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Broad-complex, but not *Ecdysone receptor*, is required for progression of the morphogenetic furrow in the *Drosophila* eye

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SUMMARY

The progression of the morphogenetic furrow in the developing *Drosophila* eye is an early metamorphic, ecdysteroid-dependent event. Although *Ecdysone receptor*-encoded nuclear receptor isoforms are the only known ecdysteroid receptors, we show that the *Ecdysone receptor* gene is not required for furrow function. *DHR78*, which encodes another candidate ecdysteroid receptor, is also not required. In contrast, zinc finger-containing isoforms encoded by the early ecdysone response gene *Broad-complex* regulate furrow progression and photoreceptor specification. *br*-encoded *Broad-complex* subfunctions are required for furrow progression and proper R8 specification, and are antagonized by other subfunctions of *Broad-complex*. There is a switch from Broad complex Z2 to Z1 zinc-finger isoform expression at the furrow which requires Z2 expression and responds to Hedgehog signals. These results suggest that a novel hormone transduction hierarchy involving an uncharacterized receptor operates in the eye disc.

Key words: *Drosophila*, Ecdysone, Morphogenetic furrow, BR-C, EcR

INTRODUCTION

Hormones play essential roles in the development of many animals, particularly during postembryonic developmental changes, such as amphibian and insect metamorphosis, and mammalian puberty. While DNA-binding nuclear receptors have been characterized for many steroid hormones, our understanding of how steroid hormone signaling directs tissues to differentiate remains rudimentary.

In *Drosophila* the ecdysteroids co-ordinate and trigger the developmental events associated with molting and metamorphosis. Ecdysone is released from the ring gland in pulses during development, and converted to 20-hydroxyecdysone (20-HE) in the fat body and target tissues (Riddiford, 1993). Pulses of ecdysteroids at the end of larval life and during pupal development direct many larval tissues to die (Jiang et al., 1997), others to remodel (Levine et al., 1995), and imaginal tissues to undergo proliferation, morphogenesis and differentiation (Fristrom and Fristrom, 1993).

A heterodimeric nuclear receptor complex for 20-HE is composed of the Ecdysone receptor (EcR) and Ultraspiracle (Usp); the EcR/Usp dimer can bind 20-HE-responsive regulatory DNA sequences, repressing transcription in the absence of hormone, and activating it in the presence of 20-HE (Cherbas et al., 1991; Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1993). Although null *EcR* or *usp* mutants die as embryos or young larvae (Bender et al., 1997; Perrimon et al., 1985), the essential roles of these genes at metamorphosis have been demonstrated by rescuing mutants through early lethal phases with inducible transgenes; both *usp* and *EcR* third instar mutants fail to execute the normal program of metamorphosis (Hall and Thummel, 1998; Li and Bender, 2000). Usp, an RXR homolog, is thought to be able to dimerize with multiple steroid receptors (Oro et al., 1990; Sutherland et al., 1995; Zelhof et al., 1995a).

Ashburner and colleagues proposed that ecdysone signaling directly activates a set of early puffs or genes. These early genes were proposed to encode factors that transduce the ecdysteroid signal and impose a temporal sequence of response by activating other, late, genes as well as regulating their own transcription (Ashburner et al., 1974). In support of this model, the early gene *Broad complex* (*BR-C*; *br* – FlyBase) was found to be required for the expression of late genes as well as for metamorphic events in tissues other than the salivary glands (Belyaeva et al., 1980; Belyaeva et al., 1981; Stewart et al., 1972). The early genes at the *E74*, *E75* and *BR-C* loci all encode transcription factors, and have mutant phenotypes consistent with their roles as transducers of ecdysone signals (Burtis et al., 1990; Buszczak et al., 1999; DiBello et al., 1991; Fletcher et al., 1995; Fletcher and Thummel, 1995; Kiss et al., 1988; Segraves and Hogness, 1990).

The *BR-C* plays a key role in directing the appropriate, stage-specific responses to hormone signaling at pupariation, and may provide competence to cells to respond to ecdysone signals (Karim et al., 1993). *BR-C* encodes a family of

transcription factors that all possess one of four possible alternative pairs of zinc fingers (Z1-Z4), and a BTB-POZ protein interaction domain (Bardwell and Treisman, 1994; Bayer et al., 1996a; DiBello et al., 1991; Zollman et al., 1994). *BR-C* null mutants (*non-pupariating*; *npr1*, that lack all BR-C protein isoforms) are unable to pupariate, and die after a prolonged third instar, whereas mutants lacking isoform subgroups die as pupae (Kiss et al., 1988; Stewart et al., 1972). In mid and late third instar, activation of *BR-C* is less dependent on EcR and Usp function than are *E74* and *E75* (Bender et al., 1997; Hall and Thummel, 1998). In addition, *BR-C* function is required for maximal induction of *E74* and *E75* (Belyaeva et al., 1981; Karim et al., 1993; Zhimulev et al., 1982), whereas *BR-C* is not reciprocally regulated by *E74* (Fletcher and Thummel, 1995).

There is evidence to support a novel hormone response pathway in the mid-third instar. This includes the moderate transcriptional induction of *EcR*, *E74B* and *BR-C* in a *usp*-independent manner (Andres et al., 1993; Hall and Thummel, 1998; Huet et al., 1993; Thummel, 1996; von Kalm et al., 1994). The DHR78 steroid receptor (Hr87 – FlyBase) may act in this hierarchy; it is widely expressed at this time, and binds to many early puffs (Fisk and Thummel, 1995; Fisk and Thummel, 1998; Zelhof et al., 1995b). *DHR78* mutants fail to exhibit the characteristic mid-third instar pattern of hormone-dependent gene activity. Ectopic expression of DHR78 is not detrimental, suggesting that its activity may be regulated by a ligand (Fisk and Thummel, 1998).

One dramatic consequence of ecdysteroid signaling at metamorphosis is the transformation of the imaginal discs into the epidermis and peripheral nervous system of the adult fly. During pupal development, discs evert, elongate and terminally differentiate in an ecdysteroid-dependent manner (Fristrom and Fristrom, 1993). *EcR*, *usp* and *BR-C* mutants all show defects in disc eversion and elongation (Fristrom et al., 1981; Hall and Thummel, 1998; Kiss et al., 1988; Li and Bender, 2000).

Prior to overt morphogenesis, imaginal discs undergo patterning and specification of neuronal elements (Cohen, 1993). For example, in the third instar eye disc, retinal specification begins at the posterior margin and expands anteriorly, with the addition of new rows of photoreceptor clusters every two hours (Wolff and Ready, 1993). Associated with the anterior boundary of retinal patterning are a synchronized cell cycle arrest in G1 (Thomas et al., 1994; Wolff and Ready, 1991), relaxation of heterochromatic gene silencing (Lu et al., 1998), and a coordinated apical-basal contraction of cells that produces the indentation known as the morphogenetic furrow (Ready et al., 1976). The expression of Hedgehog (Hh) and possibly Decapentaplegic (Dpp) signaling proteins posterior to and in the furrow, respectively, drive the furrow anteriorly at a controlled pace (Curtiss and Mlodzik, 2000; Heberlein and Treisman, 2000).

Such early neuronal differentiation in imaginal discs is also under the control of ecdysteroids. Reduction of ecdysteroid titer in the *Drosophila* eye disc in vivo with the ecd^{ts} mutation results in irreversible furrow arrest and ommatidial disarray (Brennan et al., 1998). 20-HE also sustains furrow progression in vitro (Li and Meinertzhagen, 1995). The travelling furrow is associated with the localized expression of a reporter of ecdysteroid-dependent gene activity and of Broad-complex proteins (Brennan et al., 1998). In the *Manduca* eye disc, minimal levels of either ecdysone or 20-HE are required to sustain furrow progression; below the threshold level, the furrow reversibly stops during diapause (Champlin and Truman, 1998b). In the *Drosophila* wing disc, the sensory precursor cells in the wing margin undergo differentiation and neurite outgrowth in response to 20-HE (Schubiger and Truman, 2000). Accompanying the onset of differentiation in imaginal discs is the relaxation of heterochromatic gene silencing in imaginal tissues (Lu et al., 1998). In the eye disc, this relaxation commences at the morphogenetic furrow, and in wing, leg and eye imaginal discs, this relaxation can be induced in vitro by 20-HE (Lu et al., 1998).

Recent evidence suggests that the molecular hierarchies that transduce ecdysteroid signals in discs at these early stages are distinct from those operating in larval tissues. In the wing disc, *usp* represses *BR-C* during the mid third instar (Schubiger and Truman, 2000); however, in the whole animal, no upregulation of *BR-C* is seen at any time (Hall and Thummel, 1998). This suggests that genetic interactions in imaginal discs may be masked in studies of whole animals by effects in larval tissues, which predominate by mass. The shared dependence among imaginal discs of neural differentiation and heterochromatic derepression on ecdysteroid signaling suggests that the eye disc may be a good model for steroid hormone signaling in differentiating tissues.

In this study we report whole disc and mosaic mutant analysis to investigate the requirements for *EcR*, *DHR78* and *BR-C* during late third instar eye development. We show that neither *EcR* nor *DHR78* is required for normal furrow progression or ommatidial assembly. In contrast, *BR-C*-encoded alternative zinc finger isoforms are differentially required in these processes. We also find evidence that the pattern of BR-C expression is affected by Hh signaling.

MATERIALS AND METHODS

Drosophila stocks and generation of clones

Clones were generated using EcR^{M554fs}, a null allele (Bender et al., 1997) by gamma-irradiation (1000R ^{137}Cs) at 24-48 hours after egg-laying. Disc clones were negatively marked with both arm-*lacZ* (at 51A) and EcR antibody stains, to confirm proximal mitotic recombination and to control for perdurance of EcR protein. Adult clones were negatively marked with a p(w^+) transgene at 47A. $DHR78^2$ clones were also generated by gamma-irradiation, and marked with arm-*lacZ* for disc clones, or a p(w^+) transgene at 70ºC for adult clones. Although $DHR78^2$ is a null allele with a stop codon in the ligand-binding domain (Fisk and Thummel, 1998), it produces non-functional protein that is detected by anti-DHR78 antibody. Therefore, clones could not be confirmed by loss of anti-DHR78 staining; however, in 12 clones examined, no ommatidial disruption was seen. *y w*; $EcR^{M554fs}/EcR^{V559fs}$; $hs\text{-}EcRB2^{30.1}$/+ larvae were rescued through the molt to the third instar by repeated heat shocks and then maintained at 18°C for 4 more days. EcR protein is undetectable 12 hours after heat shock (Li and Bender, 2000). Hemizygous $y\ npr1^3\ w$, $y\ br^5\ w$, $y\ rbp^5$ and $y\ 2Bc^1$ males were identified by their yellow mouthhooks and dissected. *BR-C* subfunction mutations used: $npr1^3$ is null for all *BR-C* proteins, rbp^5 disrupts Z1 isoforms, br^5 is null for Z2 isoforms and $2Bc^1$ is null for the Z3 isoforms (Bayer et al., 1997; Belyaeva et al., 1980; DiBello et al., 1991; Emery et al., 1994; Kiss et al., 1988; Stewart et al., 1972). *BR-C*, *smo Mad*, and *Pka* clones were generated by hsFlp-induced

FRT recombination (Xu and Rubin, 1993). *FRT18 arm-lacZ*; *MKRS hsFlp*/*TM6* males were crossed to *y npr1*^{3} *w FRT18*/*FM7*, *y br*^{5} *w FRT18*/*FM7*, *y rbp*^{5} *FRT18*/*FM7* and *y 2Bc*^{1}*FRT18*/*FM7* females. *smo*^{2} *Mad*^{B1} FRT40/CyO and *PKA*^{h2} FRT40/CyO males were crossed to *w* hsFlp; arm-lacZ FRT40/CyO females. Recombination was induced by 1 hour heat shocks 24-48 hours after egg-laying.

Histochemistry

Eye discs were fixed and stained as previously described (Brennan et al., 1998). Antibodies used: rabbit anti-β-galactosidase 1:2500 (CR7001RP2; Cortex Biochem); mouse anti-β-galactosidase 1:200 (Z378A, Promega); mouse anti-EcR 11D9.6 1:100 (Koelle et al., 1991); rat anti-Elav 1:150 (Bier et al., 1988); mouse anti-BR-C core 25E9 1:100, anti-BR-C Z1 3C11 1:5 and mouse anti-BR-C Z3 9A7 1:5 (Emery et al., 1994); rabbit anti-Atonal 1:1500 (Jarman et al., 1994); and mouse anti-Cyclin B 1:100 (Knoblich and Lehner, 1993). All secondary antibodies were from Jackson Laboratories and included: HRP-goat anti-rat IgG, rhodamine-donkey anti-rat, FITC donkey anti-mouse, Cy5-donkey anti-rat, Cy5-donkey anti-mouse, FITC-goat anti-mouse, TRITC-goat anti-mouse, Cy5-goat anti-rabbit, FITC-goat anti-rabbit, and LRSC-goat anti-rabbit. In situ hybridizations were performed as previously described (Mlodzik et al., 1990; Tautz and Pfeifle, 1989). Digoxigenin-labelled antisense RNA probes specific for Z1 and Z2 zinc finger-containing BR-C isoforms were transcribed from *Eco*RI-linearized pSP64-Z1 and *Sal*I-linearized pSP65-Z2 (Bayer et al., 1996a).

RESULTS

Neither *EcR* nor *DHR78* is required for furrow initiation or progression or ommatidial cluster assembly

We examined mosaic clones and entire eye imaginal discs lacking EcR function. *EcR* clones at the posterior margin of the eye disc and in the center of the eye disc were normal or showed very minor defects (Fig. 1A-F). This suggests that *EcR* is not required for the initiation of retinal differentiation at the posterior margin, for the anterior progression of the furrow, or for proper ommatidial assembly. The mosaic discs were stained with EcR antibody (Fig. 1B,E); lack of staining in the clones demonstrated that perdurance of EcR protein was not responsible for the lack of phenotype. We note that the EcR antigen did not appear to be nuclear, as one might expect. In these same specimens, the anti-Elav antibody was capable of showing the nuclear localization of Elav – thus it appears that the fixation and detergent conditions in this experiment do allow full penetration of the IgG primary and secondary antibodies. As details of the EcR stain differ from the anti-β-galactosidase stain, it appears that this stain is not simply bleed-through from the other channel. Thus, the experiment does show a cytoplasmic location for EcR in the eye disc. We have observed some cytoplasmic localization of this antigen before, in the embryo (Koelle et al., 1991), so this is not unprecedented.

Although six *EcR* clones both posterior and anterior (not shown) to the furrow were examined, no clones spanning the furrow were recovered. To confirm that *EcR* is not required for furrow progression, and to test the possibility that *EcR* may be non-autonomously required for early events in eye development, we examined eye discs from *EcR* homozygotes that had been rescued through the molt to the third instar by a *hs-EcR* construct. Such animals show delayed wandering behavior and pupariation failure, including the lack of anterior spiracle eversion and larval cuticle hardening, and the persistence of larval organs (Li and Bender, 2000). However, in these discs, anterior progression of retinal differentiation proceeded normally. Moreover, discs were seen in which the furrow had traversed the entire eye field, reaching the antennal boundary (Fig. 1G). This represents a late stage of eye disc development that is normally only reached following pupariation, and suggests that furrow progression is uncoupled from metamorphic requirements for EcR function.

Initiation of furrow progression at the posterior margin occurs prior to salivary chromosome puff stage 1 (Brennan et al., 1998), suggesting that the ecdysteroid control of furrow progression might be mediated by the alternative mid-third instar hormone transduction hierarchy. To test whether DHR78, which has been proposed to be the critical hormone receptor at this early stage (Fisk and Thummel, 1998; Zelhof et al., 1995b), mediates the ecdysteroid regulation of furrow progression, we examined *DHR78* mutant clones. However, they displayed normal furrow progression and ommatidial assembly (Fig. 1H,I).

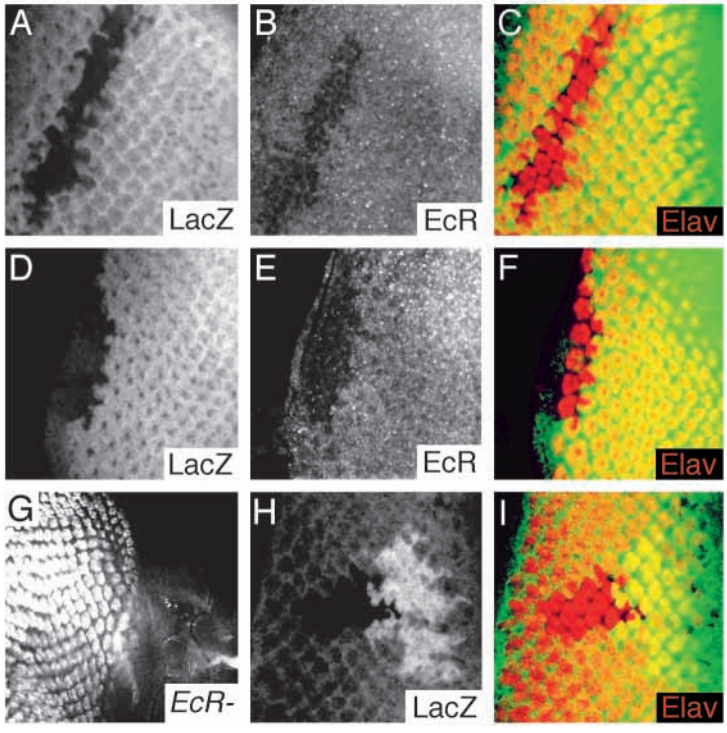

Fig. 1. Neither *EcR* nor *DHR78* is required for furrow progression or ommatidial assembly. Third instar eye imaginal discs. *EcR*^{M554fs} clones in the center of the eye disc (A-C) and at the posterior margin (D-F) are marked by loss of β-galactosidase (A,D and green in C,F) and EcR (B,E) antibody stains. Staining with an Elav antibody (red; C,F) reveals normal ommatidial morphology. An eye disc null for *EcR* (see Material and Methods) is stained with an Elav antibody (G) and shows normal ommatidial morphology, as well as advancement of the furrow to near the antennal disc boundary. A *DHR78*^{2} clone marked with β-galactosidase (H; green in I) also shows normal ommatidial morphology as seen with an Elav stain (red). Anterior is on the right.

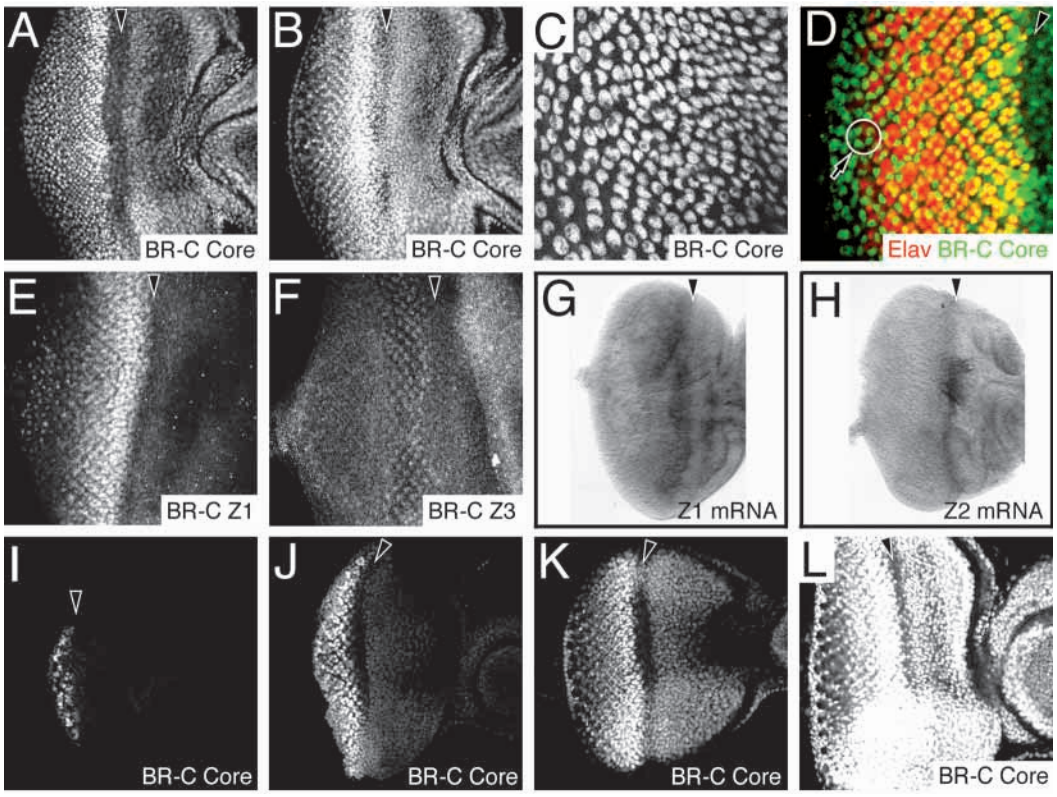

Fig. 2. *Broad-complex* expression in the eye disc. An antibody that recognizes all isoforms of Broad-complex stains the eye disc anterior in, and posterior to, the morphogenetic furrow (A,B). A and B are the same field; B is a more basal optical section to show staining in the furrow. The same antibody stains polytene nuclei in the peripodial membrane (C). Double staining of the disc with the same antibody (green) and Elav (red) in D shows that while BR-C is initially expressed in all cells in the furrow, it remains strongly expressed in photoreceptors as they differentiate. More posteriorlt, BR-C expression has diminished in photoreceptors, and is seen in the newly formed, non-Elav expressing cone cells (arrow and circle in D). An antibody that recognizes Z1-containing isoforms of BR-C is restricted to cells posterior to the furrow (E), while an antibody that recognizes Z3 isoforms lightly stains the whole disc, with slight increases in expression in newly formed photoreceptor cells (F). In situ hybridization confirms that Z1 isoforms are expressed posterior to the furrow (G), and that the BR-C expression anterior to the furrow is represented by Z2 isoforms (H). Furrows in G,H marked with arrowheads. (I-L) A time series of BR-C expression. Discs of a range of ages were stained together with the common BR-C antibody and confocal images were collected under identical conditions. BR-C expression travels with the furrow, and increases dramatically late in the third instar, corresponding to the time of the late larval ecdysteroid pulse. Anterior on the right. Arrowheads show the position of the furrow.

A BR-C isoform switch at the furrow

Despite this demonstrated lack of requirement for known or candidate ecdysteroid receptors in transducing the hormone requirement for furrow function, previous evidence suggested that the early ecdysone response gene *Broad-complex* (*BR-C*) plays a role in this process. Broad-complex proteins are expressed at the furrow in an *ecd*-dependent manner, and discs null for all *BR-C* function display defects in furrow progression and ommatidial organization (Brennan et al., 1998).

We characterized the expression pattern in the eye disc of the different zinc finger-containing BR-C isoforms using antibody and mRNA probes (Fig. 2). An antibody that recognizes all isoforms (‘core antibody’) stained cells both in and flanking the furrow, with a peak of expression just posterior to the furrow (Fig. 2A,B). BR-C is highly expressed in the nuclei of cells in the peripodial membrane (Fig. 2C). BR-C proteins appeared to be expressed in cells until they differentiated; immediately posterior to the furrow, Elav-expressing differentiating photoreceptors express BR-C, whereas more posteriorly, expression is stronger in cone cells (Fig. 2D). Staining with an antibody that detects only Z1-containing isoforms of BR-C was restricted to posterior to the furrow (Fig. 2E), and a Z3-specific antibody stained the entire disc at very low levels, with slight upregulation in photoreceptors posterior to the furrow (Fig. 2F). Although no Z2-specific antibody is available, we reasoned that the staining anterior to the furrow seen with the core antibody might be represented by Z2-containing isoforms. This was confirmed by in situ hybridization using probes specific for the Z1 and Z2 zinc fingers (Fig. 2G,H). Z2 mRNA was detected anterior to the furrow, followed by Z1 posterior to the furrow. A switch in BR-C isoforms expression from Z2 forms to Z1 forms around the time of pupariation in imaginal discs has been described (Emery et al., 1994). Our results show that this switch from larval to prepupal forms is precocious and asynchronous in the eye disc, and is associated with the morphogenetic furrow as it traverses the disc. Although levels of BR-C expression increase dramatically during the last few hours of the third instar, corresponding to the late larval ecdysone pulse, BR-C expression still remains highest near the furrow (see time series in Fig. 2I-L).

npr1 and *br* are required for proper ommatidial assembly

Bar is a dominant mutation causing premature arrest of the furrow, which results in the deep anterior nick in the adult eye (compare wild type in Fig. 3A with +/Bar in Fig. 3B; Kojima et al., 1991). As *Bar* has the dominant effect of stopping the furrow early, one might expect loss-of-function mutations at other loci that normally act to promote furrow progression to be genetic enhancers of *Bar* and loss-of-function mutations in genes that normally antagonize the furrow to act as genetic

suppressors of *Bar*. Thus, we chose to examine genetic interactions between *BR-C* sub loci and *Bar*.

We found that mutants defective for different *BR-C* subfunctions displayed unexpected heterogeneity in their genetic interactions with *Bar*, suggesting that the role of the *BR-C* in the regulation of furrow function might be complex. *BR-C* has several recessive lethal complementation groups that correspond to mutations that remove the function of all or individual zinc finger-containing isoforms subgroups. *npr1* mutations lack all function, whereas *rbp*, *br* and *2Bc* mutant groups correspond to the loss of Z1-, Z2-, and Z3-containing isoforms, respectively (see Table in Fig. 3; Bayer et al., 1997; Crossgrove et al., 1996; DiBello et al., 1991; Emery et al., 1994; Liu and Restifo, 1998; Sandstrom et al., 1997). Both *npr1/Bar* and *br/Bar* eyes were significantly smaller than *+/Bar* (Fig. 3C,D), indicating a dominant enhancement of the *Bar* furrow-stop phenotype, consistent with the earlier reports that the *BR-C* was required for furrow progression (Brennan et al., 1998). However, *br/Bar* eyes were smaller than *npr1/Bar*, suggesting that the *BR-C* might encode isoforms that act antagonistically during furrow progression, so that the effect of losing isoforms that positively regulate furrow progression is more severe than losing all isoforms. This idea is supported by the observation that *rbp/Bar* and *2Bc/Bar* eyes are larger than *+/Bar*, suppressing the phenotype, and possibly representing furrow-antagonistic functions of *rbp*- or *br*-encoded BR-C isoforms (Fig. 3E,F).

Hemizygous males of all *BR-C* mutant groups survived through the third instar, and the eye discs of these males displayed defects consistent with the genetic interactions with *Bar*. *npr1*/Y discs showed ommatidial disorganization, and signs of furrow failure, including mature ommatidial clusters at the furrow (Fig. 3G, Brennan et al., 1998). *br*/Y discs showed a much more dramatic failure of furrow progression, as well as ommatidial disorganization (Fig. 3H). *rbp*/Y and *2Bc*/Y discs did not show any defects (Fig. 3I,J). We note that while *rbp* does interact strongly with *Bar* it shows no eye disc defect alone – it may be that *rbp* is a redundant function.

br impairs furrow progression more severely than *npr1*; both required for R8 patterning

Disc clones lacking individual *BR-C* subfunctions were generated to further characterize the mutant phenotypes (Fig. 4). Both *npr1* and *br* clones displayed defects in ommatidial organization, including the wrong number of photoreceptors in clusters (Fig. 4A,B). *rbp* and *2Bc* clones appeared normal (Fig. 4C,D).

To investigate the stages at which *BR-C* function is required for normal third instar eye development, we examined mosaic clones spanning the morphogenetic furrow (Fig. 5). In contrast to *br* clones, which were frequently associated with some slowing of furrow progression (Fig. 5B), none of over fifteen *npr1* furrow-spanning clones was associated with any retardation of the furrow (Fig. 5A). This is consistent with the hemiygous disc phenotypes that showed a more severe furrow effect in *br* than *npr1* mutants (Fig. 3). Neither *npr1* (not shown) nor *br* (Fig. 5C) clones at the posterior margin of the disc showed any visible defects in furrow initiation which is consistent with the lack of requirement for *ecd* function for furrow initiation (Brennan et al., 1998).

Both *npr1* and *br* clones were associated with ommatidial disarray, and were indistinguishable in this regard. Consistent with this, both *npr1* and *br* clones were defective in the specification of the founding R8 photoreceptors, as shown by Atonal expression (Fig. 5D-I). *atonal* is the proneural gene for photoreceptor cells, and is first expressed in a broad equivalence group of cells anterior to the furrow, and subsequently restricted to expression in the R8 founder photoreceptors (Jarman et al., 1994). A complex network of inductive and inhibitory signals mainly involving the Notch pathway specifies the R8 cells and ensures their correct spacing (Baker et al., 1996; Cagan and Ready, 1989). In both *npr1* and *br* clones, clusters of three Atonal-positive R8 cells were seen.

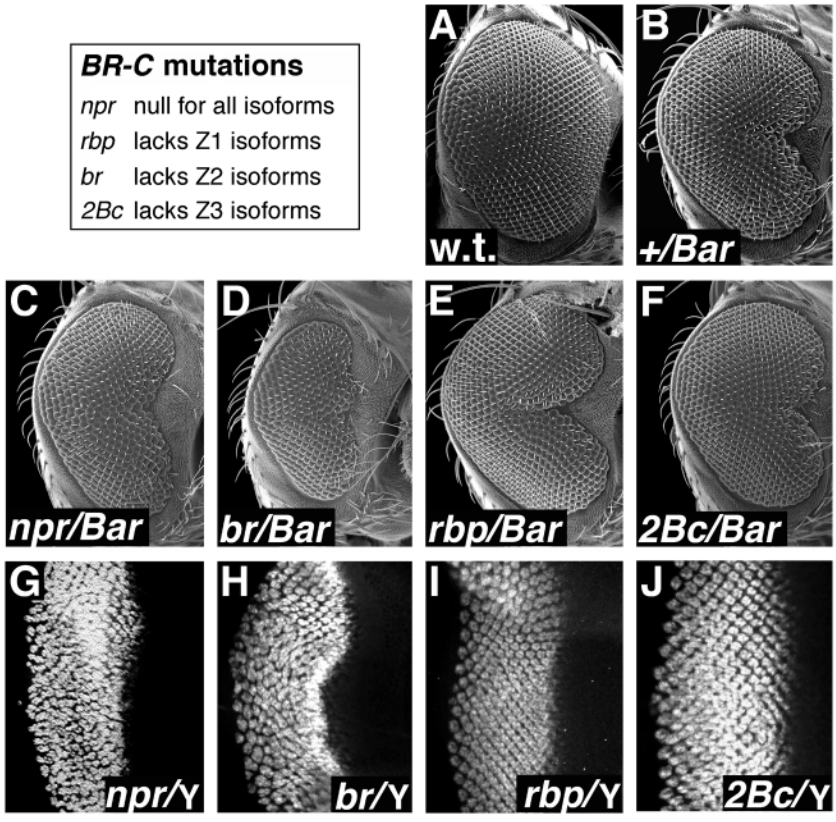

Fig. 3. *BR-C* subfunctions differentially affect furrow function.
(Top left) Correspondence between the genetic subfunctions of *BR-C* and protein isoforms; references in Materials and Methods. (A-F) Scanning electron micrographs of eyes from females of the following genotypes: (A) wild-type; (B) +/FM7 *B*; (C) $npr1^{3}$/FM7 *B*; (D) br^{5}/FM7 *B*; (E) rbp^{5}/FM7 *B*; (F) $2Bc^{1}$/FM7 *B*. *br* dominantly enhances the *Bar* phenotype to a greater degree than does *npr1*; *rbp* and *2Bc* both appear to repress *Bar.* Note that A-F are all to the same scale. (G-J) Third instar eye imaginal discs of males hemizygous for the same alleles of *npr1*, *br*, *rbp* and *2Bc*, stained for Elav. *npr* shows mild signs of furrow failure, while *br* discs are more severely affected. *rbp* and *2Bc* discs look normal. Anterior on the right.

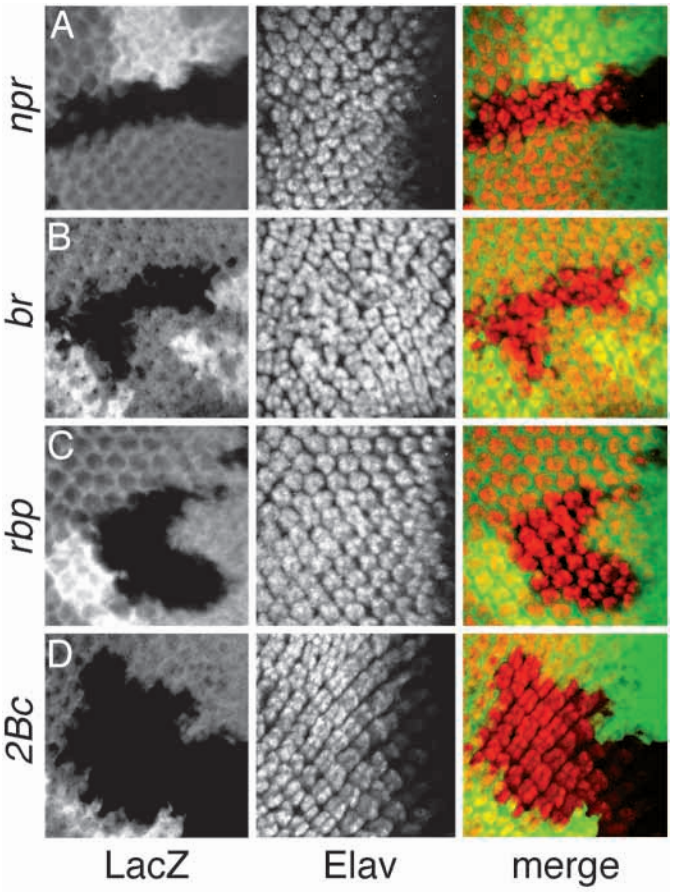

Fig. 4. *npr* and *br* are required for proper ommatidial morphology. Mutant clones for (A) $npr1^3$, (B) br^5, (C) rbp^5 and (D) $2Bc^1$ are marked with β-galactosidase (left and green in merge on the right) and stained with Elav antibody (middle panel and red in merge on the right). Both *npr1* and *br* clones display disrupted ommatidial morphology, including clusters with too many and two few photoreceptors. *rbp* and *2Bc* clones show no defects. Anterior on the right.

This suggests a failure in the lateral inhibition mechanisms that pattern these founder cells.

The cell cycle is influenced by steroid hormones, including the insect ecdysteroids (Champlin and Truman, 1998a). Cell cycle control in the *Drosophila* eye disc is essential for the orderly specification of the retinal array; the synchronous cell cycle arrest in G1 in the furrow is necessary for proper cell-cell communication (Thomas et al., 1994; Wolff and Ready, 1993). Dpp, produced from cells in the furrow, is thought to regulate cell cycle synchronization in cells anterior to the furrow (Horsfield et al., 1998; Pignoni and Zipursky, 1997). Cyclin B is expressed in cells at the G2/M transition (Edgar and Lehner, 1996). Normally, Cyclin B is expressed in unsynchronized cells far anterior to the furrow, turned off just anterior to and in the furrow and reactivated in a tight band of cells posterior to the furrow (Thomas et al., 1994; Wolff and Ready, 1991). We assessed Cyclin B expression in *br* clones. In a long narrow *br* clone that extends across the furrow, there was a delay in cessation of Cyclin B expression anterior to the furrow (Fig. 5J-L). However, close examination revealed that this was a non-autonomous defect; the greatest delay occurred just adjacent to the clone, in a region that is directly anterior to the section of the mutant clone that crosses the furrow. We stained one other *br* clone for Cyclin B that fell in this region of the eye disc and it showed an indistinguishable phenotype. This suggests that the delay in cell cycle synchronization anterior to the furrow is a secondary defect resulting from delays in events at the furrow, possibly including delayed production of Dpp.

BR-C isoform expression in mutant clones

br mutations lack the function of Z2 isoforms of the Broad-complex, which are expressed in and anterior to the furrow in the eye disc. This corresponds to the zone in which the earliest defects in clones are noted: defects in R8 photoreceptor patterning. To confirm the hypothesis that lack of Z2 isoform expression anterior to the furrow is the cause of the defects in *br* and *npr1* clones, expression of various BR-C isoforms was assessed in clones. As expected, in *npr1* clones, expression of all BR-C proteins was eliminated (data not shown). More surprising was the finding that *br* clones were unreactive to an antibody that recognizes all BR-C proteins (Fig. 6A). This suggests that the expression of Z2 isoforms anterior to the furrow is required for the subsequent expression of Z1 isoforms posterior to the furrow. Evidence of such positive autoregulation between Z2 and Z1 expression has been described (Bayer et al., 1996b; Emery et al., 1994; Karim et al., 1993). *rbp* clones also lacked expression of Z1 isoforms, as expected (Fig. 6C), yet have no defects in retinal specification (compare Fig. 6B,D), suggesting that the defects in *br* clones are due to the lack of Z2 expression, and not to the lack of Z1. Although *2Bc* clones do show Z3 protein (data not shown), this protein is presumably nonfunctional, as the $2Bc^1$ allele has been shown to be a genetic null (Emery et al., 1994).

Localized signals at the furrow and cellular differentiation state influence BR-C expression

Although *BR-C* expression is dependent on highly diffusible ecdysteroids, the pattern of *BR-C* expression in the eye disc is spatially restricted. To test the possibility that local signals that control the rate of furrow progression also influence the spatial domain of BR-C expression, we examined clones in which cells were unable to respond to the Hh and Dpp signals that drive furrow progression, or in which the Hh signaling pathway is constitutively activated (Curtiss and Mlodzik, 2000). *smoothened Mothers against dpp* (*smo Mad*) double mutant cells, which cannot transduce Hh or Dpp signals (Alcedo et al., 1996; van den Heuvel and Ingham, 1996; Wiersdorff et al., 1996), still expressed BR-C proteins near the furrow, showing that reception of these signals is not strictly required for BR-C expression (Fig. 7A-H). Aberrant nuclear migration patterns in *smo Mad* cells distorted the appearance of the BR-C staining pattern: basal optical sections showed that homozygous mutant cells resembled their heterozygous neighbors in BR-C expression levels (Fig. 7D). Neural differentiation was blocked in *smo Mad* clones (Fig. 7B,F); BR-C expression persisted in such clones far posterior to the furrow, suggesting a link between differentiation and cessation of BR-C expression (Fig. 7G,H). Constitutive activation of the Hh signaling pathway in *Pka* mutant eye disc cells generates ectopic *Mad*-dependent circular furrows radiating outwards (Pan and Rubin, 1995; Strutt et al., 1995; Wiersdorff et al., 1996). A *Pka* clone examined just posterior to the endogenous furrow showed ectopic expression

of Z1 isoforms anterior to the endogenous Z1 domain, surrounding the clone (Fig. 7I,J). This suggests that, although expression of BR-C proteins at the furrow does not require Hh or Dpp signaling, the timing of the switch from Z2 to Z1 isoforms is regulated, directly or indirectly, by these signals.

EcR and *BR-C* but not *DHR78* are required for later stages of eye development

To assess the requirements for *BR-C*, *EcR* and *DHR78* for the terminal differentiation that occurs during pupal development, we examined *white*-marked clones in the adult eye. *br* clones at the anterior margin of the adult eye showed a small nick and subtle disordering of the ommatidial array, defects that probably result from the furrow-associated defects described above (Fig. 8A). In contrast, *npr1* clones showed a scar, indicative of a requirement for non-*br*-encoded *BR-C* functions in later stages of maturation (Fig. 8B). Such defects often represent failure of differentiation, and subsequent death of the affected photoreceptors. *EcR* clones also produced scars (Fig. 8C). This suggests that *BR-C* might function downstream of *EcR* during later stages of eye development, or that the two genes function in parallel, regulating maturation events. Either model would be consistent with the demonstrated requirements for 20-HE for later events in eye morphogenesis (Li and Meinertzhagen, 1997). Homozygous loss-of-function clones of the repressor *dSin3A* also leave retinal scars (Neufeld et al., 1998), suggesting that the repressive functions of EcR may be important for proper morphogenesis. *DHR78* clones in the adult eye showed no defects (Fig. 8D).

DISCUSSION

We have shown that the ecdysteroid requirement for furrow progression and orderly ommatidial assembly in *Drosophila* eye development does not depend on the activity of two known steroid hormone receptors that are required for many of the events of metamorphosis, EcR and DHR78. Mutant *EcR* or *DHR78* clones show no defects in ommatidial specification, and eye discs from larvae lacking detectable EcR protein display normal furrow progression, although *EcR* is required for later events in eye development.

In contrast, we found that BR-C proteins, whose expression is lost in discs in which the ecdysteroid titer is reduced in vivo (Brennan et al., 1998), play important roles in third instar eye imaginal disc development. We have shown that *BR-C* function is not required for initiation of retinal patterning at the posterior margin, but is required to maintain furrow progression, for the appropriate specification of founding R8 photoreceptors in the furrow, for proper ommatidial assembly and for later maturation events. These requirements for *BR-C* are differentially met by distinct isoform-encoding genetic subfunctions of the gene. The furrow-promoting activity of *BR-C* is supplied by the *br* subfunction, which encodes Z2 isoforms. A furrow-repressing activity appears to be encoded by other *BR-C* subfunctions, as *npr1* (*BR-C* null) discs and clones show less severe impairment of furrow progression than *br*. We were not able to identify the furrow-antagonizing genetic subfunction of *npr1*; the lack of furrow acceleration in *rbp* (Z1-encoding) or *2Bc* (Z3-encoding) discs and clones may be due to redundancy with each other or

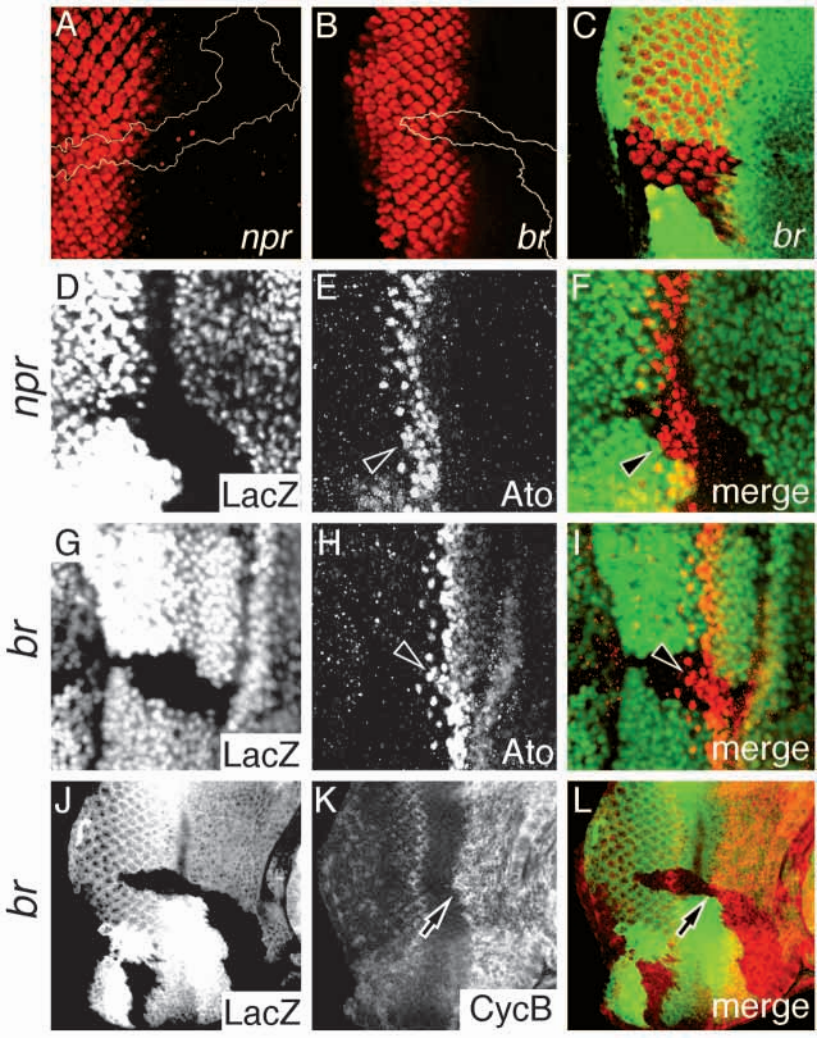

Fig. 5. *br* and *npr* functions are required for R8 specification, while only *br* functions are strongly required for furrow advancement. While no furrow-spanning *npr* clones were associated with retardation of neural specification (A), *br* clones frequently showed furrow delay (B). Clones are marked in white outline, Elav in red. Neither *npr* (not shown) nor *br* clones at the posterior margin (C) were associated with failure of photoreceptor development or furrow initiation. All clones were marked with *lacZ*, shown in green in C,F,I,L. *npr* and *br* clones were assayed for Atonal expression (red in F,I) and Cyclin B (red in L). Both *npr* (D-F) and *br* (G-I) clones showed defects in R8 specification, especially excess numbers of R8 cells in a cluster (arrowheads). A *br* clone (J-L) was associated with delays in patterns of Cyclin B expression, both in the band of expression posterior to the furrow and the cessation of expression anterior to the furrow. While the former is autonomous, the latter (arrow) is non-autonomous and likely results from delays in inductive events in the mutant tissue spanning the clone directly posterior. Anterior on the right.

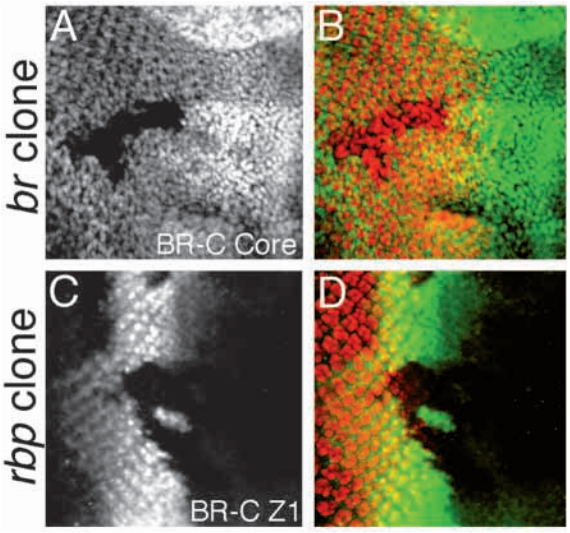

Fig. 6. BR-C isoform expression in mutant clones. (A,B) br^5 clone stained with antibody recognizing all forms of BR-C protein. Lack of staining indicates *br* clones lack both Z2 and Z1 isoforms. (C,D) rbp^5 clone stained with antibody recognizing Z1 isoforms of BR-C, shows lack of Z1 expression in clone. Elav stain (red) shows position of clone relative to furrow and indicates that lack of Z1 expression in *br* clone is not the source of ommatidial defects, because the *rbp* clone also lacks Z1, yet shows no defects. Anterior on the right.

other ecdysone-response genes, or may suggest there are other *BR-C* functions not represented by *br*, *rbp* or *2Bc*. *br* and *npr1* disc tissue was similarly impaired in proper R8 specification and subsequent ommatidial organization. Both *npr* and *br* tissue in the eye lack BR-C expression, as detected with the core antibody; the reason for the more severe furrow phenotype of *br* than *npr* is not known.

We found that a switch from Z2-containing to Z1-containing BR-C isoform expression occurs at the morphogenetic furrow. This isoform switch normally occurs in imaginal discs at pupariation, suggesting that the furrow may mark a general boundary between larval and prepupal patterns of gene expression. We also found evidence that expression of Z2 isoforms anterior to the furrow may be required for subsequent Z1 expression posterior to the furrow; *br* clones, whose primary defect is failure of Z2 isoform expression, also failed to express Z1 isoforms. The primary failure in *br* and *npr1* discs appears to occur in or anterior to the furrow, corresponding to the zone of Z2 expression, and is manifested as failure to properly specify R8 cells. Our result showing ectopic Z1 isoform expression near a *Pka* clone suggests that the switch from Z2 to Z1 isoforms may be influenced by furrow-associated expression of Hh and Dpp.

How is the ecdysteroid signal transduced during furrow progression?

We have found that EcR does not mediate the ecdysteroid requirement for morphogenetic furrow progression. The lack of a requirement for EcR in furrow progression is surprising for several reasons. EcR is expressed in the eye imaginal disc during the time of furrow progression (Fig. 1), and is required at this time in other tissues (Li and Bender, 2000). We had

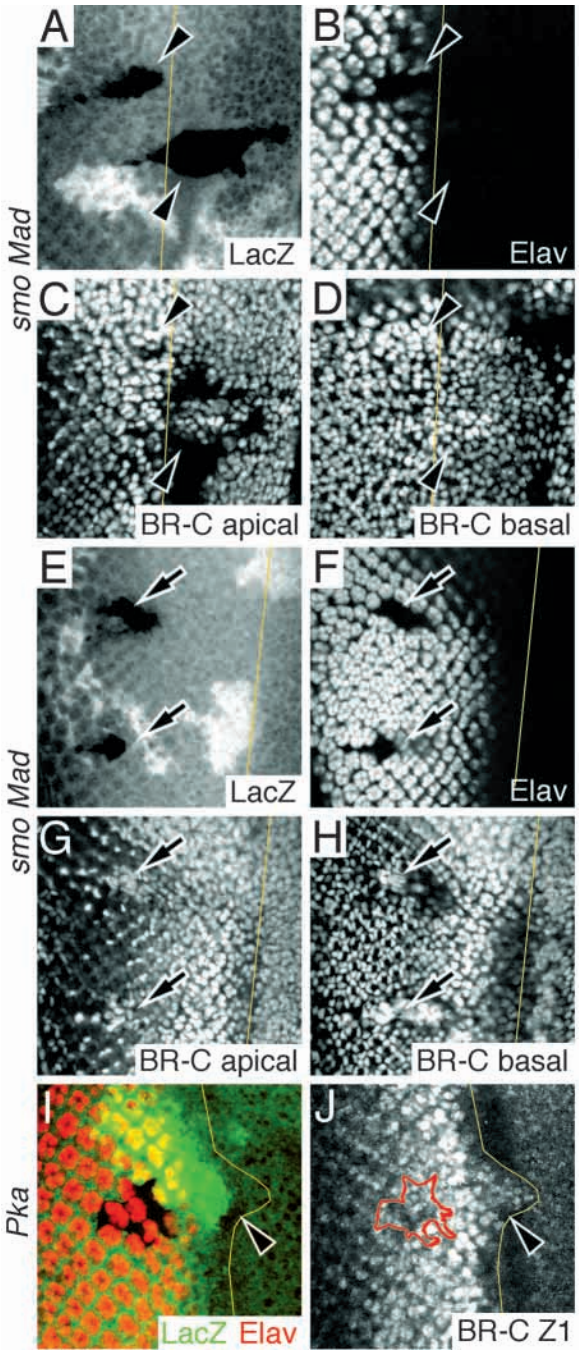

Fig. 7. Hh and Dpp signaling and state of differentiation affect BR-C expression. Approximate position of furrow indicated by yellow lines. Anterior on right. (A-D) Furrow-spanning smo^2 Mad^{B1} clones (arrowheads) show neural differentiation failure (B) and aberrant nuclear migration (compare apical and basal focal planes in C,D), but no reduced expression of total BR-C protein. (E-H) Cells in posterior smo^2 Mad^{B1} clones (arrows) have failed to differentiate (F), and show persistent BR-C staining. (I-J) PKA^{h2} clone has non-autonomously induced precocious expression of BR-C Z1 isoforms adjacent to clone (arrowheads).

previously found that a heptamer of Ecdysone Response Element (EcRE) sequences was able to drive expression of a reporter in the furrow (Brennan et al., 1998). Evidence

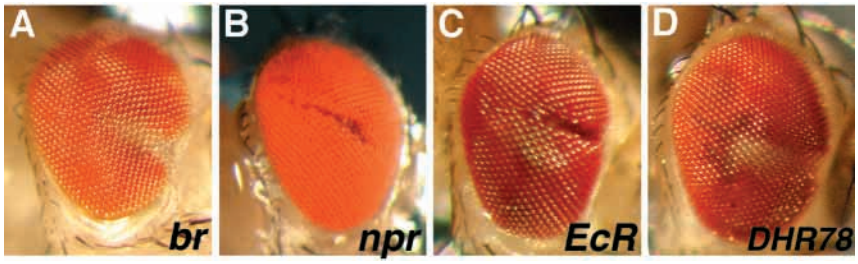

Fig. 8. *BR-C* and *EcR* are required for later events in eye development. *white*-marked clones of br^5 (A), $npr1^3$ (B), EcR^{M554fs} (C) and $DHR78^2$ (D). Anterior nick in *br* clone likely reflects furrow function, while scars in *npr1* and *EcR* clones probably represent pupal requirements. *DHR78* clone shows no defect. Anterior on the right.

suggesting that only the EcR/Usp dimer is able to activate transcription from EcREs includes that EcRE in Schneider cells is bound by a single complex that is destroyed by anti-EcR antibodies, EcR/Usp binds EcRE in the presence or absence of hormone, and transfection of EcR confers 20-HE responsiveness to cells carrying an EcRE reporter (Koelle et al., 1991; Yao et al., 1992). The lack of *EcR* requirement for furrow progression suggests either that a contribution by EcR to furrow regulation is masked by redundant contributions by another ecdysteroid receptor, or that an unidentified receptor that can also activate transcription from EcREs exclusively mediates the hormone regulation of furrow progression. Redundancy seems unlikely because loss of EcR function has strong phenotypes in almost all tissues where it is expected to function, including at the larval molts and at pupariation (Li and Bender, 2000). One candidate steroid receptor to mediate the hormonal regulation of furrow progression is DHR96; it is expressed during the mid-late third instar, and is able to bind EcREs in gel-shift assays (Fisk and Thummel, 1995).

The *EcR* clone phenotypes in both discs and adult eyes are distinct from those of *usp*, which are reported to produce furrow acceleration in the disc and abnormal rhabdomeres, but not scars, in the eye (Oro et al., 1992; Zelhof et al., 1995a). These results, along with earlier findings of distinct embryonic and early metamorphic phenotypes (Bender et al., 1997; Hall and Thummel, 1998; Li and Bender, 2000; Perrimon et al., 1985), suggest that EcR and Usp each have functions independent of the other.

Does the receptor that transduces the ecdysteroid requirement for furrow progression include Usp? *usp* retinal clones are reported to show variable degrees of furrow acceleration that correlate to some degree with stage of development; clones spanning furrows near the posterior margin show a more marked degree of furrow advancement than those examined at later stages (Zelhof et al., 1997). The ecdysteroid titer increases dramatically during the time that the furrow traverses the eye disc; it is possible that Usp represses furrow progression at the low titers prevailing near the time of furrow initiation, but that this repression is relieved by increasing hormone concentrations over the next several hours. This would be somewhat analogous with the situation in the wing disc, where Usp repression of neural development is relieved by 20-HE (Schubiger and Truman, 2000); however, Usp repression of furrow progression at low to moderate hormone levels could only be partial, because furrow progression does occur.

Regulation and function of *BR-C* during furrow progression

We previously reported that BR-C expression in the eye disc is dependent on *ecd* function, suggesting that it is regulated by ecdysteroid titer (Brennan et al., 1998). While BR-C expression in the eye disc is unlikely to be regulated by EcR, Usp may play a role. *BR-C* and *usp* disc clone phenotypes are opposite in that the former shows some furrow retardation while the latter shows a slight acceleration (Zelhof et al., 1997). Possible Usp repression of *BR-C*, or Usp regulation of BR-C isoform ratio could explain these differences. *BR-C* and *usp* eye clones also resemble each other in that both show ommatidial disarray. This similarity underscores the importance of orderly progression of the furrow for subsequent events, and may reflect an essential hormonal input that regulates timing of furrow progression (Brennan and Moses, 2000).

Local events appear to regulate BR-C expression at a number of levels. Two regulatory inputs into the switch from Z2 to Z1 expression at the furrow have been suggested: (1) the lack of Z1 expression in *br* clones suggests that prior expression of Z2 isoforms anterior to the furrow is required for subsequent expression of Z1 isoforms; (2) the ectopic Z1 expression seen surrounding a *PKA* clone suggests that Hh-dependent signaling at the furrow also promotes the switch to Z1 isoforms. Cessation of BR-C expression in eye disc cells correlates with cell-fate determination; photoreceptors diminish expression levels earlier than cone cells, and failure to differentiate in *smo Mad* cells is associated with prolonged expression of BR-C proteins. BR-C proteins are thought to confer competence to respond to ecdysteroid signals (Karim et al., 1993); in the asynchronously developing eye disc, it may be essential to spatially restrict such competence.

How might BR-C proteins exert their effects on eye development? Although ecdysteroids regulate ommatidial patterning at many stages of eye development in *Manduca* (Champlin and Truman, 1998b), our evidence suggests that the critical stage at which early eye development in *Drosophila* is regulated by Broad-complex is just anterior to and in the furrow. This corresponds to the site of Z2 expression and the zone at which the earliest defects are detected, in R8 specification. No transcriptional targets of BR-C have been identified in the eye; the aberrant specification of R8 founders suggests that targets may include elements of the Notch signaling pathway. BR-C regulation of such targets is likely to be complex, with different isoforms acting antagonistically.

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