Development 125, 2181-2191 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV2267

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

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Accepted 9 April; published on WWW 19 May 1998

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 few are still available today (Quiring et al., 1994). Flies homozygous for ey^2 or ey^R 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^2 and ey^R 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. Kloter, 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 ey^2, ey^R, so^1, so^3, so^{l1}, so^{l3}, eya^1 and UAS-*ey* stocks are described in Lindsley 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 so^1 and eya^1 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 *ci* ey^2 stock was used to generate nullo 4 embryos.

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

The pEYE-lacZ (*ey*-enhancer-lacZ) transgene was constructed by first inserting a 3.5 kb *Eco*RI fragment derived from the first intron of the *ey* gene into pBluescript (Stratagene). This fragment contains a *Kpn*I site 100 bp from its 5′ end. A 3.5 kb *Kpn*I fragment was then excised (the 3′ *Kpn*I 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* (*ey*-enhancer-*ey* 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 *Not*I-*Xba*I 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 ey^2 mutant background. Because the expressivity of the ey^2 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*/*SM1* ; ey^2/ey^2 flies were crossed with ey^2/ey^2 flies and the eye sizes of the progeny were quantitated. The eye sizes of the flies carrying pEYE-*ey* were then compared to those of the flies carrying *SM1*. The *SM1* balancer itself did not affect the expressivity of ey^2.

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

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 *Eco*RI fragment from the *ey* cDNA E10 (positions 1449-2849, Quiring et al., 1994) was subcloned into the *Eco*RI 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% × 3) and mounted in 70% Canada Balsam in methylsalicylate. In situ hybridisation to whole-mount embryos using digoxygenin-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 $MgSO_4$, 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 (Robinow and White, 1991)) in PBTB at 4°C overnight. Disc complexes were washed 6 × 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 ey^2 and ey^R 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 ey^2 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, ey^R stocks had a similar range of eye defects although with lower expressivity. In the subsequent rescue experiments and phenotypic analyses the selected ey^2 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 ey^2 mutant background and the eye sizes of ey^2 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 ey^2 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 ey^2 mutant embryos is indistinguishable from wild-

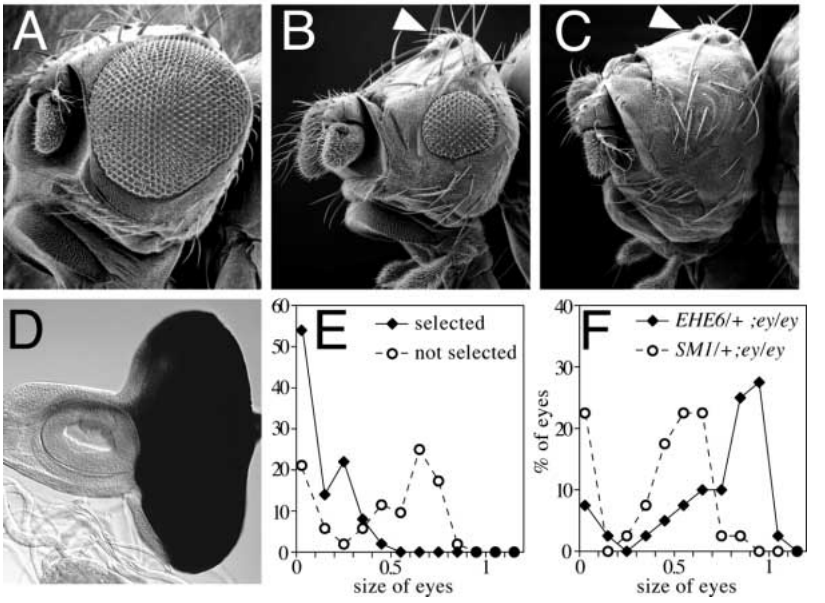

Fig. 1. The adult ey^2 phenotype and rescue by an *eyeless* minigene. (A-C) Scanning electron micrographs of heads of (A) a wild-type fly and (B,C) ey^2 flies with moderate and strong eye phenotypes, respectively. Anterior is to the left. The fly with the strong eyeless phenotype has a small head and completely lacks the compound eyes (C). Bristles normally surrounding the eye are also missing. The ocelli on the dorsal head are not affected in ey^2 flies (arrowheads). (D) The eye enhancer located in the first intron of the *ey* gene drives expression in the eye disc. β-galactosidase activity staining of an eye-antennal disc from a third instar larva carrying an *ey* enhancer lacZ transgene. β-galactosidase activity is detected in the entire eye disc (to the right), barely in the antennal disc (to the left) but not in leg or wing discs (not shown). β-galactosidase activity is not only detected anterior to the furrow, as are *ey* transcripts and protein itself (Fig. 3; Quiring et al., 1994), but also posterior to it. This might be due to perdurance of β-galactosidase protein. (E) Quantitative determination of the eyeless phenotype. The graphs show the percentage of eyes with a certain size plotted against eye size given in fractions of an average wild-type eye. In all cases $n = 50$. In the homozygous ey^2/ey^2 stock that was constantly selected for strong eyeless phenotypes, over 50% of eyes were completely missing and more than 90% of eyes were smaller than a third of the normal size. In a non-selected homozygous ey^2/ey^2 line, the distribution of eye sizes is significantly shifted towards wild type. Heterozygous $ey^2/+$ flies have eyes of wild-type size in average (not shown). (F) Rescue of the ey^2 eye phenotype by an *ey* minigene that contains the *ey* eye enhancer driving the expression of an *ey* cDNA. Flies carrying the rescue construct (EHE6) had significantly larger eyes compared to their siblings that did not (*SM1*). More than 55% of rescued eyes but only 3% of the non-rescued ones were nearly wild-type size. The non-rescued flies had a relatively weak eye phenotype, which was not due to the presence of *SM1* (not shown). The three other transgenic lines showed similar rescue effects (not shown).

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 portion 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). Eye discs with weaker phenotypes showed ectopic cell death anterior to the furrow (not shown). This cell death phenotype is very similar to those observed in so^1 and eya^1 mutants (Bonini et al., 1993; Cheyette et al., 1994).

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 *eya* 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 instar, 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 fashion with strongest expression just anterior to the furrow (Fig. 3F,H,I). 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

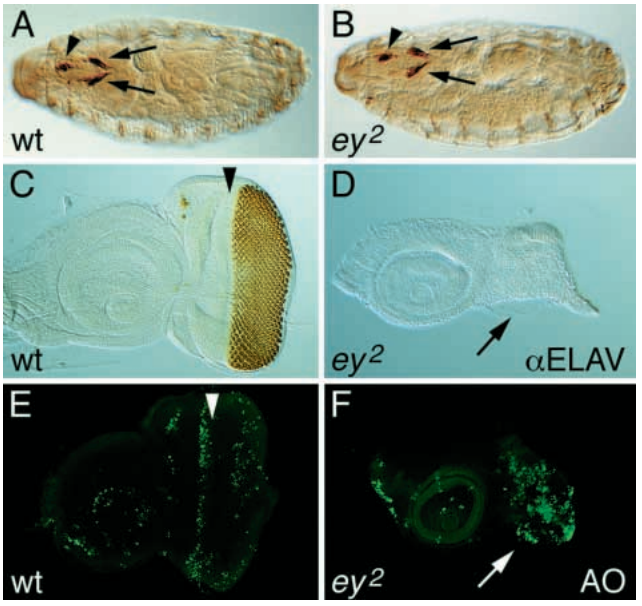

Fig. 2. The embryonic and larval eye disc phenotypes of *ey*^{2}. Left panels (A,C,E) show wild-type, right panels (B,D,F) show *ey*^{2} mutants. (A,B) Anti-β-galactosidase antibody stainings of stage-16 embryos carrying the *ey*-enhancer-lacZ reporter transgene (see also Fig. 1D). The reporter expresses high levels of β-galactosidase in the embryonic eye anlagen (arrows), which are morphologically distinct at this stage. β-galactosidase protein is also detected in a few cells associated with the anterior pharynx, a pattern that does not reflect endogenous *ey* expression (arrowheads). No difference in staining is observed between wild-type and *ey*^{2} mutant embryos. Anterior is to the left, dorsal views. (C-F) Eye-antennal imaginal discs from wandering third instar larvae. Eye portions are to the right, antennal portions to the left. (C,D) α-ELAV antibody stainings that label the clusters of developing photoreceptors (Robinow and White, 1991). (C) Posterior to the morphogenetic furrow (arrowhead) ommatidial clusters of photoreceptors are developing in wild type. (D) In *ey*^{2} mutant discs no differentiating photoreceptors are detected. In addition, the eye disc is strongly reduced in size (arrow), while the antennal portion is of normal size. (E,F) Acridine orange stainings that highlight dead cells (Spreij, 1971). (E) In wild type, dead cells are located mainly in a band just anterior to the furrow (arrowhead). (F) Massive cell death is observed in the remaining portion of the *ey*^{2} mutant eye disc (arrow). The antennal part of the disc is not affected.

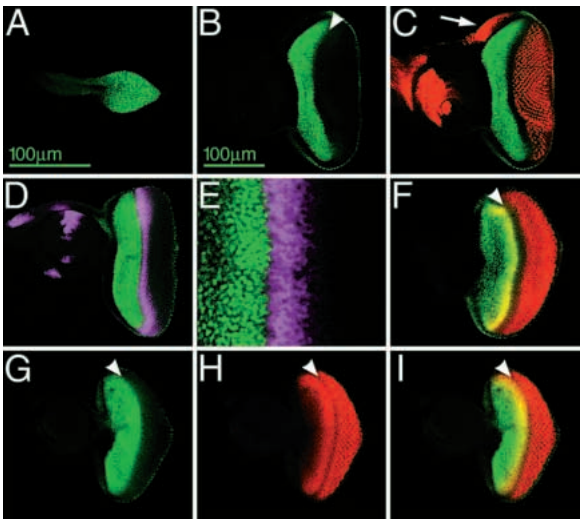

Fig. 3. Expression pattern of EY during eye imaginal disc development. In all panels anti-EY staining is green. Same orientations as in Fig. 2. (A) EY is expressed in the entire eye portion of the disc but not in the antennal part (to the left). (B) EY is expressed uniformly in the eye field anterior to the morphogenetic furrow, but its expression is downregulated at the furrow (arrowhead) and no nuclear antigen is detected in differentiating ommatidial clusters. (C) EY-Hedgehog (HH) double-staining of the disc shown in B. HH (red), monitored with an HH enhancer trap line (Lee et al., 1992), is expressed in the antennal disc, the developing photoreceptor cells and in the presumptive dorsal head region where the ocelli will form (Royet and Finkelstein, 1996; arrow). EY is not expressed in these regions. (D) Third instar eye-antennal disc double-stained for EY and DPP (pink). Expression of DPP, monitored by a reporter transgene (Blackman et al., 1991), marks the morphogenetic furrow (Masucci et al., 1990; Blackman et al., 1991). (E) Higher magnification of D showing that the expression patterns of EY and DPP abut each other at the furrow. (F) Double staining for EY and SO (red). SO is expressed posterior to the furrow (arrowhead) and in a gradient anterior to it with strongest expression just anterior to the furrow. In this region, SO and EY expressions overlap (yellow). (G-I) Eye-antennal disc stained for EY (green) and EYA (red). I shows a superposition of G and H. Similar to SO, high levels of EYA are detected posterior to the furrow (arrowhead) and in a band of cells anterior to it, where it overlaps with EY expression (I, yellow).

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*^{2}, *so*^{1} and *eya*^{1} mutant eye discs. Genetic and molecular data indicated that the *so*^{1} and *eya*^{1} 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*^{1} and *eya*^{1} mutant eye discs are still viable. Eye discs from *ey*^{2} 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*^{1} (Fig. 4G) and *eya*^{1} 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*^{2} 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*^{1} 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 *eya*^{1} 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*^{2}, *so*^{1} or

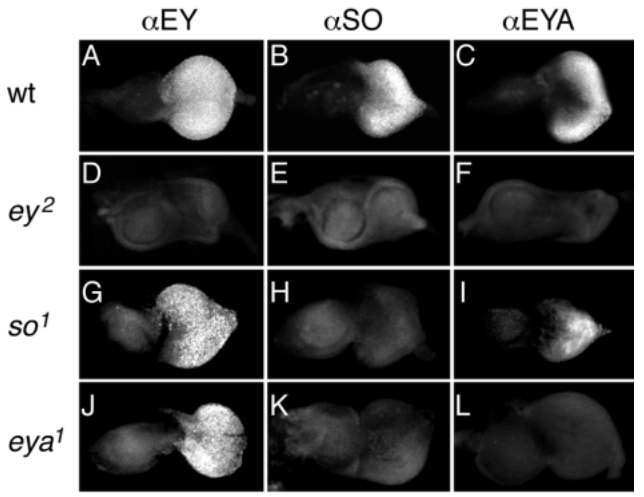

Fig. 4. Expression of EY, SO and EYA in early third instar eye discs. Same orientations as in Fig. 2. In wild-type early third instar eye-antennal discs EY expression (A) is detected in the entire eye portion of the disc and SO (B) and EYA (C) are expressed in a gradient with highest levels at the edge of the disc. In ey^2 mutant eye discs with strong phenotypes none of the three proteins could be detected (D-F). These eye discs are also reduced in size relative to the antennal portion. EY is expressed at wild-type levels in the entire eye disc of so^1 and eya^1 mutants (G,J). SO is not expressed in any of the three mutants (E,H,K). No EYA protein was detected in ey^2 or eya^1 mutant eye discs (F,L), while about half of the so^1 mutant eye discs showed low levels of EYA expression (I).

eya^1 mutants could immunoreactive material be detected with the respective antibody in the eye disc (Fig. 4D,H,L). This is consistent with ey^2, so^1 and eya^1 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,L). 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 so^1 and eya^1 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 so^1 and eya^1 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 so^1 and eya^1 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 so^1 and eya^1 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 so^1 and eya^1 mutants. Nevertheless, we found that in early to mid third instar wing discs, EY induced ectopic expression of EYA in a so^1 mutant background (Fig. 6F) and, conversely, SO was induced by EY in an eya^1 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, suggesting that *so* and *eya* are required for efficient induction of each other's expression. In summary, our results show that EY acts upstream of *so* and *eya* and requires 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. Czerny 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 ey^2 and ey^R 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 ey^2 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 ey^2 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 ey^2 mutant embryos or larvae, suggesting that ey^2 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 so^1 mutant background and SO expression in an eya^1 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, within 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*

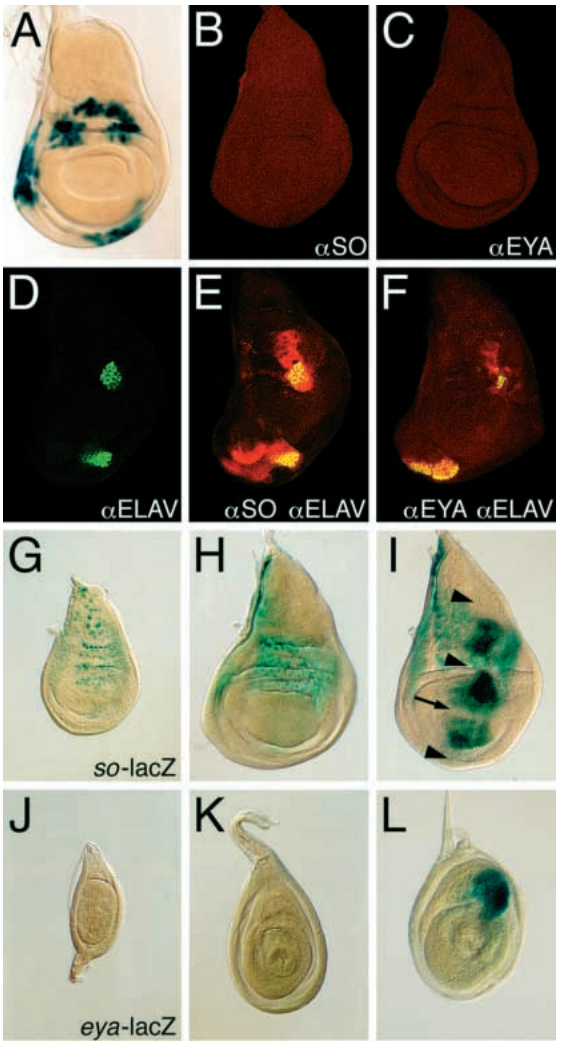

Fig. 5. EY induces ectopic expression of *so* and *eya*. (A) β-galactosidase activity staining of a late third instar wing disc expressing a UAS-lacZ reporter transgene driven by the E132 Gal4 driver (Halder et al., 1995a). (B,C) Wild-type wing discs stained for SO and EYA proteins, respectively. No immunoreactive material was detected by either antibody in the wing disc proper. We detected a nuclear protein in the peripodial membrane of the disc with the α-SO antibody (out of focus). (D-F) Wing discs ectopically expressing EY directed by the E132 Gal4 driver. The discs are double-stained for ELAV (green) and for SO (E, red) or EYA (F, red). (D,E) Same disc, D showing the ELAV pattern only. Ectopic expression of SO and EYA is observed in two domains where ectopic photoreceptor clusters develop corresponding to presumptive dorsal and ventral hinge regions. (G-L) Ubiquitous overexpression of EY induces ectopic expression of *so* and *eya* in a spatially restricted manner. β-galactosidase activity stainings of wing discs from larvae carrying a *so* enhancer trap chromosome (G-I) and of leg discs from larvae with an *eya* enhancer trap insertion (J-L). In addition, larvae in (G,I,J,L) carried the heat-inducible *ey* transgene. (G,J) β-galactosidase activity stainings of discs 83 hours after egg laying and prior to heat shocks. (G) The *so* enhancer trap is expressed in the large cells of the peripodial membrane of the wing disc but not in the disc proper. The *eya* enhancer trap is not expressed in leg (J) or wing discs (not shown) at this stage. (H,K) Stainings after heat-shocking larvae that did not carry the heat-inducible *ey* transgene. Expression patterns are unchanged by heat shock. (I,L) Stainings of discs from larvae carrying the heat-inducible transgene after six heat shocks. (I) Ectopic β-galactosidase expression of the *so* enhancer trap is detected in broad domains along the A/P boundary in wing discs. The reporter is not induced where WG is expressed, i.e. along the prospective wing margin (arrow) and in the hinge and notum (arrowheads; Baker, 1988). (L) The *eya* reporter is ectopically expressed in leg discs in a dorsal domain at the A/P boundary. In addition, the *eya* reporter is ectopically expressed in wing discs similar to *so*, and the *so* reporter is ectopically expressed in leg discs as observed for *eya* (not shown). In all panels dorsal is up and anterior is to the left.

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/toy* (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

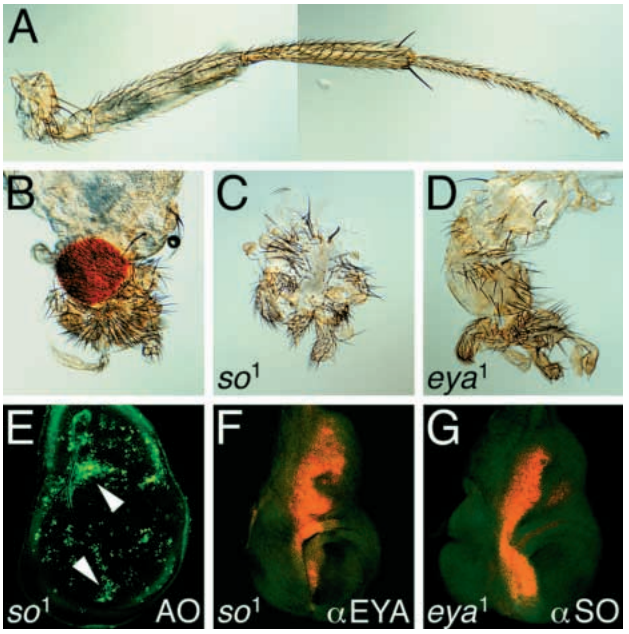

Fig. 6. SO and EYA are required for ectopic eye development and are independently induced by EY. (A) Wild-type leg. (B) Extra eye on a leg induced by dpp-Gal4 driven EY expression. The leg is abnormally short and totally deformed. Same magnification as A. dpp-Gal4 driven EY expression in a so^1 (C) or eya^1 (D) mutant background is unable to induce the development of extra eyes, but still leads to short and strongly deformed legs. (E) Ectopic expression of EY in a so^1 mutant background results in ectopic cell death in late third instar wing discs in the region where extra eyes would develop in a wild-type genetic background (arrowheads). The disc is stained with the vital dye Acridine orange. (F,G) Mid third instar wing discs of so^1 (F) and eya^1 (G) larvae ectopically expressing EY along the A/P boundary driven by dpp-Gal4. The discs are stained for EYA (F) and SO (G), respectively. Both genes are still induced by EY, independently of the function of the other gene. Both discs are double-stained with the α-ELAV antibody (green) that did not detect any developing photoreceptor cells, consistent with the observation that EY cannot induce extra eye development in so^1 and eya^1 mutants (C,D).

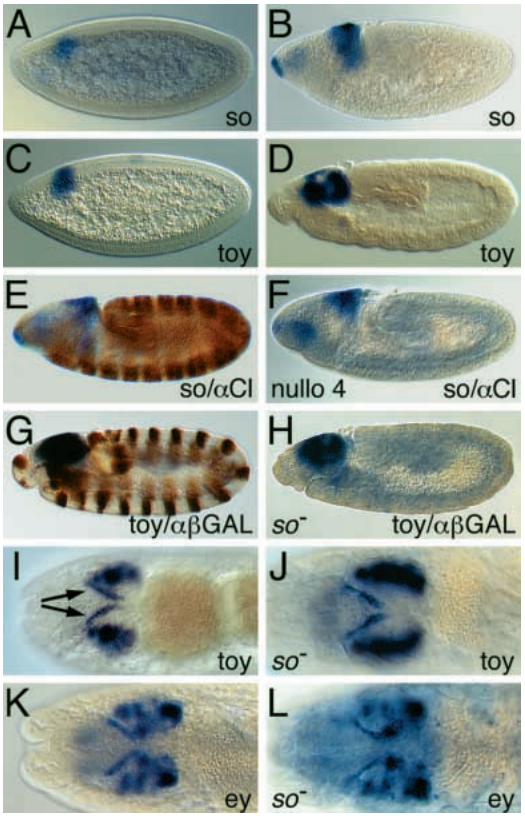

Fig. 7. *toy* and *so* act in parallel in the embryonic head. (A-D) Lateral views of wild-type embryos stained for *so* (A,B) or *toy* (C,D) transcripts. (A,C) Both genes start to be expressed at the cellular blastoderm stage in the procephalic neurogenic region (PNR) of the developing head. They continue to be expressed in the developing head and brain through germband extension (B,D). *toy* expression in the PNR is broader than that of *so*. Transcripts of *so* are also detected anterior to the stomodeal invagination at the anterior tip of the embryo in (B). (E,F) Germband extension-stage embryos stained for *so* transcripts (blue) and CI protein (brown). (E) C(4)RM *ci* ey^2 embryo (toy^+) and (F) nullo 4 embryo (toy^-, ey^-, ct^-). Transcripts of *so* are detected at normal levels in the PNR of nullo 4 embryos. Lack of CI protein allowed the identification of nullo 4 embryos (F). (G,H) Germband extension stage embryos stained for *toy* (blue) and β-galactosidase protein (brown). (G) so^-/*CyO*, *wg*-lacZ and (H) so^-/so^- embryos. *toy* is still expressed in the PNR of so^- embryos. (I-L) Dorsal views of the head region of stage-16 embryos stained for *toy* (I,J) or *ey* (K,L) transcripts. (I,K) Wild-type embryos, (J,L) so^-/so^- embryos that are also stained for β-galactosidase protein to identify the so^- mutant embryos as above. Expression of *ey* and *toy* in the V-shaped eye anlagen (arrows in I) is not affected in so^- embryos. The morphology of the eye anlagen also appears to be normal. All embryos are oriented anterior to the left. Embryos in A-H are lateral views with the dorsal side up.

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; Fujiwara 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 Eya1-3, indicating a surprisingly high degree of evolutionary conservation of the eye developmental program.

We are grateful to Seymour Benzer, Nancy Bonini, Mike Hoffmann, Graeme Mardon, Kevin Moses, Bill Gelbart, Joe O'Tousa and Larry Zipursky for fly stocks, antibodies and cDNAs. We thank Markus Dürrenberger, Jörg Hagman, Andreas Hefti and Steve Paddock for assistance with scanning electron and confocal microscopy. We thank Grace Panganiban, Markus Affolter, Nadean Brown, Kirsten Guss and Graeme Mardon for discussion and valuable comments on the manuscript and Erika Wenger-Marquardt for help with manuscript preparation. G. H. is grateful to Sean Carroll for supporting the later stages of this work. Supported by the Kantons of Basel and the Swiss National Science Foundation (W. J. G.) and by a Janggen-Poehn predoctoral fellowship (G. H.).

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