Pattern formation in *Drosophila* head development: the role of the orthodenticle homeobox gene

Julien Royet and Robert Finkelstein

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

SUMMARY

Significant progress has been made towards understanding how pattern formation occurs in the imaginal discs that give rise to the limbs of *Drosophila melanogaster*. Here, we examine the process of regional specification that occurs in the eye-antennal discs, which form the head of the adult fruitfly. We demonstrate genetically that there is a graded requirement for the activity of the orthodenticle homeobox gene in forming specific structures of the developing head. Consistent with this result, we show that OTD protein is expressed in a graded fashion across the disc primordia of these structures and that different threshold levels of OTD are required for the formation of specific subdomains of the head. Finally, we provide evidence suggesting that otd acts through the segment polarity gene engrailed to specify medial head development.

Key words: orthodenticle, *Drosophila*, imaginal discs, gradient, engrailed, pattern formation

INTRODUCTION

In the process of pattern formation, a developmental field is subdivided into discrete domains. This process has been investigated in greatest detail in studies of early *Drosophila* embryogenesis. In the syncytial blastoderm embryo, a positional coordinate system is established through the action of maternally deposited gene products (reviewed in Green and Smith, 1991; St. Johnston and Nüsslein-Volhard, 1992). These gene products form molecular concentration gradients along both the anteroposterior and dorsoventral axes of the embryo. Along the anteroposterior axis, for example, gradients of the bicoid and hunchback proteins specify the development of head, thoracic and abdominal structures (Driever and Nüsslein-Volhard, 1988; Struhl et al., 1992). Similarly, the subdivision of the embryo into territories along the dorsoventral axis is initiated by a nuclear concentration gradient of the dorsal protein (Govind and Stewart, 1991; Ip and Levine, 1992).

Because this early regional specification occurs in a syncytium, it differs from the process that must occur in multicellular tissues where the diffusion of patterning molecules is more constrained. *Drosophila* imaginal disc development represents an excellent model system for studying pattern formation in a multicellular field. Important strides have been made towards understanding how pattern is organized within the thoracic discs that give rise to adult limb structures (reviewed in Bryant, 1978; Cohen, 1993). During this process, the expression of the engrailed gene defines cell populations that constitute the posterior compartment of each of these discs (Lawrence and Morata, 1976; DiNardo et al., 1985; Kornberg et al., 1985). These posterior cells secrete hedgehog protein, causing adjacent anterior cells to produce the wingless or decapentaplegic gene products (Basler and Struhl, 1994). These proteins are thought to initiate the process of pattern formation across the two disc compartments. Additional progress has also been made in understanding the regulatory interactions among genes that continue the regional specification of the limb primordia (Campbell et al., 1993; Diaz-Benjumea and Cohen, 1993; Williams et al., 1993; Capdevila et al., 1994).

Despite these advances in understanding limb formation, the development of the head of the adult fly remains less well characterized. Most of the adult head capsule is derived from the fusion of the paired eye-antennal discs. Pattern formation in the eye-antennal discs is clearly different from that which occurs in the thoracic discs (reviewed in Cohen, 1993; Jürgens and Hartenstein, 1993). Unlike the limb discs, each eye-antennal disc is derived from multiple embryonic segments. In addition, anteroposterior compartmentalization of the eye-antennal discs occurs during the third instar larval stage, while thoracic disc compartments are established during embryogenesis (Morata and Lawrence, 1978, 1979). Although the molecular genetics of compound eye development has been studied in great detail, the genes that specify other regions of the head capsule are mostly unknown. Two exceptions are the homeotic selector genes labial and Deformed. Mutations at each of these loci cause the transformation of specific regions of the head towards an apparent thoracic fate (Lewis et al., 1980; Merrill et al., 1987, 1989).

Here, we investigate the function of the *Drosophila orthodenticle* (otd) gene in the specification of the eye-antennal imaginal discs. The *otd* gene encodes a homeodomain protein required for cell fate determination during both embryonic and imaginal development. In the embryo, otd is required for the correct development of the head and ventral midline (Finkelstein and Perrimon, 1990; Finkelstein et al., 1990). Later,
during larval development, *otd* is expressed in specific imaginal discs and required for the establishment of medial cell fates (Wieschaus et al., 1992). In particular, *otd* is required for the development of the dorsal region of the adult head, which forms from the fusion of the two eye-antennal discs.

In these studies, we analyzed the requirement for *otd* in the development of specific head structures. We demonstrate genetically that the progressive reduction of *otd* activity causes the graded loss of structures along the mediolateral axis of the head. We show that OTD protein is expressed in the region of the disc that gives rise to these head structures, and that this expression is specifically decreased by *otd* mutations that affect adult head development. In addition, we show that OTD protein is distributed in a concentration gradient across the disc primordia of these structures. Using heat-shock promoter-driven *otd* expression, we demonstrate that different *otd* levels are required for the development of different mediolateral subdomains of the developing head. Finally, we provide evidence that *otd* acts through the segment polarity gene *engrailed* to pattern the most medial region of the head.

**MATERIALS AND METHODS**

**Fly strains**

The *otd* and *oc* alleles used in this study are: the X-ray-induced allele *otd* 

\[ otd_{A101} \]

and the ethylmethane-sulfonate (EMS)-induced mutations *otd* 

\[ otd_{X86}, \quad otd_{3H13} \]

and *otd* 

\[ otd^{P087} \]

(Wieschaus et al., 1984). The allele referred to here as *oc* is the same as the *In(1)49, oc* 

\[ 49, oc^{A1} \]

allele (Bedichek, 1934). *T(1;2) oc^{p1} (referred to here as *oc* 

\[ oc_{p1} \]

and *oc* 

\[ oc_{h} \]

have been previously described (Wakimoto and Spradling, 1981; Mohler, 1984). The chromosomes carrying these mutations were maintained as FM7c or FM3 balancer stocks. The wild-type strains used were either Oregon-R or *yw*. Balancer chromosomes and other mutations are described in Lindsley and Zimm (1992). The two *lacZ* enhancer trap lines used for fate-mapping analysis were the A101 line, which labels sensory mother cells (Huang et al., 1991), and the L1 line, which labels ocelli precursor cells and cells in the lamina of the brain (Mozzer and Benzer, 1993). The *en-lacZ* and *hsp70-en E5n* lines have been described previously (Hama et al., 1990; Heemskerk et al., 1991).

**Histochemistry and immunohistochemistry**

*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 in 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 0.1 μg/ml of 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.

Larvae were grown in uncrowded conditions to obtain optimal imaginal disc morphology. Imaginal discs were dissected (attached to the larval mouthhooks) in PBS and fixed in 4% paraformaldehyde in PBS saturated with heptane for 20 minutes at room temperature. 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 (rat polyclonal OTD antiserum (described in Wieschaus et al., 1992) at 1:500, EN mAb 4D9 (Patel et al., 1989) at 1:3 and β-galactosidase (Cappel) at 1:500). The discs were then washed 3× 1 hour in PBT and incubated for 3 hours at room temperature with secondary biotinylated anti-rat or anti-mouse antibodies (horse IgG, Cappel) or with fluorescein-labeled goat anti-rat IgG or rhodamine-labeled goat anti-mouse anti-bodies (Cappel), each used at a 1:500 dilution except for the rhodamine-labeled antibody, which was used at a 1:20 dilution. For fluorescence labelling, following 3× 1 hour PBT washes, discs were mounted in 90% glycerol in PBS. For HRP staining, after 3× 1 hour washes in PBT discs were treated with biotinylated horseradish peroxidase (HPR)-avidin complex (Vector laboratories, Elite ABC Kit) in a 1:50 dilution in PBT for 1 hour and washed again 3× 45 minutes in PBT. Staining was visualized by incubating the discs in 0.5 mg DAB/ml in PBT in the presence of 0.04% H₂O₂.

Discs were viewed with Nomarski optics using a Zeiss Axioskop microscope. For confocal imaging, a Biorad MRC600 Laser sharp confocal system equipped with CoMos software (Biorad) was used.

**hsp70-otd flies and heat-shock protocol**

To generate the *hsp70-otd* flies, a 3.8 kb *otd* cDNA was cloned as an EcoRI fragment (Finkelstein et al., 1990) into the P element vector pCaSpeR-hs, which contains the *hsp70* promoter and 3′ flanking regions (obtained from Carl Thummel). This plasmid, along with the p25.7wc helper plasmid, was introduced into a *yw* recipient strain using standard methodologies (Rubin and Spradling, 1982). Surviving adults were crossed to *yw* flies and their progeny scored for w + transformants. Lines were established and mapped genetically. The transformant line used in this study (denoted HS55A) contains a P element insertion on the third chromosome maintained over a TM3 Sb balancer chromosome.

*yw; hsp70-otd/TM3 Sb or hsp70-otd En5* homozygous males were crossed to *oc* 

\[ oc_{F3} \]

females and allowed to lay eggs on molasses egg collection plates for one hour. Plates were incubated at 25°C for 22 hours, after which larvae were transferred to vials containing *Drosophila* medium (30 larvae/vial). Vials were returned to 25°C, until the indicated heat shock time period. Heat pulses were performed by immersing the vials in a 37°C water bath for the intervals described. Heads from the eclosing flies were mounted in 30% glycerol in ethanol.

**RESULTS**

The laterally symmetric adult head is largely formed from the fusion of the two eye-antennal discs. The dorsal region (vertex) of the head is occupied by a characteristic set of structural features that lie between the compound eyes (Fig. 1A). Along the mediolateral axis, each half of the head can be divided into three morphologically distinct subdomains. The medial subdomain contains the ocelli (simple eyes) and a set of characteristic sensory bristles (the large ocellar and postvocal bristles, and the small interocellar bristles). The ocelli and its associated bristles are situated on the ocellar cuticle, which is marked with small hairs but otherwise devoid of distinguishing landmarks. The second (mediolateral) subdomain lies immediately adjacent to the ocellar region on either side of the head. This subdomain contains the dorsal frons, which consists of a series of closely spaced, parallel ridges. The frons converges anterior to the ocelli, such that it delineates a triangular area surrounding the ocellar region. The third (lateral) subdomain of the dorsal head is the orbital region. The orbital cuticle, which is similar in appearance to ocellar cuticle, occupies the space between the frons and the compound eyes on either side of the head. It is also marked by a stereotypical array of macrochaetes (the orbital bristles).

**orthodenticle mutations affect adult head development in a graded fashion**

In previous studies, it was shown that the *orthodenticle* (*otd*)
homeobox gene is required during both embryonic and imaginal development. During embryogenesis, otd is required both to specify a discrete region of the developing embryonic head and to establish cell fates along the ventral midline (Finkelstein and Perrimon, 1990; Finkelstein et al., 1990; Klambt et al., 1991). Because of these early functions, null alleles of otd are recessive embryonic lethal. In addition to this early requirement, otd is necessary for the correct development of the medial regions of certain imaginal discs (Wieschaus et al., 1992). In particular, otd is allelic to ocelliless (oc). oc alleles are recessive viable mutations that cause the loss of the ocelli and surrounding structures (Bedichek, 1934; Finkelstein et al., 1990; Wieschaus et al., 1992). The oc mutations that have been analyzed at the molecular level are associated with lesions 3’ to the otd gene (Finkelstein et al., 1990) and appear to affect imaginal but not embryonic development.

To better understand the function of otd in adult head development, we compared the effects of mutations at the otd locus in detail. In previous work, it was shown that homozygous mitotic clones of strong otd alleles cause, depending on their location, the complete loss of either the ocelli or the frons in a cell autonomous fashion (Wieschaus et al., 1992). Consistent with this observation, flies hemizygous for a strong oc allele (oc<sup>91</sup>/Y) almost completely lack medial and mediolateral head structures (compare Fig. 1A and D). In the medial region, the ocelli are absent, as well as the surrounding postvertical, ocellar and interocellar bristles. In the mediolateral domain, the frons is almost entirely deleted, with only small lateral patches

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**Fig. 1.** Graded effects of otd mutations on adult head development, (A) Wild-type (Oregon R). The cuticle between the compound eyes can be divided into three morphological domains: the ocellar cuticle, the ridged frons cuticle and the orbital cuticle. The ocellar cuticle, which is in the most medial position, contains the three ocelli (oc) and associated macrochaetes (the postvertical bristles, pvb, and ocellar bristles, ocb) and microchaetes (the interocellar bristles,ioc). The ocellar domain is flanked by the ridged cuticle of the frons (fr). The space between each compound eye and the frons is occupied by orbital cuticle, which carries the three orbital bristles (orb). Orbital and ocellar cuticle cannot be morphologically distinguished. (B) otd<sup>DA101</sup>+. otd<sup>DA101</sup> is a null allele caused by a deletion that removes the otd gene (Finkelstein et al., 1990). The medial region of the head is most affected by this genotype. The median ocellus is displaced anteriorly and is smaller than usual. The pattern of interocellar and ocellar bristles is disturbed (note that one of the ocellar bristles is absent). Also note the increased distance between the two lateral ocelli and between the two postvertical bristles as compared to wild-type. The ocellar cuticle is expanded laterally and towards the anterior of the head. The ridges of the frons are not as well organized as in A. The orbital cuticle and orbital bristles are not affected. (C) oc<sup>2</sup>/Y. The characteristic structures of the medial region, including the ocelli and the interocellar, ocellar and postvertical bristles are absent. Only disorganized macrochaetes are now present in this region (open arrows). The more medial portion of the frons is also missing. A few lateral ridges of frons cuticle remain, delimiting the border between the orbital cuticle and the medial region. (D) oc<sup>91</sup>/Y. As in C, the ocelli and associated bristles are deleted. In this genotype, there are no remaining macrochaetes in the medial region. More of the frons cuticle is deleted than in C. Frons (fr) remains only in small lateral patches and in more anterior regions. The orbital bristles (orb) are slightly moved towards the midline of the head. Anterior is up in all panels. Scale bar shown in (A) (all panels are the same scale): 100 μm.
remaining. The lateral orbital region is slightly expanded medially and the only bristles now present on top of the head are the orbital bristles.

Flies hemizygous for a weaker \( oc \) allele (\( oc^{1} \)) show a similar but not identical head phenotype (Fig. 1C). The ocelli and their associated bristles are again absent with disorganized bristles now present in positions more anterior and more lateral than those normally occupied by the ocellar and postvertical bristles. In this case, however, more frons cuticle remains, delimiting the border between the orbital cuticle and the medial region. In \( oc^{1} \) flies, the orbital cuticle is usually wild-type in size and appearance.

A still weaker head phenotype is exhibited by flies that are heterozygous for embryonic lethal \( otd \) alleles. We have previously noted that \( otd \) is haploinsufficient and that heterozygotes show alterations in the ocellar region (Wieschaus et al., 1992). We have now analyzed this heterozygous phenotype in detail for three \( otd \) alleles (\( otd^{A101} \), \( otd^{D87} \) and \( otd^{H1} \)). In each case, the medial domain is the primary region affected (Fig. 1B). The ocelli are present, but the ocellar lenses are sometimes smaller than normal, with the median ocellus always the most affected. In addition, deletions or duplications of the postvertical and interocellar bristles generally occur. Finally, the cuticle that surrounds the ocelli (which is indistinguishable from the orbital cuticle near the compound eyes), expands both laterally and anteriorly. The frons is still present in its usual mediolateral position, but appears slightly disorganized.

The mildest alterations are shown by flies heterozygous for \( oc \) alleles. In this case, the dorsal region of the head is largely indistinguishable from that of a wild-type fly. The only change is a variable disturbance in the arrangement and number of the interocellar bristles and occasionally an alteration of the pattern of the postvertical bristles (not shown). This phenotype is similar to that observed in flies homozygous for the weakest known \( oc \) allele (i.e. \( oc^{ab} \); not shown).

The phenotypic series revealed by our analysis is, from weakest to strongest: \(+/+\) (wild type) \( > oc^{ab}/oc^{ab} > oc^{1}+/+ > oc^{1}loc^{1} > otdd+/+ > otddloc^{ab} > otddloc^{H1} \). In this series, progressively severe allelic combinations affect progressively lateral head structures. In \( oc^{ab}/oc^{ab} \), \( ocl^{+} \), or \( otdd+/+ \) heads, medial structures are primarily affected. In \( oc^{1}loc^{1} \), \( ocl^{H1}loc^{ab} \) or \( otddloc^{ab} \) genotypes, both medial and mediolateral structures are deleted.

**Analysis of OTD expression in the developing eye-antennal imaginal disc**

The genetic results described above suggested a graded requirement for \( otd \) along the mediolateral axis of the head. The lateral domain (the orbital cuticle and bristles) does not appear to require \( otd \) activity since it is largely unaffected by the genotypes tested. The mediolateral domain (the ridged frons cuticle) shows a requirement for \( otd \) activity and is progressively reduced (in the medial to lateral direction) by increasingly severe mutations. Finally, the medial domain is most sensitive to the reduction of \( otd \) activity, with the mildest allelic combinations primarily affecting the ocelli and their associated bristles.

In order to understand the molecular mechanism underlying these phenotypes, we analyzed OTD protein expression in this region of the eye-antennal disc. In particular, we examined the relationship between this expression and the primordia for the structures affected by \( oc \) and \( otd \) mutations. Haynie and Bryant (1986) previously established a fate map for the eye-antennal disc by transplanting small disc fragments into host larvae and observing the structures that develop. This fate map, although very useful in determining the relative positions of various anlagen, gives only approximate information about their precise locations. This problem is compounded by the morphological changes that the eye-antennal discs undergo during larval development.

To overcome these problems, we used two \( lacZ \) enhancer trap strains that permit the localization of the primordia of specific head structures. In the first line (L1), the \( lacZ \) gene is expressed in precursor cells of the ocelli as well as in compound eye precursors (Mozer and Benzer, 1993). When early third instar (75-85 hours after egg laying (h AEL)) eye-antennal discs from this strain are stained for \( \beta \)-galactosidase activity, only the photoreceptor cells of the compound eye are labeled (Fig. 2A). As disc development proceeds (85-100 h AEL), \( lacZ \) expression becomes clearly detectable in the precursors of the median and lateral ocelli (Fig. 2B). Since the median ocellus forms from the fusion of the two eye-antennal discs, its primordium in each disc includes fewer cells (Birmingham, 1942). Between 100 and 110 h AEL, the medial region of the disc containing these primordia folds inward, forming a flap. During this ‘flap stage’, the ocelli precursor cells are situated near the inner edge of the flap (Fig. 2C). Finally, at the end of third instar larval development (110-120 h AEL), the flap unfolds and the ocelli return to their original spatial orientation within the disc (Fig. 2D).

To visualize the positions where specific bristles arise, we used an enhancer trap line (A101) which labels the nuclei of sensory precursor cells (Huang et al., 1991). This line was not used to visualize ocelli precursor cells, since \( lacZ \) expression does not begin until late in the third instar stage. In late third instar discs, \( \beta \)-galactosidase staining is observed in the putative precursors of the lateral and median ocelli, as well as in the precursors of the postvertical, ocellar and orbital bristles (Fig. 2E). To confirm the identities of these precursor cells, we cultured third instar eye-antennal discs from this strain in vitro. In the presence of the hormone ecdysone, the eye-antennal discs will fuse and correctly form the adult head capsule (Milner and Haynie, 1979). \( lacZ \) expression in head capsules formed in culture using A101 discs was consistent with the marked precursors giving rise to the ocelli and sensory bristles as described above (Fig. 2F).

In the ocellar region, the \( lacZ \) expression pattern in discs from the A101 strain is essentially consistent with the fate map deduced by Haynie and Bryant. In third instar eye-antennal discs, increasingly medial adult head structures are derived from increasingly medial regions of the disc. The precursors of the most medial sensory structures, the ocelli and the ocellar bristles, are located close to the medial edge of the disc. The laterally situated orbital bristle precursors are located more centrally in the disc and form a pattern similar to their final pattern on the adult head. We presume that the mediolateral frons precursors (which cannot be detected with this line) are located between the precursors for the ocelli and those of the orbital bristles. The \( lacZ \) expression pattern also provides more precise information about the anteroposterior positions of the ocelli and bristle precursors.
Having identified the relative mediolateral positions of the precursors for these head structures, we examined the location of OTD expression with respect to these positions. In the eye-antennal disc, we have previously shown that OTD protein is expressed in the ocellar region, in the first antennal segment and in cells posterior to the morphogenetic furrow (Wieschaus et al., 1992). Immunohistochemical staining had suggested that OTD levels might not be uniform across the region of the head vertex primordia (see, for example, Fig. 4A). To examine protein expression levels with greater sensitivity, we repeated the antibody staining experiments using fluorescently labeled secondary antibodies and confocal microscopy. These experi-

Fig. 2. Localization of the precursors of the ocelli and sensory bristles using the L1 and A101 enhancer trap lines. Third instar eye-antennal discs were dissected and stained with X-gal to detect β-galactosidase activity. (A-D) The L1 line. (A) Early third instar eye-antennal disc (between 75 and 85 h AEL). Staining is present posterior to the morphogenetic furrow but not in the ocellar region. (B) Early/mid-third instar disc (85-100 h AEL). Precursors of the median (m) and lateral (l) ocelli begin to be visible in the mediolateral region at this stage. The dot indicated by an arrowhead is not part of the disc epithelium. (C) Mid-third instar disc at the ‘flap’ stage (100-110 h AEL) (see text). The precursors of the ocelli are present near the edge of the flap (indicated by white arrows). (D) Late third instar disc (110-120 h AEL). The flap has unfolded and the two ocelli have returned to their earlier mediolateral location. (E,F) The A101 line (110-120 h AEL). (E) In this line, lacZ is expressed in neural precursor cells (see text). Staining is present in the lateral ocellus (l) and in the half of the median ocellus derived from each disc (m). Precursors of the postvertical (pvb), ocellar (ocb) and orbital bristles (orb) can also be visualized. (F) 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 then fixed and stained with X-gal. Precursors of the ocelli and of the ocellar, orbital and postvertical bristles can be identified in their correct positions on the dorsal head. In all panels, anterior is up. In panels (A-E) medial is to the right. Scale bar shown in E corresponds to 100 μm (all panels are to the same scale).
OTD protein is expressed in a graded fashion in the ocellar region of the developing eye-antennal disc. (A-L) Wild-type third instar larval discs stained with antibodies to OTD, followed by FITC-labeled secondary antibodies. (B,C,F,G,J,K) Fluorescence intensity was visualized using pseudo-color imaging, where white and red indicate the highest and lowest staining levels detected respectively. C, G and K are higher magnification views of B, F and J. (D,H,L) The quantitation of staining intensity within the ocellar primordium. (M-P) A third instar disc from the L1 enhancer trap line stained with a combination of antibodies to OTD and β-galactosidase, followed by FITC-labeled and rhodamine-labeled secondary antibodies. (A-C) Early third instar disc (75-85 h AEL). Staining is visible in the ocellar region (large arrow), in subretinal cells (small arrow) and along the medial edge of the antenna segment (arrowhead). OTD protein levels are graded across the head primordia. Highest fluorescence intensities (white and purple) are in the most medial region of the disc. Lowest staining levels are observed in presumptive lateral head regions. (E-G) Mid-third instar disc (100-110 h AEL). (‘flap’ stage). OTD expression can be seen throughout the flap, with highest levels near the medial edge (now in a more internal position of the disc). Expression gradually decreases in the direction of more lateral anlagen. (I-K) Late third instar disc (110-120 h AEL). The flap has unfolded, such that highest OTD concentrations are again in the outer region of the disc. Highest staining levels are seen in the area that will give rise to the most medial structures (e.g. the ocelli). (D,H,L) Pixel intensity along a line traversing the ocellar region in C, G and K respectively. (M) Late third instar disc (110-120 h AEL) stained for both OTD and lacZ expression. OTD expression is visualized. (N) The same disc as M, with lacZ expression visualized in the anlagen of a lateral ocellus. Staining corresponding to the median ocellus is not visible in this view. (O) Merger of the images in M and N. (P) Pseudo-color image corresponding to OTD staining in panel M. In A-C, E-G, I-K and M-P anterior is up. Scale bars: A,B; E,F; I,J (shown in A upper left): 50 μm; C,G,K and M-P (shown in M upper left): 50 μm.

ments show that throughout the third instar larval stage, OTD staining in the ocellar region is not uniform. In an early third instar disc, the cells in the medial region of the ocellar domain show stronger staining than cells in more lateral regions (Fig. 3A-D). This graded distribution is also observed in a third instar disc at the ‘flap’ stage (Fig. 3E-H). The inner edge of the flap, which will form the most medial region of the adult head, shows the highest OTD expression. Expression gradually decreases in the direction of the primordia of more lateral structures. This graded distribution is still present in late third instar discs after the flap has unfolded (Fig. 3I-L). In order to directly correlate OTD expression levels and position on the fate map, we stained third instar eye-antennal imaginal discs for both OTD protein and lacZ expression in the ocelli-specific marker line L1. As shown in Fig. 3M-P, the ocelli, which are in the medial head domain, are derived from regions of high
OTD protein expression. Double staining was not performed with the A101 line, since lacZ expression in this strain is present at very low levels.

**ocelliless mutations cause loss of OTD expression in the head primordia**

Next, we examined the effect of *ocelliless* mutations on OTD expression and eye-antennal disc development. During the third instar stage, the normal folding process of the eye-antennal disc is perturbed in *oc^1* discs. In most mutant discs, the flap that would normally form (see above) develops only in the posterior region of the disc, causing a bump to appear in the medial region (see Fig. 5D). This bump is likely caused by aberrant folding of the disc rather than by overproliferation since BrdU staining shows no abnormal cell division in this region (J. Royet, unpublished results). In *oc^1* discs, OTD expression is reduced or absent in the medial region, but is still present in more lateral regions (Fig. 4B). This expression is consistent with the loss of ocelli and the presence of a significant amount of residual frons cuticle in *oc^1* heads. OTD expression in the primordia of the antenna and compound eye is normal in *oc^1* discs. As expected from the more severe adult head phenotype of *oc^an1* flies, OTD protein is almost entirely absent from the head vertex primordia of *oc^an1* discs (Fig. 4C). Only weak staining remains, probably accounting for the very small amount of residual frons cuticle seen in *oc^an1* heads (see Fig. 1D).

**Increasing levels of OTD expression rescue increasingly medial head structures**

The fact that different *otd* allelic combinations affect head development in a graded fashion combined with the existence of an OTD protein concentration gradient suggested that different levels of OTD might be required for the development of different head structures. To test this hypothesis, we induced ubiquitous OTD expression at specific times during the development of *oc^1* larvae using the heat-inducible hsp70 promoter.

hsp70-driven OTD expression was induced at a series of time points separated by 2 hours and covering the first, second and third instar larval stages. We found that almost complete phenotypic rescue of the *oc^1* head phenotype could be achieved only when OTD expression is induced in third instar larvae between 92 and 94 h AEL. The rescue window (which is prior to the onset of lacZ expression in the L1 marker strain) is quite narrow, with heat shocks initiated at 90 or 96 hours of development producing limited or no rescue. Since the head phenotypes produced by *otd* mutations are cold sensitive (not shown), we also tested whether heat pulses alone could induce rescue. No rescuing effect was found in larvae that do not carry the hsp70-otd transgene.

When the ‘rescued’ head phenotypes are examined in detail, it is evident that the restoration of wild-type head structures is a graded process. In particular, longer heat shocks are required to rescue more medial head structures. As described earlier, *oc^1* mutant heads exhibit only a few ridges of frons near the lateral orbital regions. Following a 30 minute heat pulse, the number of frons ridges increases, resulting in a partial or complete restoration of the mediolateral region. In a fraction of these flies, ectopic frons also occupies the medial region of the head (Fig. 5B). In this case, the usual medial head structures (ocelli and associated bristles) are absent and frons cuticle covers the entire region between the compound eyes. Only a 45 minute heat shock produces significant rescue of wild-type mediolateral head structures (Fig. 5C). In these rescued flies, frons cuticle is restricted to the mediolateral domain and the medial region is almost normal in appearance, containing the ocelli and the postvertical, ocellar and interocellar bristles.
Consistent with the idea that different concentrations of OTD protein are required to rescue different subdomains, we observed that the level of OTD expression is clearly correlated with the length of the heat pulse. After a 30 minute heat shock, weak staining can be detected throughout the disc, leaving the endogenous OTD expression pattern still clearly apparent (compare Fig. 5D and E). In contrast, a 45 minute pulse induces significantly higher OTD levels that render endogenous OTD expression more difficult to detect (Fig. 5F). Under these conditions, we see no evidence for the restoration of a mediolateral gradient of OTD expression across the head vertex primordia. The heterogeneity of staining among discs and the abnormal morphology in the ocellar region of $\text{oc}^{-1}/Y$ discs prevent the precise comparison of OTD levels following heat shock with the values found in the normal OTD gradient. However, after a 45 minute heat pulse, OTD expression does not appear to be higher in the mediolateral domain and ectopic frons appears in the medial region of the head (white arrow). The orbital cuticle remains unchanged. (C) Head of a transgenic $\text{oc}^{-1}/Y$; $\text{hsp70-otd}^{+/+}$ fly following a 45 minute heat shock at 94 h AEL. In the medial domain, the postvertical bristles and the two lateral ocelli are now present. The ridges of the frons are restricted to their usual mediolateral location. The median ocellus and orbital bristles are also visible, although at slightly altered positions. This phenotype resembles that seen in $\text{otd}^{+/+}$ flies (see Fig. 1B). (D-F) Eye-antennal discs from $\text{oc}^{-1}/Y$; $\text{hsp70-otd}^{+/+}$ larvae heat shocked for different time periods and stained with OTD antibodies. Third instar larvae were heat shocked for 0 (D), 30 (E) or 45 minutes (F) at 37°C. One hour after the end of the heat pulse, discs were fixed and labeled with OTD antibodies as described in Materials and Methods. The arrow indicates the 'bump' characteristic of $\text{oc}^{-1}$ discs. In A-C, anterior is up. In D-F, anterior is up and medial to the right. Scale bars. A,B (shown in A), 100 μm; C, 25 μm; D-F (shown in F), 50 μm.

Within the rescue window defined (92-94 h AEL), the exact time of OTD induction does not appear to be critical. When single heat pulses of either 30 or 45 minutes are initiated at different times within this period, the phenotypic results are unchanged. This suggests that, rather than there being distinct windows required for rescuing frons or ocelli, it is the level of OTD protein that is important for specifying these two regions. Finally, we examined the easily visible structures within the medial domain in order to quantitate the extent of rescue more precisely. In particular, we scored the rescue of the postvertical bristles (the most lateral structures within the medial domain) and of the lateral ocellar lenses (Table 1). A 15 minute heat shock (at 94 hours of development) produced no increase in the appearance of either of these structures. Following a 30 minute heat pulse, 33% or 3% of the flies have one or two postvertical bristles, respectively (compared to 17% or 0% of heat-shocked control flies). This heat pulse never caused the appearance of ocellar lenses. 45 minutes of OTD induction resulted in the appearance of both lateral ocellar lenses in 16% of the flies scored. The most medial head structures, the ocellar
flies lacking the transgene were heat shocked for 45 minutes, 83% had no postvertical bristles (pvb) and lateral ocellar lenses (oc) observed on the resulting flies. For each time point, at least 50 fly heads were scored. The last row sums to greater than 100%, since the flies in the last column (2pvb+2oc) were also counted in the previous column (2pvb). When control flies lacking the transgene were heat shocked for 45 minutes, 83% had no postvertical bristles, 17% had a single postvertical bristle and no ocellar lenses are produced (not shown).

<table>
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<th>Length of heat pulse (minutes)</th>
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oc1/Y; hsp70-otd/+ larvae were heat shocked for 15 (A), 30 (B), or 45 (C) minutes at 94 h AEL. The effects of the heat pulse were quantitated by counting the postvertical bristles (pvb) and lateral ocellar lenses (oc) observed on the resulting flies. For each time point, at least 50 fly heads were scored. The last row sums to greater than 100%, since the flies in the last column (2pvb+2oc) were also counted in the previous column (2pvb). When control flies lacking the transgene were heat shocked for 45 minutes, 83% had no postvertical bristles, 17% had a single postvertical bristle and no ocellar lenses are produced (not shown).

bristles and medial ocellus, return at still lower frequencies. OTD induction also produced some rescue of the 'flap defect' in third instar ocelliless discs, which varied in its extent in different discs (not shown). These results again indicate that increasing OTD expression is required to rescue progressively medial head structures.

**engrailed** is a candidate *otd* target gene in ocelli formation

In order to identify other molecules that function in patterning the vertex of the head, we studied genes known to be expressed in this region and analyzed their possible interactions with *otd*. Here, we focus on the segment polarity gene *engrailed* (*en*). In the third instar eye-antennal disc, EN protein, in addition to being expressed in the posterior antennal compartment and in the developing compound eye, is present in a distinct patch of cells situated in the medial region of the head primordium (Fig. 6A). This EN expression is not present in early third instar discs (75-85 h AEL) and hence appears well after *otd* expression, which is easily detectable in second instar eye-antennal discs (J. Royet, unpublished results). Using an *en-lacZ* enhancer trap line (Hama et al., 1990), we show that *lacZ* expression, which is easily detectable in second instar eye-antennal discs. In the genital disc, for example, *otd* also appears to be expressed non-uniformly in the medial ocellar cuticle, suggesting that *en* is expressed in the ocelli primordia of the disc (Fig. 6D). This has been confirmed by double staining with the L1 ocelli marker (not shown).

Since this medial head region is lost in *oc1* flies and since *hsp70*-driven *otd* expression can rescue the ocelliless phenotype, we asked whether *otd* might act through *engrailed* to induce ocelli formation. We obtained several results that suggest that this is indeed the case. First, in *oc1* mutant discs, *en* expression is lost or greatly reduced (Fig. 6B). Second, when *otd* expression is induced under the conditions that allow rescue of the ocelliless phenotype (i.e. a 45 minute heat shock within the 92-94 h AEL rescue window), we observe significant restoration of the *en* ocellar spot shortly after the heat pulse in at least 10% of discs (Fig. 6C). Finally, using an *hsp70-en* chromosome to induce EN expression in *oc1* flies, we find that a one hour heat pulse administered at approximately 100 h AEL causes the reappearance of ocelli (Fig. 6E). Heat pulses administered earlier, during the *otd* rescue window, fail to restore ocelli. Taken together, these results strongly suggest that *otd* acts through *en* in ocelli formation. It is interesting to note that other medial head structures (i.e. the ocellar and postvertical bristles) as well as the frons cuticle are not rescued by heat shock-induced EN expression. Since EN expression is only able to restore ocelli, *otd* must act through additional target genes to establish other head structures.

**DISCUSSION**

In previous work, it was shown that the *otd* homeobox gene is required for the correct specification of medial cell fates during both embryonic and imaginal development. Here, we show that specific threshold levels of *otd* expression are in fact necessary to establish different subdomains along the mediolateral axis of the adult head. By gradually decreasing *otd* dosage genetically, we demonstrate that medial structures are lost first, followed by the graded loss of increasingly lateral regions. Conversely, when *otd* levels are progressively increased in an *ocelliless* mutant background, there is a graded restoration of increasingly medial head structures.

The rescue experiments, depicted schematically in Fig. 7, also indicate that different threshold levels of OTD protein are required to establish the three domains of the dorsal head. The medial region, for example, appears capable of assuming three alternative fates. In *oc1* mutant flies, the medial domain is occupied by cuticle that lacks medial or mediolateral head structures (e.g. the ocelli and frons respectively). We believe that this region, in the absence of normal OTD expression, has become transformed to a more lateral (i.e. orbital) fate. Following 30 minutes of OTD induction, the medial domain is capable of assuming a second fate, characterized by the ectopic appearance of frons cuticle. After 45 minutes of induction, the medial region assumes a third phenotype, characterized by normal medial head structures.

Consistent with these results, we show that OTD protein is expressed in a concentration gradient across the ocellar region of the eye-antennal disc. In particular, increasingly medial regions of the disc express gradually increasing levels of OTD. Although we have focused here on the eye-antennal disc, it is also possible that *otd* functions in a graded fashion in other imaginal discs. In the genital disc, for example, OTD protein also appears to be expressed non-uniformly in the medial region and *otd* mitotic clones produce apparent changes in cell fate (Wieschaus et al., 1992).

The results described can be explained by three possible models. In the first, different OTD levels would be required simply for cell proliferation or cell viability in each subdomain of the developing head. In the absence of sufficient OTD expression, precursor cells for that region would either die or fail to proliferate. We think that this interpretation is unlikely for several reasons. First, acridine orange and BrdU staining reveal no evidence of significant cell death or cell overproliferation in the ocellar region of *oc1* and *oc3a1* eye-antennal discs (J. Royet, unpublished results). In addition, although *oc3a1* discs express almost no *otd* protein throughout the head capsule primordium, the heads of *oc3a1* flies are not reduced in size along the mediolateral axis. This is consistent with previous analysis showing that *otd* mutant clones in the medial head region are normal in size, but lack all medial head structures, suggesting a change in cell fate rather than the loss of cells (Wieschaus et al., 1992).
In the second possible model, OTD levels would be both necessary and sufficient to determine the fates of cells in each subdomain of the head. We believe that this is unlikely, since ubiquitous, high level OTD expression does not produce medial structures across the head vertex. Therefore, we believe that OTD concentration, although important, is not sufficient to fully specify regional head identities.

Instead, we favor a third model in which additional positional signals, in conjunction with *otd*, are required to define specific subdomains of the head. This requirement would cause a restriction of the developmental potential of precursor cells within each subdomain. For example, the mediolateral (frons) region may not be capable of being transformed to a medial fate because of the absence of an additional medial cue. As a result, ectopic OTD (or EN) expression does not produce ectopic medial cuticle. Consistent with this idea, specific segment polarity genes are expressed in restricted patterns along the mediolateral axis of the head primordia (J. Royet, unpublished results). *hedgehog*, for example, is specifically expressed in the medial region beginning at very early stages of eye-antennal disc development. We are currently testing whether *hedgehog* or other segment polarity genes act, along with *otd*, to define the identities of these subdomains.

Finally, we have begun an analysis of the pathway of *otd* function in adult head development. We provide evidence that, in the medial subdomain of the head, *engrailed* acts as an *otd* target gene. *EN* protein expression in this region appears well after OTD expression and is dependent upon it. Heat shock-induced OTD expression, which rescues the *ocelliless* phenotype, restores medial *EN* expression soon after the heat pulse. Finally, the induction of *EN* is also capable of restoring ocelli in the same mutant background, although at a later time.

Fig. 6. *engrailed* is a candidate *otd* target gene in ocelli formation. (A-C) Third instar eye-antennal discs (98 h AEL) labeled with antibodies to *engrailed*. Arrows indicate the ocellar region. (A) Wild-type disc. *EN* protein is expressed in the primordium of the ocelli (see text). (B) *oc* disc. *EN* expression in the ocellar region is absent. (C) *oc*; *hsp70-otd* disc. Induction of OTD partially restores *engrailed* expression. Eye-antennal discs from *oc*; *hsp70-otd* larvae were heat-shocked for 45 minutes at 93 h AEL to induce OTD expression, fixed 5 hours later and stained with antibodies to *en*. (D) Adult head from an *en-lacZ* enhancer trap line stained with X-gal, showing *lacZ* expression in the ocellar cuticle. (E) Adult head derived from an *oc*; *hsp70-en* larva heat-shocked for one hour at 100 h AEL to induce *engrailed* expression. In this head, a single ocellus is restored (arrow). In all panels anterior is up. For A-C, medial is on the right. Scale bars, A-C (shown in A), 50 μm; D,E, 50 μm.
window of disc development. en does not appear to completely mediate otd function, since it rescues ocelli, but not other structures within the medial subdomain. Although the verification of the role of en in this region will require the analysis of en mutant clones, our results strongly suggest that the en gene acts downstream of otd in head formation.

It is interesting to note that regulation of en by otd also occurs during embryonic head development (Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990). In otd mutant embryos, the expression of both en and wingless is lost in specific segments of the anterior head. It has been hypothesized that, in this region, these segment polarity genes are in fact direct targets of the head gap genes otd, empty spiracles and button head (reviewed in Cohen and Jurgens, 1991; Finkelstein and Perrimon, 1991). It will be important to determine if such a mechanism might also apply to the development of the vertebrate brain, where expression of the Engrailed1 gene is contained within the larger domains of expression of the OTD-related genes Otx1 and Otx2 (Davidson et al., 1988; Davis and Joyner, 1988; Simeone et al., 1992). Defining the molecular genetic hierarchy that governs head formation in Drosophila is likely to result in more general insights about pattern formation during development.

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REFERENCES

Jurgens, G. and Hartenstein, V. (1993). The terminal regions of the body


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