**hedgehog, wingless and orthodenticle specify adult head development in**

**Drosophila**

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**SUMMARY**

The adult head capsule of *Drosophila* forms primarily from the eye-antennal imaginal discs. Here, we demonstrate that the head primordium is patterned differently from the discs which give rise to the appendages. We show that the segment polarity genes *hedgehog* and *wingless* specify the identities of specific regions of the head capsule. During eye-antennal disc development, *hedgehog* and *wingless* expression initially overlap, but subsequently segregate.

This regional segregation is critical to head specification and is regulated by the *orthodenticle* homeobox gene. We also show that *orthodenticle* is a candidate *hedgehog* target gene during early eye-antennal disc development.

Key words: *hedgehog*, *wingless*, *orthodenticle*, *Drosophila*, imaginal discs, head development

**INTRODUCTION**

Analysis of *Drosophila* development has shown that pattern formation is achieved differently in the head and trunk regions of the early embryo (reviewed by Cohen and Jurgens, 1991; Finkelstein and Perrimon, 1991). In the blastoderm, a genetic cascade activates the pair-rule genes, which subdivide the trunk into parasegmental units (reviewed by Ingham, 1988; Pankratz and Jackle, 1993). This subdivision is indicated at the molecular level by the activation of the segment polarity genes. In the cephalic region, however, there is no evidence that pair-rule genes are involved in segmentation. Instead, it has been proposed that anterior segmentation and segment polarity gene activation are directly initiated by the gap-like genes *buttonhead* (*btld*; Cohen and Jurgens, 1991).

The molecular distinction between the cephalic and trunk regions is also demonstrated by subsequent interactions among genes of the segment polarity class. In the early embryonic trunk, the segment polarity genes *hedgehog* (*hh*) and *engrailed* (*en*) are co-expressed in a single row of cells in each parasegment, while adjacent anterior cells express *wingless* (*wg*; DiNardo et al., 1985; Ingham et al., 1985; Kornberg et al., 1985; van den Heuvel et al., 1989; Gonzalez et al., 1991; Lee et al., 1992). Later in embryogenesis, the maintenance of *wg* expression requires *hh*, while *hh* and *en* expression depend on *wg* (reviewed by Martinez Arias, 1993). In specific anterior head regions, however, not all cells that express *hh* express *en*, and *wg* expression is *hh*-independent (Tabata et al., 1992; Ingham and Hidalgo, 1993; Mohler, 1995). These and other observations indicate that segment polarity gene regulation is achieved differently in different embryonic subdomains.

The segment polarity genes also act during imaginal development to pattern the adult fly. Recent analyses of this process have focused primarily on the imaginal discs that give rise to the adult appendages (reviewed by Cohen, 1993; Blair, 1995; Campbell and Tomlinson, 1995). During early development of the limb discs, posterior compartment cells are specified in part through their expression of *en* (Morata and Lawrence, 1975; Kornberg et al., 1985). Posterior cells secrete hedgehog protein, thereby inducing neighboring cells of the anterior compartment to produce either the *wg* or *decapentaplegic* (*dpp*) gene products (Basler and Struhl, 1994; Capdevila et al., 1994; Tabata and Kornberg, 1994; Felsenfeld and Kennison, 1995). These two secreted molecules are subsequently involved in patterning the limb primordia (Campbell et al., 1993; Couso et al., 1993; Struhl and Basler, 1993; Basler and Struhl, 1994; Diaz-Benjumea et al., 1994).

Just as embryonic development is differentially regulated in the head and trunk, various observations suggest that the adult head is specified differently from the thoracic appendages. The head capsule is derived from specific primordia within the eye-antennal imaginal discs which, as their name indicates, also give rise to the compound eyes and antennae (Haynie and Bryant, 1986). Unlike the thoracic discs, each of which originate from a single embryonic segment, each eye-antennal disc is formed from multiple embryonic head segments (Hartenstein and Jan, 1992; Jurgens and Hartenstein, 1993). In addition, the early eye-antennal discs, in contrast to the thoracic discs, do not express *en* (Hama et al., 1990). Only the anterior primordium of the eye-antennal disc becomes compartmentalized, and this occurs significantly later than in the thoracic discs (Morata and Lawrence, 1978, 1979). No compartmentalization has been detected in the anlage of the head capsule.

We have investigated the molecular mechanism of adult head specification. We have focused on the head vertex, which is the region that lies between the compound eyes. We show that in the imaginal primordium of the medial head vertex, *hh*
and en are co-expressed, while developing lateral regions express wg. In contrast to what occurs in the thoracic discs, hh expression precedes that of en, and wg expression is hh-independent. Through both loss of function and ectopic expression experiments, we demonstrate that hh and wg specify regional identity across the head. Although the domains of hh and wg expression initially overlap, they later separate during eye-antennal disc development. This regional segregation, which is critical for head specification, is controlled by the otd homeobox gene. Finally, we present evidence suggesting that otd is a target of hh during early disc development.

**MATERIALS AND METHODS**

**Drosophila strains**

The wild-type strain used was Oregon-R. The allele referred to here as oc<sup>391</sup> is the same as T1(2) oc<sup>391</sup> which has been described previously (Wakimoto and Spradling, 1981; Mohler, 1984). The wg<sup>X2</sup>-alleles specifically affect wg function during imaginal development and is homozygous pupal lethal (Cousso et al., 1993). The allele wg<sup>FL1114</sup> produces a protein that is not secreted at 25°C but is essentially functional at 17°C (Baker, 1988; Bejsovec and Martinez Arias, 1991; Gonzalez et al., 1991). The disheveled and fused alleles used were dsh<sup>M20</sup> (Perrimon and Mahowald, 1987) and fu<sup>1</sup> (Bussen et al., 1988). The temperature sensitive allele hh<sup>h1</sup> and the hh-lacZ enhancer trap line hh<sup>P30</sup> have been described by Lee et al. (1992). The other enhancer trap lines used were wg-lacZ (Kassis et al., 1992), en-lacZ (Hama et al., 1990), neu-A101 (which labels sensory mother cells and is referred to here as A101; Huang et al., 1991), and the LI line (which labels ocellar precursor cells and regions of the developing brain; Mozer and Benzer, 1993). Balancer chromosomes and other mutations referred to are described by Lindsley and Zimm (1992).

**Histochemistry and immunohistochemistry**

Larvae were grown under uncrowded conditions to obtain optimal imaginal disc morphology. Imaginal discs were dissected in PBS (attached to the larval mouth hooks) and fixed in cold 4% paraformaldehyde in PBS. Discs were washed briefly once in methanol, 3× 5 minutes in PBT (PBS + 0.1% Tween-20) and incubated overnight at 4°C with appropriate primary antibodies preadsorbed against fixed embryos.

The following primary antibodies were used: rat polyclonal anti-otd antiserum (described by Wieschaus et al., 1992), rabbit polyclonal anti-wg antiserum (van den Heuvel et al., 1988), an anti-engrailed antibody (Lee et al., 1992). Anti-sense and sense probes were produced by riboprobe transcription from plasmids containing the indicated cDNA sequences, and were hybridized to whole discs as described by Van Vactor (1992). The detection of endogenous β-galactosidase was carried out using the Elite ABC Kit (Vector laboratories, Elite ABC Kit) at a 1:50 dilution in PBT for 1 hour and washed 3× 45 minutes in PBT. Staining was visualized by incubating the discs in the presence of 0.04% H<sub>2</sub>O<sub>2</sub> and 0.5 mg DAB/ml in PBT supplemented, for anti-wg staining with 0.8 ml/l of a 8% CuCl<sub>2</sub> solution. Discs were then washed 3× 5 minutes in PBT, mounted in 90% glycerol in PBS and viewed under Nomarski optics using a Zeiss Axioskop microscope.

**RESULTS**

The vertex of the adult head is laterally symmetric and is formed by the fusion of the two eye-antennal discs. Each half of the vertex can be subdivided into distinct mediolateral subdomains, each characterized by specific structural elements (Fig. 1A). The medial subdomain contains the ocelli (simple eyes) and characteristic surrounding sensory bristles. These structures lie on the triangular ocellar cuticle, which is marked with small hairs. Lateral, adjacent to the medial region, is the dorsal frons cuticle, which is easily recognized as a series of closely spaced, parallel ridges devoid of macrochaetae. Lateral to the frons is the orbital cuticle, which lies adjacent to the compound eyes and also contains a defined array of macrochaetae.

Using the lacZ enhancer trap line A101 to visualize the precursor cells of the ocelli and sensory bristles (Huang et al., 1991), we have precisely localized the primordia of head vertex structures on the developing eye-antennal discs (Fig. 1B,C; Royet and Finkelstein, 1995). As shown, the precursors of the most medial head structures (e.g. the ocelli and ocellar bristles) are situated near the medial edge of each of the two discs, where fusion occurs to form the head capsule. More lateral head structures, like the orbital bristles, are derived from cells that lie more towards the center of each disc, near the primordium of the compound eye. The results obtained using this enhancer trap line are consistent with a more approximate fate map derived from previous transplantation analysis (Haynie and Bryant, 1986).

**Initially overlapping domains of hh and wg expression segregate during imaginal development**

To obtain clues about the roles of the segment polarity genes in head formation, we analyzed the expression of hh, wg and en in the developing eye-antennal disc. For these experiments, we used antibodies to the en and wg proteins, and a hh-lacZ reporter construct which reproduces the imaginal expression pattern of the endogenous hh gene (Lee et al., 1992).

In the early third instar eye-antennal disc, hh is expressed in a medial patch of cells in the region of the head vertex primordium (Fig. 2A). wg protein is also expressed in this region, in addition to being present in cells on the opposite side of the disc and in the antennal anlage (Fig. 2B). At this early stage, the expression domains of hh and wg overlap in the head vertex primordium (not shown). In addition, no en protein is detectable in this region of the early third instar disc (Fig. 2C). This differs from the early coexpression of en and hh in the posterior compartments of the antennal anlage and of the thoracic discs (Brower, 1986; Lee et al., 1992; Mohler and Vani, 1992; Tashiro et al., 1993; Tabata and Kornberg, 1994).

**Generation of Act<sup>wg</sup> and Tuba1<sup>hh</sup> clones in imaginal discs**

Flies homozygous for the hsp70-flp gene were crossed to flies homozygous for either the Act5C<sup>y+</sup><sup>wg</sup> (Struhl and Basler, 1993) or the Tuba1<sup>y+</sup><sup>hh</sup> (Basler and Struhl, 1994) construct, and progeny were subjected to heat shock during either the first or second instar larval stage as described. To obtain ectopic clones in the adult head, hsp70-flp; Act5C<sup>y+</sup><sup>wg</sup> and hsp70-flp; Tuba1<sup>y+</sup><sup>hh</sup> larvae were heat-shocked for 20 minutes at 32°C or 30 minutes at 35°C respectively.
It also indicates that hh expression in the head primordium does not require en at this stage of development.

During the third larval instar, the hh and wg expression domains change sharply such that they no longer overlap, but are instead spatially separated. In the late third instar disc, hh expression becomes restricted to a medial subdomain of the vertex primordium (Fig. 2D). At this stage, wg expression has disappeared from this region, and is now confined to two patches flanking it (Fig. 2E). Double-labeling experiments confirm that hh expression persists within the region where wg expression has been lost (Fig. 2G). The hh and wg expression domains are now separated by a region 5-10 cells in width where neither gene is expressed. This differs from the thoracic discs and the early embryo, where hh- and wg-expressing cells are more directly juxtaposed. By this later stage of disc development, en protein expression has appeared in the medial region where hh is expressed (Fig. 2F).

To localize the expression domains of hh, wg and en with respect to the primordia of specific head structures, we used the L1 lacZ enhancer trap line which specifically labels the precursor cells of the ocelli (Mozer and Benzer, 1993). Double-labeling experiments using this line indicate that, in the late third instar eye-antennal disc, hh expression coincides with the medial subdomain that includes the ocellar precursor cells (Fig. 3A). Similar double-staining using wg antibodies shows that these medial precursor cells lie in the region from which wg protein expression has been eliminated (Fig. 3B).

To directly correlate segment polarity gene expression and adult head structures, we examined the heads of flies from enhancer trap strains. As predicted by the patterns of disc expression, β-galactosidase activity is precisely restricted to the medial ocellar domain in the heads of both en- and hh-lacZ flies (Fig. 3C and D). In wg-lacZ flies, lacZ expression is present in the lateral orbital region, as well as in the ptilinium, a structure immediately anterior to the frons (Fig. 3E). This suggests that the two patches of wg expression in the head vertex primordium of the late third instar disc (see Fig. 2E) correspond to the anlagen of these structures. On the adult head, wg-expressing cells of both the ptilinium and the orbital cuticle lie immediately adjacent to the frons but not in the frons itself. This suggests that, in the late third instar disc, the gap between the medial hh expression domain and the more lateral wg domains reflects the frons anlage, which does not express hh, wg, or en.

hh and wg are required for mediolateral head specification

The regionally restricted expression patterns of hh and wg in both the late third instar eye-antennal disc and the adult head suggest that these genes are involved in the specification of medial and lateral head structures respectively. To test this idea, we manipulated hh and wg activity during imaginal development and examined the effects on adult head formation.

To reduce hh function during disc development, we utilized a temperature sensitive hh allele (hh12; Lee et al., 1992). When larvae homozygous for this allele are maintained at the restrictive temperature during third instar development, the resulting flies completely lack medial head structures, including the ocelli and medial sensory bristles (Fig. 4A; Ma et al., 1993). Unlike ocelliless (oc) mutations, which cause the loss of these structures but leave the medial cuticle essentially unchanged in appearance (see below), reduced hh function causes the ocellar cuticle to be replaced by frons spanning the entire head vertex (Fig. 4A). The distance between the compound eyes remains approximately unchanged, suggesting either a medial to lateral cell fate transformation or the loss of medial precursor cells followed by overproliferation of the adjacent

![Fig. 1. Fate-mapping of adult head structures on the eye-antennal imaginal disc. (A) Adult head vertex (wild-type). The region of the head capsule between the compound eyes can be subdivided into three morphologically distinct domains. The medial domain contains the medial and lateral ocelli (mo and lo) and associated sensory bristles (one of the two ocellar bristles is indicated (ob)). Flanking the ocellar region is the ridged cuticle of the dorsal frons (fr). Between the frons and the compound eye is the orbital cuticle, which exhibits a characteristic pattern of ridged cuticle (orb). (B) β-galactosidase expression in a late third instar (120 hours after egg laying, h AEL) eye-antennal disc from the A101 enhancer trap strain. In this line, lacZ is expressed in neural precursor cells. Staining is present in the presumptive lateral ocellus, in the half of the median ocellus derived from each disc, and in the precursors of the ocellar and orbital bristles (abbreviations as in A). Identities of precursor cells were deduced by comparison to the fate map (Haynie and Bryant, 1986) and by following lacZ-expressing cells during disc fusion in vitro (see C). (C) lacZ expression in the A101 line after fusion of the two eye-antennal discs in vitro. Pairs of eye-antennal discs (attached to the larval mouthhooks) from late third instar larvae were cultured in the presence of ecdysone. After 12 hours at 25°C, the discs fuse, forming adult head capsules. Heads were fixed and stained with X-gal. Precursors of the ocelli and of the ocellar and orbital bristles can be identified in their correct positions on the dorsal head (abbreviations as in A). In all panels, anterior is up. In B medial is to the right. Scale bars: A, 100 μm; B,C (shown in B), 50 μm.
frons cuticle. Adult flies homozygous for a partial loss of function fused (fu) allele show a similar head phenotype (Fig. 4B), indicating that hh acts through a signalling pathway similar to that which functions in the embryonic epidermis and thoracic discs (reviewed by Perrimon, 1995). When wg activity is similarly reduced during third instar development, the region of the head which is affected is complementary to that seen for hh. For these experiments, we used a temperature sensitive heteroallelic combination (wgIL1114/wg CX3) which specifically reduces wg imaginal function. When raised at 17°C, flies of this genotype develop wild-type head structures. However, when such larvae develop at the restrictive temperature (25°C) during the second half of the third instar, the resulting adult flies fail to eclose and develop abnormal head structures. Examination of these flies reveals that both lateral (orbital) and mediolateral (frons) head regions are lost (Fig. 4C). The result of this loss is that the ocelli lie immediately adjacent to the compound eyes.

This loss of lateral head structures is accompanied by expansion of the medial ocellar cuticle and of the ocelli themselves. The ocellar cuticle of these flies can be three times larger, and the ocelli five times larger, than those of wild-type adults. Similar head phenotypes are produced by partial loss-of-function mutations affecting other genes of the wg signaling pathway such as disheveled (Fig. 4D; J. Royet, unpublished observations). These results suggest that normal wg activity is necessary not only for the development of lateral and mediolateral head structures, but is also required to restrict the size of the medial region. It should also be noted that since presumed frons precursor cells do not express wg in the late third instar disc (see above), wg either functions at an earlier stage of development, or acts non-autonomously in patterning the mediolateral region of the head.

As described, the loss of either hh or wg function results not only in the loss of a specific domain of the head, but also in the expansion of the adjacent domain. We tested next whether this expansion was correlated with an expansion in domains of gene expression. First, we examined hh expression in eye-antennal discs from larvae in which wg activity has been reduced as described above. Consistent with the expansion of the ocellar cuticle, we find that the medial hh expression domain is significantly larger than the wild-type domain.

Fig. 2. Regional restriction of hh, wg and en expression during eye-antennal disc development. (A-C) Early third instar discs (75-85 h AEL). (D-G) Late third instar discs (110-120 h AEL). (A,D) hh-lacZ discs labeled with anti-β-galactosidase antibodies. (A) In addition to the posterior compartment of the antennal disc (black arrowhead) and the developing compound eye (white arrowhead), hh is expressed in the primordium of the head vertex (arrow). (D) Later, hh expression is restricted to a small region of the vertex primordium near the medial edge of the disc (see also Fig. 3). (B,E) Wild-type discs labeled with anti-wg antibodies. (B) In the early disc, wg is expressed in a wedge-shaped domain in the antennal anlage (black arrowhead) and in two patches corresponding to the anlagen of the head vertex (arrow), and of the shingle cuticle (white arrowhead). (E) Later, wg expression in the vertex primordium becomes excluded from the medial region and persists in two patches corresponding to future lateral head structures (black arrowhead) and to the anterior ptilinium (black arrow). (C,F) Wild-type discs labeled with anti-en antibodies. No en protein can be detected in the head vertex primordium in early discs (arrow in C), although it is clearly detectable in the cells of the posterior compartment of the antennal disc (black arrowhead). Expression appears in an hh-like pattern during the third larval instar (F). (G) hh-lacZ disc labeled with anti-wg (black) and anti-β-galactosidase (brown) antibodies. The wg and hh expression domains are mutually exclusive but not adjacent in the vertex primordium. This differs from the antennal region where hh and wg expression are directly juxtaposed (black arrow). In all panels, medial is to the right. Scale bar (shown in A) 50 µm.
(compare Figs 4F and 2D). In such discs, the hh domain expands towards the primordium of the compound eye in accordance with the lateral enlargement of the ocellar region in wg mutant flies. A similar expansion is seen for en expression (not shown).

We also examined wg expression following hh reduction. In eye-antennal discs derived from hh^{P12} larvae grown at the restrictive temperature during the third instar, the wg antennal domain is sharply reduced (compare Figures 4E and 2E). This is consistent with previous reports that hh activates wg at the anteroposterior boundaries of the leg and antennal discs (Basler and Struhl, 1994; Diaz-Benjumea et al., 1994). However, no such effect is seen on the two patches of wg expression in the region of the head vertex. In contrast, wg expression in these patches increases in intensity and expands, causing the two domains to become interconnected (Fig. 4E). This expansion results in a reduction in size of the ‘wg-free’ domain in the medial region of the disc. These results show that, consistent with the previous phenotypic observations, hh and wg act to restrict each other’s expression during eye-antennal disc development. They also indicate that in the head vertex primordium, unlike in the leg and antennal discs, wg expression does not require hh.

**hh and wg instruct regional identity in the head**

The above results demonstrate that hh and wg are required for medial and lateral head development. We next asked whether expressing these segment polarity genes outside their respective expression domains is sufficient to reprogram cell fate. To address this question, we used the flip-recombinase technique (Struhl and Basler, 1993) to generate clones of cells that ectopically express hh or wg during disc development. We then examined the phenotypes of such clones (which are marked by yellow bristles) on the adult head cuticle.

The most obvious phenotypic consequence of expressing hh outside the medial head region is the induction of ectopic ocelli at more lateral locations (Fig. 5A, B). These ocelli, which are often larger than normal, are found at various positions across the head vertex within both the frons and the orbital cuticle. In larger hh clones, frons cuticle is sometimes disrupted and replaced by ocellar-like medial cuticle exhibiting these ectopic ocelli. Clones that lie in the medial region, where hh is normally expressed, induce no obvious morphological changes aside from occasional enlargement of the ocelli. Interestingly, we found that ocelli could also be induced in the shingle cuticle of the lateral ptilinium, which lies outside the head vertex (not shown).

We also used the flip-recombinase method to induce clones of ectopic wg expression. In this case, we found that clones in the medial head domain show an invasion of mediolateral frons cuticle into the ocellar region (Fig. 5C). This ectopic frons cuticle is associated with a reduction of the ocelli and a loss of ocellar bristles. However, clones recovered in the lateral orbital region, where wg is normally expressed, show no structural abnormalities (Fig. 5C). Clones in the mediolateral region were also normal, consistent with the role proposed earlier for wg in establishing frons cuticle.

In summary, ectopic expression of either hh or wg is sufficient to reprogram cell fates on the developing head capsule. Misexpression of hh induces ectopic medial structures (ocelli) at more lateral locations. Misexpression of wg produces more laterally placed structures (frons) in the medial region. Because of the low density of bristles on the dorsal head, the boundaries of the regions of misexpression are difficult to assess, and hence, conclusions regarding the cell autonomy of these phenotypes are not possible. In each case however, ectopic expression appears to cause the loss of the normal pathway of regional

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**Fig. 3. hh, wg, and en expression demarcate the primordia of adult head structures.** (A) L1; hh-lacZ disc labeled with anti-β-galactosidase antibodies. Because of extremely strong lacZ expression in the L1 line (which specifically labels the developing ocelli), the precursor cells of the medial and lateral ocelli (mo and lo) can be detected near the boundaries of the hh expression region (white arrow). hh expression is in the primordium of the ocellar domain. (B) L1 disc labeled with anti-wg (black) and anti-β-galactosidase (brown) antibodies. The brown staining corresponds to the precursor cells of the medial and lateral ocelli (mo and lo). These cells lie in the region that does not express wg. The two patches of wg protein expression are indicated by white and black arrowheads. (C-E) Adult heads from enhancer trap lines stained with X-gal. (C) en-lacZ and (D) hh-lacZ are expressed in the medial ocellar cuticle (arrow). (E) wg-lacZ. Staining is present within the orbital region (arrowhead) and the ptilinium (arrow), but is absent from the frons. Staining is also observed in the periphery of the ocelli. This staining appears late in development, since it is not observed in early pupal eye-antennal discs. In A and B, medial is to the right. In C-E, anterior is up. Scale bars: A, B (shown in A), 50 μm; C-E (shown in C), 100 μm.
otd regulates the regional localization of hh and wg expression

As has been described previously, the otd homeobox gene is required for the development of all medial and mediolateral head structures (Wieschaus et al., 1992; Royet and Finkelstein, 1995). otd mutations that permit embryonic development but specifically eliminate otd expression in the head vertex primordium of the eye-antennal disc are also referred to as oc alleles (Bedichke, 1934; Royet and Finkelstein, 1995). The strongest oc mutation (oc^3^) causes the loss of both ocellar structures and frons cuticle but does not affect the lateral orbital region (Fig. 6E). Consistent with this effect, otd protein is expressed across the anlagen of both medial and mediolateral head structures in the wild-type third instar eye-antennal disc (Fig. 6A; Wieschaus et al., 1992; Royet and Finkelstein, 1995) and is almost totally absent from the head vertex primordia of oc^3^ eye-antennal discs (Royer and Finkelstein, 1995).

To explore potential interactions between otd and the segment polarity genes hh and wg, we first compared their regions of expression during eye-antennal disc development. In the early third instar disc, otd, like hh and wg, is expressed in the region of the head vertex primordium (Fig. 6A). We have shown previously that otd protein expression is graded across ocelli (o) to be directly adjacent to the compound eyes. Some residual frons is present in the more anterior region of the head (fr), which disappears with a stronger heat pulse (not shown). Note that the ocellar cuticle and the ocelli themselves (particularly the lateral ocelli) are larger than in wild-type flies (compare with Fig. 1A). (D) dsh^M20/dsh^M20 head vertex. The phenotype is similar to that shown in C. Note the dramatic expansion of the lateral ocellus on the right. (E) Eye-antennal disc from hh^ts2^ larvae grown at 28°C during the second half of the third larval instar and stained with anti-wg antibodies. Although wg expression in the antennal region, which is hh-dependent, is greatly reduced (arrowhead), wg staining in both the orbital (thick arrow) and ptilinium (thin arrow) primordia is expanded and more intense than in wild-type discs (compare to Fig. 2E). (F) Late third instar eye-antennal disc from wg^l114/l114; hhlacZ/hhlacZ larvae grown at the restrictive temperature (25°C) and stained with X-gal. The hh ocellar domain is larger than in wild-type discs and expands inward towards the compound eye (arrow; compare to Fig. 2D). In A-D, anterior is up. In E and F, medial is to the right. Scale bars: A-D (shown in A), 100 μm; E and F (shown in E), 50 μm.

**Fig. 4.** hh and wg are required for regional specification of the adult head. (A) hh^ts2^ larvae were grown at the restrictive temperature (28°C) for 12 hours during the third larval instar. The resulting flies lack medial head structures, including the median ocellus (white arrow) and the ocellar and postvertical bristles. In addition, they exhibit reduced lateral ocelli (open arrow) and fewer interocellar microchaetes. Frons cuticle (fr), which is normally restricted to the mediolateral region, is now also present in the region normally occupied by the ocelli (white arrow). The aberrant appearance of the frons results from the fact that the heat shock treatment is pupal lethal and the resulting pupal heads, which are not rigid, are easily disrupted when mounted. (B) fu^1/fu^1 head vertex. As in A, medial head structures are absent, including the ocelli and associated bristles. Ridged frons (fr) cuticle now occupies the medial region (white arrow). (C) wg^l114/l114; wg^CX3^ larvae were grown at the restrictive temperature (25°C) during the second half of the third larval instar. The heads of the resulting flies lack both lateral (orbital) and mediolateral (frons) structures, causing the
now replaced by ocellar-like cuticle (arrow). (C) Ectopic clone of wg on the head vertex. The clone, marked by yellow bristles (white arrows), has induced a partial loss of a lateral ocellus (open arrow) and an expansion of the frons within the medial ocellar region (black arrow).

The results we have described show that _otd_ expression is required both to eliminate _wg_ and to maintain _hh_ expression in the medial region of the disc.

### Ectopic _hh_ activates _otd_ during eye-antennal disc development

The results we have described show that _hh_, _wg_ and _otd_ pattern the head vertex primordium during third instar eye-antennal disc development. It is therefore important to understand the regulatory relationships among these genes during earlier stages of imaginal development. In the ectopic _hh_ expression experiments, we were surprised to find that _hh_ could induce ocellus formation in the shingle cuticle, a region of the head that lies outside the normal domain of _otd_ expression. Because _otd_ activity is essential for ocelli development, we suspected that ectopic _hh_ might induce _otd_ expression.

To test this hypothesis, we examined _otd_ protein distribution following the induction of ectopic _hh_ expression by the flp-recombinase technique. We found that ectopic _hh_ indeed induces patches of _otd_ expression outside the wild-type _otd_ expression domain (Fig. 7A,B). These patches are not randomly distributed across the eye-antennal disc. Instead, they are restricted to a mediolateral zone extending across the disc, and are never found in the anlagen of the antenna or compound eye. This zone includes the primordium of the shingle cuticle (Haynie and Bryant, 1986), where ectopic ocelli were observed in the flp- _hh_ experiments described above. When ectopic _wg_ expression was similarly induced using the flp-recombinase method, no additional _otd_ expression is

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Fig. 5. Ectopic expression of _hh_ and _wg_ alters regional head specification. Clones of cells ectopically expressing _hh_ and _wg_ were induced during the second larval instar as described in Materials and Methods. (A,B) Clones expressing ectopic _hh_ on the head vertex. Yellow macrochaetes marking the clones are indicated with arrowheads. _hh_ clones in the ocellar region have no phenotypic effect (A,B). However, if _hh_ is ectopically expressed in more lateral regions, such as the frons (A) or the orbital region (B), supernumerary ocelli (labelled O') are observed (white arrow and dashed line respectively). The _hh_ clone in B crosses the entire frons, which is

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the head vertex anlage, with highest levels present in the medial region, and progressively lower levels laterally (Royet and Finkelstein, 1995).

In the late third instar disc, _otd_ continues to be expressed in a graded fashion across the entire head vertex primordium (Fig. 6B) by the time when _hh_ and _wg_ expression have become regionally segregated. At this later stage, _hh_ has become restricted to the medial portion of the _otd_ domain, where _otd_ protein levels are highest. The patch of _wg_ expression corresponding to presumptive orbital cuticle is immediately adjacent to the _otd_ expression region (not shown). The result of these patterns of spatial expression is the generation of three domains of gene expression across the head primordium, in which _otd_ and _hh_, _otd_ alone, and _wg_ alone are expressed. As has been discussed, these domains appear to correspond to the anlagen of the medial (ocellar) region, the mediolateral (frons) region, and the lateral (orbital) region respectively. It should also be noted that, within the vertex primordium, _wg_ expression disappears from the region where _otd_ protein levels are highest.

Next, we examined the effect of _oc_ mutations on the expression of _hh_ and _wg_. We found that in _oc^361_ eye-antennal discs, _wg_ expression fails to disappear from the medial region, and instead persists across the entire primordium of the head vertex (compare Fig. 6C and 6D). Consistent with this observation, we found that _wg_ is also ectopically expressed in the medial region of the heads of _oc^361_ flies (Fig. 6F). _hh_ expression, however, is either lost or greatly reduced in _oc^361_ discs (Fig. 6G), suggesting that _otd_ is required for the maintenance of _hh_ expression in the medial region. The result of these effects is a disc lacking both _hh_ and _otd_, in which _wg_ is expressed across the entire vertex primordium. These results show that _otd_ is required both to eliminate _wg_ and to maintain _hh_ expression in the medial region of the disc.
detected (not shown). These results suggest that *otd* expression in the head vertex primordium may be activated by *hh* during normal imaginal development.

**DISCUSSION**

**Pattern formation in the primordium of the head vertex**

Here, we show that the segment polarity genes *hh* and *wg* specify regional identity across the head vertex primordium of the eye-antennal disc. Reduction of *hh* activity during the third larval instar causes the loss of the medial domain, which includes the ocelli, medial sensory bristles, and underlying ocellar cuticle. Loss of *wg* causes the deletion of both the mediolateral frons cuticle and the lateral orbital region. In addition, ectopic expression of either gene can respecify cell fates in the adjacent domain of the head. Ectopic *hh* expression can induce ocellus formation in the frons, while ectopic *wg* generates frons cuticle in the ocellar region.

The molecular regulatory relationships described here are quite different than, for example, the interdependence of *hh* and *wg* expression in the embryonic trunk segments. In the vertex primordium, the elimination of *wg* function leads not to the loss, but instead, to the expansion of the *hh* and *en* expression domains (Fig. 4F and J. Royet, unpublished observations). In a reciprocal fashion, the elimination of *hh* activity appears to increase the field of action of *wg*. This suggests that *hh* and *wg* each act not only to initiate a specific developmental pathway, but also to inhibit the pathway of the adjacent domain. The ability of either gene, when ectopically expressed, to redirect cell fates also suggests such a competitive mechanism of cell fate determination. The ability of *wg* to prevent the expansion of the ocellar region may be analogous to its role as a negative regulator of morphogenetic furrow progression across the developing compound eye (Ma and Moses, 1995).

**The *otd* homeobox gene is required for the segregation of the *hh* and *wg* expression domains**

As we have described, *hh* and *wg* specify regional identity in
should also be noted that the repression of repression (not shown). It does not show expression but do not show which reduce rather than eliminate imaginal development. In the absence of critical to pattern formation during head development. By which these domains become spatially separated is therefore by which these domains become spatially separated is therefore critical to pattern formation during head development.

We show here that otd, which functions in embryonic head formation, is also required for hh and wg regionalization during imaginal development. In the absence of otd, wg expression fails to disappear from the medial head, and hh expression is lost. We do not believe that this failure in wg repression results simply from the loss of hh. Specific otd allelic combinations, which reduce rather than eliminate otd expression, permit hh expression but do not show wg repression (not shown). It should also be noted that the repression of wg by otd in the eye-antennal disc differs from what occurs in the cephalic region of the embryo. In the embryo, otd is required for both wg and hh expression in the anterior head (Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990a; Mohler, 1995). There, however, otd positively regulates wg, which is in turn required for the maintenance of hh (Mohler, 1995).

The function of otd in the eye-antennal disc is not limited to its role in hh and wg regionalization. Since oc mutations cause the loss of both medial and mediolateral cell fates, otd must also be required for the correct specification of these domains. In the medial region, we have shown that otd acts at least partially through en during the formation of the ocelli (Royet and Finkelstein, 1995). Since reduction of hh also leads to the loss of en expression in the medial region (not shown), both otd and hh are required for medial fate specification.

The molecular hierarchy in the early head primordium

Our analysis has focused on pattern formation during the third instar stage of larval development. An important area for future investigation will be the molecular events that precede this stage. We have shown here that ectopic hh expression can activate otd outside its normal expression domain. This suggests that otd may be a hh target gene during early disc development. We have also demonstrated, however, that otd is required for the maintenance of hh expression. These results imply the existence of a regulatory feedback loop between the two genes. This mutual regulation is reminiscent of the proposed interaction between Sonic hedgehog and HNF-3β in the vertebrate notochord and floor plate (Echelard et al., 1993; Ang and Rossant, 1994).

There are two other implications of the ability of ectopic hh expression to activate otd. Since this activation occurs only within a specific region of the eye-antennal disc, there must be additional molecular requirements either for otd activation within this region or for otd repression outside it. We are currently investigating the possibility that dpp, which is expressed in a complementary fashion to otd in the early eye-antennal disc (J. Royet, unpublished observations), prevents otd expression during early development. In addition, as shown in Fig. 7, the ectopic otd expression induced by hh is graded in intensity. This suggests that hh may be responsible for generating the concentration gradient of otd protein that normally exists across the head vertex primordium. We have shown previously that this gradient is important for disc patterning (Royet and Finkelstein, 1995).

Homologues of hh, wg, and otd (Sonic hedgehog, Wnt-1 and the Otx genes) are expressed in anterior regions of the developing vertebrate embryo (reviewed by Bally-Cuif and Wassef, 1995). Sonic hedgehog, for example, is expressed in a specific ventral region of the developing mouse forebrain (Echelard et al., 1993; Rubenstein et al., 1994), while Wnt-1 and Otx expression overlap near the mes/metencephalic boundary (Bally-Cuif et al., 1995). It will be interesting therefore to determine whether any of the regulatory relationships we have described are conserved in vertebrates. Further analysis of the molecular hierarchy governing Drosophila head development is likely to provide insight regarding anterior patterning in other animals.

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