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orthodenticle activity is required for the development of medial structures in the larval and adult epidermis of *Drosophila*

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Summary

Lethal alleles of *orthodenticle* (=*otd*) cause abnormalities in the embryonic head that reflect an early role in anterior pattern formation. In addition, *otd* activity is required for the development of the larval and adult epidermis. Clonal analysis of both viable and lethal alleles shows that the adult requirement for *otd* is restricted to medial regions of certain discs. When *otd* activity is reduced or removed, some medial precursor cells produce bristles and cuticle characteristic of more lateral structures. Similar medial defects are observed in the larval epidermis of embryos homozygous for lethal *otd* alleles. Antibodies to *otd* recognize a nuclear protein found at high levels in the medial region of the eye antennal discs, the leg discs, the genital discs and along the ventral midline of the ventral epidermis of the embryo. These results suggest that the *otd* gene product is required to specify medial cell fates in both the larval and adult epidermis.

Key words: *Drosophila* gap gene, homeodomain, imaginal disc.

Introduction

Anterior-posterior patterning in the *Drosophila* embryo is controlled by a hierarchy of transcription factors that define increasingly smaller spatial domains within the final pattern (Nüsslein-Volhard and Wieschaus, 1980; Ingham, 1988). In one of the first steps of this hierarchy, the zygotic genome responds to the level of maternally supplied *bicoid* protein by establishing broad stripes of "gap" gene expression. Each gap gene is expressed in a defined region of the blastoderm and its product is thought to direct subsequent expression of pair-rule genes, segment polarity genes and homeotic genes in that region. In addition to their early role in anterior-posterior patterning, many gap genes are expressed in defined spatial patterns after the blastoderm stage (e. g., *Krüppel*, Gaul et al., 1987, Gaul and Weigel, 1991; *hunchback*, Tautz et al., 1987; *tailless*, Pignoni et al., 1990). The significance of these later, more complex expression patterns and their relationship to the role of the genes during early segmentation is not well understood.

In this paper, we focus on the putative gap gene *orthodenticle* (=*otd*). In homozygous embryos, lethal *otd* alleles cause pattern deletions in the antennal and preantennal regions of the head (Wieschaus et al., 1984; Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990). In mutant *otd* embryos, precursor cells in these regions no longer express genes characteristic of those segments, suggesting that *otd* is required for their proper developmental programming (Cohen and Jurgens 1990, Finkelstein and Perrimon, 1990). At the blastoderm stage, *otd* is expressed in a circumferential stripe of cells at the anterior end of the egg (Finkelstein et al., 1990). This early domain of expression includes the precursors for the antennal and preantennal regions affected in mutant embryos. Sequence analysis indicates that *otd* encodes a homeodomain-containing protein and thus probably functions as a transcription factor during early development (Finkelstein et al., 1990). Based on these results, it has been argued that *orthodenticle* functions like a gap gene in defining antennal and preantennal segments in the head.

In addition to its early role as a putative gap gene in the anterior region of the embryo, *otd* is required at later times in development. Homozygous *otd* embryos show abnormalities in the specification of medial cells in the central nervous system (Finkelstein et al., 1990; Klämbt et al., 1991). Moreover, in the ventral epidermis of the larva, the denticle bands that mark the anterior margin of each embryonic segment are reduced in size and in the types of pattern elements that they contain. *orthodenticle* is also required for the development of imaginal discs. Flies homozygous for *ocelliless* (= *oc*) mutations survive to adult stages but show deletions of sense organs in the adult head. Because *oc* alleles fail to complement lethal *otd* mutations, the ocellar defects are assumed to reflect a requirement for *otd* during development of the eye-antennal disc. However, the two *oc* mutations result in molecular alterations outside the known coding region of the gene (Finkelstein et al., 1990), and thus it is not clear how

these adult defects relate to the requirements for *otd* gene activity.

To study the *otd* requirement during postblastoderm stages, we have examined the pattern defects of *oc* homozygous adults in greater detail. We have also made mosaics to analyze the effects of lethal *otd* alleles in developmental stages after embryogenesis and to determine the autonomy of the mutant phenotype. By comparing the regional specificity detected in this mosaic analysis to the distribution of *otd* protein in discs, we postulate a general role for *otd* in the development of ventral medial structures in adult epidermis. The requirement for *otd* activity in imaginal discs was then compared to the requirement in the postblastoderm embryo, using phenotypic analysis of homozygous embryos, antibody staining and mosaic analysis. We argue that, in addition to its function in head specification, *otd* also plays a central role in the formation of medial structures in the embryonic epidermis. This requirement may be directly analogous to its role in later disc development and may also be related to its role in patterning of the embryonic central nervous system.

Materials and methods

Fly stocks and genetics

The original allele of *ocelliless* (oc^1) is associated with *In(1)oc*, a small inversion with break points in 7F1-2 and 8A1-2. As a homozygote, it is viable in males and females, but causes female sterility and defects in the head capsule. The sterility is due to the 7F break point which disrupts a cluster of chorion-specific genes in that region (Spradling and Mahowald, 1981). The head defects are due to the 8A break point and are uncovered by deficiencies of that region. A similar head phenotype is produced in males hemizgous for $oc^{\gamma a1}$, a translocation allele with an X-chromosomal break point also in 8A1 (Wakimoto, personal communication, Finkelstein et al., 1990). *trans*-heterozygotes of *oc* and any of the three lethal *otd* alleles are fertile females, but show the head defects characteristic of *oc*. The two lethal *otd* alleles used in this study (otd^{XC86}, otd^{YH13}) are ethylmethane sulfonate induced and their isolation is described in Wieschaus et al., (1984). Both show strong embryonic phenotypes close to that produced by deletions of the region, although the phenotype of otd^{XC86} is somewhat more variable. Meiotic mapping experiments indicate that both otd^{XC86} and otd^{YH13} lie about 0.11-0.14 map units proximal to the *oc* break point (Wieschaus, unpublished observations). Other mutations and balancers used in these studies are described in Lindsley and Grell (1968) or Lindsley and Zimm (1986).

Production of genetic mosaics using mitotic recombination

Clones homozygous for *oc* or *otd* were produced in the imaginal discs by irradiating female larvae of the following genotypes: *y w* oc^1 f^{36a}/+, *y w* oc^1 f^{36}/*M(1)*o^{sp}, *y w* otd^{XC86} f^{36}/*M(1)*o^{sp} or *y w* otd^{YH13} f^{36}/*M(1)*o^{sp}. Embryos from the appropriate crosses were collected on Petri dishes containing apple juice agar egg-laying medium and allowed to develop to the appropriate age. They were irradiated as young larvae (40 hours ± 12 hours after ovoposition, approximately 1.0 krad, Gammator Model B). After the irradiation, they were transferred to bottles containing standard fly food and allowed to develop to adult stages. Flies of the appropriate genotypes were collected and temporarily stored in 1:4 glycerin/ethanol. The flies were then boiled briefly in 10% potassium hydroxide, washed in water and mounted on slides in Faure's medium. Flies were scored for the presence of bristles marked with *yellow* and $forked^{36a}$. The positions of clones were recorded on diagrams of the different regions of the body. These procedures do not allow accurate description of clones in regions not carrying bristles. Although *oc* and *otd* clones were also homozygous for *white*, the eye and ocellar pigments are destroyed by the preparation procedures. It was therefore not possible to use that marker to identify clones in the ommatidia or ocelli.

Production and analysis of otd *gynandromorphs*

To produce *otd* gynandromorphs, *R(1)*w^{vC} males (Hinton, 1955) were mated to females heterozygous for a marked *otd* chromosome (*y w* otd^{YH13} f^{36a} or *y w* otd^{XC86} f^{36a}) and an X-chromosome balancer (*FM7c*). Loss of the ring-X in the *R(1)*w^{vC}/*y w* otd^{YH13} f^{36a} or *R(1)*w^{vC}/*y w* otd^{XC86} f^{36a} progeny results in large clones of marked *otd* cells in an otherwise heterozygous individual. *otd* gynandromorphs that survived to adult stages were counted and stored in 1:3 glycerin/ethanol. They were mounted in Faure's medium, following the procedures described above for the mitotic recombination experiments. The number of gynandromorphs mounted on the slides (Table 4) differs slightly from the numbers recorded when the progeny of each cross was counted (Table 3). The difference most likely reflects counting errors made in the initial progeny counts. In each experiment, the surviving FM7c gynandromorphs served as a control for ring-X loss and recovery of the *otd* mosaics. Cuticle preparations of embryos derived from the same cross were prepared in Hoyer's medium for microscopic examination, following the procedures described in Wieschaus and Nüsslein-Volhard (1986).

Production of antibodies to otd

To generate antibodies to the *otd* protein a *Hin*cII-*Hin*cII restriction fragment (extending from nucleotides 983 to 2329 of the *otd* cDNA described in Finkelstein et al., 1990) was cloned into a T7 expression vector (pAR 3038; Rosenberg et al., 1987). The resulting plasmid was transformed into *E. coli* strain BL21(DE3) which was grown and induced with IPTG according to standard procedures (Sambrook et al., 1989). Bacterial *otd* protein was isolated by SDS-PAGE, the gel band resuspended in Freund's adjuvent and injected into Long-Evans rats. The immunization regimen consisted of an initial injection followed by two boosts at four weeks and six weeks, respectively. The resulting sera were preabsorbed against fixed *Drosophila* embryos and used at a final dilution of 1:250 or 1:500.

Morphology, antibody staining and in situ hybridizations

Embryonic cuticles were prepared as in Wieschaus and Nüssein-Volhard (1986), as modified by Struhl (1989). Embryos were stained with *otd* antibody according to the protocol of Patel et al. (1989) and imaginal discs according to the protocol of Carroll and White (1989). In some experiments, the *otd* antiserum was preabsorbed against old embryos (which express very low levels of *otd*) or imaginal discs. Neither preabsorption procedure affected the staining patterns observed in embryos or discs. In most experiments, preabsorption to embryos was used. Antibody staining was visualized using the Vecta-stain ABC kit (Vector Labs), with the ad-dition of 0.04% nickel chloride. In situ hybridizations to imaginal discs were done using intact discs, following the protocol of Tautz and Pfeifle (1989), as modified by Phillips et al. (1990).

Results

ocelliless *homozygotes show autonomous pattern transformations restricted to a small region of the eye-antennal disc*

The *Drosophila* ocelli are three simple light-sensitive lenses

located on the dorsal midline at the top of the adult head (see Bryant, 1978 for review of adult cuticular morphology). The ocellar region is formed by the fusion of the right and left eye-discs and the most medial of the three ocelli is formed by contributions from both discs. The ocelli are associated with a fixed pattern of four large macrochaetes (the ocellar and the postvertical bristles) and 8-10 smaller microchaetes (Fig. 1A). The cuticle immediately surrounding the ocelli is contiguous with the occipital region on the back of the head. Immediately lateral to the ocelli, the head cuticle forms the frons (=F) and shows the fine ridges and medial orientation of hairs characteristic of that structure. A second cluster of bristles (the orbital bristles=OR) separates the frons laterally from the compound eye. The ocelli, frons and occipital region all lie on the right-left midline of the head and will be referred to as medial structures.

Females homozygous for the original *ocelliless* allele survive at high frequency to adult stages, but show obvious abnormalities in the ocellar region of the head capsule (Fig. 1B). Slightly stronger defects are observed in individuals *trans*-heterozygous for *oc* alleles and small deletions of the region, or in *trans*-heterozygotes for *oc* and lethal alleles of *otd*. Although *otd* alleles are generally recessive over a wild-type chromosome, they become partially dominant in individuals heterozygous for *Minute* mutations. The defects range from the loss of occasional ocellar bristles in *oc*/*M(1)o*^{sp} individuals to large-scale pattern deletions in *otd/M(1)o*^{sp} heterozygotes. The partial dominance of *otd* phenotypes suggests that the ocellar region is sensitive to the dosage of *otd*. The stronger phenotypes of *otd* in all these combinations indicate that *oc*, while allelic to *otd*, may retain partial *otd* function in the eye disc.

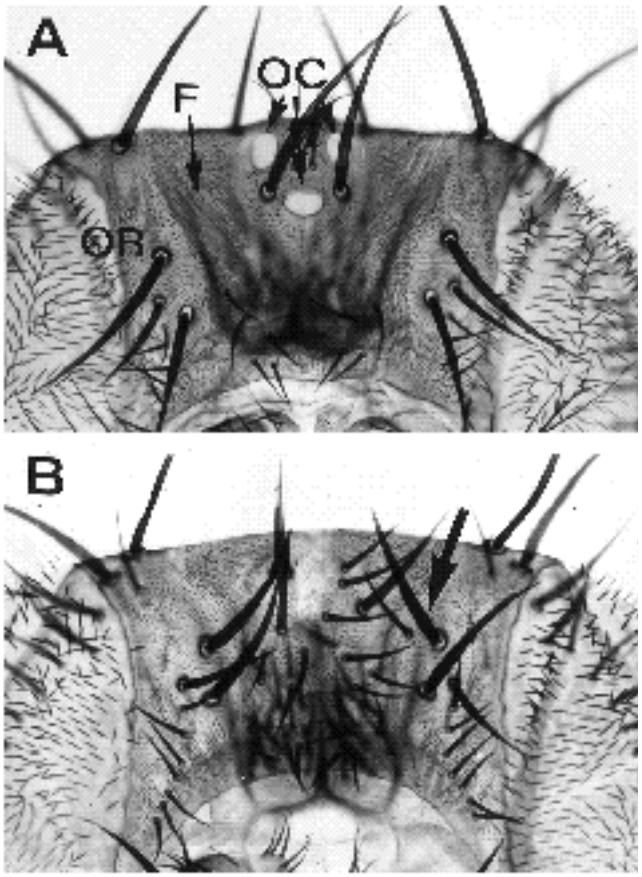

Fig. 1. The *ocelliless* phenotype in the cuticle of the adult head. (A) In the medial region of a normal (oc^1/+) adult head, the three ocelli (OC) and adjacent ocellar bristles are flanked by the ridged frons cuticle (F). Lateral to the frons are the orbital bristles (OR) of the eye. (B) In an $oc^1/oc^{\gamma a1}$ adult, the ocelli and much of the adjacent cuticle is missing. The frons region lacks its typical ridged morphology and shows additional ectopic bristles. A comparison with the wild-type head (A) indicates that the orbital region is also somewhat expanded toward the midline (arrow).

The pattern abnormalities in *oc* individuals are restricted to the medial regions of the head. In the weakest *oc* phenotypes, the ocellar region is smaller and the right and left extensions of the frons meet at the dorsal midline. In slightly stronger phenotypes, the ocellar abnormalities are associated with reductions in the adjacent occipital region. In the strongest phenotypes, the frons-type cuticle immediately lateral to the ocellus is also missing. In all moderate to strong phenotypes, deletion of medial elements is associated with the formation of ectopic bristles. These bristles have a size and pattern similar to those found in the orbital region and suggest that the pattern deletions may be associated with a transformation of some medial cell identities to more lateral fates. In addition, lateral regions of *oc/otd* individuals, although normal in pattern, are somewhat expanded in size. For example, the distance from the dorsal-most orbital macrochaete and the margin of the compound eye is 30% greater than the comparable distance in wild type.

To determine how these pattern alterations arise, we used mitotic recombination to produce clones homozygous for *oc* cells in otherwise (*oc*/+) normal heads. In most regions of the head, the clones were detected with normal frequencies and showed normal morphology. The only regions to show any deviation from normal morphology were the ocellar and occipital regions and the frons (Table 1). In 9 of the 191 heads, the ocelli and the associated bristle region were deleted. The frequency is similar to the frequency of *yellow forked* clones observed in that region in the non-*ocelliless* controls, supporting the view that the defects in *oc*/+ heterozygotes are associated with homozygosity for *oc*. Although the markers and preparation procedures used in this experiment do not mark defective ocellar regions which lack bristles, in 6 of the 9 cases the defects were associated with *y f* clones in immediately adjacent regions. This association is highly significant, given the overall low frequency of clones in these regions (13/191, Table 1). Similar defects were observed when *oc* homozygous clones were produced in a *Minute* heterozygous background (Table 1, Fig. 2). Here again the defects were frequently associated with *y f* bristles in structures normally adjacent to the ocellus.

In these experiments, we never observed ocellar bristles that were homozygous for *y f* and thus for *oc*. The failure to detect such clones suggests that the requirement for *oc* activity in the ocellar region is autonomous. Alternatively, it might be argued that the absence of such clones reflected a *non-autonomous* effect of mutant cells in a nearby region, cells that because of their proximity and the large clone size

Table 1. *Homozygous* oc/otd *clones in eye disc derivatives*

Genotype	Number of heads examined	Number of clones found	*y f* clones in			Defects in		
			OC	dFR	OR	OC	dFR	OR
y w oc^{1} f^{36a}/+	191	45	0	8	6	9	5	0
y w f^{36a}/+	243	47	9	3	12	0	0	0
y w otd^{YH13} f^{36}/M	113	59	0	6	6	n.d.	7	0
y w f^{36a}/M	116	58	19	12	9	0	0	0
y w oc^{1} f^{36}/M	200	71	0	10	15	9	9	0

Abbreviations: OC, ocellar region; dFR, dorsal frons; OR, orbital region; *M, M(1)o^{sp}*.

n.d. = defects in the ocellar region of *Minute* heterozygotes were not determined due to the strong dominant enhancing effect of the *Minute* mutation (see text).

are always mutant when the ocellar bristles are mutant. To investigate that possibility, we examined individuals that had been irradiated late in larval development (at 94 hours after ovoposition). Such irradiation induces clones that are very small. More than half such clones mark only a single bristle. None of the 164 clones detected in these experiments, however, were recovered in the ocellar region, although other regions of the head were marked by these clones very frequently (15 to 25 times, depending on the region examined). The failure to detect even small late clones in the ocellar region confirms the autonomous requirement for *oc* in the ocellar region and indicates that this requirement continues until late in development.

The only other regions of the head affected by *oc* homozygous clones were the frons and the occipital cuticle adjacent to the ocellus on the back of the head. Clones in the frons showed large clusters of ectopic bristles resembling the orbital-type bristles described above for the *oc* homozygotes. (Fig. 2A,B). Based on their location, we conclude that clones giving rise to these bristles arose in precursors for the frons, but that the homozygous cells were shifted in their fates, presumably to form structures of more lateral character (see Discussion). This transformation is strictly autonomous and the adjacent wild-type cells were never observed to participate in these ectopic structures. Moreover, the presence of a clone in the frons had no obvious nonautonomous effect on patterning in other regions of the head.

Imaginal requirements for ocelliless/orthodenticle *activity are restricted to the derivatives of the eye-antennal and genital discs*

Outside the head, the morphology of *oc* homozygous adults is generally normal and *oc^{1}/Df* females are fully viable and fertile. Because lethal *otd* alleles cause death in homozygous embryos, the requirement for *otd* product may be broader than is indicated by the *oc* adult phenotype. To test whether elimination of *otd* product would have more drastic effects on disc development, young larvae heterozygous for lethal *otd* alleles were irradiated to produce marked clones homozygous for the mutation. The *otd* chromosomes were marked with *yellow* and *forked^{36a}* and the clones induced in a *Minute* background to increase their size and to give them a growth advantage over the neighboring heterozygous cells in the same disc. Homozygous *otd* clones were recovered at control frequencies in all discs, indicating that the wild-type gene is not required for cell viability during imaginal growth (Table 2). In the thorax and abdomen, the clones were normal in morphology, respected the appropriate compartment boundaries and were of a size comparable to the controls. In the head, *otd* clones behaved similarly to those observed in experiments using *oc$^{1.}$* Because of the enhancing effect of *Minute* on the *otd* phenotype, the ocellar regions of most of the heterozygous heads were missing bristles or showed other moderate defects. Individuals with *otd* clones in those regions (Fig. 2C,D) could still be recognized because of the large clusters of *y f* ectopic bristles similar in morphology to those observed in experiments using the original *oc* allele.

In an earlier description of *oc^{1}* (Beatty, 1949), a fraction of the homozygotes were found to lack parovaria, internal genital structures derived from the medial region of the genital disc. In our stocks, this defect is somewhat variable, with about half of the *oc^{1}/oc^{ya1}* females (6/12) lacking parovaria, and others showing defects in the size or number of parovaria. More striking abnormalities were observed when homozygous *otd* clones were induced in the genitalia. All clones in the female vaginal plates were abnormal. Most frequently the right and left plates were fused along the midline resulting in no vaginal opening. These defects were associated with missing internal genital structures such as spermathecae. The missing structures, like the parovarian defects described for *oc* homozygotes, represent deletions in the medial regions of the genital disc. In these cases, and in less defective cases as well, the rows of thorn-like bristles on the adjacent vaginal plates were duplicated (Fig. 2E,F), suggesting that loss of *otd* may be associated with an expansion of lateral cell fates.

In the following section, we show that *otd* is expressed in leg discs, in a region that gives rise to adult structures on the ventral midline. However, no defects were observed in *otd* clones induced in these regions. While there are no bristles situated on the midline of the adult, we did observe numerous examples of marked *otd* bristles immediately adjacent to the midline (i. e. , the sternomedials and posterior medials of the second leg, the BH region of the first leg). In all cases, these and the adjacent cuticle were morphologically normal with no obvious signs of pattern deletions or duplications. The normal development of clones in the leg suggests that any requirement for *otd* in the leg discs is very subtle (see Discussion).

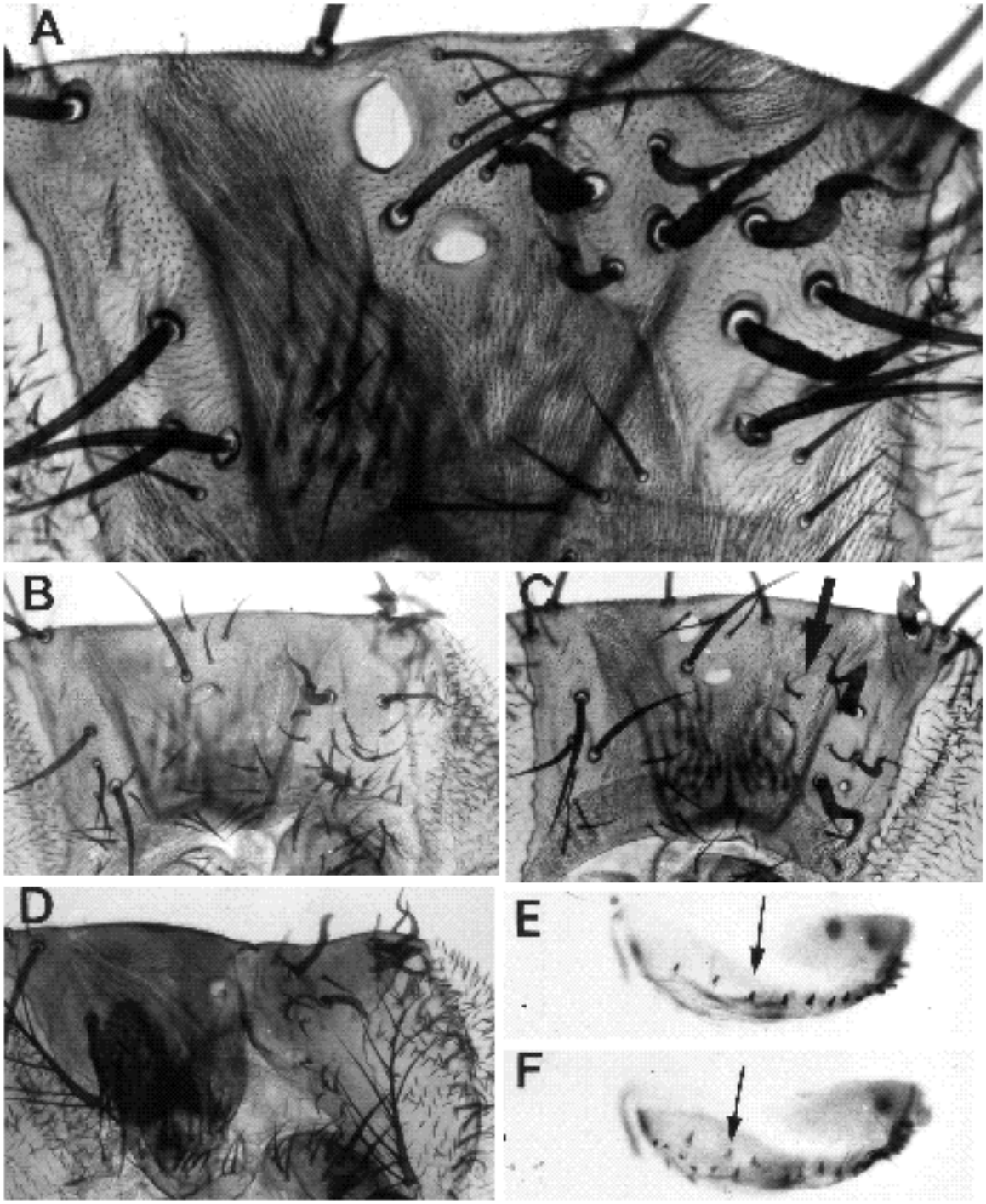

Fig. 2. Clones homozygous for the *oc* and *otd* are associated with defects in medial pattern elements. (A) A *y* oc^1 *f* clone in the right orbital region of an oc^1/M heterozygote is associated with a loss of the ocelli and frons on that side of the head. Ectopic *y (oc) f* bristles in the region normally occupied by the frons are continuous with the orbital bristles and similar to them in morphology. (B) A smaller *y oc f* clone showing expansion of the orbital region. (C) A clone homozygous for otd^{XC86} causing a small patch of ectopic bristles in the frons (arrow). (D) A clone homozygous for otd^{YH13}, showing a total deletion of the ocellar region and frons as well as the formation of large ectopic bristles in their place. Note the strong dominant effect of heterozygosity for the *Minute* mutation on the side of the head without the clone. (E) Normal vaginal plates carry only a single row of thorn-like bristles (arrow). (F) Induction of a *y* otd^{YH13} clone in the vaginal plate primordia is associated with an expansion of the row of thorn bristles (arrow). This clone was also associated with a deletion of internal genital structures and vaginal ducts.

Table 2. *Recovery of* otd *clones in adult derivatives of various imaginal discs*

Genotype	Eye	Labial	Humerus	Wing	Leg	Tergite 1-7	Sternite 2-7	Genitalia	Analia
Control	58	4	9	33	80	126	25	7	8
(N=116)	(25)	(2)	(4)	(18)	(11)	(8)	(2)	(6)	(7)
otd^{YHI3}	59	7	8	28	103	130	44	12	6
(N=115)	(25)	(3)	(4)	(14)	(15)	(8)	(3)	(11)	(5)
otd^{XC86}	30	5	10	14	78	100	28	7	7
(N=100)	(15)	(3)	(5)	(7)	(13)	(7)	(2)	(7)	(7)

Values in parentheses are the frequencies of clones per 100 discs. N = Number of adults examined. Control genotype = *y* w^A f^{36}/*M(1)*o^{sp}; otd^{YH13} genotype = *y w* otd^{YH13} f^{36}/*M(1)*o^{sp}; otd^{XC86} = *y w* otd^{XC86} f^{36}/*M(1)*o^{sp}.

otd *protein is expressed in ventral medial epidermis of the embryo, as well in medial regions of the eye antennal disc, the leg disc and the genital disc*

To examine the distribution of *otd* gene product in discs, we generated rat polyclonal antiserum to *otd* protein (Material and methods). The resultant serum appears to be specific for *otd* protein. When used to stain embryos, it shows a pattern similar to that previously published for *otd* RNA (Finkelstein et al., 1990, Fig. 3A,B) and does not stain embryos hemizygous for a small deficiency of the region (otd^{JA101}, Fig. 3C). *otd* protein is localized to the nucleus, as is expected for a putative transcription factor. At the blastoderm stage, *ortho - denticle* is expressed in a broad band in the anterior region of the embryo (Finkelstein et al., 1990; Finkelstein and Perrimon, 1990; Fig. 3A). At later stages of embryonic development, *otd* protein is also observed along the ventral midline (Fig. 3B). Earlier descriptions of this late expression pattern focused on *otd* expression in the nervous system (Finkelstein et al., 1990). Our *otd* antibody indicates that this ventral staining also includes the cells of the ventral epidermis. A stripe of ectoderm 3-4 cells wide on either side of the ventral midline shows staining that persists through germband shortening and early stages of dorsal closure.

When applied to eye antennal discs, the antiserum identified an antigen localized in a broad band on the medial side of the eye disc, extending into the antennal primordia on its medial side (Fig. 3D). Within the eye disc, the staining includes the region that gives rise to the ocellus and frons (Haynie and Bryant, 1988). Staining was observed in the medial region of both the male and female genital discs (Fig. 3E,F). The right and left genital discs are fused into a single structure situated on the ventral midline. The medial region of the fused disc gives rise to internal genital structures and is flanked laterally by the primordia for the external vaginal and anal plates. The levels of high expression thus correspond to the genital region shown by our clonal analysis to be affected by loss of *otd* activity. In addition, as mentioned above, nuclear staining was observed in the leg disc epithelium (Fig. 3G,I). The staining was restricted to a crescent of cells adjacent to the peripodial membrane in the medial posterior quadrant of the disc and extended into the nuclei of the adjacent peripodial membrane. The position of this staining is analogous to the medial staining observed in the eye-antennal disc and the genital disc.

The patterns of *otd* expression in the medial regions of the eye, leg and genital discs described above were confirmed using probes for *otd* RNA (data not shown). Strong staining was also observed in differentiating photoreceptor cells in the posterior region of the eye (Fig. 3D), in the large nuclei that underlie them, and in nuclei distributed along the nerve cord that extends from the center of each leg disc to the central nervous system (Fig. 3H). The specificity of this staining is difficult to evaluate. Following in situ hybridization of *otd* DNA probes to imaginal discs, we do see weak staining in the ommatidial region, but none in the nerve cord of the leg disc. The failure to detect quantitatively comparable *otd* RNA expression in either disc might indicate that the photoreceptor and nerve cord staining is not specific for *otd*, or it might reflect an accessibility problem in our in situ assay. Because our preparation procedures do not allow detection of *white otd* clones in the ommatidia (Material and methods), our clonal analysis would not have detected a requirement in the photoreceptor cells. Finally we also detect low level staining in discrete regions of the wing and haltere discs. This staining is significantly weaker and not detected with probes for *otd* RNA. It may therefore reflect cross-reactivity of our antisera.

Postblastoderm requirements for otd *in the embryonic epidermis*

The observation that *otd* is expressed in the ventral epidermis encouraged us to re-examine of the cuticle phenotype observed in late *otd* embryos. The phenotype of *otd* homozygous embryos is most obvious in the head region and presumably reflects the role of the gene during early segmentation. *otd* embryos also show abnormalities in the denticle pattern of the anterior abdomen. In wild-type embryos, the first and fourth denticle rows provide the most distinctive morphological landmarks within the denticle band itself (Fig. 4A). They are the only denticles that point anteriorly and their location along the anterior-posterior axis marks the insertion of the ventral medial musculature (Szabad et al., 1978). In the anterior region of the abdomen, these rows do not extend through the entire width of the belt, and thus they provide landmarks for both medial-lateral as well as anterior-posterior positional values. In the anterior abdomen of *otd* homozygotes, the first and fourth denticle rows are deleted and, as a consequence, all the remaining denticles point in the same posterior direction, hence the name “orthodenticle” (Fig. 4B). The abnormalities in belt morphology are not, however, restricted to the first and fourth rows. The entire pattern at the ventral midline is distorted and the normal attachment sites of the ventral musculature are gone (N. P.,

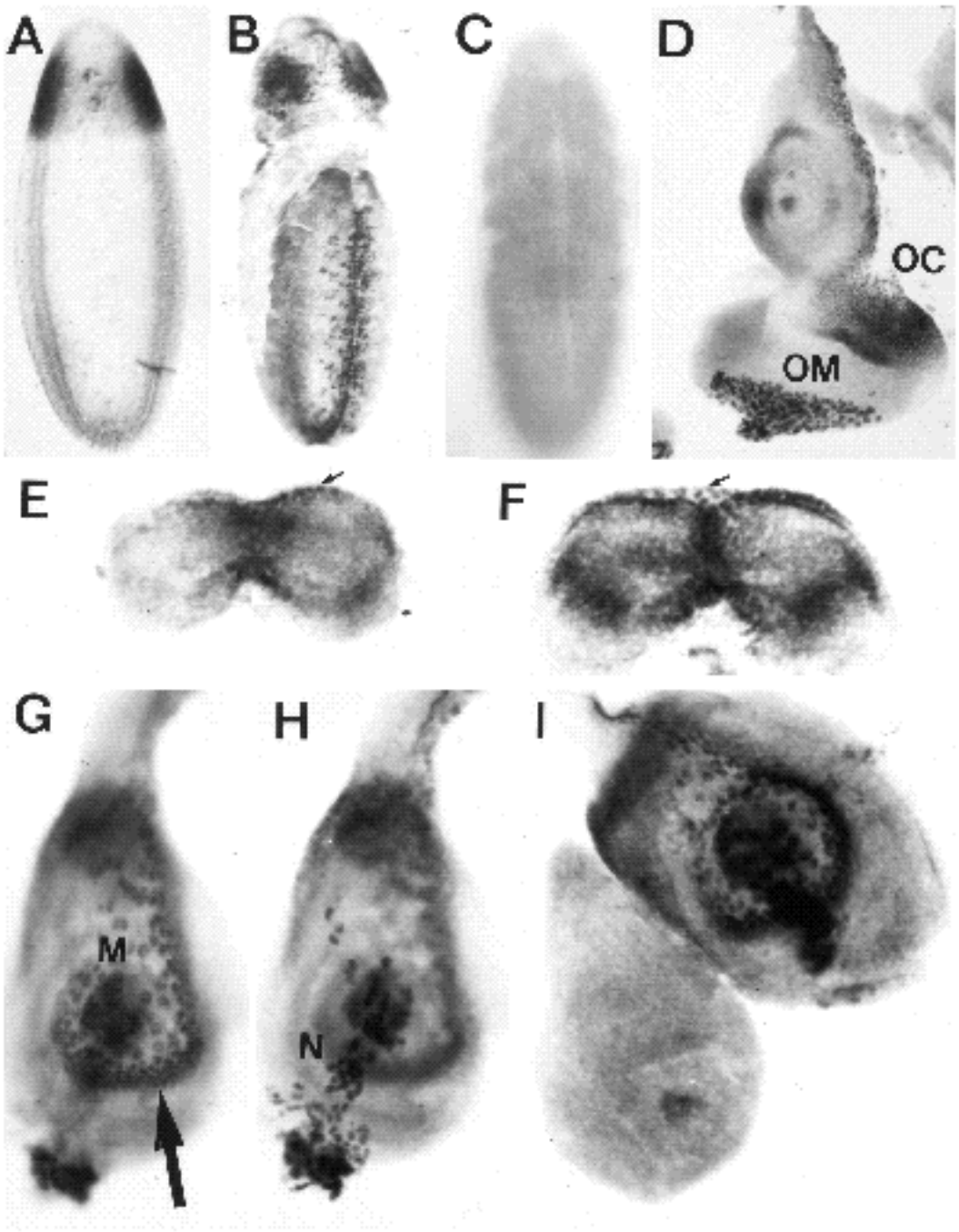

Fig. 3. Antisera to *otd* recognizes a nuclear antigen expressed in a defined spatial pattern in embryos and in imaginal discs. (A) *otd* antibody staining in a cellular blastoderm embryo shows localization of *otd* protein to a band in the head region of the embryo. (B) *otd* antibody staining of extended germband-stage embryos shows accumulation of *otd* protein in a band of epidermal cells along the ventral midline. (C) Embryos hemizygous for *otd* deletions (Df(1)JA101) do not show *otd* staining, confirming the specificity of the antisera for *otd* product. (D) Eye-antennal imaginal disc stained with antibody to *otd* protein. The nuclear staining is restricted to cells of the medial side of the disc and is particularly intense in the region of the head primordia that gives rise to the ocelli and frons (OC). Nuclear staining is also observed in the developing ommatidia (OM). (E) Nuclear localization (arrow) of *otd* protein in the medial region of the female genital disc. (F) Nuclear localization (arrow) of *otd* staining in the male genital disc. Lower (G) and upper (H) views of the second leg disc, showing nuclear staining of *otd* protein in a medial crescent (arrow) of cells adjacent to the peripodial membrane (M) and in the nerve cord (N). (I) Comparative staining patterns in the third led disc and adjacent haltere disc. Note the strongly staining crescent of *otd* localization in the leg disc and the relative lack of staining in the haltere disc.

unpublished observations). The cuticular phenotype is less pronounced in the posterior abdomen, although this may reflect the difficulty in detecting medial deletions in those segments where our medial landmarks (the first and fourth denticles rows) extend into the lateral pattern of the belt (see Discussion).

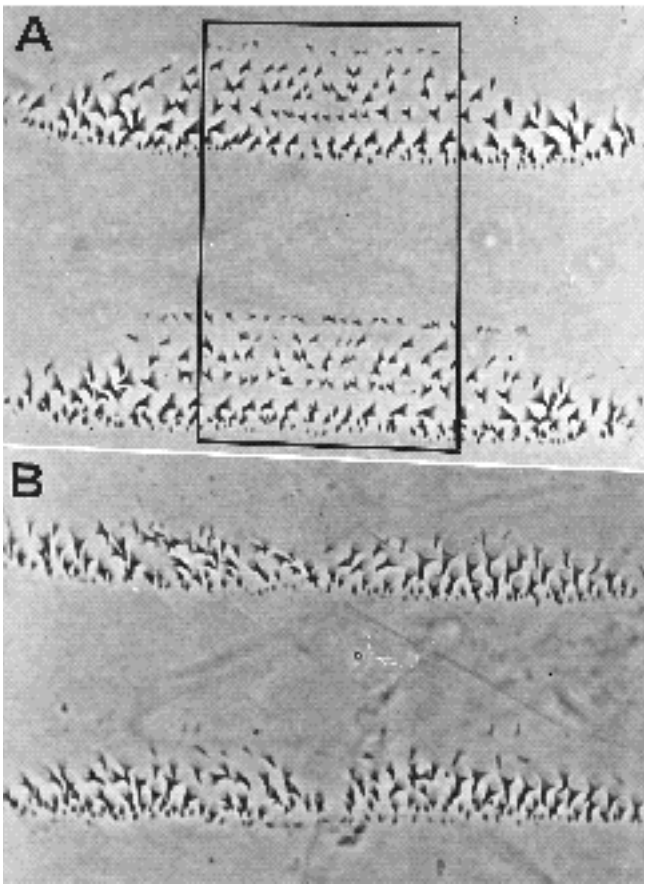

Fig. 4. Comparison of the ventral cuticular pattern of wild type and embryos homozygous for *otd*. (A) Pattern of denticles in the third and fourth denticle bands of a wild-type larva. Note the large size and posterior orientation of the denticles in the lateral region of the belt. The rectangle indicates the medial region of pattern deleted in *otd* mutant embryos. (B) Pattern of ventral denticles in the third and fourth denticle bands in an otd^{YH13} mutant embryo. The pattern consists of denticles with the size and polarity found in the lateral regions of normal denticle belts. Note the absence of the anteriorly pointing first and fourth denticle rows.

In order to study the relationship between *otd* embryonic lethality and the adult defects observed in *ocelliless*, we made gynandromorphic mosaics (Hotta and Benzer, 1972; Hall et al., 1976). To generate mosaic embryos with various distributions of *otd* tissue, we mated ring-X ($R(1)w^{vC}$) males to females heterozygous for *FM7* and one of two different *otd* alleles (XC86 and YH13). The *otd* chromosomes were marked with *y* and f^{36a} and thus any mosaics surviving to the adult stage could be identified based on the presence of these markers in the adult cuticle. The cross also generates *FM7* mosaics. These should arise at the same frequency as *otd* mosaics and serve as an internal control for the frequency of ring-X loss and the viability of the mutants.

Surviving *otd* gynandromorphs were obtained with both alleles tested, but only at 25-37% the frequency found in the controls (Table 3). To determine whether this reduced recovery was due to elimination of mosaics with particular distributions of mutant and wild-type tissue, we examined the surviving adults to determine the frequency with which adult structures were mutant (Table 4). All 196 *otd* mosaics that survived to adult stages had eye-antennal discs which were entirely *otd/+* in genotype, arguing for an absolute autonomous requirement for the wild-type *otd* allele in the precursors for the adult head. This requirement correlates with the *otd* expression observed in the head region at the blastoderm stage and is consistent with "gap-gene-like" role for *otd* in blastoderm patterning. Loss of gene activity in this region at early stages may account for the head defects observed in *otd* homozygotes (Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990). Within any given segment, cells of *otd* genotype were also more frequently found in the dorsally derived discs than in those of ventral origin (Table 4, compare wing and leg, or tergites and sternites). The dorsal-ventral bias in the recovery of adult mosaics suggests an additional requirement for *otd* close to (but not identical to) the primordia for the ventral discs and histoblasts. This ventral requirement correlates with the expression of *otd* protein in the ventral epidermis and the requirement for *otd* in forming ventral-medial cuticle structures. Lastly, all surviving mosaics had at least some *otd/+* cells in their genital disc derivatives. This may reflect a separate requirement for *otd* in the posterior embryo, or it may only be a reflection of the ventral requirement, given the extreme ventral-medial position of the genital primordia (Gehring et al., 1976; Schüpbach et al., 1978; see Discussion).

Discussion

The most striking feature of all *oc/otd* phenotypes is their regional specificity. When homozygous mutant, most of the embryo and disc derivatives have normal morphology. Aside from the blastoderm requirement for *otd* in embryonic head development, the epidermal phenotypes in both the larva and the adult fly indicate a specific role for the gene in the development of ventral-medial structures. Reductions of *otd* activity result in loss of medial pattern elements and possibly a redirection of medial precursor cells to more lateral fates. During the development of larval and adult epidermis, *otd*

Table 3. *Recovery of surviving* otd *gynandromorphs: adult survival from the cross* y w otd f/FM7×R(1)w^{vC}/Yy^{+}

			FM7/R(1)w^{vC}		*y w otd f^{36a}/R(1)w^{vC}*	
	Fm7/Yy+	*FM7/0*	not mosaics	mosaics	not mosaics	mosaics
otd^{YH13}	417	59	198	297	147	56
otd^{XC86}	655	79	263	437	224	139
Total	1072	138	461	734	371	195

Table 4. *The* otd *Lethal Focus: The number of cases among the surviving adult gynandromorphs in which specific discs were mutant or mosaic for* otd *and the "sturt" distance between the primordium of that disc and the nearest lethal focus of* otd

genotype		Eye	La	Hu	W	L1	L2	L3	Tg1	Tg2	Tg3	Tg4	Tg5	Tg6	Tg7	S2	S3	S4	S5	S6	S7	Gen	An
otd^{XC86}	male	0	45	56	24	6	15	17	23	43	51	52	62	60	58	40	47	49	51	55	52	0	0
	mosaic	0	13	3	40	46	45	37	66	39	42	43	39	30	7	6	22	21	15	8	2	6	2
	sturts	0	19	21	23	10	14	13	20	23	26	27	30	27	21	17	21	22	21	21	19	2	1
otd^{YH13}	male	0	7	12	8	1	3	1	3	11	13	13	22	20	19	10	21	19	13	21	15	0	0
	mosaic	0	7	1	8	13	12	11	17	12	11	20	17	12	1	1	2	6	2	1	1	2	0
	sturts	0	9	11	10	6	8	6	10	15	16	20	26	22	17	9	19	19	21	18	13	2	0

Data for otd^{XC86} are based on examination of 276 sides of 138 adult mosaics, data for otd^{YH13} are based on 116 sides of 58 adult mosaics. The frequency of XO genotype (maleness average) in the FM7 control mosaics was 33%, based on examination of 144 FM7 mosaics from the XC86 cross. The sturt distances to the lethal focus ("sturts") were calculated as described in Hotta and Benzer (1974). Abbreviations: Eye, eye-antennal disc; La, labial disc derivatives; Hu, humerus; W, wing; L, leg; Tg, tergite; S, sternite; Gen, genitalia; An, analia.

protein is expressed in these medial precursor cells, consistent with an autonomous role in establishing or maintaining medial cell fates.

otd *mutants cause the transformation of medial structures to more lateral cell fates*

The most obvious effect of the adult viable alleles of *otd* is an elimination of the ocellar pattern elements in the head. When these patterns elements are missing, fields of disorganized bristles often appear in their place. Such ectopic bristles were also observed in our clonal analysis and, judging from the *y f* markers, were always *otd* in genotype, indicating that they arise in cells that have lost *otd* activity. In the frons, the ectopic bristles clearly represent changes in cell fate, given that frons cells normally do not form bristles. Similar fate transformations may also account for disorganized patterns of bristles observed in the ocellar region of *oc* homozygotes. We interpret these ectopic bristles as arising from transformations of the identities of medial cells to more lateral fates. Their designation as lateral (i. e., orbital) is tentative and is based on the morphology of the bristles and trichome of the underlying cuticle. It is consistent with the observation that the cuticle carrying these ectopic bristles is continuous with the orbital cuticle of the eye and often shows the same polarity of trichomes (Fig. 2A). It is not necessary that all medial cells in an *oc* individual be transformed to lateral fates. Many may simply die or not form differentiated cuticle. Moreover, we cannot strictly rule out an alternative possibility, that the ectopic bristles arise from an overproliferation of the lateral (orbital) precursors. This possibility seems less likely to us, given that *otd* clones in the orbital region can be perfectly normal and are only associated with the formation of ectopic bristles when they extend into the frons. Although we do observe an enlargement of the orbital region in *oc* flies, the altered pattern might also be explained if medial cells, which would normally not give rise to orbital bristles, had been shifted to more lateral fates.

Lateral shifts in cell fates may also occur in the genital disc. Induction of clones often results in the elimination of medial structures (e. g., the vaginal duct and other internal genital structures), while the row of thorn bristles in the more laterally situated vaginal plates is expanded. The increase in number of thorn bristles is probably too small to account for all the medial structures lost, arguing again that some medial cells are lost as a consequence of reduced *otd* activity, rather than invariably being transformed to lateral fates. The one apparent discrepancy between *otd* expression and function is found in the leg. There are no obvious abnormalities in the legs of *oc* individuals (or in *otd* mosaics), despite medial expression of *otd* RNA and protein in these discs. This may however simply reflect the absence of morphological markers in these regions that would allow the distinction between medial and more lateral fates.

In *otd* mutant embryos, the defects along the ventral midline offer an obvious correspondence to the medial defects observed in adults. Although the denticle belt phenotype was originally described as deletions of the first and fourth rows, in fact the entire medial region of the belt is affected in mutant embryos. The interpretation that we favor is that the *otd* gene product is required for establishing or maintaining ventral-medial cell fates in the epidermis. In mutant embryos, cells along the ventral midline would be shifted to more lateral fates. The overall size of each denticle belt is maintained, but medial elements are not present and elements immediately lateral to them expanded. This is consistent with the morphology of the belts shown in Fig. 3E. In the anterior abdomen, the most obvious consequence of this shift would be the deletion of the first and fourth denticle rows, since these rows do not extend far laterally. In the posterior segments, where the first and fourth rows normally extend into the lateral domain, the shift in fate might not remove all anteriorly pointing denticles and would therefore be less obvious. This might explain the apparent decrease in strength of the denticle belt phenotype along the anterior-posterior axis of the animal.

Analysis of the distribution of *otd* cells in our surviving adult gynandromorphs argues strongly for a requirement at the anterior end of the embryo. This may be related to the early requirement for *otd* in the formation of head structures. We also observed a clear dorsal bias in the recovery of *otd* cells which may reflect a requirement for *otd* in the development of ventral-medial structures. This requirement is consistent with the observed distribution of *otd* RNA and protein in the precursors for the larval epidermis. The width of this band corresponds roughly with the size of the medial pattern deletion observed in mutant embryos. We also suspect that the low recovery of surviving adult mosaics with mutant genitalia may also reflect this same ventral requirement. The genitalia arise from paired primordia very close the ventral

midline (Schüpbach et al., 1978). This proximity makes it very likely that at least some portion of the genital disc will be wild-type whenever the ventral focus has sufficient wild-type cells to allow for embryonic viability.

The correspondence of adult and larval functions of otd

In *Drosophila*, epidermal development occurs in two phases. The vast majority of cells in the embryo are destined to form structures of the larval body. Development of these cells occurs very rapidly. Cell division ceases by about 10 hours of embryogenesis, and differentiation of defined cuticle elements has already begun by 15 hours. The larva hatches 22 hours after fertilization. The second phase of epidermal development involves only those cells that will give rise to adult structures after pupation. During middle stages of embryogenesis, a small number of adult precursor cells are set aside in each segment as primordia for imaginal discs and histoblasts. These cells proliferate extensively during larval and early pupal stages. They differentiate their final pattern during pupation, after the larval cells have histolysed.

Although the larval and imaginal primordia share certain common features in their segmental organization and cell lineage, the relationship of the patterning process to cell proliferation is different. Thus it is unclear how similar the underlying genetic and cellular mechanisms will be. Although some of the genes which control patterning in the embryo also appear to function during larval stages in the imaginal discs, it is not always easy to extrapolate functional requirements between larval and adult periods. For example, segment polarity genes like *wingless* and *armadillo* are required for anterior-posterior patterning in the embryo, but seem to be more directly required in the ventral-distal patterning in the adult (Baker, 1988; Peifer et al., 1991). Similarly the *decapentaplegic* gene plays a role in dorsal-ventral patterning in the embryo (Irish and Gelbart, 1987), but is required for the formation of distal structure in the adult (Posakony et al, 1991). These examples involve proteins that are thought to be secreted (e. g., *wingless*, van den Heuvel et al., 1989; *decapentaplegic* , Padgett et al., 1987) or are localized near the cell surface (e. g., *armadillo*, Riggleman et al., 1990; Peifer and Wieschaus, 1990). Such proteins may play a role in patterning processes which depend on cell communication. Interestingly, the spatial requirements in larval and imaginal epidermis seem to parallel each other more precisely when the gene in question encodes a transcription factor, particularly one containing a homeodomain. This parallel has been proposed to reflect a feedback mechanism that maintains cells in particular determined states (Garcia-Bellido, 1975). Once such a "selector" gene is expressed in a given region of the epidermis, its activity is maintained in the progeny of those cells at subsequent stages of development. Obvious examples of genes thought to function in this way are the homeobox genes *engrailed* (Kornberg, 1981) and *Deformed* (Kuziora and McGinnis, 1988) .

As a putative gap gene, *otd* is unique in that it encodes a homeodomain protein. Like previously identified gap genes, its expression is not restricted to early embryogenesis. *otd* is also expressed in the central nervous system at later stages, where it plays an important role in determining the development of the midline lineages (Finkelstein et al., 1990; Klämbt et al., 1991). Unlike most gap genes, its later expression includes epidermal cells in the embryo. *otd* is also the only gap gene known to be required during the development of the adult epidermis. Like other homeodomain proteins that function in both the embryonic epidermis and in imaginal discs, *otd* activity plays a spatially homologous role in both tissues. In each case, it is essential for programming cells to medial fates. It is required in medial structures of the embryonic epidermis and in the medial regions of specific imaginal discs. More detailed studies may reveal whether the parallel expression patterns represent a continued maintenance of expression established early in the embryo.

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