Eyeless initiates the expression of both *sine oculis* and *eyes absent* during *Drosophila* compound eye development

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SUMMARY

The *Drosophila* Pax-6 gene *eyeless* acts high up in the genetic hierarchy involved in compound eye development and can direct the formation of extra eyes in ectopic locations. Here we identify *sine oculis* and *eyes absent* as two mediators of the eye-inducing activity of *eyeless*. We show that *eyeless* induces and requires the expression of both genes independently during extra eye development. During normal eye development, *eyeless* is expressed earlier than and is required for the expression of *sine oculis* and *eyes absent*, but not vice versa. Based on the results presented here and those of others, we propose a model in which *eyeless* induces the initial expression of both *sine oculis* and *eyes absent* in the eye disc. *sine oculis* and *eyes absent* then appear to participate in a positive feedback loop that regulates the expression of all three genes. In contrast to the regulatory interactions that occur in the developing eye disc, we also show that in the embryonic head, *sine oculis* acts in parallel to *eyeless* and *twin of eyeless*, a second Pax-6 gene from *Drosophila*. Recent studies in vertebrate systems indicate that the epistatic relationships among the corresponding vertebrate homologs are very similar to those observed in *Drosophila*.

Key words: *Drosophila*, Eye development, *eyeless*, Pax-6, *sine oculis*, *eyes absent*

INTRODUCTION

The *Drosophila* eye is a hexagonal array of approximately 750 ommatidia, each containing eight photoreceptor and eleven accessory cells (reviewed by Wolff and Ready, 1993). The eye develops from a small number of cells that are set aside in the embryo (Younoussi-Hartenstein et al., 1993). These cells form the eye part of the eye-antennal imaginal disc and proliferate during the larval stages. The stereotyped array of ommatidia is generated beginning early in the third instar larva, when a wave of pattern formation, marked by an indentation called the morphogenetic furrow, moves across the eye disc in a posterior to anterior direction (Ready et al., 1976). Anterior to the furrow cells are undifferentiated, whereas posterior to it cells are sequentially recruited into ommatidial clusters and start to differentiate (Tomlinson and Ready, 1987). However, it is anterior to the furrow where cells are initially determined to become retinal cells. While our understanding of the molecular events that occur in and posterior to the furrow, such as pattern formation, ommatidial assembly and cell differentiation, has advanced dramatically in recent years (reviewed by Bonini and Choi, 1995; Dickson, 1995; Heberlein and Moses, 1995; Freeman, 1997; Kumar and Moses, 1997), relatively little is known about events occurring in front of the furrow.

One of the genes acting anterior to the morphogenetic furrow is *eyeless* (*ey*) (Quiring et al., 1994; Halder et al., 1995a). *ey* is a key player in the specification of eye tissue, since targeted expression is sufficient to induce the development of extra eyes on wings, legs and antennae (Halder et al., 1995a). *ey* encodes a member of the Pax-6 family of transcription factors and contains two DNA binding domains, a homeodomain and a paired domain (reviewed by Macdonald and Wilson, 1996; Callaerts et al., 1997). *ey* is expressed in the eye anlagen as early as they can be detected in the embryo (Quiring et al., 1994). In the subsequent larval stages, *ey* continues to be expressed in the eye disc, first throughout the eye disc, later only anterior to the furrow. In addition, *ey* is expressed in the ventral nerve cord, in the optic lobes and in other discrete domains of the brain.

Several mutant alleles of *ey* were isolated (see Lindsley and Zimm, 1992), of which only a few are still available today (Quiring et al., 1994). Flies homozygous for *ey* or *ey* have reduced eyes or are completely eyeless. These two alleles have been analyzed molecularly (Quiring et al., 1994). Both mutations are caused by insertions of transposable elements into the first intron of *ey*. These insertions disrupt an eye-specific enhancer, thereby abolishing detectable *ey* expression in the embryonic eye primordia and in the developing eye discs (Quiring et al., 1994). Therefore, *ey* and *ey* are amorphic or severely hypomorphic for *ey* function in the eye disc. Together
with the finding that ey can switch on the eye developmental program and induce the formation of extra eyes, these results show that ey acts high up in the genetic cascade regulating eye development. However, little is known about the identity of subordinate target genes that implement the eye inducing activity of ey.

Genes known to be expressed and required during early eye development are candidate ey targets. Three such genes are sine oculis (so), eyes absent (eya) and dachshund (dac). The so gene encodes a homeodomain protein that is required for the development of the entire visual system including the compound eye, the ocelli, the optic lobe and the larval photoreceptor organ known as Bolwig’s organ (Cheyette et al., 1994; Serikaku and O’Tousa, 1994, Pignoni et al., 1997). eya encodes a novel nuclear protein involved in compound eye, ocellar and other developmental processes such as the development of the somatic gonadal precursors (Bonini et al., 1993, 1997; Leiserson et al., 1994; Boyle et al., 1997; Pignoni et al., 1997). In the developing eye disc both genes are expressed in a graded fashion, with highest levels of expression at the posterior of the disc from late second (eya) and early third (so) instar stages onwards, i.e. before the morphogenetic furrow forms (Bonini et al., 1993, 1997; Cheyette et al., 1994). Loss of function of either of these genes results in extensive cell death anterior to the furrow and subsequently in flies with reduced eyes or no eyes at all. Both genes are also required posterior to the furrow (Pignoni et al., 1997). dac encodes a novel nuclear protein that is expressed at the edge of the eye disc prior to furrow formation, in a pattern very similar to that of so and eya. dac is required for furrow initiation and loss-of-function mutations in dac transform eye tissue into head cuticle. During furrow propagation, dac is expressed anterior to, within and posterior to the furrow and is required for proper ommatidial assembly (Mardon et al., 1994). dac is also essential for leg development (Mardon et al., 1994).

eya physically interacts with so and dac and ectopic expression of eya or dac alone or synergistically in combinations of eya with so or dac induces extra eye formation and ey expression (Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997; Shen and Mardon, 1997). In addition, ey, eya and dac are induced and required during extra eye development induced by these genes, suggesting that they act together in a positive feedback loop at some point during compound eye development (Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997; Shen and Mardon, 1997). In this paper we show that the ey mutant phenotype is very similar to that of so and eya. We demonstrate that ey function is required for the expression of so and eya in the eye disc. On the other hand, so and eya functions are dispensable for ey expression. In addition, so and eya are independently induced and required during the development of ey induced extra eyes. Taken together, these results indicate that during normal eye development ey acts upstream of so and eya and either directly or indirectly induces their initial expression anterior to the furrow.

We recently identified a second Pax-6 gene of Drosophila, designated twin of eyeless (toy) (T. Czerny, G. Halder, P. Callaerts, U. Klöter, W. J. Gehring and M. Busslinger, unpublished). toy is initially expressed in a defined region in the head of the early embryo and is later expressed in the eye discs, the optic lobes and other parts of the nervous system. This embryonic expression pattern is very similar to that of so (Cheyette et al., 1994; Serikaku and O’Tousa, 1994), and we therefore investigated the epistatic relationships among toy, ey and so in the embryonic head. In contrast to the situation in the eye disc, we found that neither toy nor ey is required for activation of so expression in the embryo, nor is so necessary for toy or ey expression. Thus, toy/ey and so function in parallel in the early embryo.

Homologs of ey, so and eya are expressed in developing vertebrate eyes. These observations lead to the hypothesis that parts of the eye developmental programs are conserved between flies and vertebrates, despite the large differences in morphology of their eyes (Halder et al., 1995b). We discuss the relationships of toy, ey, so and eya during Drosophila eye development and compare our results to recent findings in vertebrates.

**MATERIALS AND METHODS**

**Fly stocks**

The eyP, eyP, soP, soP, soP, soP, eyP and UAS-ey stocks are described in Lindsay and Zimm (1992), Quiring et al. (1994), Cheyette et al. (1994), Heitzler et al. (1993), Bonini et al. (1993) and Halder et al. (1995a). Chester (1971) had noted that larval crowding reduced the expressivity of the ey mutation. Therefore, in order to control for larval density, we grew only 50-100 larvae per vial. The soP and eyP mutant stocks showed high penetrance and expressivity of the eyeless phenotype and required only little selection to maintain their phenotypic strength. The C(4)RM ct eyP stock was used to generate null 4 embryos.

**Construction of pEYE-lacZ, pEYE-ey and pHSE, and generation of transgenic flies**

The pEYE-lacZ (eyP-hancer-lacZ) transgene was constructed by first inserting a 3.5 kb EcoRI fragment derived from the first intron of the ey gene into pBluescript (Stratagene). This fragment contains a KpnI site 100 bp from its 5’ end. A 3.5 kb KpnI fragment was then excised (the 3’ KpnI site is in the polylinker of pBluescript) and cloned into HZ50PL. This fragment spans about two thirds of the first intron of ey and contains a small portion of exon 3 at its 3’ end. The pEYE-ey (eyP-hancer-eyP cDNA) construct contains the same 3.5 kb ey enhancer fragment followed by the hsp70 minimal promoter and the full-length embryonic ey cDNA (Quiring et al., 1994). The SV40 polyadenylation sequence from pUAST (Brand and Perrimon, 1993) was inserted downstream of the ey cDNA. Detailed description of the construction of pEYE-ey is available on request. pHSE (Heat-shock eyeless): full-length embryonic ey cDNA was cloned into phsCaspeR as a NotI-Xbal fragment. Flies were transformed as described by Rubin and Spradling (1982). The recipient strain was y w.

**Rescue and quantitative determination of eye size**

In order to assay for rescue activity, four stable pEYE-ey transformants were each independently crossed into an eyP mutant background. Because the expressivity of the eyP eye phenotype depends on the genetic background, it was necessary to compare sibling flies carrying and lacking the rescue transgene. To do so, pEYE-ey/SMI; eyP/eyP flies were crossed with eyP/eyP flies and the eye sizes of the progeny were quantitated. The eye sizes of the flies carrying PEGE-ey were then compared to those of the flies carrying SMI. The SMI balancer itself did not affect the expressivity of eyP.

To determine eye sizes, anesthetized female flies were observed under a stereomicroscope and pictures were captured using an attached video camera. The surface area of the photographed eye was measured and expressed in the fraction of the size of an average

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This document contains scientific content and descriptions of experiments involving Drosophila melanogaster, a model organism commonly used in genetic and developmental studies. It highlights the importance of certain genes in eye development, particularly focusing on the roles of ey, so, and eya. The text discusses the expression patterns of these genes and how they are involved in the development of different eye structures. Additionally, it covers the methodologies used in genetic studies, such as the construction of transgenic flies and the quantification of eye size. The reference to ey, so, and eya suggests a focus on understanding the genetic basis of eye development in Drosophila and its potential implications for vertebrate eyes.
Oregon R wild-type eye. Because compound eyes are not flat, the eye sizes are underestimated with that method and the differences in eye sizes thus appear slightly smaller than they actually are.

Preparation of anti-EY antibody
The 1.4 kb EcoRI fragment from the ey cDNA E10 (positions 1449-2849, Quiring et al., 1994) was subcloned into the EcoRI site of pGEX-2T (Smith and Johnson, 1988), a glutathione-S-transferase fusion vector. The resulting plasmid encodes a fusion protein of glutathione-S-transferase and EY (amino acid positions 455-838). The E. coli strain JM109 carrying this plasmid expressed a novel fusion protein of the predicted size. This protein was purified according to the method of Smith and Johnson (1988), except that the induction was performed at 18°C overnight instead of at 37°C. Rats were immunized intracutaneously at multiple sites with about 100 μg of fusion protein in complete Freund’s adjuvants, followed by three boosts of 100 μg fusion protein in incomplete Freund’s adjuvants every 3 weeks.

Histology
Immunohistochemistry of whole-mount embryos was performed as described in Frasch et al. (1987) and Lawrence and Johnston (1989). After fixing and blocking, embryos were incubated with the anti-β-galactosidase antibody (Cappel; 1:1000 dilution) at 4°C overnight. Embryos were stained according to the directions of the Vectastain ABC kit (Vector Laboratories), using biotinylated secondary antibodies. Preparations were dehydrated in a graded ethanol series (70%, 90%, 100% x 3) and mounted in 70% Canada Balsam in methylsalicylate. In situ hybridisation to whole-mount embryos using digoxigenin-labeled DNA probes was performed according to Tautz and Pfeifle (1989) with modifications (a detailed protocol is available on request). For double stainings, the antibody staining was completed before starting the in situ hybridization procedure.

Antibody stainings of imaginal discs were carried out as follows. Larvae were dissected in cold PBS and fixed in PLP (McLean and Nakane, 1974) for 45 minutes on ice or in PEM (100 mM Pipes, pH 6.9, 2 mM MgSO4, 1 mM EGTA, 4% formaldehyde) for 30 minutes on ice. Disc complexes were then washed 4 times for 15 minutes in PBT (PBS with 0.3% Triton X-100) on ice and blocked in PBTB (PBS with 0.3% Triton X-100 and 3% BSA) for at least 30 minutes at 4°C. Disc complexes were incubated with the primary antibody (rat α-EY 1:600, mouse α-EYA 1:2 (Bonini et al., 1993), mouse α-SO 1:300 (Cheyette et al., 1994), mAb α-βGal (Promega) 1:1000, rabbit α-βGal (Cappel) 1:1000, rat α-ELAV 1:30 (Robinson and White, 1991)) in PBTB at 4°C overnight. Disc complexes were washed 6 x 20 minutes in PBTB at 4°C and incubated with the secondary antibody for 2 hours at room temperature or overnight at 4°C. Secondary antibodies used were from Jackson ImmunoResearch Laboratories and included the following F(ab’)2 fragments from donkey: DTAF α-rat (1:200-1:1000), Cy5 α-mouse (1:500-1:2000), DTAF α-rabbit (1:200-1:1000), Cy3 α-rabbit (1:500-1:2000). After secondary antibody incubation, disc complexes were washed as above and discs were dissected and mounted in Vectashield (Vector Labs).

Cell death was visualized by dissecting larvae in 1.6 μM Acridine orange in PBS and viewing the dissected discs with a fluorescence microscope (Spreij, 1971). X-Gal staining for β-galactosidase activity in imaginal discs was performed as described in Hiromi and Gehring (1987). For scanning electron microscopy flies were dehydrated in an acetone series, critical-point dried, sputter-coated with 15-20 nm gold and examined with 6-12 kV acceleration potential.

Heat-shocking larvae
First instar larvae that hatched within a period of 1 hour were collected and aged to the desired stage. Heat shocks were then given for 45 minutes at 38°C. The induction of ectopic eyes was most efficient when six heat shocks were given at 4-hour intervals beginning 83 hours after egg laying. Heat shocks beginning earlier resulted in lethality. For β-galactosidase activity stainings, larvae were then allowed to recover for 24 hours before dissection.

RESULTS
Rescue of the eyeless mutant eye phenotype
The ey2 and eyR stocks obtained from the stock centers were phenotypically almost indistinguishable from wild type and had apparently accumulated a significant number of phenotypic suppressors (Morgan, 1929). In order to obtain stronger phenotypes, we systematically exchanged the first, second and third chromosomes with Oregon R wild-type chromosomes and isogenized the ey mutant fourth chromosome. This treatment dramatically improved the penetrance and expressivity of the eyeless phenotype. Nevertheless, these stocks still showed some variability in eye size (Fig. 1A-C,E). In the ey2 stock with the strongest phenotype, more than 50% of eyes were completely missing and more than 90% were smaller than a third of the normal size. No eyes were larger than half the normal size (Fig. 1E). We did not observe any defects in the ocelli (Fig. 1B,C, arrowheads). In general, eyR stocks had a similar range of eye defects although with lower expressivity. In the subsequent rescue experiments and phenotypic analyses the selected ey2 stock was used.

Before analyzing the ey mutant phenotype in more detail, we wanted to confirm that the eyeless phenotype is caused by mutation in ey. We took advantage of a 3.5 kb enhancer fragment derived from the first intron of the ey gene to build a rescue construct with the embryonic ey cDNA. This enhancer drives expression specifically in the developing eye (Fig. 1D; Quiring et al., 1994). After transformation into flies, four stable transformants were each independently crossed into an ey2 mutant background and the eye sizes of ey2 flies carrying or lacking the rescue construct were quantitated. For each of the four transgenes, the flies carrying the rescue constructs had significantly larger eyes than their siblings without the transgene. For example, 55% of the flies with the rescue transgene EHE6 had nearly wild-type eye sizes, while only 3% of the non-rescued flies had wild-type eyes (Fig. 1F). These experiments confirm that the cause of the ey2 phenotype is a defect in ey gene expression in the eye imaginal discs.

The anlagen of the eye are formed normally in eyeless mutant embryos
Medvedev (1935) argued, based on his studies on growth rate and size of the eye discs, that in ey mutant embryos fewer cells might be recruited into the eye anlagen. Since ey is expressed in the embryonic eye primordia and this expression is lost in ey mutants (Quiring et al., 1994), such a scenario is possible. We therefore analyzed the development of the embryonic eye anlagen making use of an ey-eye enhancer lacZ reporter. This reporter contains the same enhancer fragment used for the rescue constructs described above. In wild-type embryos, the transgene drives β-galactosidase expression in part of the morphologically distinct eye primordia (Fig. 2A, arrows). During larval stages, β-galactosidase is continuously expressed in the eye discs and in parts of the brain (Fig. 1D, and data not shown). The position and number of cells that express this reporter in ey2 mutant embryos is indistinguishable from wild-
type embryos (Fig. 2B). We conclude that the anlagen of the eye are formed in ey\(^2\) mutant embryos. Therefore, defects in the first steps of eye development are not the major cause of the eyeless phenotype.

**Extensive cell death in eyeless mutant eye discs**

In contrast to the normal appearance of the eye anlagen in ey\(^2\) embryos, the morphology of eye-antennal imaginal discs from late third instar ey\(^2\) mutant larvae is highly abnormal with the eye part being strongly reduced (Fig. 2C,D). The antennal part is not affected. Staining for differentiating photoreceptors failed to show any evidence of ommatidial cluster formation in most ey\(^2\) mutant eye discs (Fig. 2D). Previous work suggested that the ey\(^2\) phenotype was a result of cell death in third instar eye discs (Fristrom, 1969). To assess cell death we stained eye discs with the vital dye Acridine orange (Spreij, 1971). A low level of cell death is normally observed in wild-type eye discs, mainly in the region just anterior to the furrow (Fig. 2E; Fristrom, 1969; Spreij, 1971; Wolff and Ready, 1991). In contrast, eye discs from third instar ey\(^2\) larvae displayed massive cell death in the remainder of the eye discs (Fig. 2F).

**eyeless function is required for eye disc expression of sine oculis and eyes absent but not vice versa**

To gain insight into the epistatic relationships among ey, so and ey\(\alpha\) we first compared their expression patterns in eye discs. EY expression in the eye disc starts in the embryo (Quiring et al., 1994) and is later observed in the entire eye disc of late second and early third instars (Fig. 3A). During subsequent development, EY expression is strong in the region anterior to the furrow and downregulated in differentiating cells (Fig. 3B,C). We detected very little, if any, expression posterior to the furrow or in the region of the developing ocelli in third instar eye discs with our polyclonal antibody or by in situ hybridisation (Fig. 3B,C and data not shown). At the furrow, the expression patterns of EY and Decapentaplegic (DPP) abut each other, indicating that EY expression is downregulated just before cells enter the furrow (Fig. 3D,E).

EYA and SO start to be expressed in eye discs later than EY. In contrast to EY, neither SO nor EYA is expressed in the eye anlagen of stage-16 embryos (Bonini et al., 1993; Cheyette et al., 1994; our own observations). Expression of EYA and SO in the eye disc starts in the late second and early third instars, respectively (Bonini et al., 1993; Cheyette et al., 1994). At these stages, both genes are expressed in a gradient with strongest expression at the posterior of the eye disc (Fig. 4, Bonini et al., 1993; Cheyette et al., 1994). Later, when the furrow moves across the eye disc, SO and EYA are expressed in a graded manner with strongest expression at anterior to the furrow (Fig. 3F,H,1). In this region the expression pattern of EY overlaps with those of SO and EYA (Fig. 3F,I). However, in the most anterior part of the eye disc only EY is detected at high levels (Fig. 3F-I). Unlike EY, SO and EYA continue to be expressed posterior to the furrow. Both genes are also expressed in the region of the differentiating ocelli (Bonini et al., 1993; Cheyette et al., 1994). In summary, EY is expressed...
in the eye disc from embryonic stages onwards, until cells enter the furrow and start to differentiate, while SO and EYA start to be expressed later, and cells begin to express increasing levels of SO and EYA as the furrow moves across the eye disc. These results are consistent with ey acting upstream of so and eya during eye disc development.

To test this possibility, we analyzed gene expression in ey, so and eya mutant eye discs. Genetic and molecular data indicated that the so and eya alleles are amorphic or severely hypomorphic in the developing eye (Bonini et al., 1993, 1997; Cheyette et al., 1994; Leiserson et al., 1994). Because massive cell death is observed in late third instar eye discs of all three mutants, gene expression analysis at this stage is not possible. We therefore studied expression patterns in early third instar eye discs (Fig. 4). At this stage all three genes are expressed (Fig. 4A-C) and cells in the so and eya mutant eye discs are still viable. Eye discs from ey mutants, however, already show first signs of morphological abnormalities (Fig. 4D-F), indicating that ey function is required prior to this stage. In eye discs of so (Fig. 4G) and eya mutants (Fig. 4J), EY is expressed normally, indicating that the functions of so and eya are not required for EY expression. On the other hand, neither SO nor EYA expression is observed in ey mutant eye discs (Fig. 4E,F). This demonstrates that ey function is required for eye disc expression of SO and EYA. In about half of the so mutant eye discs weak EYA immunoreactivity was detected, suggesting that so may not be required for EYA expression (Fig. 4I). Expression of SO was not seen in ey mutant eye discs (Fig. 4K). However, because SO and EYA are expressed in nearly identical patterns and because both genes are required for cell viability, these results are not conclusive. Below we describe other experiments that address the epistatic relationships between so and eya. Finally, in neither ey nor
eya¹ mutants could immunoreactive material be detected with the respective antibody in the eye disc (Fig. 4D,H,L). This is consistent with ey², so¹ and eya¹ being amorphic alleles in the eye field.

In summary, our data show that ey acts earlier than and upstream of so and eya in the developing eye disc and that so and eya functions in the eye disc appear to be dispensable for ey expression.

**eyeless induced extra eyes express and require sine oculis and eyes absent**

To further investigate the epistatic relationships among ey, so and eya, we examined gene expression in developing extra eyes induced by Gal4-directed ectopic expression of ey (Fig. 5; Brand and Perrimon, 1993; Halder et al., 1995a). In wild-type third instar larvae SO and EYA are not expressed in the wing disc proper (Fig. 5B,C). However, in wing discs that develop ey-induced extra eyes, both genes are ectopically expressed in and surrounding developing photoreceptor clusters (Fig. 5D-F). These results indicate that ey acts upstream of so and eya during extra eye development.

In order to investigate the dynamics and the spatial restriction of the induction of so and eya expression, we ubiquitously expressed ey in a temporally controlled manner using a heat-inducible transgene. Expression of so and eya was monitored by assaying lacZ expression of so and eya enhancer-traps (Bonini et al., 1993; Cheyette et al., 1994). Ubiquitous expression of ey was induced starting at 83 hours after egg laying during the mid third instar stage. At that time neither so nor eya are expressed in the wing disc proper (Fig. 5G, not shown) and eya is not expressed in leg discs (Fig. 5J). Two heat shocks induced only weak ectopic expression of so and eya, did not induce extra eye formation in adult flies and just barely affected their morphology. This suggests that higher or prolonged levels of ey may be required to efficiently reprogram cells into the eye developmental pathway. Consistent with this, induction of extra eyes was efficient when larvae carrying the heat-inducible ey transgene were heat-shocked six times. Such animals readily induced ectopic expression of so and eya (Fig. 5H,I,K,L) and nearly 100% of pharate adult flies developed extra eyes. Although EY was expressed ubiquitously, induction of both genes was confined to regions close to the A/P boundary that do not express WG but DPP (Fig. 5I,LL). Thus, ey alone is not sufficient to induce so and eya but only those cells that are close to a source of DPP appear competent to express so and eya in response to ey.

The finding that ey positively regulates so and eya transcription raised the possibility that so and eya may be required downstream of ey for ectopic eye formation. Indeed, targeted expression of ey was unable to induce ectopic eye development in so¹ and eya¹ mutant backgrounds (Fig. 6A-D), although ectopic EY protein was produced (not shown) and functional as inferred from its deleterious effects (Fig. 6C,D). Consistent with the lack of ectopic eye production, no ectopic photoreceptors develop in wing discs of so¹ and eya¹ mutants following targeted expression of EY (Fig. 6F,G, and data not shown).

We took advantage of the ectopic induction of SO and EYA by EY to find out whether ey activates so and eya in parallel and independently of one another or whether induction of one gene depends upon the function of the other one. As discussed above, the cell death phenotypes observed in the eye discs of so¹ and eya¹ make such an analysis difficult in the eye discs. We reasoned that by expressing ey ectopically we might be able to bypass those requirements for cell viability. However, in late third instar larvae, ectopic EY expression in so¹ and eya¹ mutant backgrounds caused ectopic cell death in wing discs (Fig. 6E) and resulted in strongly reduced and deformed adult structures (Fig. 6C,D). Apparently, ey is able to completely reprogram wing cells into the eye developmental pathway even if that leads to cell death, as is the case in so¹ and eya¹ mutants. Nevertheless, we found that in early to mid third instar wing discs, EY induced ectopic expression of EYA in a so¹ mutant background (Fig. 6F) and, conversely, SO was induced by ey in an eya¹ mutant background (Fig. 6G). Therefore, both genes appear to be independent targets of EY. However, the ectopic expression was weaker than that induced in a wild-type background and requires more prolonged levels of EY to find out whether EY activates so and eya downstream of eya¹. In summary, our results show that ey activates so and eya and their function during ectopic eye induction.

**Pax-6 and sine oculis act in parallel in the Drosophila embryo**

In addition to its function in the developing compound eye, so is required for the formation of the entire visual system, including the optic lobes of the brain and the larval photoreceptor organs or Bolwig’s organs. In blastoderm-stage embryos, so is expressed in a dorsal domain of the head region that gives rise to those structures (Cheyette et al., 1994; Serikaku and O’Tousa, 1994; Fig. 7A,B). Whether this region
also includes the primordia of the eye discs is unknown and no so transcripts are detected in the eye discs when they become morphologically discernible towards the end of embryogenesis (Cheyette et al., 1994; our own observation). We have recently isolated a second Pax-6 gene from Drosophila, designated twin of eyeless (toy), which is expressed in the developing head from the blastoderm stage onwards (Fig. 7C,D; T. Czerney et al. unpublished). ey, in contrast, starts to be expressed at germ band extension (Quiring et al., 1994). The early expression of toy overlaps so expression in the head and we thus wanted to investigate their epistatic relationship. Cytologically, toy maps close to ey on the fourth chromosome. Since no mutations in toy have been identified thus far we took advantage of a compound fourth chromosome to generate nullo 4 embryos that lacked both toy and ey functions. Such embryos expressed so at normal levels in the head, indicating that toy is not required for so expression in the embryonic head (Fig. 7E,F). Similarly, toy is expressed in an appropriate pattern in embryos homozygous for a null allele of so (Fig. 7G,H). Therefore, so and toy appear to act in parallel during the development of the embryonic head of Drosophila. Later in development, so null embryos express toy and ey in the eye anlagen indicating that so is not only dispensable for that expression but also for the initial formation of the eye anlagen (Fig. 7I-L).

DISCUSSION

**eyeless acts anterior to the morphogenetic furrow**

The loss of adult eye structures in ey2 and ey8 results from cell death of the eye imaginal disc during larval stages. Staining with the vital dye Acridine orange revealed massive apoptosis anterior to the morphogenetic furrow in these mutants (Fristrom, 1969; this study). We found that a small proportion of early third instar eye discs from stocks with the most penetrant eyeless phenotype were already reduced in size as compared to wild type. In line with these observations Chen (1929) and Medvedev (1935) found the earliest manifestation of the eyeless phenotype in the second instar, 48 hours after egg laying. But, in contrast to what Medvedev postulated, our data suggest that ey is not required for the initial formation of the eye anlagen in the embryo. Nor is, as was argued by Chen, the smaller size of the eye discs (only) due to a proliferation defect, since the amount of 5-bromodeoxyuridine (BrdU) incorporation into replicating DNA is not significantly different between wild-type and ey2 mutant eye discs (data not shown). It thus appears that the eye discs can form and grow without ey, but that later the cells cannot differentiate and die by apoptosis.

It is conceivable that the ey2 allele is not totally amorphic for ey function in the developing eye. However, we have not detected any residual ey expression in the developing eyes of ey2 mutant embryos or larvae, suggesting that ey2 is at least a very strong hypomorphic allele. To fully answer the question of how early ey functions during eye development will require the isolation and characterization of null mutations. Similarly, the question of whether ey is required for the development of the ocelli awaits isolation and analysis of ey null alleles.

In accordance with the mutant phenotype, ey is expressed in the entire eye disc anterior to the morphogenetic furrow throughout development. At the furrow, ey expression abuts the expression of dpp. dpp expression is directly induced by the posterior Hedgehog (HH) signal (reviewed by Heberlein and Moses, 1995), suggesting that ey expression is downregulated in cells that receive the HH signal. We have not been able to detect ey transcripts or EY protein in cells posterior to the furrow in third instar eye discs. This downregulation of ey expression is essential for normal eye development, since ectopic expression of ey using sev-Gal4, GMR-Gal4 and other Gal4-lines that drive expression posterior to the furrow caused eye phenotypes ranging from a severe roughening to the complete loss of eyes (data not shown). EY thus interferes with the later differentiation of retinal cells although it activates the eye developmental program at earlier stages of development.

**sine oculis and eyes absent are downstream targets of eyeless**

Our analysis showed that so and eya are ectopically induced by targeted expression of ey in wing and leg imaginal discs. Furthermore, so and eya are required during ey directed ectopic eye development. Therefore, ey acts upstream of so and eya during extra eye development. Several lines of evidence indicate that ey also acts upstream of so and eya during normal eye development. First, ey is expressed earlier than so and eya in the eye discs. Second, ey function is required for the expression of so and eya in eye discs, but not vice versa. Third, ectopic eyes appear to develop in the same way as the normal compound eyes as indicated by gene expression patterns and histology (Halder et al., 1995a; this study, and data not shown).

Therefore, we conclude that ey acts upstream of so and eya during normal eye development and either directly or indirectly induces their expression anterior to the furrow. More recent studies in our laboratory indicate that so transcription is indeed directly activated by EY (T. Niimi et al., unpublished).

In our ectopic expression system, EY was able to induce EYA expression in a so1 mutant background and SO expression in an eya1 mutant background, indicating that so and eya are independent targets of EY. Thus, both genes may be direct targets of EY activity, rather than one being indirectly activated by EY through the other one. Loss-of-function alleles of either so or eya show massive cell death anterior to the furrow. This is very similar to the ey phenotype and suggests that so and eya are important mediators of ey function in the eye disc.

Notably, EY is expressed anterior to the furrow only, whereas so and eya are expressed anterior to, and posterior to the furrow. If EY directly activates so and eya transcription, it would account for the initial expression of so and eya anterior to the furrow only. Cheyette et al. (1994) have argued that so expression is autoregulated in the eye disc. Therefore, after initial induction by EY, SO may maintain its own expression. A similar situation could pertain for EYA.

The expression patterns of ey and those of so and eya only partially overlap anterior to the furrow. While ey is expressed in all eye progenitor cells anterior to the furrow from embryonic stages onwards, neither so nor eya transcripts are detected in the eye discs at the end of embryogenesis and high levels of so and eya expression start later, during early third and second instars respectively. In addition, both genes are initially expressed in a gradient from posterior to anterior. Only as the furrow moves across the eye disc do all ey-expressing cells induce high levels of so and eya expression. Therefore, while ey is necessary, it is not sufficient to induce so and eya.
expression. Thus a factor that acts in conjunction with ey may exist. Similarly, we found that ubiquitous expression of EY induced ectopic expression of so and eya preferentially along the A/P boundary in wing discs and in a dorsal domain at the A/P boundary in leg discs. This induction was not observed close to cells that secrete the WG signaling protein (Baker, 1988), consistent with the finding that WG inhibits furrow initiation and progression (Ma and Moses, 1995; Treisman and Rubin, 1995).

Reciprocal regulation between eyeless and other genes involved in early eye development

It has recently been shown that SO, EYA and DAC form protein complexes and that DAC and EYA are able to induce extra eye development when expressed alone (Bonini et al., 1997; Shen and Mardon, 1997) and when expressed in combinations do so synergistically (DAC/EYA, SO/EYA) (Chen et al., 1997; Pignoni et al., 1997). ey is ectopically induced and required during extra eye development directed by these genes. Similar to our results, these authors also showed that dac and eya are induced and required during ey driven ectopic eye development (Bonini et al., 1997; Chen et al., 1997; Shen and Mardon, 1997). Apparently ey, so, eya and dac function in a feedback loop and may act together to control early eye development. It appears therefore that once ey induces the expression of so, eya and dac in the developing eye disc, they upregulate each others expression, possibly to stabilize the system and to fully implement the eye developmental program. Consistent with that hypothesis we found that so and eya functions are required for the induction of high levels of eya and so gene expression.

Insect compound eyes versus vertebrate single lens eyes

Homologs of ey/ho (Pax-6), so and eya are active during vertebrate eye development, suggesting that vertebrates and flies may use conserved genetic pathways during eye development (reviewed by Halder et al., 1995b; Macdonald and Wilson, 1996; Callaerts et al., 1997; Oliver and Gruss, 1997). The overall expression pattern of Pax-6 during vertebrate and Drosophila eye development is strikingly similar. In vertebrates, Pax-6 is expressed initially in a large area of the head neural ectoderm and the overlying surface ectoderm that gives rise to the lens and nasal placodes (Krauss et al., 1991; Walther and Gruss, 1991; Püschel et al., 1992; Li et al., 1994; reviewed in Callaerts et al., 1997). During further eye development, Pax-6 expression progressively becomes restricted to the developing optic vesicle, lens and cornea. In Drosophila, toy is initially expressed in a broad domain of the
embryonic head and continues to be expressed in the eye discs (Fig. 7, T. Czerny et al., unpublished data). ey expression in the developing eye starts later, when the eye anlagen form after gastrulation (Quiring et al., 1994). In addition, it appears that in both flies and vertebrates, Pax-6 expression in the developing eye is directly downregulated by HH signaling (this study; Ekker et al., 1995; Macdonald et al., 1995; Li et al., 1997), providing a parallel in the regulation of Pax-6 as well.

Not only the expression patterns, but also the phenotypes of loss of Pax-6 function in the developing eyes are similar. In Small eye mutant mice and rats that lack Pax-6 function, the optic vesicles form but do not develop further (Hogan et al., 1988; Hill et al., 1991; Fujimura et al., 1994; Grindley et al., 1995; Quinn et al., 1996). Similarly the eye anlagen do form in ey^2 mutant Drosophila but then degenerate. Thus, ey most likely is not required for the initial formation of the eye anlagen, but for their specification. Whether this is also the case for toy remains to be seen.

Three eya homologs were found in mouse, all of which are expressed in the developing eye (Abdelhak et al., 1997; Duncan et al., 1997; Xu et al., 1997; Zimmerman et al., 1997). Similar to their Drosophila counterparts, they appear to be expressed later than Pax-6 in the lens placode and/or optic vesicle. The expression of Eya1 in the lens placode requires Pax-6 function (Xu et al., 1997), suggesting that in vertebrates as in Drosophila, Pax-6 induces the initial expression of Eya1.

In the mouse, one of the so homologs, Six3, is expressed in
the developing eye (Oliver et al., 1995). Six3 starts to be expressed early in the anterior neural plate including the region of the forebrain from which the optic vesicles form. In the developing eye, however, it is expressed later than Pax-6. Six3 expression subsequently occurs in the optic vesicle, the optic stalk and later strongly in the developing neural retina. Six3 is also induced in the developing lens. There is strong genetic evidence indicating that Six3 acts downstream of Pax-6 during eye development, which is very similar to the situation in Drosophila (G. Goudreau and P. Gruss, unpublished).

At the present time, we cannot explain why ey and mouse Pax-6 can induce ectopic eye development in Drosophila, whereas ectopic expression of Pax-6 in Xenopus did not induce ectopic retinal development (Hirsch and Harris, 1997) and only resulted in the induction of ectopic lenses (Altmann et al., 1997) rather than complete eyes. However, there may be different Pax-6 isoforms or cofactors required for retina and lens development. In summary, the expression patterns and hierarchical relationships between toy, ey, so and eya are comparable to a large extent to those of Pax-6, Six3 and Eya1 lens development. In summary, the expression patterns and different Pax-6 isoforms or cofactors required for retina and resulted in the induction of ectopic lenses (Altmann et al., 1997).
morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate. Development 124, 603-615.


