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Ecdysone pathway is required for furrow progression in the developing *Drosophila* eye

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SUMMARY

In *Drosophila*, secretion of the steroid hormone ecdysone from the prothoracic ring gland coordinates and triggers events such as molting and metamorphosis. In the developing *Drosophila* compound eye, pattern formation and cell-type specification initiate at a moving boundary known as the morphogenetic furrow. We have investigated the role of ecdysone in eye development and report here that the ecdysone signaling pathway is required for progression of the morphogenetic furrow in the eye imaginal disc of *Drosophila*. Genetic disruption both of the ecdysone signal in vivo with the *ecdysoneless*^{1} (*ecd*^{1}) mutant and of ecdysone response with a *Broad-Complex* mutant result in disruption of morphogenetic furrow progression. In addition, we show that ecdysone-dependent gene expression, both of a reporter of transcriptional activity of the Ecdysone Receptor and of the Z1 isoform of the Broad Complex, are localized in and close to the furrow. These results suggest that, in the morphogenetic furrow, temporal hormonal signals are integrated into genetic pathways specifying spatial pattern.

Key words: *Drosophila*, Morphogenetic furrow, Retina, Ecdysone, Steroid hormone, Broad-Complex

INTRODUCTION

The steroid hormone ecdysone is secreted from the ring gland of *Drosophila* and other insects in a series of pulses during larval and pupal development, triggering events such as molts, entry into the pupal stage and eversion of the imaginal discs (Riddiford, 1993). A heterodimer of the Ecdysone Receptor (EcR) and the Ultraspiracle protein (Usp) can function as a receptor for ecdysone (Koelle et al., 1991; Yao et al., 1993). The hormone-receptor complex is able to activate transcription of a number of loci including some tissue-specific structural genes and other targets known as 'early genes'(Natzle, 1993; Fletcher and Thummel, 1995). The early genes encode transcription factors that both activate 'late genes' and repress their own transcription resulting in sequential cascades of molecular events that temporally regulate responses to the ecdysone signal (Ashburner, 1974, 1990; Thummel, 1996).

Several mechanisms contributing to the tissue and temporal specificity of response to ecdysone have been described. The EcR protein has three isoforms, with distinct patterns of expression in larval and imaginal tissues which are likely to confer tissue specificity (Talbot et al., 1993). Moreover, mutations affecting individual isoforms have different phenotypic effects (Bender et al., 1997). Target genes have different promoter sensitivities to ecdysone and different transcript lengths, both thought to be strategies to control the strength and immediacy of response to an individual pulse of ecdysone (Karim and Thummel, 1992). Although Usp is the only known dimerization partner of EcR, it is not required in all tissues during metamorphosis (Oro et al., 1992), suggesting that other pairing partners might exist, providing another means of regulating the cellular response. Indeed, the nuclear receptor DHR3 interacts with EcR in vitro and in vivo in cultured cells (White et al., 1997). Furthermore, complex cross-regulatory interactions among ecdysone-induced and ecdysone-repressed transcription factors modulate the cellular response and control stage specificity (Karim et al., 1993; Woodard et al., 1994; Thummel, 1996; Lam et al., 1997; White et al., 1997).

Mutations have been isolated that affect several steps in the ecdysone signaling pathway. We have used two of these as reagents in the studies reported here. A temperature-sensitive mutation in *ecdysoneless* (*ecd*^{1}) reduces ecdysone titer in vivo up to 20-fold, but does not eliminate it completely (Garen et al., 1977; Berreur et al., 1984). Genetic mosaic analysis has shown that *ecdysoneless* is required in the ring gland (and ovary) and the mutation is thus likely to affect either ecdysone synthesis or release (Henrich et al., 1987). The *Broad-Complex (BR-C)* lies in an early ecdysone-induced polytene puff and *BR-C* transcription is directly activated by the receptor hormone complex (Ashburner, 1974; DiBello et al., 1991). The *BR-C* encodes a family of proteins related by alternative RNA processing, each possessing a common core domain and one of four pairs of zinc fingers (Z1-Z4) (DiBello et al., 1991;

Bayer et al., 1996). The BR-C proteins are expressed differentially in many tissues around the time of metamorphosis (Emery et al., 1994). Mutations that affect single isoforms have been recovered (Belyaeva et al., 1980; Kiss et al., 1988; Emery et al., 1994; Bayer et al., 1997), and the *nonpupariating-1* (*npr-1*) mutation is a null mutation that disrupts all of the Broad-Complex proteins (Stewart et al., 1972; Kiss et al., 1976; Bayer et al., 1997). The *BR-C* loci are early genes required for maximal induction of other early genes as well as their own transcription before pupariation and play an important role in metamorphic responses to ecdysone (Karim et al., 1993).

Pattern formation and cell-type specification in the compound eye begin in the eye-antennal imaginal disc in the late stages of larval life and are completed during metamorphosis in the pupa. Each facet or ommatidium of the eye has 20 cells, including a cluster of 8 photoreceptors, which are arranged in a highly ordered array (Dietrich, 1909; Waddington and Perry, 1960). Assembly of the eye is initiated at the posterior margin of the presumptive eye field in the imaginal disc and proceeds anteriorly, adding successive rows of photoreceptor cell clusters (Ready et al., 1976). The morphogenetic furrow, a visible indentation in the disc, which marks the anterior front of this wave of differentiation, sweeps across the disc epithelium over about 2 days (Ready et al., 1976). Anterior to the furrow there is unpatterned mitotic proliferation. In the furrow, the cells' division cycles become synchronized with all cells arrested in G_1 and with a tightly localized final cell division following the furrow (Ready et al., 1976; Wolff and Ready, 1991; Thomas et al., 1994; de Nooij and Hariharan, 1995).

The precise spatial pattern in the eye is generated through a sequence of localized inductive signals. The signals that initiate the morphogenetic furrow at the posterior margin and propel it across the first 10-12 columns appear to be different from those that instruct the continued propagation of the furrow across the latter two-thirds of the disc. The first phase is likely to be *hedgehog* (*hh*)-independent and requires *decapentaplegic* (dpp, Heberlein et al., 1993; Ma et al., 1993; Wiersdorff et al., 1996; Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997). Recently published work suggests that *hedgehog* does act before furrow initiation, perhaps much earlier, to establish the eye field boundary (Domínguez and Hafen, 1997; Royet and Finkelstein, 1997). However, there does not appear to be a requirement for *hh* at the time of furrow initiation as it is neither required then (shown by the use of a temperature-sensitive mutation) nor expressed at the posterior margin at that time (Ma et al., 1993). The later progression of the furrow is driven by the secretion of Hedgehog from newly determined photoreceptor neurons, instructing cells anterior to the furrow to enter the differentiation phase (Heberlein et al., 1993; Ma et al., 1993).

Cells in the furrow constrict, producing very narrow apical profiles, and group into 'rosettes' (Wolff and Ready, 1991) which later resolve into 5-cell 'preclusters' (Ready et al., 1976). The establishment and spacing of these clusters involves signaling via the Notch pathway, which acts together with other factors to focus the initially broad expression of the proneural transcription factor Atonal to single founding photoreceptor cells (the future R8 cells, Cagan and Ready, 1989; Jarman et al., 1994; Baker and Zitron, 1995; Baker et al., 1996; Dokucu et al., 1996). The photoreceptor cells and accessory cells are recruited and patterned in a defined temporal sequence (Tomlinson and Ready, 1987). These sequential steps produce a gradient of maturity in the disc as a whole, with young clusters recently formed in the furrow being smaller and containing fewer differentiated cells than older clusters located farther posterior (Wolff and Ready, 1993) During ommatidial assembly, ligands such as Spitz and Bride-of Sevenless and their receptors Egfr and Sevenless work through the Ras/MAP Kinase pathway, in combination with a complex pattern of preexisting transcription factors to trigger the acquisition of cell identities in each ommatidium (Krämer et al., 1991; Simon et al., 1991; Wassarman et al., 1995; Freeman, 1997; Kumar and Moses, 1997; Tio and Moses, 1997).

Others have reported a requirement for the EcR dimerization partner Usp in retinal development (Zelhof et al., 1997). These authors report a very slight and variable acceleration of the morphogenetic furrow when the effect is summed over many columns in large usp^- mosaic clones. In the usp^- clones, extra photoreceptor cells are specified early, on the anterior face of the preclusters. We interpret differentiation of these extra cells (usually two) as the primary effect of loss of Usp function, and the acceleration of the furrow as a secondary effect. These extra cells are likely to express Hedgehog (as do the others), and this increase in titer may be responsible for the reported furrow acceleration (Zelhof et al., 1997).

In this study, we have examined the function of ecdysone signaling in the developing eye of *Drosophila*. Organ culture experiments have shown a requirement for supplemental ecdysone in the medium to support furrow progression in the eye disc (Li and Meinertzhagen, 1995). We have found that the progression of the morphogenetic furrow in the whole animal is regulated by the ecdysone signaling pathway and can be disrupted by withdrawal of ecdysone. We show that ecdysone-dependent gene expression, both of a reporter of EcR activation and of *BR-C*, an early gene in the ecdysone response hierarchy, are localized to the furrow, and that the loss of *BR-C* function also disrupts the furrow. In addition, we see effects of ecdysone withdrawal both earlier and later in eye development.

MATERIALS AND METHODS

Drosophila stocks and temperature-shift regimes

The wild-type stock used was *Canton-S*. Temperature-shift experiments were performed on flies carrying the ecd^1 (Garen et al., 1977) mutation in *trans* to a deletion for the region, *Df(3L)R-G7* (Sliter et al., 1989). Larvae from the cross of $ecd^1 \times$ *R-G7/TM6Tb* were exposed to 30°C for 24 hours. Long larvae of genotype ecd^1/*R-G7* were picked for immediate dissection. EcRE:*lacZ* flies were a gift of S. Stowers and D. Hogness and carry a construct with a heptamer of Ecdysone Receptor-binding sites (EcREs) from the *hsp27* promoter driving expression of β-galactosidase (Koelle et al., 1991; White et al., 1997). *npr-1*, a null mutation for the *BR-C*, was a gift of C. Thummel. Hemizygous *y npr-1 w* 3rd instar males were identified by their yellow mouth-hooks and dissected.

Histology

Eye discs were prepared (as described by Tomlinson and Ready, 1987). Discs were examined by light microscopy or by laser scanning confocal microscopy. Primary antibodies were: mouse mAb 22C10 (gift of L. Zipursky and S. Benzer, Fujita et al., 1982), rat anti-Elav

(from University of Iowa, Developmental Studies Hybridoma Bank, Bier et al., 1988), rabbit anti-β-galactosidase (Cortex Biochem CR7001RP2), rabbit anti-Atonal (gift of Y.-N. Jan and A. Jarman, Jarman et al., 1994), rabbit anti-Hedgehog ATA7 (gift of A. Taylor and P. W. Ingham, Fietz et al., 1995), mouse anti-BR-C Z1 3C11 (gift of G. Guild, Emery et al., 1994), mouse anti-Ecdysone Receptor 11D9.6, mouse anti-Ecdysone Receptor-A 15G1a, mouse anti Ecdysone Receptor-B1 AD4.4 (all gifts of D. Hogness, Koelle et al., 1991), and mouse anti-Cyclin B (gift of P. O'Farrell and N. Yakubovich, Knoblich and Lehner, 1993). Secondary antibodies were Cy5-conjugated goat anti-rabbit (Jackson Labs, 111-176-003), FITC-conjugated donkey anti-mouse (Jackson Labs, 715-095-151), LRSC-conjugated donkey anti-rat (Jackson Labs, 712-085-153), TRITC-conjugated goat anti-mouse (Jackson Labs 115-025-003), goat anti-mouse IgG-HRP conjugate (Bio-Rad, 170-6516), goat anti-rat IgG-HRP conjugate (Jackson Labs, 112-035-003) and a Vectastain kit for rabbit HRP detection (Vector Laboratories, PK-6101). Cytoplasmic actin was detected with FITC-conjugated phalloidin (Molecular Probes, F-432). S-phase cells were detected by 5-bromo-2′ deoxyuridine incorporation, (BrdU, as described by Wolff and Ready, 1991) using Sigma BrdU (catalog no. B-5002) visualized with anti-BrdU (Becton Dickinson, 347-580). Acridine orange staining was performed as described by Bonini et al. (1993). 30 μm cross-sections were prepared by embedding discs in Histoprep (Fisher, SH75-125D) and sectioning on a freezing microtome. Adult eyes were prepared for scanning electron microscopy (as described by Ready et al. (1976).

RESULTS

Withdrawal of ecdysone disrupts eye development

The *ecd*^{1} mutation is a hypomorphic temperature-sensitive allele thought to affect ecdysone synthesis or release in the ring gland (Garen et al., 1977; Henrich et al., 1987). Homozygous *ecd*^{1} flies show some eye defects when shifted to 30˚C for 24 hours during their final larval instar (data not shown). However, consistent with the partial loss of function associated with *ecd*^{1} (Berreur et al., 1984), these phenotypes are more pronounced when this mutation is placed in *trans* to a deletion for the region *(Df(3L)R-G7)*, and all data shown here are from such heterozygotes. When larvae are returned to the permissive temperature (18˚C) after a 24 hour exposure to 30˚C during the third instar, and allowed to mature, their adult compound eyes are disrupted, with anterior nicks in the retinal tissue (compare Fig. 1A to B). These nicks are similar to those previously reported (Redfern and Bownes, 1983). We also found that larvae that were exposed to 30˚C for 24 hours during the second instar had more severe defects in adult eye morphology, showing significant intrusions of cuticle tissue into the eye field (data not shown).

We focused our investigations on the defects resulting from withdrawal of ecdysone during the third instar because this is the principal time of pattern formation and early differentiation in the eye disc. To characterize the developmental disruption more directly, we examined the imaginal discs that give rise to the adult eyes with anterior loss of retinal tissue. Discs were dissected from late 3rd instar *ecd*^{1}*/(Df(3L)R-G7)* larvae immediately following a 24 hour exposure to 30˚C (to be referred to as *ecd-ts* discs). We visualized two neural antigens, Elav and 22C10, in wild-type (Fig. 2A,C) and *ecd-ts* discs (Fig. 2B,D) and observe that the mutant phenotype is consistent with the furrow moving much more slowly than normal, or with total arrest: relatively mature clusters at the furrow and an absence of earlier stages. This is similar to the phenotype seen with *hh* loss of function and other genotypes that arrest the furrow (Heberlein et al., 1993; Ma et al., 1993). Clusters behind the furrow have continued to mature, but new clusters have not been recruited, with the result that mature clusters with as many as five cells, and with well-grown axons, are seen right at the furrow. A wild-type furrow progresses at about the rate of one column every 1.5 hours at 25˚C (Basler and Hafen, 1989) and somewhat faster at 30˚C. Possibly because the *ecd*^{1} allele is not a null, the furrow does not stop immediately upon exposure to the non-permissive temperature. Comparison of the overly mature clusters at the furrow in the *ecd-ts* condition with the wild-type gradient of maturity (Fig. 2A-D) reveals that the most mature anterior clusters in the *ecd-ts* discs resemble wild-type clusters about 6 columns behind the furrow. The aberrant clusters appear to be about 8-9 hours old, and thus their constituent cells would have been several cell diameters anterior to the furrow at the beginning of the 24 hour temperature shift. These cells would have participated in the events common to all cells in the furrow before the furrow finally arrested. Other disruptions include clusters with single cells posterior to larger clusters and altered cluster morphology. In addition, the discs were occasionally smaller than in wild-type discs (up to 25% smaller). Normally cells in the furrow have very small profiles indicative of apical constriction and, in *ecd-ts* discs, these profiles have broadened, suggesting that the furrow is not only slowed or stopped, but is also dysmorphic (Fig. 2E,F).

To examine early events in the furrow, we examined the expression pattern of *atonal* (*ato*), the proneural gene for the R8 photoreceptor cell, the founder of the ommatidium (Tomlinson and Ready, 1987; Jarman et al., 1994). Like the products of other proneural genes, Ato is first expressed broadly in an equivalence group of cells and subsequently narrows to single cells before disappearing (Jarman et al., 1995; Baker et al., 1996; Dokucu et al., 1996). Due to the reiterative nature of retinal development, both phases of expression can be seen at once, with a wide zone of staining in the furrow and single cell staining in R8 founder cells posterior to the furrow (Fig. 2G). In all *ecd-ts* discs, the wide band of Ato expression in the furrow disappears (Fig. 2H). In some of the discs, the later R8-specific expression has also

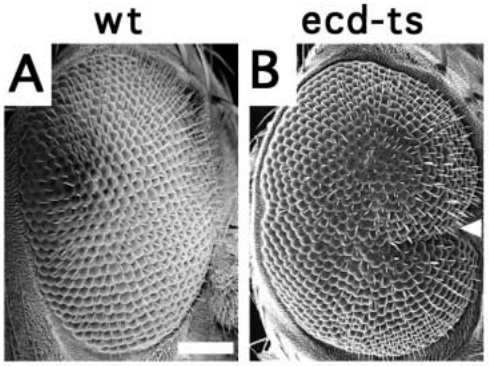

Fig. 1. Anterior eye nick in *ecd-ts* flies. (A,B) SEM of adult eyes. (A) Wild-type; (B) *ecd-ts*. White arrowhead in B shows anterior nick (see text) Scale bar, 100 μm. Anterior to the right.

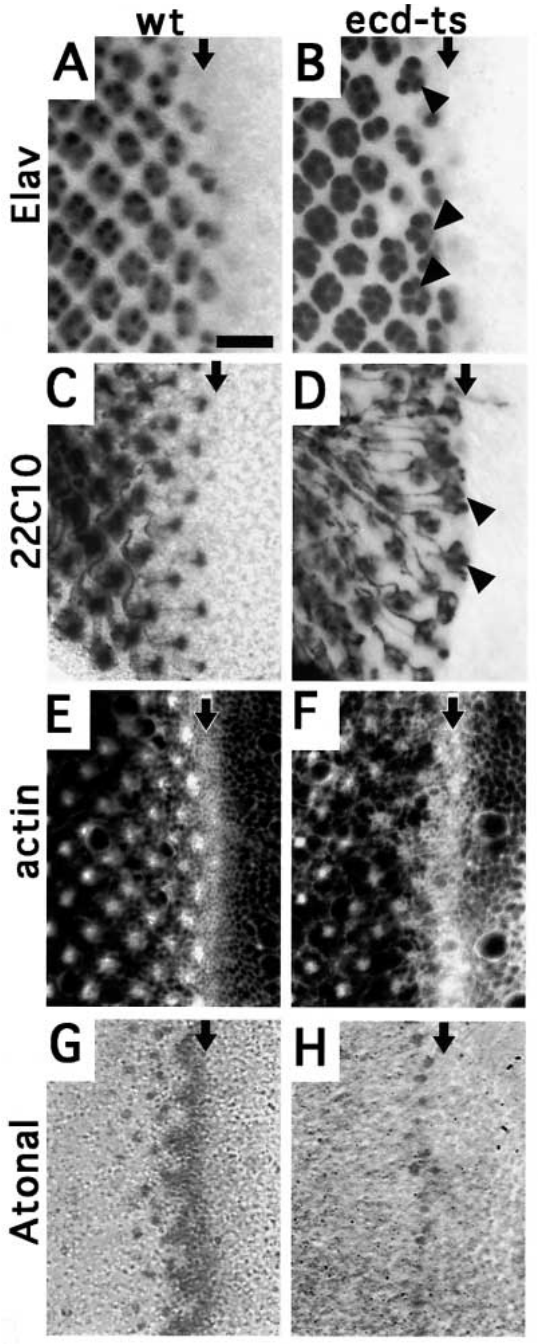

Fig. 2. Disruptions in eye development in *ecd-ts* eye discs. Third instar eye imaginal discs. (A,B) Elav; (C,D) mAb 22C10; (E,F) actin; (G,H) Atonal. (A,C,E,G) Wild-type; (B,D,F,H) *ecd-ts* (see text). Black arrowheads in B,D show large, mature clusters close to the furrow (compare to similar columns in A,C). Arrows indicate the furrow. Scale bar, 10 μm. Anterior on the right. Note that the *ecd-ts* condition disrupts furrow progress by all markers shown here.

gone (data not shown). We suggest that, once ecdysone signaling is disrupted, no new cells begin to express *ato*, but that cells already expressing this gene complete the normal expression sequence. The variability of these data may be due to the known leaky nature of the ecd^1 allele (see above and Berreur et al., 1984). These results confirms a failure of recruitment of cells into the photoreceptor differentiation pathway, further supporting our interpretation of this phenotype as a failure of furrow progression.

Cell cycle regulation is affected in the *ecd-ts* furrow

Normally the cells' mitotic division cycle becomes synchronized in the furrow. Ahead of the furrow, cells proliferate randomly but, in the furrow, all cells are held in G_1 arrest. This is followed by a tight band of DNA synthesis and mitosis in all those cells not included in preclusters in the furrow (Ready et al., 1976; Wolff and Ready, 1991). We sought to determine whether the *ecd-ts* disruption of the furrow includes this cell cycle regulation. We tested this by two methods and both showed that, while the prefurrow general proliferation is not visibly altered, cell-cycle synchrony in the furrow is lost.

The first method was to use bromodeoxyuridine (BrdU) incorporation to label S-phase cells (Gratzner, 1982; Wolff and Ready, 1991). We observe a general loss of BrdU incorporation in *ecd-ts* eye discs, in particular in the zone just posterior to the furrow (compare Fig. 3A and B). The second method was to stain for Cyclin B (CycB) expression, which labels cells at the G_2/M transition (Edgar and Lehner, 1996). In wild-type, CycB is expressed generally anterior to the furrow and in a tight band just posterior to it (see arrowhead in Fig. 3C). In *ecd-ts*, the anterior CycB expression remains, but the tight, postfurrow band of CycB is lost (Fig. 3D). Thus both approaches show results consistent with a loss of cell cycle regulation in the furrow of *ecd-ts* discs. This supports the hypothesis that the eye disc phenotypes seen after loss of ecdysone are not due to general failure of disc cell proliferation, but rather to specific effects on the furrow. In addition, we have examined the pattern of cell death in the disc using acridine orange staining and find that no excess cell death is associated with ecdysone withdrawal (data not shown). This result differs from previous work (Redfern and Bownes, 1983) which reported cell death in imaginal discs of ecd^1 homozygotes that had been exposed to 29˚C for 2 days. It may be that generalized cell death is an effect seen in this genotype only after longer periods of exposure to 29˚C.

The results thus far have shown that the morphogenetic furrow requires ecdysone during the middle phase of progression across the eye disc. Mechanisms controlling the earliest phases of furrow initiation and progression are believed to be different from the those controlling later phases, so we investigated whether the sensitivity of the furrow to ecdysone titer is restricted to the later phases. By staining early 3rd instar *ecd-ts* eye discs with 22C10, we found that even the earliest phases of furrow progression are sensitive to ecdysone (Fig. 4A,B). Overly mature photoreceptor clusters with well-extended axonal projections can be found at the anterior edge of differentiation, just as in older discs (compare with Fig. 4 with Fig. 2D).

Hedgehog expression is affected posterior to the *ecd-ts* furrow

Progression of the furrow beyond the first ten columns is driven by Hedgehog, expressed in the differentiating clusters and diffusing forward to induce anterior cells to enter the furrow. Two eye-specific *hedgehog* alleles (hh^1 and hh^{fse}) cause the furrow to arrest about a third of the way across the eye field

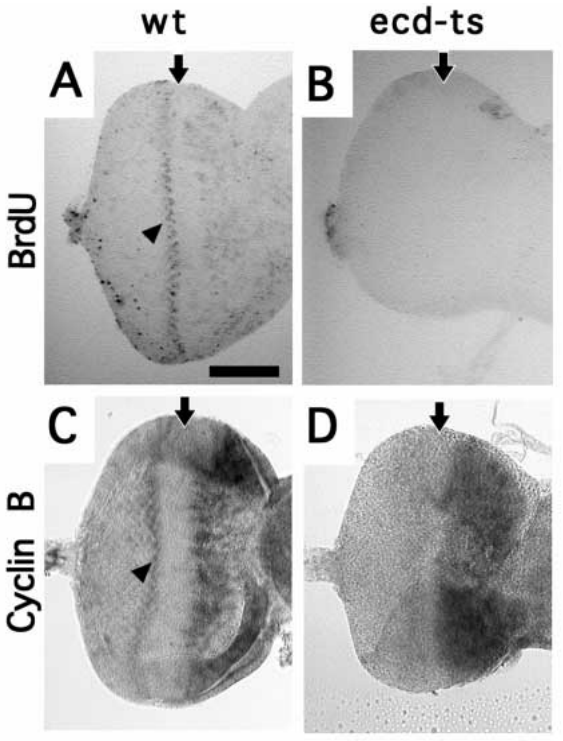

Fig. 3. Cell-cycle synchronization at the furrow is disrupted in *ecd-ts* discs. Third instar eye imaginal discs. (A,B) BrdU incorporation; (C,D) Cyclin B. (A,C) Wild-type; (B,D) *ecd-ts* (see text). Arrows indicate the furrow. Black arrowheads in A,C show co-ordination of cell cycle posterior to the furrow. Arrowhead in A shows S-phase cells and in C shows cells at G_2/M transition. Scale bar, 100 μm. Anterior on the right. Note that the *ecd-ts* condition affects cell-cycle synchronization at the furrow.

(Heberlein et al., 1993; Ma et al., 1996), and when Hedgehog is removed with a temperature-sensitive allele, only the first several rows of photoreceptor clusters are formed, yielding a 'Bar' shaped eye (Ma et al., 1993). We examined whether the furrow failure in *ecd-ts* discs might be associated with loss of Hedgehog expression.

Disc pairs were separated after fixation and stained separately with anti-Ato and anti-Hh antibodies. In all larvae where Ato expression was impaired in the typical *ecd-ts* manner, Hh protein in the contralateral disc was greatly reduced far below normal levels (Fig. 5). Hh protein appears to be lost uniformly across the disc, contrasting with Ato whose domain of expression is progressively narrowed from the anterior.

Ecdysone receptor reporter activity correlates with mid-phase furrow movement

To determine the time at which the furrow initiates relative to known ecdysone-mediated developmental events, we examined polytene chromosome puffs and eye imaginal discs from larvae at several times in the third instar. We found that furrow initiation occurs prior to puff stage 0 (data not shown), corresponding to the pre-wandering stage of the third instar, when ecdysone titers are very low.

We used a transgenic construct to reveal the domain of gene expression that is directly regulated by EcR in the developing eye. This construct contains a heptamer of Ecdysone Receptor-binding sites (EcREs, ecdysone response elements) from the *hsp27* promoter driving expression of β-galactosidase and provides the most direct assay currently available of the EcR in situ in the living disc (EcRE:lacZ, Koelle et al., 1991). In mid third instar discs, β-gal is restricted to a zone in and anterior to the furrow (Fig. 6A-D,J). This domain then travels with the furrow until the end of the third larval instar, when it becomes ubiquitous (as hormone titer rises at the end of larval life, Fig. 6K,L). In younger discs, we find no β-galactosidase expression until the furrow has produced 10 to 12 columns of ommatidia (Fig. 6E-J). The EcRE:*lacZ* may not represent the complete domain of transcriptional activation by the Ecdysone Receptor in the eye disc; a similar construct placed upstream of a different promoter did not drive larval or pupal ecdysone-dependent gene expression in transgenic flies (Antoniewski et al., 1996), suggesting that outside of the endogenous promoter environment, such a construct may not report the entire range of transcriptional activity. Our results, however, do show clear spatially restricted ecdysone-responsive transcriptional activation in the eye disc.

We thought that the simplest explanation for this spatial restriction of EcRE:*lacZ* activity would be a restricted expression pattern of the EcR itself, and thus we examined the distribution of the EcR protein. However, antibodies that recognize both the A isoform of EcR (the form that predominates in imaginal discs) and a common EcR domain (Talbot et al., 1993) show uniform staining in the eye disc (data not shown). This is also true of the one known dimerization partner of EcR, Usp (Zelhof et al., 1997). Thus the localized expression of the EcRE:*lacZ* reporter construct is not likely to be due to restricted expression of the known components of the ecdysone receptor.

A BR-C protein is expressed near the furrow and is required for furrow progression

To investigate how the ecdysone signal might be transduced in the eye disc, we examined the expression and role of *BR-C*, an early ecdysone response gene complex known to play an important role in metamorphic responses to ecdysone (DiBello et al., 1991; Karim et al., 1993; Emery et al., 1994; Bayer et al., 1997). With an antibody specific to the Z1 finger-

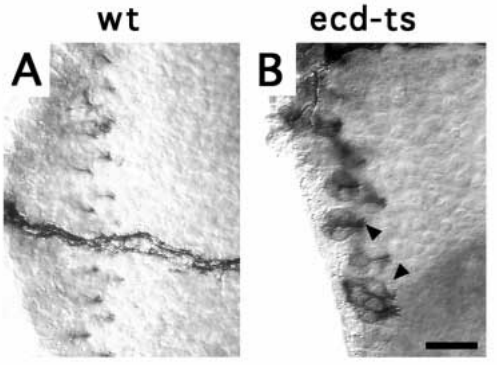

Fig. 4. Early sensitivity of furrow progression to ecdysone titer. Early third instar eye imaginal discs stained with mAb 22C10. (A) Wild-type; (B) *ecd-ts*. Black arrowheads in B indicate overly mature clusters. Compare B with Fig. 2D. Scale bar, 10 μm. Anterior to the right. Note that *ecd-ts* condition can disrupt the furrow early.

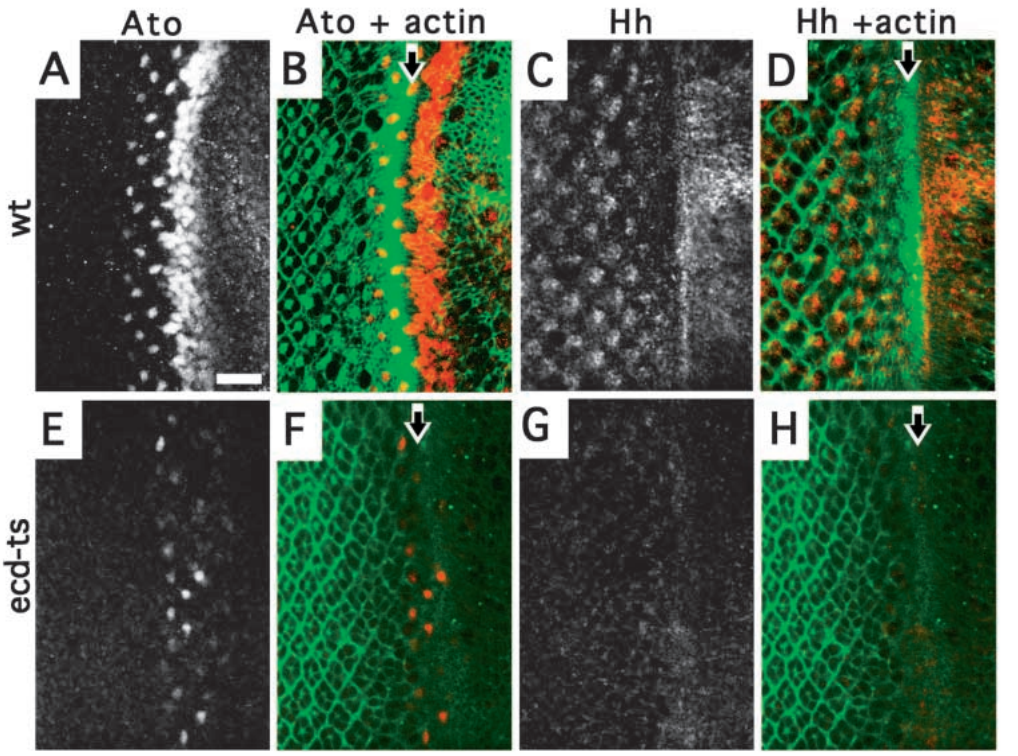

Fig. 5. Hh and Ato expression are affected differently by *ecd-ts*. Third instar eye imaginal discs. (A-D) Wild-type; (E-H) *ecd-ts*. (A,E) Atonal; (C,G) Hedgehog. (B,D,F,G) Merged images of cytoplasmic actin (shown in green) and the Atonal (B,F) or Hedgehog (D,H) image from the immediate left (shown in red). Scale bar, 10 μm. Anterior on the right. Arrows indicate the furrow. Note that: *ecd-ts* condition reduces or eliminates both Ato and Hh. Hh expression falls uniformly across the disc, whereas Ato expression is lost from anterior to posterior.

containing forms of this protein (the isoform expressed more strongly in differentiating imaginal tissues, Emery et al., 1994), we found that this protein also localized near to the furrow (Fig. 7). Z1-containing isoforms of this protein begin to be expressed just anterior to the furrow and reach maximal levels posterior to the furrow, following in time the activation of EcRE:*lacZ* as visualized by β-galactosidase staining just anterior to the furrow. This suggests that, although *BR-C* is immediately downstream of the Ecdysone Receptor, there is a delay in its maximal expression as compared with the expression of EcRE:*lacZ*, which may reflect autoregulation (Karim et al., 1993) or post-transcriptional control of expression (Bayer et al., 1996). Both cross-sections (Fig. 7C) and whole mount stainings (Fig. 8A) show that, in addition to its furrow domain of expression, BR-C Z1 is also expressed ubiquitously in the peripodial membrane. BR-C Z1 expression both near the furrow and in the peripodial membrane is greatly reduced when ecdysone titer is reduced in *ecd-ts* flies (Fig. 8).

Males hemizygous for *npr-1* (an allele null for all *Broad-Complex* functions), exhibit failures of furrow progression and photoreceptor recruitment (Fig. 9A,B). Comparison of the *ecd-ts* and *npr-1* Elav- and Atonal-staining patterns reveals some similarities and differences (Figs 2B,H, 9A,B). Both display the furrow arrest phenotype of mature clusters at the anterior front of differentiation, and both have abnormal clusters with incorrect cells numbers and degree of rotation. However, in *npr-1*, the broad furrow domain of Ato expression is reduced as in *ecd-ts*, but additional defects not seen in *ecd-ts* include aberrant patterns of focused R8 staining, including a triplet of three Ato-expressing cells together. These patterns may reflect defects in the mechanisms for spacing the proneural precursors, or in selecting the R8 cell from an equivalence group (Dokucu et al., 1996). The correlation between effects of removal of ecdysone on furrow progression and *BR-C* expression and the effects of direct removal of BR-C function suggest that BR-C may mediate a subset of the ecdysone effect in the eye, but may also have independent functions, as does Usp (Zelhof et al., 1997).

DISCUSSION

We have shown that elements of the ecdysone signaling pathway are required for *Drosophila* eye development during morphogenetic furrow progression with three lines of evidence: two mutations in the pathway, ecd^1 (synthesis or release of ecdysone) and *npr-1* (*Broad-Complex*, response to ecdysone) affect furrow progression, and, thirdly, ecdysone-dependent gene expression moves with the furrow (the EcRE:*lacZ* reporter and BR-C Z1). Withdrawal of ecdysone using the temperature-sensitive ecd^1 allele causes the furrow to slow or stop, and the period of ecdysone sensitivity appears include both early and middle phases of furrow progression. Cell-cycle regulation at the furrow is disrupted as shown by BrdU incorporation and CycB expression, and the apical constriction of cells in the furrow is relaxed as shown by actin staining. Examination of photoreceptor cluster morphology and maturity posterior to the furrow using neural-specific antibodies shows that, in *ecd-ts* discs, there is a lack of immature stages, a phenotype also seen in other mutations such as *hh*, where the furrow has stopped. We also saw aberrant cluster morphologies, which may be due to secondary effects of furrow disruption or to distinct effects of ecdysone withdrawal. We found that the expression of two proteins important for patterning at the furrow are disrupted in different ways: successive phases of Atonal expression near the furrow are lost progressively, whereas Hedgehog expression appears to be lost uniformly across its normal domain posterior to the furrow.

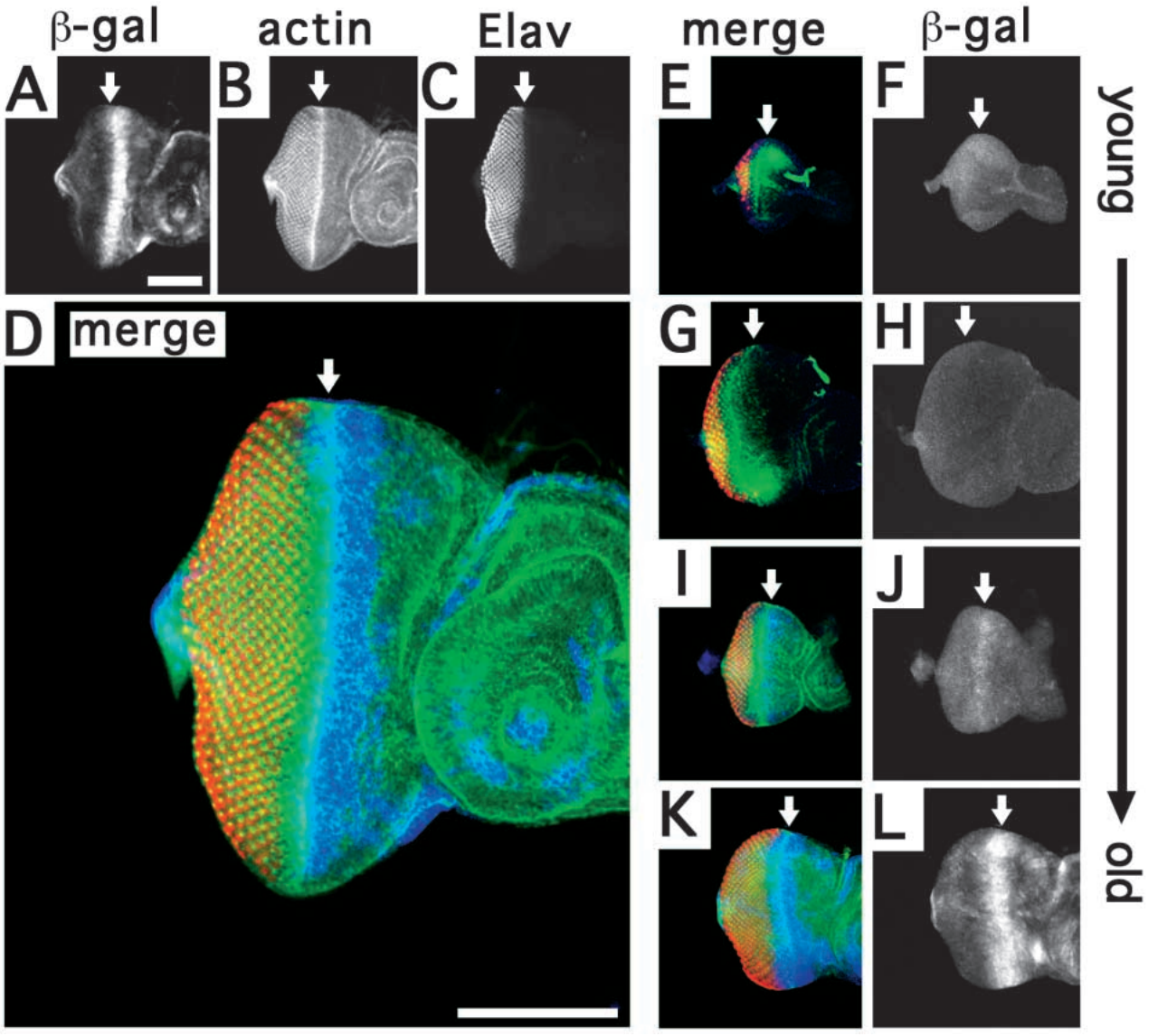

Fig. 6. A reporter of ecdysone regulated transcriptional activity in the furrow over time. Third instar eye imaginal discs carrying EcRE:*lacZ* reporter. (A-D) Late third-instar disc. (A) β-galactosidase; (B) cytoplasmic actin; (C) elav; (D) merge of A-C with β-galactosidase in blue, actin in green and Elav in red. (E-L) A time series of third instar eye discs with early third instar in E,F and late third instar in K,L. (E,G,I,K) Merged images with the same color scheme as D. (F,H,J,L) The same discs with the β-galactosidase stain shown alone. Arrows indicate the furrow. Scale bars are 100 μm. All panels except D are shown to the same scale. Anterior to the right. Note that there is no β-galactosidase expression during the early progression of the furrow, then β-galactosidase expression travels with the furrow.

Following the early phase of furrow progression, expression of the reporter construct EcRE:*lacZ* is spatially restricted to in and just anterior to the furrow. It is likely that one mediator of the ecdysone signal is the Z1 isoform of the Broad Complex, because it is expressed at the furrow and its expression is greatly reduced in *ecd-ts* discs. BR-C null mutants (*npr-1*) also display a disrupted furrow phenotype.

ecd^{1} has been reported to affect the development of several adult structures, including 'nicking in the anterior margin of the eye' (Redfern and Bownes, 1983) and, with hindsight, this may now be seen as evidence of a furrow defect. The furrow represents a moving boundary between anterior undetermined cells and posterior cells that have entered the differentiation pathway, and signaling across this boundary from posterior photoreceptors induces the first stages of determination in anterior cells (Ma et al., 1993). Also occurring at the furrow is lateral cell-cell communication in which the founder photoreceptor cells of each cluster are specified with the correct spacing (Ready et al., 1976; Wolff and Ready, 1991; Ellis et al., 1994; Dokucu et al., 1996). Thus the furrow is a site of integration of a systemic temporal hormonal signal into the genetic pathways that generate spatial pattern.

Integration of the ecdysone signal into eye development

We present a simple model for the integration of ecdysone into the known morphological and genetic events of furrow progression and eye development (Fig. 10). In response to

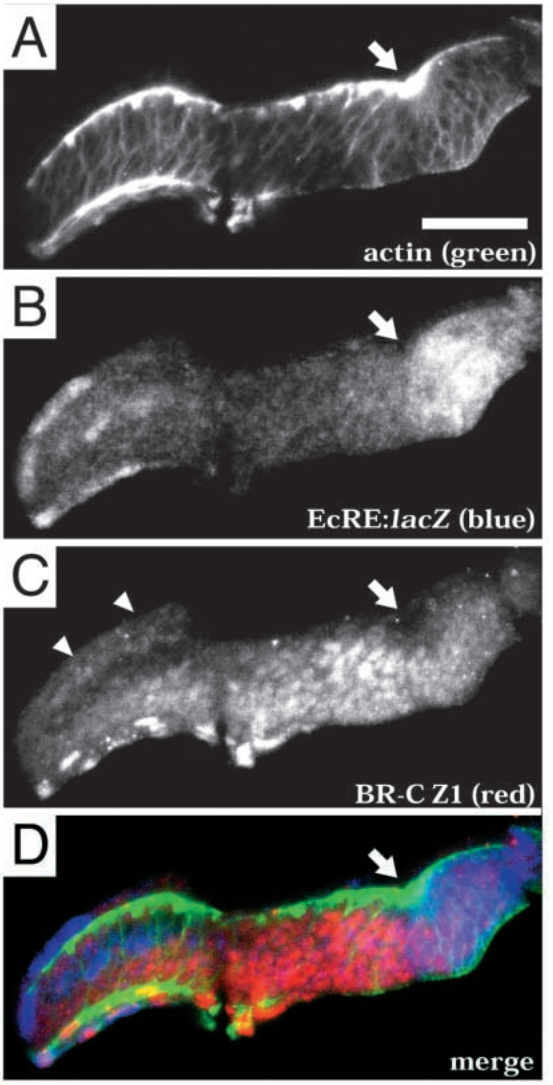

Fig. 7. BR-C Z1 expression. Third instar eye imaginal discs carrying the EcRE:*lacZ* construct shown in cross-section. (A) Cytoplasmic actin; (B) β-galactosidase; (C) Z1 isoform of Broad-Complex; (D) merge of A-C with actin in green, β-galactosidase in blue and BR-C Z1 in red. Arrowheads in C indicate BR-C Z1 staining in the peripodial membrane. Arrows indicate the furrow. Scale bar: 50 μm. Anterior on the right. Note that while EcRE-driven β-galactosidase is expressed just ahead of the furrow, BR-C Z1 is expressed slightly later, just behind the furrow.

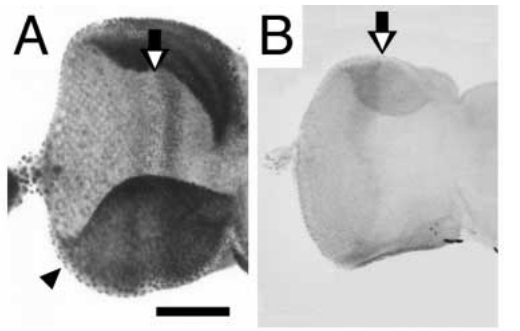

Fig. 8. BR-C Z1 expression in the eye is greatly reduced when ecdysone is withdrawn. Third instar eye imaginal discs stained with antibody that recognizes Broad Complex isoforms carrying Z1 zinc fingers. (A) Wild-type; (B) *ecd-ts*. Arrowhead in A indicates staining in the peripodial membrane. Arrows indicate furrow. Scale bar, 50 μm. Anterior to the right.

ecdysone, the Ecdysone Receptor is activated anterior to the furrow and stimulates the transcription of *BR-C*, and perhaps other early genes. The immediate transduction of the signal most likely occurs through a hierarchy of response similar to that discovered through the puffing response involving the sequential activation of numbers of transcription factors (Thummel, 1996). These transcription factors then activate genes directly involved in furrow progression. It is likely that there are multiple targets of such transcription factors in the eye disc (in the salivary glands there are over 100 late genes, Thummel, 1996). Although the ecdysone signal may first be received in cells anterior to the furrow, any delay in transduction of the signal (such as the delayed maximal induction of BR-C Z1) would mean that the cells would be behind the furrow by the time some of the effects on transcription of genes directing eye development were exerted. Loss of ecdysone, then, fails to stimulate the ecdysone response genes, and consequently genes needed for furrow progression and eye morphogenesis are not transcribed, and the furrow arrests. The defects in ommatidial assembly, morphology and rotation that are apparent in *ecd-ts* discs (Fig. 2B,D), and also in *npr-1* discs (Fig. 9), are irregular and variable, and we think that they may not be direct effects of loss of ecdysone or of BR-C function, but rather secondary consequences of a severe disruption of eye development that accrue during the 24 hour temperature shift.

Two lines of evidence suggest that *hedgehog* may be one target of ecdysone-dependent transcriptional regulation in the eye. Although the normal range of Hedgehog expression is wider than that of Atonal (about 10 rows visible by antibody staining, data not shown) and starts about 2 rows posterior to the furrow, approximately where Ato is lost from R8 cells, Hedgehog expression is not lost sequentially row by row, but appears to be reduced all at once. *ecd-ts* animals, which still retain residual R8 Ato staining in one disc, have lost most or all Hh in the contralateral disc. This, however, does not represent a failure in transition from the proneural (Ato-expressing) to neural fate, since neural antigens Elav and 22C10 are expressed in abundance in photoreceptor precursors behind the furrow. Indeed, abrupt loss of Hh could explain the progressive loss of Ato starting from the posterior, because without the Hh signal, cells anterior to the furrow might not be induced to enter the proneural phase of differentiation.

Also consistent with the possibility of regulation of *hh* by ecdysone-responsive transcription factors is the timing of the expression of EcRE:*lacZ*. Furrow initiation and progression through the first several columns are controlled by different signaling pathways than furrow progression beyond this point; initiation is *dpp*-dependent, whereas *hh* propels the furrow after about the first ten columns (Ma et al., 1993; Wiersdorff et al., 1996; Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997). EcRE:*lacZ* expression also becomes associated with the furrow at about the same stage as the furrow is thought to come under control of *hh*. We do not propose that *hh* regulates

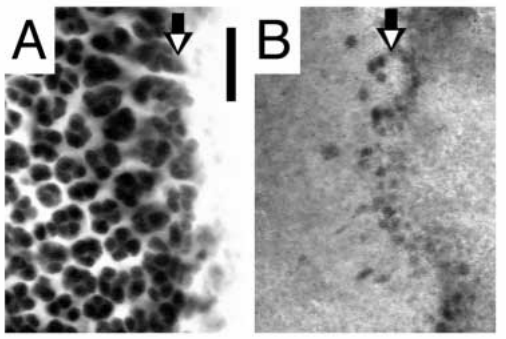

Fig. 9. Broad Complex mutants display furrow phenotypes. Third instar discs hemizygous for *npr-1* null mutation in *BR-C*. (A) Elav; (B) Atonal. Scale bar, 10 μm. Anterior to the right. Note that discs mutant for *BR-C* have phenotypes with some similarities to discs that have lost ecdysone (compare with Fig. 2B,H).

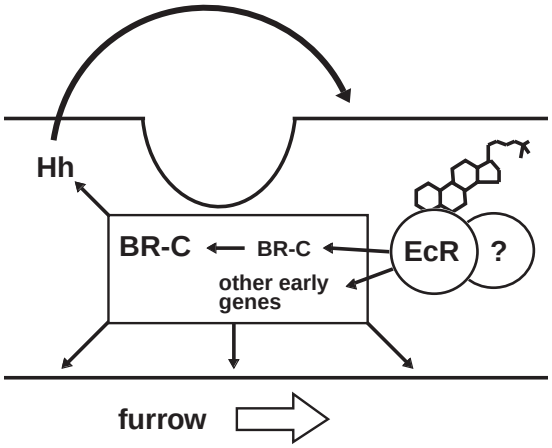

Fig. 10. Model for the action of ecdysone at the morphogenetic furrow. The functional ecdysone receptor, with the EcR protein and transcriptional co-activator, is complexed with hormone and activates transcription ahead of the furrow. Immediate targets include *BR-C* and possible other 'early genes'. BR-C expression does not reach maximal levels until behind the furrow, possibly because of post-transcriptional regulation or autoregulation. The early gene products then activate transcription of a number of targets in cells both ahead of, in and behind the furrow. This includes regulation of *hh* in cells behind the furrow. In the absence of ecdysone, the early genes are not activated, and genes needed for furrow progression are not transcribed: furrow progression fails. Anterior to the right.

EcRE:*lacZ*, but rather that the timing of some aspects of ecdysone signaling may coincide with this stage of furrow progression. Our finding that the sensitivity of furrow progression to ecdysone titer begins earlier than the association of EcRE:*lacZ* expression with the furrow may suggest that integration of the ecdysone signal into mechanisms driving furrow progression is achieved by different means early and late in progression, or that EcRE:*lacZ* is not revealing all phases of ecdysone-dependent transcription in the eye disc, a phenomenon reported in other tissues (see Results section and Antoniewski et al., 1996).

Spatial restriction of ecdysone-dependent activity in the eye

Both EcR (see above) and its dimerization partner Usp (Zelhof et al., 1997) have uniform distributions in the eye imaginal disc. It is not clear how a diffusible hormone such as ecdysone could be spatially restricted. What, then, could account for the furrow-specific activity that we see both with a reporter of the ecdysone response element and in the expression pattern of BR-C, which is regulated by ecdysone? One possibility is that a uniformly distributed hormone precursor (such as ecdysone itself) could be locally modified to a more active form (such as 20-hydroxyecdysone) to produce a spatially restricted elevated level of pathway signaling. A second possibility is that a prepattern of proteins in the disc localizes the ecdysone response, much as pre-existing factors in cells determine what the nature of the molting response to a given ecdysone pulse will be (Woodard et al., 1994; Thummel, 1996).

The marked differences in degree of tissue organization ahead of and behind the furrow lead us to predict that the mechanisms restricting anterior and posterior ecdysone responsiveness posteriorly will be different. Several mechanisms of negative regulation of nuclear hormone receptors are known to act at multiple levels. For example, some orphan nuclear receptors appear able to compete for DNA-binding sites with EcR-Usp dimers or titrate away either Usp or EcR, preventing their heterodimerization and ability to activate transcription in the presence of ecdysone (Thummel, 1995; White et al., 1997). Seven-up (Svp) is one such protein and is suspected of forming an inactive heterodimer with the Ecdysone Receptor (Zelhof et al., 1995). Svp is expressed in the eye disc behind the furrow where it is required for correct cell fate specification of the photoreceptors in each cluster (Mlodzik et al., 1990; Hiromi et al., 1993). Since it is only expressed in a subset of the cells in each cluster (photoreceptors R1, R3, R4, R6), Svp could not inhibit EcR/Usp dimerization in all of the cells in the posterior field, but perhaps it represents one component of such a mechanism. Another newly discovered class of negative regulators includes the SMRT and NCoR proteins, identified in 2-hybrid screens with vertebrate nuclear receptors (Chen and Evans, 1995; Hörlein et al., 1995). These proteins bind to DNA-bound unliganded receptors and actively interfere with transcription, thus transforming the receptors into repressors. While *Drosophila* homologs of these proteins have not yet been reported, such proteins could play a role in spatial restriction of ecdysone activity in the disc when ecdysone titer is at the low levels that precede the late third instar pulse.

It has been reported that the EcR dimerization partner Usp normally acts to limit the rate of furrow progression: *usp* loss-of-function mosaic clones show a slight acceleration of furrow progression (Zelhof et al., 1997). Loss of function of ecd^1 and *BR-C* have the opposite effect, namely to arrest the furrow. We propose, then, that Usp does not act directly in the pathway of ecdysone control of furrow progression, further suggesting that EcR may have other dimerization partners in the eye disc.

Within most tissues examined, Broad-Complex proteins are expressed uniformly within a given tissue type at a particular time (Emery et al., 1994). The only reported exceptions to this are in the eye imaginal disc described here and in oocyte follicle cells (Deng and Bownes, 1997). The Z1 isoform of BR-C is expressed early in oogenesis in all follicle cells but, by stage 10 of oogenesis, is restricted to two groups of lateral-

dorsal-anterior cells that will make the dorsal appendages. The early widespread expression may be ecdysone-regulated, but the later spatially restricted phase is regulated by the Egfr/Ras and Dpp/BMP signaling pathways. Similar signaling molecules are highly active in regulating both furrow progression and ommatidial assembly in the eye, thus raising the intriguing possibility of complex feedback interactions between ecdysone signal-transducing proteins and cell signaling functions at the furrow to localize the ecdysone response. Additionally, eye imaginal discs have been reported to have significantly lower levels of *BR-C* transcripts than other discs (Bayer et al., 1996), which may now be seen to be due to spatial restriction of its expression within the eye disc.

Ecdysone control of furrow progression

Our results raise the question of why the progression of the furrow should be under the control of ecdysone. A previous study found that the furrow accelerates up to 65% in the late stages of progression, a phenomenon that could reflect sensitivity to ecdysone titer (Basler and Hafen, 1989). What adaptive value could this sensitivity to ecdysone concentration have for furrow progression in *Drosophila* if, when the ecdysone titer drops, the furrow stops irreversibly and the eye is severely malformed? One role for ecdysone that has not been reported in *Drosophila*, but has been in other dipterans, is in mediating entrance and exit from pupal diapause, a period of arrested development that permits insects to survive adverse environmental conditions (Nijhout, 1994). It is possible that the sensitivity to ecdysone reflects an ability in some ancestral species to arrest the furrow and then restart it in the context of a diapause. Indeed, such an ability has been reported in *Manduca sexta* (Champlin and Truman, 1998). The retention of such a sensitivity in a modern *Drosophila* species could be due to lack of selection against it, to a practical difficulty in uncoupling one component of a regulatory system from another and disabling it, or to an unreported ability of *Drosophila* to enter diapause under non-laboratory conditions.

Several experimental advantages of *Drosophila* have permitted extensive investigation of the hormonal regulation of development. Studies on puffing patterns in polytene chromosomes revealed a complex hierarchy of gene response to the hormone ecdysone, and genetic and molecular approaches have permitted elucidation of many of the molecules and interactions that confer temporal and tissue specificity of response. The discovery of ecdysone activity in the developing eye offers two additional advantages. Because the knowledge of genetic networks controlling eye development is extensive, the eye is an excellent system for the study of the integration of hormonal signals into tissue-specific developmental pathways. Furthermore, the re-iterative nature of eye development in *Drosophila* translates temporal patterns of gene expression into spatial patterns, which both permits easier visualization of hierarchies and facilitates mosaic analysis.

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