal head capsule. As noted earlier, wg is also expressed in the pri-
Primordia in the Drosophila eye-antennal imaginal disc

Previous studies demonstrated a requirement for primordium in a cell autonomous fashion. In addition, inhibi-
activation of the otd wg activates otd in the dorsal head primordium the position and size of this primordium.
mordium of the gena, which lies in the ventral region of the eye disc. A similar process of mutual repression may specify the position and size of this primordium.

**wg activates otd in the dorsal head primordium**

In the early vertex primordium, hh, wg and otd expression approximately coincide (Royet and Finkelstein, 1996). This raises the question of the regulatory relationships among these genes. We showed previously that ectopic hh expression activates ectopic otd and wg expression in a specific region of the eye-antennal disc (Royet and Finkelstein, 1996; J. Royet, unpublished results). However, the results described above using a temperature-sensitive hh allele suggest that endogenous wg and otd expression in the vertex primordium do not require hh.

We have also demonstrated that wg is epistatic to otd in the pathway of dorsal head formation. As we have shown, ectopic activation of the wg pathway induces otd expression in the eye primordium in a cell autonomous fashion. In addition, inhibition of the wg pathway in the vertex anlage eliminates otd expression. Combined, these observations strongly suggest that otd is a wg target gene in the vertex anlage.

Loss of dpp activity induces ectopic wg and otd expression in both the eye and antennal primordia. In the antennal disc, however, zw3 mutant clones do not express otd (J. Royet, unpublished observations). This suggests that the activation of the wg pathway may be necessary but not sufficient for otd induction. It also indicates that the eye-antennal disc contains underlying patterning information independent of dpp and wg signaling.

**Early hh expression is required to define the eye primordium**

Previous studies demonstrated a requirement for hh in mor-
phogenetic furrow progression and ommatidial differentiation. We propose that, in addition to this later function, hh plays an early role in the global patterning of the eye-antennal disc. In the absence of hh function, dpp is not expressed in the posterior eye disc. As a result, both wg and otd are ectopically expressed, causing an eye-to-vertex transformation. Consistent with this function, we found that hh is expressed at the posterior margin of the eye anlage significantly before furrow initiation. Since we detected this expression using a hh-lacZ reporter strain, we cannot be sure of the time window during which HH protein is normally expressed. It is possible that hh expression in this region occurs early in eye-antennal disc development, and that the expression we see just before furrow initiation results from perdurance of the β-galactosidase protein.

We propose that wg and otd expression in the eye-antennal discs are inherited from the embryo, where the two genes are expressed in segments from which these discs are derived. The almost ubiquitous expression of the two genes programs the early disc to the vertex fate. Later, hh expression in the posterior region of the future eye disc induces dpp expression along the margins of the eye primordium. dpp represses wg, permitting the formation of the eye primordium (see schematic in Fig. 7).

In vertebrates, homologues of the genes analyzed in this study are expressed in the developing head and brain (reviewed in Rubenstein et al., 1994; Tickle, 1995). Have the genetic reg-
ulatory relationships demonstrated here been evolutionarily conserved? The link between wg and otd expression is one example of a regulatory interaction that may have been retained in vertebrate head development. Itoh and Sokol recently showed that injection of Xenopus dsh or Wnt8 mRNA induces Otx2 expression, resembling the induction of otd by the wg pathway that we have observed (Itoh and Sokol, 1997). Para-
doxically, however, microinjection of the zw3 homologue glycogen synthase kinase also induces Otx2 in frogs (Itoh et al., 1995). Further studies will be required to elucidate which elements of the signaling cascade described here have been conserved in higher animals.

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