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

Julien Royet and Robert Finkelstein

University of Pennsylvania School of Medicine, Department of Neuroscience, 215 Stemmler Hall, Philadelphia PA 19104, USA

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 *orthodenticle (otd), empty spiracles (ems),* and *buttonhead (btd;* 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 differently 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, which each 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 antennal 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 $oc^{\gamma a1}$ is the same as $T(1;2)\ oc^{\gamma a1}$ which has been described previously (Wakimoto and Spradling, 1981; Mohler, 1984). The wg^{CX3} allele specifically affects *wg* function during imaginal development and is homozygous pupal lethal (Couso et al., 1993). The allele wg^{IL1114} 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^{M20} (Perrimon and Mahowald, 1987) and fu^{1} (Busson et al., 1988). The temperature sensitive allele hh^{ts2} and the *hh-lacZ* enhancer trap line hh^{P30} 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 L1 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., 1989), an anti-engrailed mAb 4D9 (Patel et al., 1989), and an anti-β-galactosidase mAb (Cappel). After incubation in primary antibody, discs were washed 3× 1 hour in PBT and incubated for 3 hours at room temperature with secondary biotinylated anti-rat, anti-mouse or anti-rabbit antibodies (Cappel). After 3× 1 hour washes in PBT, discs were treated with biotinylated horseradish peroxidase (HRP)-avidin complex (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_2O_2 and 0.5 mg DAB/ml in PBT supplemented, for anti-wg staining with 0.8 ml/ml of a 8% $CuCl_2$ 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.

lacZ expression in enhancer trap line-derived eye-antennal discs and adult fly heads was monitored using the β-galactosidase substrate X-gal as described by Brand and Perrimon (1993). To examine eye-antennal disc development in vitro, late third instar larval discs were dissected and incubated in the culture medium described by Schneider (1964) in the presence of 0.1mg/ml β-ecdysone (Sigma). After overnight culturing at 25°C, the newly fused eye-antennal discs were fixed in 1% glutaraldehyde in PBS and stained with X-gal.

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

Flies homozygous for the *hsp70-flp* gene were crossed to flies homozygous for either the Act5C<y+<wg (Struhl and Basler, 1993) or the Tuba1<y+<hh (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<y+<wg and hsp70-flp; Tuba1<y+<hh larvae were heat-shocked for 20 minutes at 32ºC or 30 minutes at 35ºC respectively.

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. Laterally, adjacent to the medial region, is the dorsal frons cuticle, which is easily recognized as a series of closely spaced, parallel ridges devoid of macrochaetes. Lateral to the frons is the orbital cuticle, which lies adjacent to the compound eyes and also contains a defined array of macrochaetes.

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).

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 *en-lacZ*, *hh-lacZ*, and *wg-lacZ* 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 (hh^{ts2}; 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

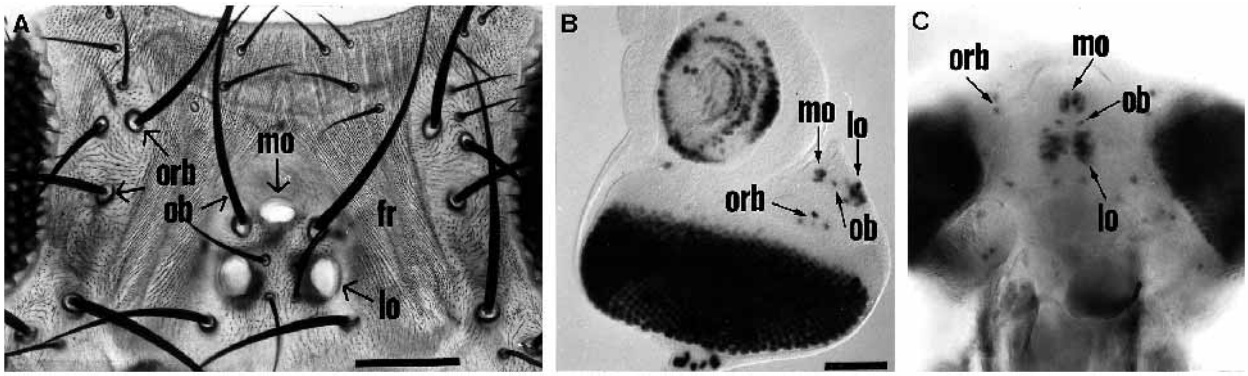

Fig. 1. Fate-mapping of adult head structures on the eye-antennal imaginal disc. (A) Adult head vertex (wild-type). The region of the head capsule between the compound eyes can be subdivided into three morphologically distinct domains. The medial domain contains the medial and lateral ocelli (mo and 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 orbital bristles (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.

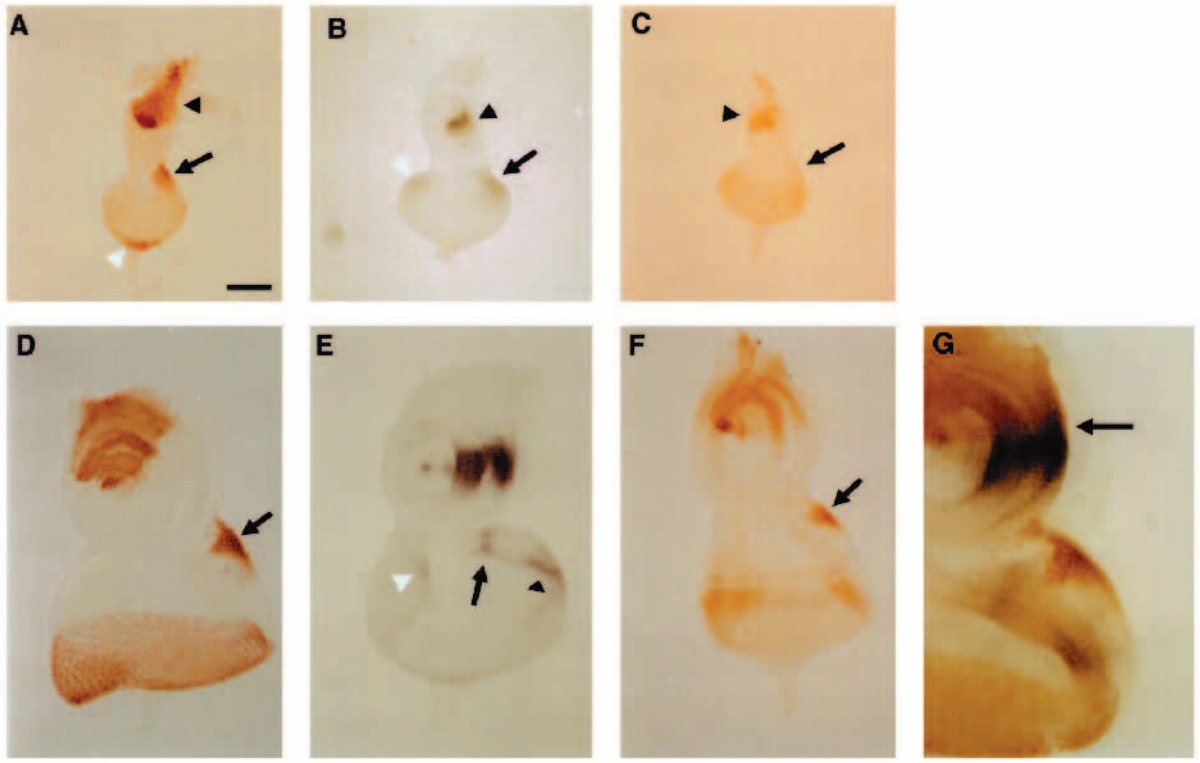

Fig. 2. Regional restriction of *hh*, *wg* and *en* expression during eye-antennal disc development. (A-C) Early third instar discs (75-85 h AEL). (D-G) Late third instar discs (110-120 h AEL). (A,D) *hh-lacZ* discs labeled with anti-β-galactosidase antibodies. (A) In addition to the posterior compartment 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.

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 heteroalleleic combination (wg^{IL1114}/wg^{CX3}) which specifically reduces *wg* imaginal function. When raised at 17ºC, 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 time 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

(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^{ts2} 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 respecify cell fate. To address this question, we used the flp-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 flp-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 respecify cell fates on the developing head capsule. Misexpression of *hh* induces ectopic medial structures (ocelli) at more lateral locations. Misexpression of *wg* produces more lateral head 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 not possible. In each case however, ectopic expression appears to cause the loss of the normal pathway of regional

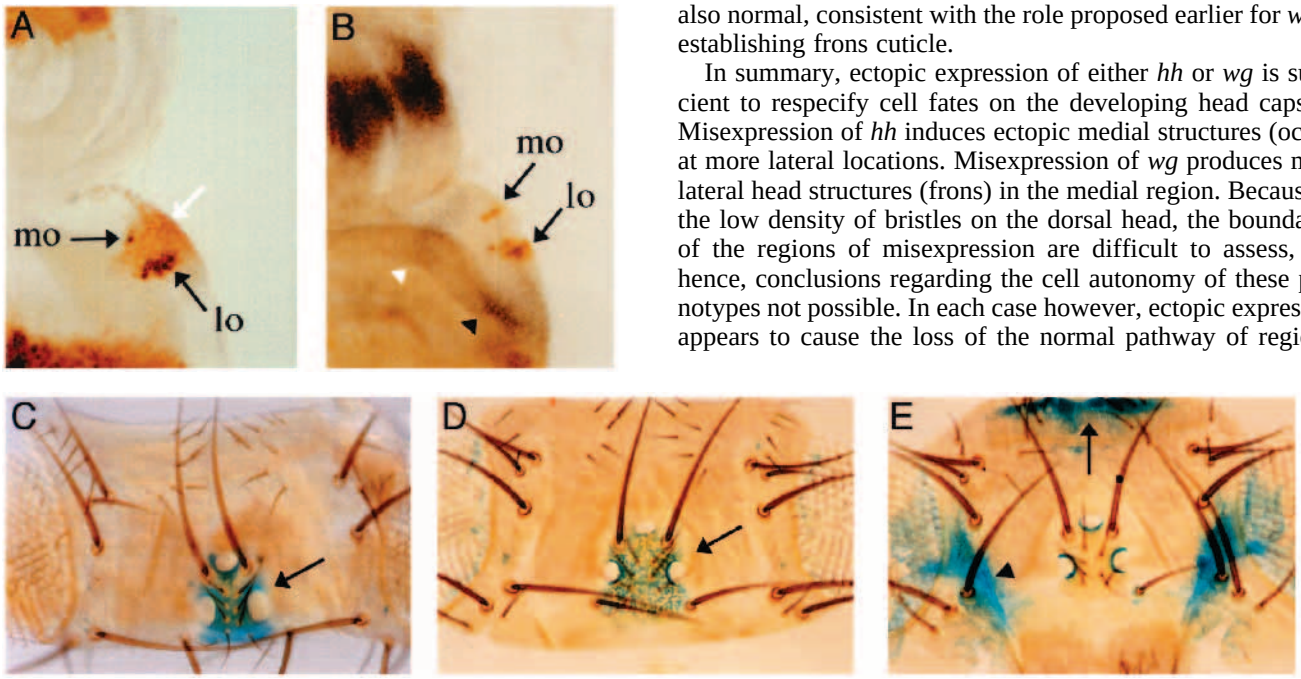

Fig. 3. *hh*, *wg*, and *en* expression demarcate the primordia of adult head structures. (A) L1; *hh-lacZ* disc labeled with anti-β-galactosidase antibodies. Because of extremely strong *lacZ* expression in the L1 line (which specifically labels the developing ocelli), the precursor cells of the medial 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.

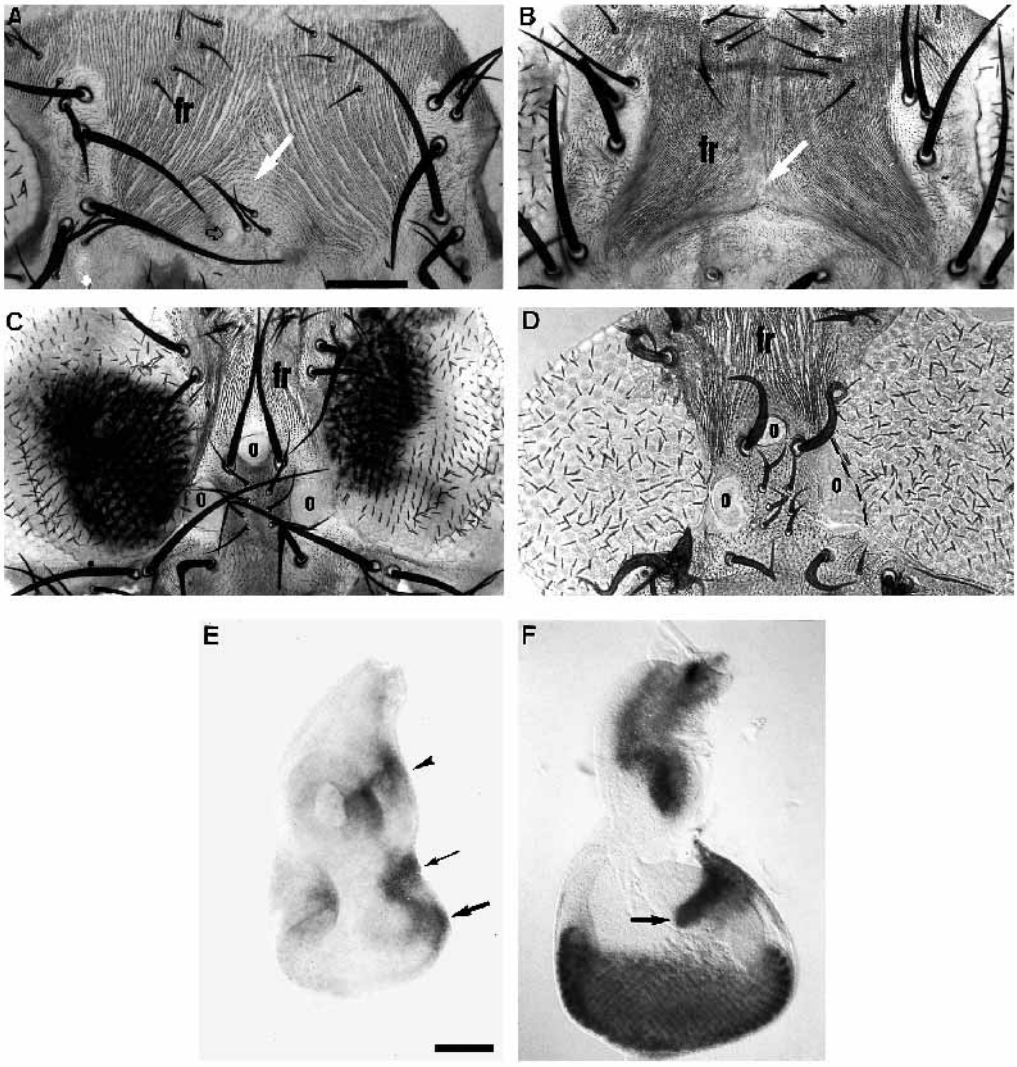

Fig. 4. *hh* and *wg* are required for regional specification of the adult head. (A) hh^{ts2} larvae were grown at the restrictive temperature (28°C) for 12 hours during the third larval instar. The resulting flies lack medial head structures, including the median ocellus (white arrow) and the ocellar 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^{IL1114}/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 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^{IL1114}/wg^{CX3}; *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.

specification, and its replacement by the pathway of an adjacent region of the head.

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 (Bedichek, 1934; Royet and Finkelstein, 1995). The strongest *oc* mutation ($oc^{\gamma a1}$) 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. 6AB; Wieschaus et al., 1992; Royet and Finkelstein, 1995) and is almost totally absent from the head vertex primordia of $oc^{\gamma a1}$ eye-antennal discs (Royet 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

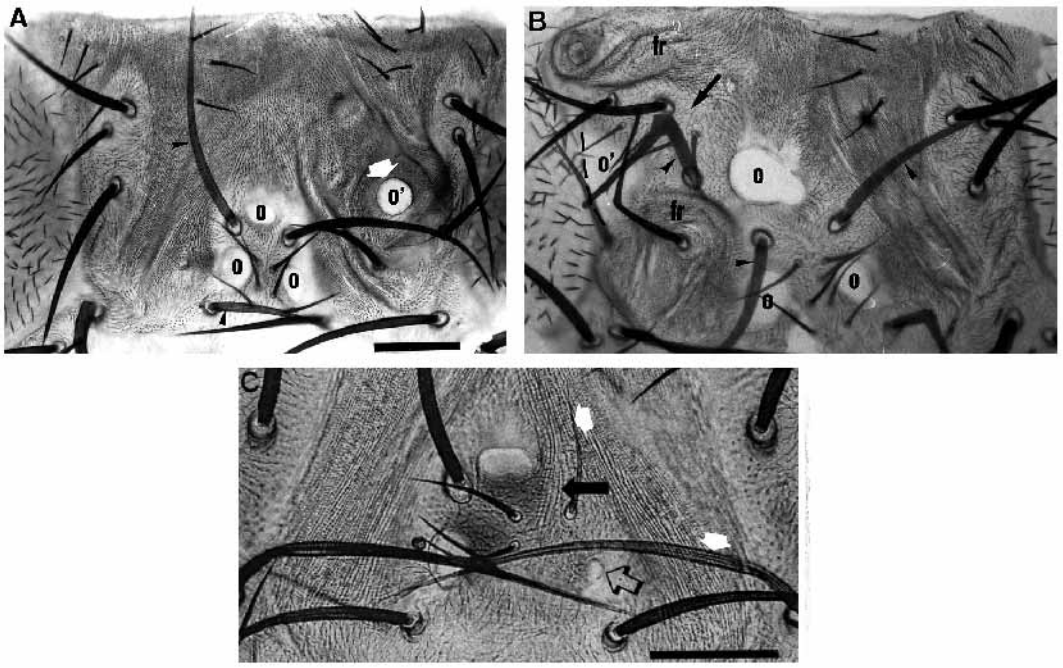

Fig. 5. Ectopic expression of *hh* and *wg* alters regional head specification. Clones of cells ectopically expressing *hh* and *wg* were induced during the second larval instar as described in Materials and Methods. (A,B) Clones expressing ectopic *hh* on the head vertex. Yellow macrochaetes marking 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 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). Clones in the frons or the orbital region show no morphological abnormalities (not shown). The low density of bristles on the head vertex and the fact that bristles often move outside of clonal borders makes the precise localization of these borders impossible. It is therefore difficult to assess whether the phenotypic effects observed are due to cell-autonomous or non cell-autonomous effects. In all panels, anterior is up. Scale bars: 100 μm.

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 $oc^{\gamma a1}$ 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 $oc^{\gamma a1}$ flies (Fig. 6F). *hh* expression, however, is either lost or greatly reduced in $oc^{\gamma a1}$ 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.

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

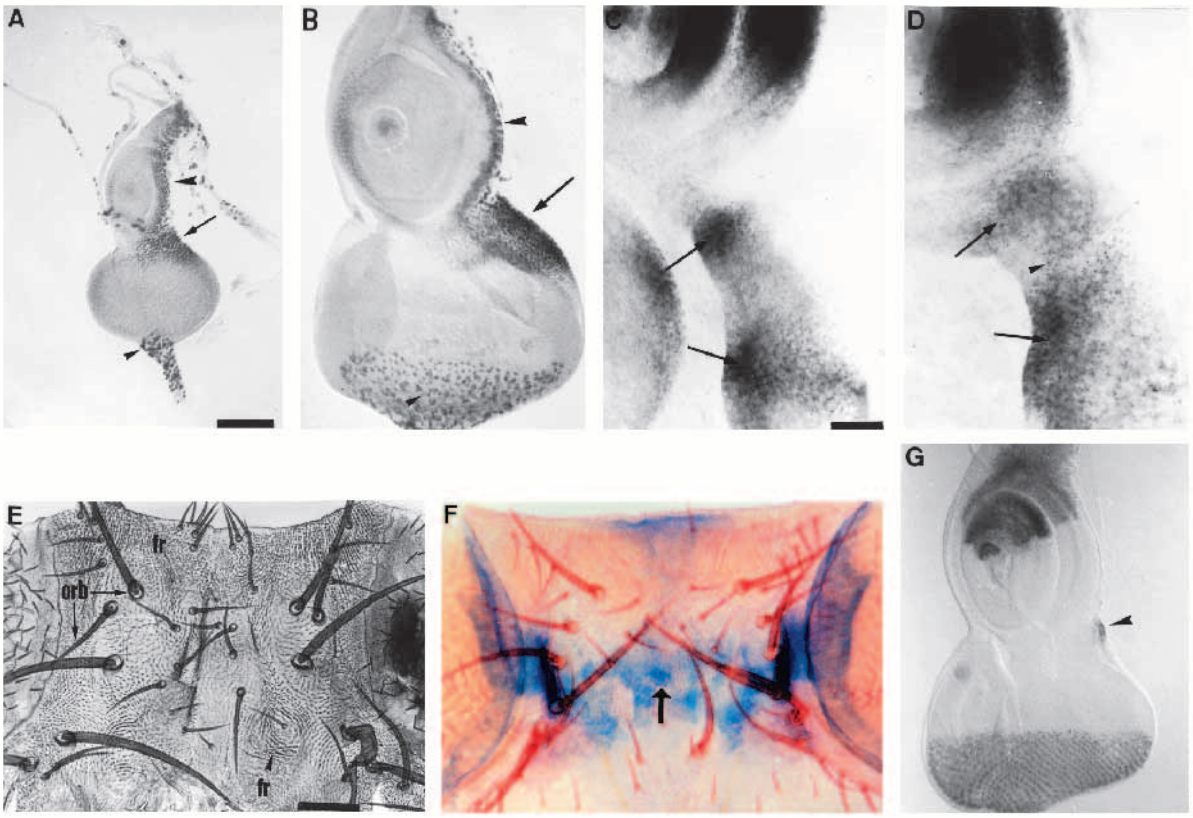

Fig. 6. *otd* is required for the regional segregation of *hh* and *wg* expression. (A,B) otd antibody labeling of early (A) and late (B) third instar eye-antennal discs. otd is expressed in a graded fashion, with highest protein concentrations present in the most medial region of the head vertex primordium (arrow). otd is also expressed in the first antennal segment (large arrowhead) and in the subretinal cells of the compound eye (small arrowhead). (C,D) wg antibody staining of wild-type (C) and $oc^{\gamma a1}$/Y (D) eye-antennal discs. In a wild-type disc (C), characteristically punctate wg protein expression can be seen in the anlagen of the ptilinium and the orbital region (arrows), but is excluded from the region between them. In the absence of otd (D), ectopic wg expression appears between these two primordia (arrowhead). (E,F) Adult head vertices of $oc^{\gamma a1}$/Y (E) and $oc^{\gamma a1}$/Y; *wg-lacZ* (F) flies stained with X-gal. Medial head structures (the ocelli) and mediolateral structures (frons cuticle) are absent on $oc^{\gamma a1}$/Y heads (E), with residual frons remaining only in more anterior regions (fr). The orbital bristles (orb) are normal but shifted slightly towards the midline of the head. *wg* expression is not restricted to the lateral orbital cuticle of $oc^{\gamma a1}$/Y flies (F), but expands into the medial head region (arrow). (G) anti-β-galactosidase labeling of an $oc^{\gamma a1}$/Y; *hhlac-Z/hh-lacZ* eye-antennal disc. Only very weak residual staining can be observed in the medial ocellar primordium (arrowhead). In A-D and G, medial is to the right. In E and F, anterior is up. Scale bars: A, B and G (shown in A), 50 μm; C, D (shown in C), 25 μm; E, F (shown in E), 100 μm.

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

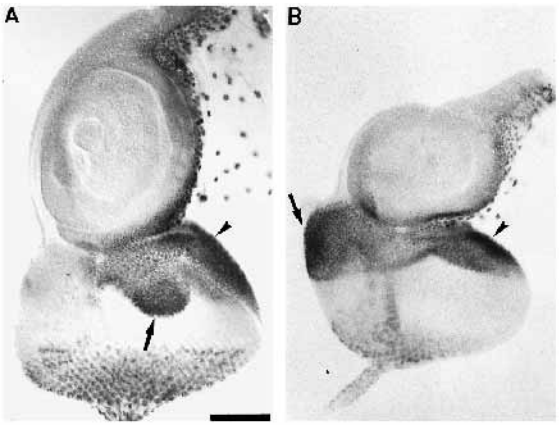

Fig. 7. Ectopic *hh* activates *otd* expression during early disc development. (A,B) *hsp70-flp ; Tuba1<y+<hh* larvae were heat shocked for 30 minutes at 35°C during the first larval instar. 48 hours later, third instar eye-antennal discs were dissected and stained with antibodies to otd. Apart from the normal staining in the head vertex primordium (arrowhead) and the first antennal segment, ectopic otd staining can be seen in a region close to the ocellar primordium (A, arrow) or on the opposite side of the disc (B, arrow) in the primordium of the shingle cuticle. Note that in both cases, ectopic otd expression is not uniform, but graded in intensity.

spatially distinct territories of the developing head. Initially, however, the expression domains of *hh* and *wg* overlap in the vertex primordium of the eye-antennal disc. The mechanism 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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