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A role for Ultraspiracle, the *Drosophila* RXR, in morphogenetic furrow movement and photoreceptor cluster formation

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SUMMARY

Many of the same genes needed for proper eye and limb development in vertebrates, such as *hairy*, *hedgehog*, *patched* and *cyclic AMP-dependent protein kinase A*, are responsible for patterning *Drosophila* imaginal discs, the tissues that will give rise to the adult cuticle structures. This is well demonstrated in the control of morphogenetic furrow movement and differentiation in the eye imaginal disc. We report that *ultraspiracle*, the gene encoding the *Drosophila* cognate of the Retinoid X Receptor, is required for normal morphogenetic furrow movement and ommatidial cluster formation. Examination of the expression of genes involved in regulating the furrow suggests that *ultraspiracle* defines a novel regulatory pathway in eye differentiation.

Key words: *Drosophila*, eye development, *ultraspiracle*, RXR, morphogenetic furrow

INTRODUCTION

The *Drosophila* eye is composed of an ordered array of 800 unit eyes called ommatidia. These develop from an epithelial monolayer known as the eye imaginal disc. Differentiation of ommatidial clusters occurs in a wave that moves from the posterior margin of the eye field toward the anterior (for review see Thomas and Zipursky, 1994). The margin between the anterior undifferentiated region and the posterior region is marked by an apical-basal constriction of cells, forming a groove in the monolayer known as the morphogenetic furrow (Ready et al., 1976). Cells anterior to the furrow have no set developmental fate. As cells pass into and through the furrow they are organized into 5-cell preclusters (Wolff and Ready, 1991). Upon exiting from the posterior of the furrow, these 5 cells differentiate while others divide and become integrated into the existing preclusters to form the precursors of each individual adult ommatidium (Tomlinson and Ready, 1987).

The propagation of the furrow is dependent on *hedgehog* (*hh*), which is expressed posterior to the furrow (Heberlein et al., 1993; Ma et al., 1993). *decapentaplegic* (*dpp*), a member of the TGF-β family, is expressed within the furrow and serves as a molecular marker of the morphogenetic furrow (Blackman et al., 1991). Ectopic expression of *hedgehog* anteriorly leads to the induction of *dpp* expression and an ectopic furrow. Hh acts by antagonizing the activity of *patched* (*ptc*) and *cyclic AMP-dependent protein kinase A* (*Pka-C1*). *Ptc* and *Pka-C1* normally repress *dpp* expression anteriorly (Heberlein et al., 1995; Ma and Moses, 1995; Pan and Rubin, 1995; Strutt et al., 1995) and mutations in *ptc* and *Pka-C1* lead to ectopic anterior expression of *dpp*.

The anterior movement of the morphogenetic furrow and neuronal differentiation is also regulated by four helix-loop-helix (HLH) proteins, Hairy (H), Extramacrochaetae (Emc), Atonal (Ato) and Daughterless (Da). Clones of cells mutant for either *h* or *emc* alone show no or very subtle defects in eye development, but double mutant clones show advanced furrow movement and premature neuronal differentiation (Brown et al., 1995). Ectopic anterior expression of *hh*, in addition to inducing a new furrow and *dpp* expression, leads to Hairy expression ahead of the new furrow (Heberlein et al., 1995). *atonal* and *da* expression are markers of, and are possibly causal of, the first neuronal differentiation seen in the eye disc. *ato* expression begins just anterior to *dpp* expression and appears to be in every cell. *da* is expressed anterior of the morphogenetic furrow and shows higher expression within the furrow. As the furrow passes, *ato* and *da* expression become limited to the cells that will become the R8 photoreceptors and thereby establish the position and spacing of ommatidial clusters (Brown et al., 1995; Jarman et al., 1994).

Ultraspiracle (Usp) is the *Drosophila* cognate of RXR (Oro et al., 1990). Like RXR, it forms heterodimers with vertebrate receptors including RAR, the Thyroid Hormone Receptor and the Vitamin D Receptor, as well as with the *Drosophila* Ecdysone Receptor and DHR38 (Sutherland et al., 1995; Yao et al., 1993). The complex phenotype of *usp* suggests roles in several signaling pathways (Oro et al., 1992). We have previously demonstrated that *usp* mutations alter aspects of terminal differentiation in the *Drosophila* eye. The phenotypes include abnormal rhabdomere development as well as loss of expression of Rh-4, an R7 photoreceptor specific opsin (Oro

et al., 1992; Yao, 1994 and unpublished results). In the course of studying these phenotypes, we examined the earliest stages of differentiation in third instar eye imaginal discs. We show here that *usp* is required for proper progression of the morphogenetic furrow and subsequent organization of the ommatidial clusters. Our data suggest that *usp* defines a third pathway for regulation of furrow movement separate from the *hairy*/*emc* and *patched*/*Pka-C1* pathways. We propose that *usp* represses the propensity of anterior cells to enter into the 'furrow fate' in response to signals coming from near or posterior to the morphogenetic furrow and that loss of *usp* function allows precocious differentiation at the anterior margin of the furrow.

MATERIALS AND METHODS

Drosophila stocks and transgenic fly lines

The generation and detection of mitotic clones in imaginal discs was carried out using the FLP/FRT/myc system as described (Xu and Rubin, 1993). Enhancer trap or reporter lines were first crossed to either *w usp*^{3} *f* FRT18A/*w usp*^{3} *f* FRT18A; *λ10 Tb*/TM3 or *y w usp*^{4} FRT18A/*y w usp*^{4} FRT18A; *λ10 Tb*/TM3. *λ10* indicates an 8 kb *usp*$^+$ genomic transgene inserted on the third chromosome (Oro et al., 1992). Males that contained the reporter and were of the general genotype *usp* FRT18A; *λ10 Tb* were crossed to *π-myc M(1)o*^{Sp} FRT18A/FM7; *hsFLP38*/*hsFLP38* or *N-myc M(1)o*^{Sp} FRT18A/FM7; *hsFLP38*/*hsFLP38*. For the *emc h* experiments, a *dpp 3.0*/*dpp 3.0*; *emc*^{1} *h*^{c1} FRT80/ TM6B stock (a gift from Nadean Brown) was crossed to *hsFLP22*/*hsFLP22*; π-*myc* FRT80/π-*myc* FRT80. In experiments with both *usp* and *emc h*, first and second instar larvae were subjected to a 1 hour heat shock at 37°C to induce mitotic recombination via expression of the Flipase. *usp*^{3} and *usp*^{4} are mis-sense mutations in the DNA-binding domain which affect DNA binding in conjunction with the Ecdysone Receptor. They are inferred to be strong hypomorphic alleles (Henrich et al., 1994; Oro et al., 1992) (and unpublished results). *usp*^{3} and *usp*^{4} mutant clones give identical phenotypes in the eye disc. *pGMR-usp* was constructed by inserting a *usp* cDNA into the *Eco*RI site of pGMR (Hay et al., 1994) and introduced into flies as described (Boggs et al., 1987). Similar results are observed using either of two *pGMR-usp* insertions into the third chromosome.

Immunohistochemistry

Fixing and staining procedures have been described (Xu and Rubin, 1993). Primary antibodies: mouse anti-Usp diluted 1:5 (gift from F. Kafatos), rabbit anti-lacZ (Jackson Labs) diluted 1:1500, rabbit anti-Atonal (gift from Y. N. Jan) diluted 1:1000, mouse anti-myc (Oncogene Science) diluted 1:100, rabbit anti-hairy (gift from S. Carroll) diluted 1:200, rat anti-Elav (Developmental Studies Hybridoma Bank) and rabbit anti-cyclin A diluted 1:1000 (gift from D. Glover). Secondary antibodies: Texas Red-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit and goat anti-rat (Jackson Labs) diluted 1:100. Confocal images were collected on a Nikon/Biorad confocal microscope and imported into Adobe Photoshop for presentation.

RESULTS

Phenotypic analysis of *usp* in the eye imaginal disc

Usp is expressed in all cells of the eye imaginal disc (Fig. 1A). The apparent higher expression just anterior to the morphogenetic furrow is due to the intrinsic physical characteristics of the eye imaginal disc. Similar nuclear staining is seen when assayed by the DNA stain DAPI (data not shown). To determine the role of *usp* in the development and differentiation of the *Drosophila* eye, we created clones of cells that are mutant for *usp* using the FLP/FRT system (Xu and Rubin, 1993) and examined the consequences using expression of individual genes or proteins as markers of events related to eye differentiation. All phenotypes observed are rescued by the presence of an 8 kb wild-type *usp* transgene (Fig. 2G-I).

In *usp*$^-$ clones that lie posterior to the morphogenetic furrow, we see abnormal arrangement of developing photoreceptor clusters (Fig. 2A-F). Photoreceptor clusters are irregularly spaced, resulting in gaps between the clusters. Similar results are seen with multiple markers of cell fate (Fig. 2, data not shown). With each marker used, including an antibody against ELAV (Bier et al., 1988), which marks each of the eight photoreceptors, we see that clusters still contain the appropriate number of cells (data not shown).

Not only are ommatidial clusters misaligned in mutant regions, mutant cells posterior to the furrow differentiate prematurely (Fig. 2A-C). Using the *spalt*-expressing enhancer trap line 2-3602 (our unpublished results), we see labeled clusters of five cells starting five to six rows posterior to the furrow in wild-type regions, whereas in the *usp*$^-$ clones they appear three rows from the furrow. Even though 5-cell clusters appear prematurely in the *usp*$^-$ regions, the number of 2-3602-expressing cells and their relative arrangement within the clusters appears normal.

When *usp* function is absent, morphogenetic furrow movement is accelerated, resulting in a furrow, which bends anteriorly within and around the *usp*$^-$ region (Figs 2D-F, 3D-F). The furrow can become advanced by as many as three to four rows of ommatidial clusters. Bending is observed using markers of differentiation (Fig. 2D-F) or markers that are expressed within the furrow (Fig. 3D-F). The effect is progressive and thus dependent on the shape and size of the clone. As observed in Fig. 3A-C, when the furrow is just entering the posterior region of the clone, there is no anterior bending of the furrow. As the morphogenetic furrow passes through a large posterior-anterior spanning clone, the bending anteriorly is more prominent. Slight furrow displacement continues even after the furrow has re-entered the wild-type region (Fig. 2A-C, data not shown).

Furrow displacement is not caused by excess proliferation of *usp* cells

The anterior bending of the furrow might result from an excess of cells in the *usp*$^-$ clones. For example, the premature differentiation seen in *usp*$^-$ clones may include an acceleration of the

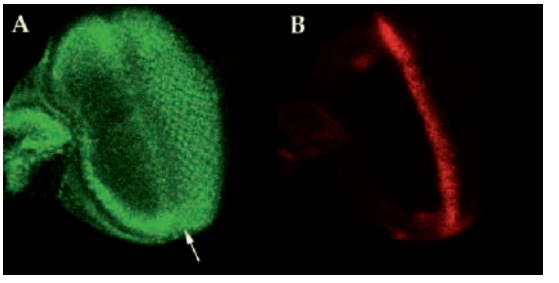

Fig. 1. Expression of *usp* in the eye imaginal disc. (A) Usp is expressed across the eye imaginal disc. The arrow marks the furrow, as shown in B by *dpp-lacZ* expression (Blackman et al., 1991).

final cell divisions that occur posterior of the furrow (Ready et al., 1976). Thus the bending of the furrow would not be caused by loss of *usp* anterior to or within the furrow region but rather by a mechanical pushing of the furrow as a result of an increase in the number of cells in mutant regions posterior to the furrow. There are several reasons to discard this hypothesis.

A 'pushing effect' from extra cell proliferation might be expected to lead to expansion and deformation in all directions, thus creating a non-autonomous disruption of the pattern of cells in all directions outside of the clonal boundary. We would expect to see not only an anterior displacement of the furrow but also a displacement of wild-type ommatidial clusters lying dorsally, ventrally and posterior of the *usp*$^-$ clones. We have not seen this effect in any *usp*$^-$ clones. The disruption of the ommatidial pattern ends at the clonal boundary.

To examine directly the possibility of increased cell density, we used DAPI stain to mark nuclei of both wild-type and mutant cells. In clones anterior to, intersecting or posterior to the morphogenetic furrow, we see no increase in cell density (Fig. 4A-C). Thus, neither deformation of adjacent lateral regions nor an increase in cell density are seen in or around *usp*$^-$ clones.

We also characterized the density of actively cycling cells by using the expression of cyclin A as a marker of cells in the S and G_2 phases of the cell cycle. Cyclin A expression is normal in clones that intersect the morphogenetic furrow (Fig. 4D-F and data not shown). In *usp*$^-$ clones that lie posterior to the morphogenetic furrow, there appear to be fewer cyclin A-positive cells than in adjacent wild-type regions (Fig. 4D-F). We interpret this to be a indication that there are fewer, not more, cycling cells in older *usp*$^-$ regions posterior to the morphogenetic furrow.

patched and *Pka-C1* activity is normal in a *usp*$^-$ clone

The disordered arrays of clusters and altered furrow advancement are similar to phenotypes characterized for other pathways that regulate furrow movement and the early steps of differentiation and may implicate Usp as a yet unidentified member of these signaling pathways. Given the ability of RXR-containing dimers, including Usp-containing dimers, to repress transcription (Chen and Evans, 1995; Horlein et al., 1995 and unpublished data), it is possible that Usp may be involved in the repression of anterior differentiation by *Ptc* and *Pka-C1*. To test this hypothesis, we asked if *dpp* expression, a marker of *Ptc* and *Pka-C1* activity, occurs anterior to the furrow in *usp*$^-$ clones (Heberlein et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). *Ptc* and *Pka-C1* normally repress *dpp* expression anteriorly and mutations in *ptc* and *Pka-C1* lead to ectopic anterior expression of *dpp*. If Usp protein acts downstream of *Ptc* and *Pka-C1* to repress gene expression, or if Usp acts upstream of *ptc* and *Pka-C1* to allow their expression, then loss of *usp* function should lead to an increase in *dpp* expression anterior to the furrow as is observed in *ptc*$^-$ and *Pka-C1*$^-$ clones.

As can be seen, in *usp*$^-$ clones there is no anterior expansion or ectopic expression of *dpp* (Fig. 3A-F and data not shown) and *dpp* expression continues to follow the furrow as it bends anteriorly within *usp*$^-$ clones (Fig. 3D-F). The deformed morphogenetic furrow also encompasses some adjacent *usp*$^+$ cells, similar to what is seen in Fig. 2D-F, almost certainly as a result of the non-cell autonomous action of Hh in induction of differentiation. We have also examined expression of an *hh* enhancer trap in *usp*$^-$ clones. As can be seen in Fig. 5, the appropriate relationship between *hh* expression and the morphogenetic furrow is seen, with *hh* expression beginning posterior to the furrow. This is true even in situations in which the furrow is anteriorly displaced. These results demonstrate that *usp* is not required for *ptc* and *Pka-C1* expression and that *ptc* and *Pka-C1* functions do not involve *usp* activity to repress *dpp*

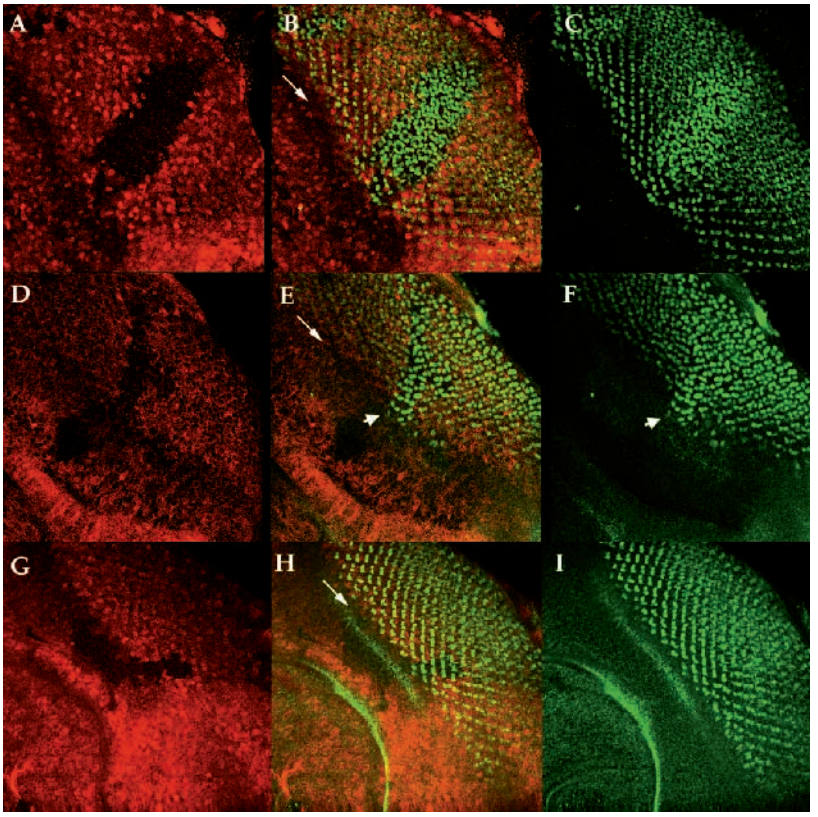

Fig. 2. Analysis of *usp*$^-$ clones in third instar eye imaginal discs. All discs are oriented with anterior to the lower left. An arrow marks the furrow. In this and subsequent figures, each row of images shows a single imaginal disc. In each row, the left panel shows expression of a myc epitope (red) which is linked to *usp*$^+$. Loss of myc staining indicates a group of *usp*$^-$ cells. The right panel of each row shows expression of the marker of interest (green) and the center panel shows a fusion of the first and third images. (A-C) *spalt* expression as represented from the *lacZ* enhancer trap 2-3602. (D-F) Expression of the *lacZ* enhancer trap AE127, which marks the expression pattern of the *seven-up* gene. This disc contains a clone intersecting the morphogenetic furrow. The short arrow indicates a position at which differentiation has occurred prematurely (more anteriorly) as judged relative to the position of AE127-expressing cells in wild-type tissue. The region of advanced differentiation corresponds to a region in which the morphogenetic furrow is advanced as well. (G-I) A disc containing a *usp*$^-$ clone that intersects the morphogenetic furrow, in a genetic background containing one copy of the *usp* genomic region inserted on the third chromosome (Oro, 1992a).

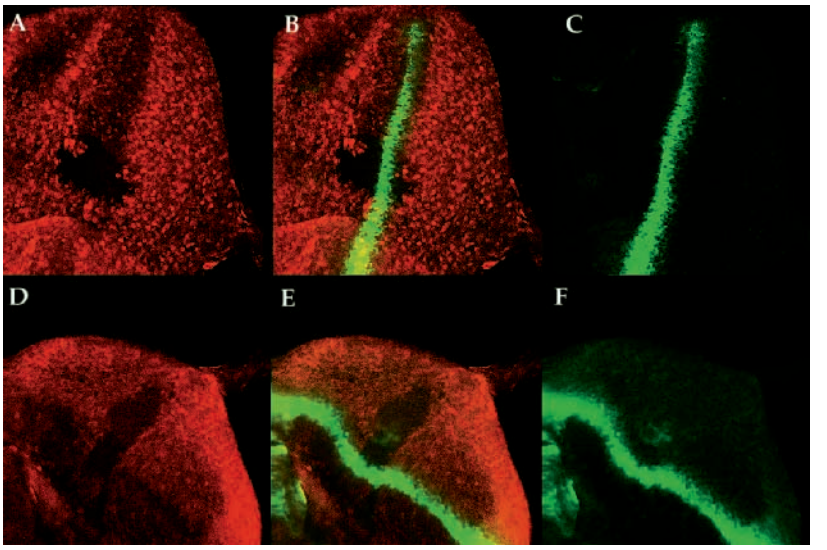

Fig. 3. *dpp* expression in *usp*⁻ clones. Each row of images shows a single imaginal disc as described in Fig. 2. (A-C) A *usp*⁻ clone intersecting the morphogenetic furrow. Anterior is to the left. (D-F) A large *usp*⁻ clone intersecting the morphogenetic furrow. Anterior is to the lower left corner.

expression. This strongly suggests that *usp* and *ptc* and *Pka-C1* define independent pathways controlling furrow movement.

HLH activity is unchanged and leads to normal initial precluster formation

hairy (*h*) and *extramacrochaetae* (*emc*), two genes encoding helix-loop-helix proteins represent a second set of genes regulating anterior movement of the morphogenetic furrow. To test whether *usp* is required for *hairy* transcription and thus is a molecule involved in this signaling pathway, we looked at Hairy expression in *usp*⁻ clones. As can be seen, Hairy is normally expressed in a stripe just anterior to the region in which *atonal* is expressed. This pattern is unchanged in *usp*⁻ cells (Fig. 6A-C). Since the *hairy emc* mutant phenotype is only seen when both *hairy* and *emc* are mutant, the normal expression of Hairy in a *usp* mutant clone indicates that the *usp* phenotype is not a consequence of the loss of *hairy* and *emc* expression. In addition, *usp* is expressed normally in *hairy*⁻ *emc*⁻ cells (data not shown). These data are consistent with a model in which *usp* and *hairy*/*emc* are part of separate pathways for the control of furrow movement.

As a test of the hypothesis that *usp* and *hairy* are in different pathways, we examined the effect of *usp* mutations on one likely target of down regulation by hairy, *atonal* (Brown et al., 1995; Jarman et al., 1994). In addition, analysis of the initial post-furrow pattern of Ato expression in *usp* clones allowed us to determine if the disarrayed ommatidial clusters seen in *usp*⁻ clones result from gross errors in the initial patterning of the R8 photoreceptor precursors. To test these possibilities, we examined the expression of Ato in *usp*⁻ clones. In Fig. 7A-C, a *usp*⁻ clone intersects the normal Atonal-expressing region just anterior to the morphogenetic furrow with no obvious anterior expansion of Atonal expression. In addition, the limitation of Atonal expression to the presumptive R8 photoreceptor cells in the region of the morphogenetic furrow occurs with apparently normal kinetics in *usp*⁻ clones (Fig. 7D-F). Examination of Ato expression patterns in *usp* mutant regions well posterior to the furrow indicates that the array and spacing of R8 cells is normal (Fig. 7G-I), even though the apparent ommatidial arrays are abnormal. Thus, Usp does regulate the kinetics or position of *atonal* expression, and the loss of Usp function does not alter the patterning of the R8 precursors.

hairy and *emc* do not act downstream of *ptc* or *Pka-C1* in regulation of *dpp*

The data presented indicate that *usp* acts in a pathway independent of both *ptc*/*Pka-C1* and *hairy*/*emc*. Small differences in phenotype between *ptc* or *Pka-C1* clones and *hairy emc* clones suggest that *ptc*/*Pka-C1* and *hairy*/*emc* define separate pathways for control of furrow movement (Brown et al., 1995; Heberlein et al., 1995; Ma and Moses, 1995; Pan and Rubin, 1995; Strutt et al., 1995). In order to test this directly, we again used *dpp* as a marker of the function of the *ptc*/*Pka-C1* pathway.

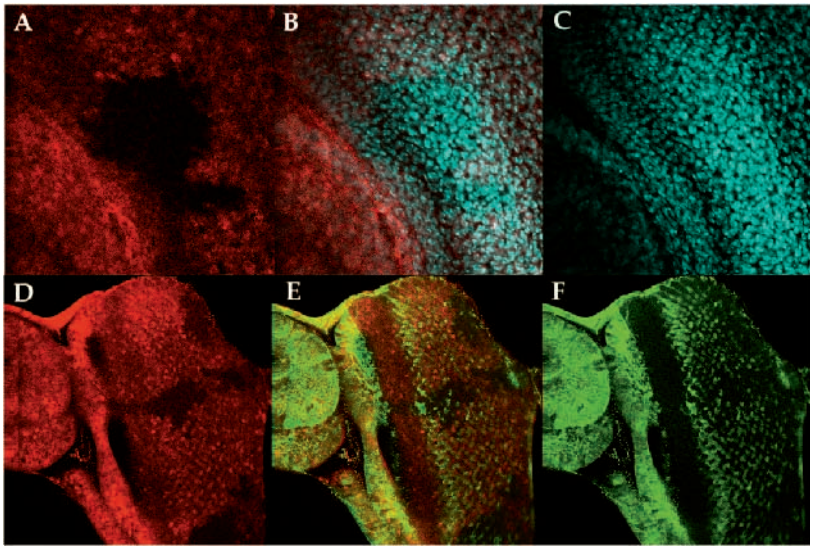

Fig. 4. DAPI staining and Cyclin A expression in *usp*⁻ clones. Each row of images shows a single imaginal disc as described in Fig. 2. (A-C) A usp⁻ clone intersecting the morphogenetic furrow stained with DAPI. Anterior is to the left. (D-F) Cyclin A staining in *usp*⁻ clones in the eye imaginal disc. Anterior is to the lower left corner.

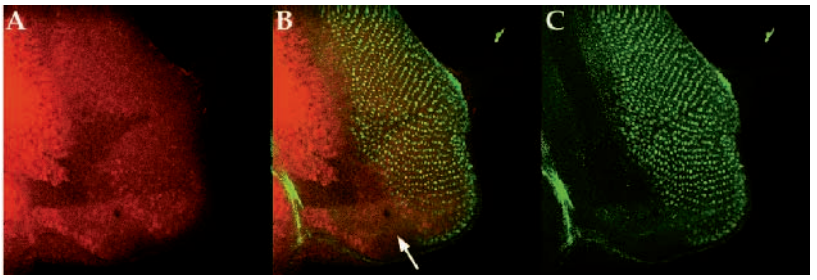

Fig. 5. *hedgehog* expression in *usp*$^{-}$ clones. The arrow marks the morphogenetic furrow. The images are arranged as described in Fig. 2. A large clone intersects the morphogenetic furrow. *hedgehog* expression is marked by β-galactosidase expression from an *hedgehog* enhancer trap. Anterior is to the left.

If *hairy* and *emc* function in the same *ptc*/*Pka-C1* pathway that regulates *dpp*, then loss of *hairy* and *emc* should lead to ectopic expression of *dpp* in the region anterior to the morphogenetic furrow, just as occurs in *ptc* or *Pka-C1* mutant clones. As can be seen, *hairy emc* mutant clones, which intersect the furrow from the posterior, have an anteriorly shifted furrow with *dpp* expression in the furrow (Fig. 8A-C). In a clone that crosses the furrow from posterior to anterior, there may be the beginning of furrow advancement or a small anterior expansion of *dpp* expression, but *dpp* expression does not fill the anterior region of the clone as would have happened in a *ptc* or *Pka-C1* clone (Fig. 8D-F). Clones that lie entirely anterior to the furrow do not show *dpp* expression (data not shown). These phenotypes are different from those of *ptc* or *Pka-C1* mutant clones. Thus, we conclude that *ptc*/*Pka-C1*, *hairy*/*emc* and *usp* each define a separate regulatory pathway acting to control events related to the rate of morphogenetic furrow movement.

usp is required anterior to, within, or just posterior to the furrow for proper eye development

From the data presented, it is not clear whether *usp* functions in the posterior differentiated region, in the anterior undifferentiated region, or both. To distinguish among these possibilities, we have constructed a transgene, *pGMR-usp*, which leads to high level *usp* expression posterior to the furrow as verified by anti-Usp antibody staining (Fig. 9A) (Hay et al., 1994). Examination of *usp*$^{-}$ clones in a *pGMR-usp* background reveals that photoreceptor clusters within the *usp*$^{-}$ clone are irregularly organized, even though Usp is being expressed at high levels from *pGMR-usp* (Fig. 9B-G). Some residual anterior deformation of the furrow can be seen (Fig. 9E-G). These results indicate that the levels or position of Usp expression from *pGMR-usp* are insufficient to rescue the *usp* mutant phenotype. Since *pGMR-usp* gives relatively high Usp expression in most regions posterior to the furrow (Fig. 9A), the simplest interpretation of these results is that *usp* normally functions anterior to, within or just posterior to the morphogenetic furrow.

DISCUSSION

Usp functions to repress differentiation and furrow movement

Previous results from our laboratories demonstrate a requirement for Usp, the *Drosophila* RXR, in the development of the adult compound eye (Oro et al., 1992). The results in this paper demonstrate that Usp plays a role from the earliest stages of cell determination and differentiation. Regions of the eye imaginal disc that lack *usp* show more rapid morphogenetic furrow movement, earlier post-furrow differentiation, as judged by expression of cell-type-specific markers, and abnormal ommatidial cluster formation and alignment.

Although a *usp*$^{+}$ genomic fragment rescues the *usp* phenotypes, these phenotypes are not rescued by above normal expression of Usp posterior to the morphogenetic furrow. This leads us to conclude that the array of *usp* mutant phenotypes observed in the early stages of eye differentiation result from loss of *usp* activity in the region anterior to or within the morphogenetic furrow.

From the observation that events proceed quicker in the absence of *usp* function, we infer that Usp, directly or indirectly, inhibits or slows the rate of furrow movement and differentiation. A direct inhibitory action of Usp, perhaps in conjunction with a non-Usp dimerization partner, is consistent with our observations that Usp/EcR dimers can repress basal level transcription (unpublished observations) and with experiments demonstrating a repressive function for unliganded RXR and some of its dimerization partners (Chen and Evans, 1995; Horlein et al., 1995).

usp, *ptc*/*Pka-C1* and *hairy*/*emc* define separate regulatory pathways for repression of furrow movement

In addition to *usp*, two other sets of genes have been shown to slow the rate of morphogenetic furrow movement. These are *ptc*/*Pka-C1* (Heberlein et al., 1995; Ma and Moses, 1995; Pan and Rubin, 1995; Strutt et al., 1995) and *hairy*/*emc* (Brown et al., 1995). This raises the question as to whether these define separate regulatory pathways or whether they all act in a single, linear cascade. Our results, when coupled to the results of others, show that *ptc*/*Pka-C1*, *hairy*/*emc* and *usp* define separate pathways inhibiting furrow movement.

First, we show that neither *hairy*/*emc* nor *usp* mutant regions in the anterior portion of the eye disc show complete ectopic

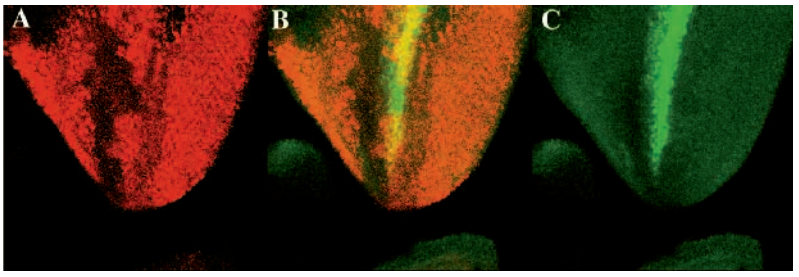

Fig. 6. Hairy expression in *usp*$^{-}$ clones. The row of images shows a single imaginal disc as described in Fig. 2. Hairy protein is revealed by rabbit anti-Hairy antibodies. A *usp* mutant clone intersects the region of Hairy staining and the morphogenetic furrow from the anterior. Anterior is to the left.

expression of *dpp* as is observed in regions mutant for *ptc* or *Pka-C1*. Thus, *hairy*/*emc* and *usp* can be separated from the *ptc*/*Pka-C1* pathway. Second, our results show that *hairy* is not required for Usp expression and *usp* is not required for Hairy expression. Finally, our results show that *atonal*, a target for repression by *hairy*/*emc*, is expressed normally, relative to the morphogenetic furrow, in *usp* mutant regions, even though mis-expression of Atonal protein occurs in *hairy*/*emc* mutant regions (Brown et al., 1995). Thus we conclude that not only are the *hairy*/*emc* and *usp* pathways separate from the *ptc*/*Pka-C1* pathway, they are separate from each other.

That *usp* defines a separate pathway controlling furrow movement does not explain the mechanism by which it acts. The progressive nature of furrow bending in *usp* clones suggests that small increments of furrow advancement are compounded as the furrow moves through the *usp* mutant regions. These incremental advancements could broadly reflect two possibilities. (1) The loss of *usp* function could result in increased sensitivity of anterior cells to a signal generated in the differentiating posterior region or very near the furrow (Strutt et al., 1995). Under such a model, with both the *ptc*/*Pka-C1* and *h*/*emc* pathways still functioning, we can infer that *usp* must normally function relatively close to the morphogenetic furrow in repressing the response to such a differentiation factor. (2) As a consequence of more rapid differentiation of *usp*$^{-}$ cells just posterior to the furrow, higher levels of a differentiation-inducing factor may be generated, leading to earlier commitment to the furrow fate and anterior bending of the furrow. Under this model, hedgehog protein is the obvious candidate for such a molecule. In *usp* mutant clones, the relationship between the furrow and the *hedgehog*-expressing cells is maintained and the relative level of *hedgehog* expression is normal. In addition, our studies show that the earliest stages of Atonal expression within and posterior to the furrow are normal. Given that two different markers of the earliest stages of post-furrow differentiation appear normal, we suggest that it is more likely that *usp* modulates the response to a signal in the anterior or furrow region rather than modulating the generation of a signal in the posterior region.

usp is required for ommatidial cluster formation

Within *usp*$^{-}$ clones, ommatidial clusters are misaligned with respect to each other and with respect to adjacent wild-type tissue. Our experiments suggest that this phenotype results from loss of *usp* function anterior to, within or just posterior to the morphogenetic furrow. This suggests that *usp* may function in the earliest stages of ommatidial cluster formation. The initial determination of the number, positioning and spacing of ommatidial clusters is the designation of single cells as the precursors of the R8 photoreceptor cells of each individual ommatidium. Precluster formation then occurs around these R8 precursor cells. Using Atonal expression as a marker of R8 determination and position, we see normal kinetics for R8 specification with the same density of R8 precursors as seen in nearby wild-type regions. In older mutant regions of the disc, the Atonal-expressing cells fall in a normal array comparable to that seen in adjacent wild-type regions, even though other markers of differentiation show that clusters are abnormal in their alignment to each other.

One possibility is that the disruption of patterning is due to the premature or abnormal differentiation and recruitment of cells into ommatidial clusters that is seen in mutant clones. The exact nature of the premature differentiation events is not clear. The pattern of expression of the 2-3602 enhancer trap is consistent with it being a marker of R1, R3, R4, R6 and R7 differentiation. If so, the pattern of expression in *usp*$^{-}$ clones suggests that R1, R6 and R7 enter ommatidial clusters prematurely. Alternatively, the 'mystery cells' (Wolff and Ready, 1993) that are normally eliminated from ommatidial preclusters may remain and differentiate as 2-3062-expressing cells. If this is the case, these modified mystery cells must take on the relative positions of the normal 2-3602-expressing cells and inhibit recruitment of the cells otherwise destined to become the 2-3602-expressing cells. In either case, based on the DAPI and cyclin A staining and premature appearance of molecular markers, it is possible that cells taking on photoreceptor fates, and possibly some of the non-neuronal accessory cells, may be

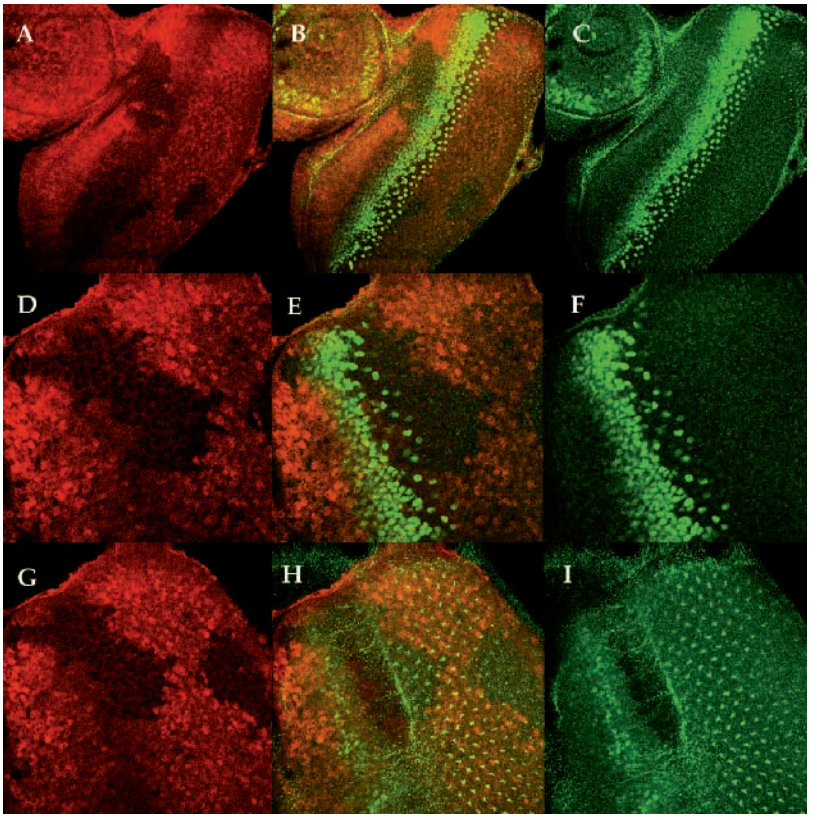

Fig. 7. Atonal expression in *usp*$^{-}$ clones. Each row of images shows a single imaginal disc as described in Fig. 2. (A-C) Atonal expression in a *usp*$^{-}$ clone that intersects and extends anterior to the morphogenetic furrow. Anterior is to the upper left corner. (D-F) Atonal expression in a *usp*$^{-}$ clone that intersects and extends posterior to the morphogenetic furrow. An additional clone is visible in the posterior region of the eye disc. (G-I) The same *usp*$^{-}$ clones as in D-F. The confocal image is of a focal plane at which the presumptive R8 cells are clearly visible.

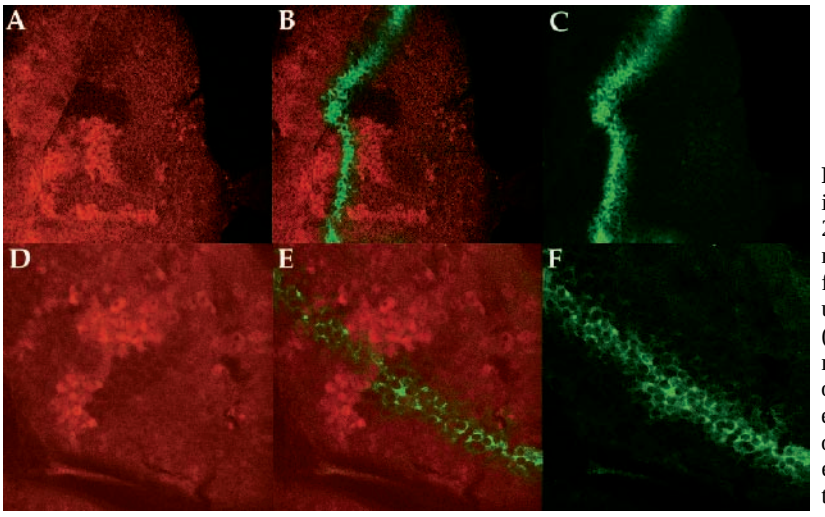

Fig. 8. *dpp* expression in h^{-}/emc^{-} clones. Each row of images shows a single imaginal disc as described in Fig. 2. (A-C) A *hairy emc* mutant clone just posterior to the morphogenetic furrow. Anterior advancement of the furrow is evident from the expression of β-galactosidase under the control of a *dpp* enhancer. Anterior is to the left. (D-F) A *hairy emc* mutant clone, which intersects the morphogenetic furrow from the anterior. The beginnings of anterior advancement or a slight expansion of *dpp* expression is seen, but expression of β-galactosidase occurs only in the region of the furrow and does not extend to the anterior border of the clone. Anterior is to the lower left.

recruited into or retained within the preclusters before the synchronous round of cell division that occurs posterior of the furrow (Ready et al., 1976). This could result in too few uncommitted cells being present at later stages for adding the final non-neuronal cells to clusters, thus resulting in the poor relative organization of the mutant ommatidial clusters.

usp signaling pathways: insights from vertebrate limb development

We conclude that *usp*, the *Drosophila* RXR, defines a novel pathway for control of morphogenetic furrow movement and differentiation. That *usp* may function anterior to the furrow to modulate the response to a factor arising posterior to or very near the morphogenetic furrow raises a number of questions including what signaling pathway involves *usp* and what, if anything, does *usp* tell us about the role of RXRs in vertebrate limbs? Results from *Drosophila* and the high degree of conservation between the *Drosophila* eye and vertebrate limbs lead us to suggest a possible answer to these questions.

Others have inferred the existence of a non Dpp 'competency' factor functioning anterior to the morphogenetic furrow (Burke and Basler, 1996; Strutt et al., 1995). Work on vertebrates has identified a positive feedback loop between *sonic hedgehog* (*shh*) expression in the zone of polarizing activity and fibroblast growth factor (FGF) expression in the Apical Ectodermal Ridge such that Shh induces FGF and FGF induces Shh (Laufer et al., 1994; Niswander et al., 1994; Vogel et al., 1996; for a review see Cohn and Tickle, 1996). Recent studies have identified both a *Drosophila* FGF and FGF receptors, with one FGF receptor, *heartless*, being expressed in a region at or near the morphogenetic furrow (Emori and Saigo, 1993). We therefore propose the possibility that a *Drosophila* FGF, acting via *heartless*, may be the 'competency' factor identified by others and that *usp* may function to down regulate the response to FGF in regions anterior to the morphogenetic furrow. If so, this may suggest a similar role for RXR in controlling the boundary between FGF- and Shh-expressing cells in vertebrate limb buds.

In conclusion, we have shown the involvement of an RXR family member in regulating the anterior/posterior boundary, the morphogenetic furrow, in the developing *Drosophila* eye

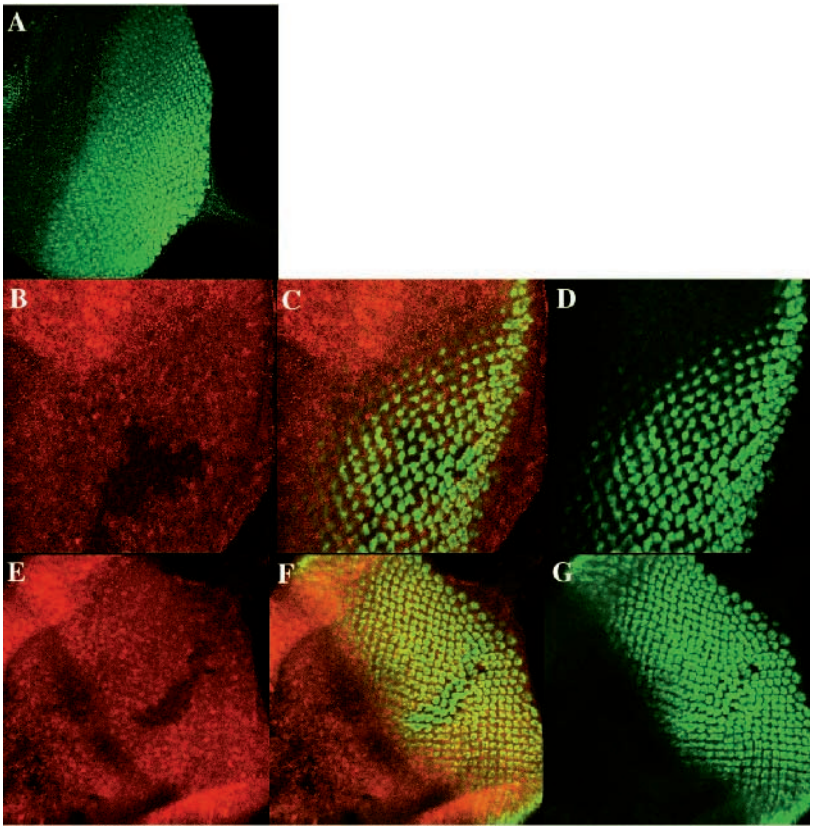

Fig. 9. *usp* is needed anterior to or within the furrow for proper eye development. (A) Usp expression in an eye disc carrying the transgene *pGMR-usp*. (B-D) a usp^{-} clone, in a genetic background of *pGMR-usp*, lying posterior to the morphogenetic furrow and stained with an antibody against ELAV. Anterior is to the lower left corner. (E-G) a second usp^{-} clone, in a genetic background of *pGMR-usp*, lying posterior to the morphogenetic furrow and stained with an antibody against ELAV.

and for regulation of posterior differentiation and ommatidial cluster formation. We have also shown that the function of Usp in these events defines a previously unrecognized pathway for the control of furrow movement and cell differentiation. Given the high degree of similarity between *Drosophila* and vertebrate regulatory pathways, these results have important implications for future studies of vertebrate eye and limb development.

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