Cell, Vol. 91, 881–891, December 26, 1997, Copyright ©1997 by Cell Press

The Eye-Specification Proteins So and Eya Form a Complex and Regulate Multiple Steps in Drosophila Eye Development

Francesca Pignoni,* Birong Hu,*
Kenton H. Zavitz,* Jian Xiao,* Paul A. Garrity,*
and S. Lawrence Zipursky*†‡§
*Department of Biological Chemistry
†Howard Hughes Medical Institute
‡Molecular Biology Institute
The School of Medicine
University of California, Los Angeles
Los Angeles, California 90095

Summary

***sine oculis* (*so*) and *eyes absent* (*eya*) are required for Drosophila eye development and are founding members of the mammalian *Six* and *Eya* gene families. These genes have been proposed to act with *eyeless* (*Pax6*) to regulate eye development in vertebrates and invertebrates. *so* encodes a highly diverged homeobox transcription factor and *eya* encodes a novel nuclear protein. We demonstrate that So and Eya (1) regulate common steps in eye development including cell proliferation, patterning, and neuronal development; (2) synergize in inducing ectopic eyes; and (3) interact in yeast and in vitro through evolutionarily conserved domains. We propose that an So/Eya complex regulates multiple steps in eye development and functions within the context of a network of genes to specify eye tissue identity.**

Introduction

Recent studies suggest that the genetic program regulating eye development has been conserved during evolution. Mutations in Drosophila (*eyeless*, *ey*), mouse (*Small eye*), and human (*Aniridia*) *Pax6* genes lead to defects in eye development, whereas misexpression of the Drosophila or murine *Pax6* genes drives ectopic compound eye development in other fly tissues (e.g., the wing, leg, and antenna) (Hill et al., 1991; Ton et al., 1991; Glaser et al., 1994; Quiring et al., 1994; Halder et al., 1995). Three genes, *sine oculis* (*so*), *eyes absent* (*eya*), and *dachshund* (*dac*) have been proposed to act downstream of *ey* (Quiring et al., 1994; Shen and Mardon, 1997). *so* and *eya* are founding members of gene families in mouse and humans. The *so* homologs, called *Six* genes, contain a diverged homeodomain and a conserved region, the Six domain, which may contribute to DNA-binding specificity (Serikaku and O'Tousa, 1994; Cheyette et al., 1994; Oliver et al., 1995a; Oliver et al., 1995b; Kawakami et al., 1996a; Kawakami et al., 1996b). *eya* encodes a novel nuclear protein (Bonini et al., 1993). It shares a C-terminal domain of homology with vertebrate Eya proteins (Eya domain) (Xu et al., 1997a; Zimmerman et al., 1997). The mammalian *Eya* and *Six* genes are expressed in overlapping patterns in the developing mouse, including the eye primordium, and have been postulated to act with *Pax6* in a regulatory pathway controlling the development of eye and other tissues (Oliver et al., 1995b; Kawakami et al., 1996b; Xu et al., 1997a). A recently identified murine homolog of *dac* is also expressed in the developing eye (G. Mardon, personal communication). This conservation suggests that an understanding of the molecular and genetic circuitry underlying compound eye development in the fly will provide important insight into the mechanisms regulating mammalian eye development.

The compound eye of Drosophila comprises an array of some 750 simple eyes or ommatidia (reviewed by Wolff and Ready, 1993). Each ommatidium contains a precise number and arrangement of 11 cell types, including 8 photoreceptor neurons (R cells). The eye forms during the third instar of larval development from a columnar epithelium, the eye imaginal disc. Following a proliferative phase, a depression in the apical surface, called the morphogenetic furrow (MF), appears along the posterior edge of the eye disc (MF initiation). The MF then sweeps anteriorly across the disc (MF propagation), leaving in its wake developing ommatidial clusters. MF initiation and propagation require the *decapentaplegic* (*dpp*) and *hedgehog* (*hh*) signaling pathways, respectively (Heberlein et al., 1993; Ma et al., 1993; Wiersdorff et al., 1996; Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997). In the third larval instar, *dpp* is expressed along the posterior and lateral edges of the eye disc (Blackman et al., 1991). Misexpression of *dpp* at the anterior margin leads to the duplication of an entire eye disc, arguing that *dpp* may also pattern the disc prior to MF initiation (Pignoni and Zipursky, 1997). *hh* is both necessary and sufficient for MF propagation; as new ommatidia form they synthesize Hh, thereby inducing cells immediately anterior to the MF to initiate ommatidial development (Heberlein et al., 1993, 1995; Ma et al., 1993). Since *dpp* and *hh* play widespread roles in development (Kingsley, 1994; Hammerschmidt et al., 1997), other genes must confer the eye tissue identity upon the primordium and, hence, determine the eye-specific response to these signals.

The discovery that *ey* induces ectopic eyes led Gehring and coworkers to propose that *ey* is the master regulator of eye development, conferring eye identity upon tissue (Halder et al. 1995). In this model, *ey* lies at the apex of a genetic cascade that controls, either directly or indirectly, subordinate genes that execute the eye program. However, since *ey* functions in other tissues, additional factors must act in combination with *ey* to confer eye identity. Like *ey*, misexpression of *dac* in other imaginal discs induces eye tissue (Shen and Mardon, 1997), and loss-of-function mutations, though not eye-specific, lead to an eyeless phenotype (Mardon et al., 1994). Consistent with an early role in eye development, *ey* is expressed prior to MF formation. As the MF progresses anteriorly, *ey* disappears behind it (Quiring et al., 1994). *dac* encodes a novel nuclear protein expressed along the posterior and lateral margins of the eye disc prior to MF initiation and throughout the eye primordium during MF propagation. Through loss-of-function studies, *dac* has been shown to be required

§To whom correspondence should be addressed.

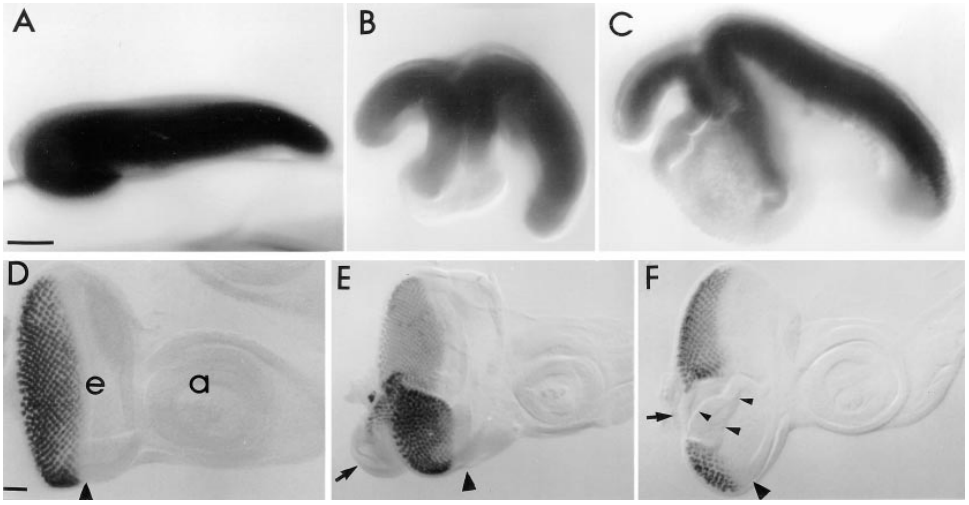

Figure 1. *so* and *eya* Control Cell Proliferation in the Eye Disc

(A–C) Third instar eye discs viewed in whole mount from the posterior end. Apical surface is up and dorsal to the left. This orientation facilitates visualization of overgrown regions within the eye disc and the boundary between mutant and wild-type tissue. Scale bar, 25 μm.

(A) Wild-type eye disc stained for a constitutive *lacZ* marker (see Experimental Procedures).

(B) so^3 mutant clone (*lacZ* negative tissue) and (C) so^1 mutant clone (anti-So antibody negative). The unstained mutant tissue bulges out of the disc epithelium due to overproliferation (see text and Experimental Procedures).

(D–F) Third instar eye-antennal discs viewed from the apical surface with posterior to the left and ventral down. The discs were stained with the neuron-specific anti-Elav antibody to visualize developing ommatidia. The position of the MF is indicated by arrowheads. Scale bar, 25 μm.

(D) Wild-type eye-antennal disc complex (e, eye disc; a, antennal disc).

(E and F) Cells in so^3 (E) and eya^{clift1} (F) mutant clones (arrows) do not differentiate into neurons as assessed using the neuron-specific anti-Elav antibody. As visualized by Nomarski optics from the apical surface, overproliferation is seen as additional folds in the epithelium (small arrowheads).

for MF initiation and neuronal development, but not for MF propagation (Mardon et al., 1994). Molecular epistasis studies suggest that *dac* acts downstream of *dpp* and *ey* (Mardon et al., 1994; Shen and Mardon, 1997; Pignoni and Zipursky, 1997).

As a step toward further dissecting the genetic program regulating eye specification, we have explored the role of *so* and *eya* in eye development. Prior to MF initiation, both genes are expressed along the posterior and lateral edges and at decreasing levels toward the central region of the disc. During MF propagation, *eya* and *so* remain expressed anterior to, within, and posterior to the MF. Defects in neuronal development and massive cell death in the developing eye have been reported for both *so* and *eya* mutants (Bonini et al., 1993; Cheyette et al., 1994). In this paper we demonstrate that *so* and *eya* play a key role in eye specification, regulating multiple steps including cell proliferation in the undifferentiated epithelium, MF initiation and propagation, and neuronal development. Genetic and molecular studies indicate that these genes function together. Comisexpression reprograms other imaginal discs to form ectopic eyes, and So and Eya directly interact through evolutionarily conserved domains. We propose that So and Eya and, by extension, their mammalian homologs, function as transcription factor complexes in an evolutionarily conserved program of eye development. The extensive cross-regulation between eye-specification genes at the level of transcription (*ey*, *dac*, *so*, and *eya*) and direct protein–protein interactions (Eya/So, this paper; Dac/Eya, Chen et al., 1997 [this issue of *Cell*]) argues that a network of interacting genes controls eye tissue specification.

Results

so and *eya* Function at Multiple Steps in Eye Development

That *so* and *eya* are continuously expressed in the eye disc from early steps in patterning to neuronal differentiation suggested that these genes might function at multiple steps in development. *so* and *eya* activities were assessed at different developmental stages using (1) eye-specific alleles, (2) mitotic recombination to induce homozygous mutant cells at different stages of development, and (3) selective temporal and spatial expression of *so* and *eya* transgenes to rescue mutant phenotypes.

so *and* eya *Regulate Growth*

Patches of cells homozygous for either the null so^3 mutation or the strong loss-of-function eya^{clift1} allele (i.e., mutant clones) were produced by mitotic recombination. Clones were induced at an early stage of development in an otherwise wild-type eye disc. so^3 and eya^{clift1} mutant cells overproliferate and fail to differentiate into neurons (Figures 1B, 1E, and 1F). Mutant clones retain their epithelial organization and lead to abnormal folding of the disc. Cells in these clones subsequently die (data not shown).

In the eye disc, as in many other tissues, differentiation is accompanied by the cessation of cell proliferation. Indeed, all cells arrest in G1 in the MF. Thus, overgrowth could result from a failure of cells to arrest in G1 and differentiate. Overgrowth of so^3 and eya^{clift1} mutant clones, however, is observed prior to the coordinated G1 arrest (data not shown). Hence, this phenotype reflects a loss of proliferation control in the undifferentiated epithelium. Alterations in proliferation do not appear to be

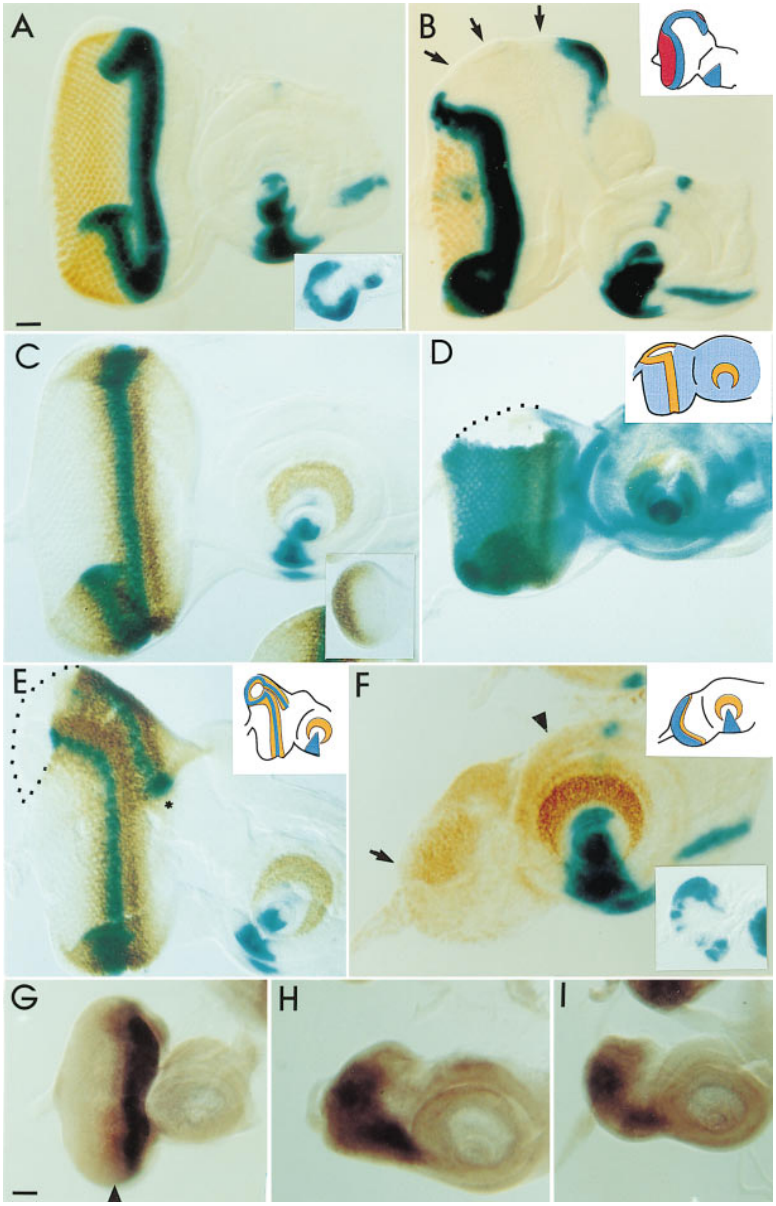

Figure 2. *so* and *eya* Are Required for the Expression of the MF-Initiation Factors *dpp* and *dac*

MF initiation does not occur in so^3 (B) and eya^{clift1} (see Figure 1F) clones encompassing the margins or in so^1 and eya^2 discs (Bonini et al., 1993; Cheyette et al., 1994). The expression of *ey* and the MF-initiation markers *dpp-lacZ* and Dac were assessed in so^3 and eya^{clift1} mutant clones and so^1 and eya^2 discs. See Experimental Procedure for generation and analysis of mutant clones. The insets in the upper right-hand corner of (B), (D), and (E) show the expected pattern of *dpp* (B and E) and Dac (D and E) expression in discs bearing *so* or *eya* mutant clones if these markers were expressed in the mutant tissue (see Mardon et al., 1994); the expression patterns would appear as a combination of the late/MF propagation (i.e., within the wild-type regions of the disc) and the early/MF-initiation patterns (i.e., within the mutant clones). The color code roughly matches the markers used.

(A) Wild-type third instar eye-antennal disc stained for Elav (brown) to visualize the developing ommatidial array and for *dpp-lacZ* (blue). Inset: Robust *dpp-lacZ* expression is observed along the posterior and lateral margins in early third instar discs prior to MF initiation.

(B) *dpp-lacZ* expression at the posterior margin is disrupted by an so^3 mutant clone marked by the absence of Elav staining (arrows).

(C) Wild-type mid-third instar eye-antennal disc stained for Dac (brown) and for the *dpp-lacZ* reporter (blue). Inset: Dac is expressed along the posterior and lateral margins in early third instar discs prior to MF initiation.

(D) A so^3 mutant clone along the lateral margin (dotted line). The clone is marked by the absence of a constitutive *lacZ* marker (see Figure 1 and Experimental Procedures) and does not express detectable levels of Dac.

(E) An eya^{clift1} clone along the posterior margin (dotted line) does not express detectable levels of *dpp-lacZ* (blue) or Dac (brown). Owing to the position of the mutant clone splitting the eye field, a second MF (asterisk) propagates from the dorsal margin toward the center of the disc.

(F) so^1 mutant disc stained for Dac (brown) and *dpp-lacZ* (blue). The disc was overstained to detect low levels of Dac expression in the eye disc. If *dpp* and Dac were expressed as in wild type, overlapping patterns of blue and brown staining would appear along the posterior and lateral margins of the disc as shown in the inset in the upper right corner. Dac expression is lower than normal, and *dpp-lacZ* is not detectable in the eye disc (arrow), whereas neither marker is affected in the antennal disc (arrowhead). Lower right inset: *dpp-lacZ* expression is patchy in early third instar so^1 discs (compare to wild-type disc shown in inset [A]).

(G–I) Eye-antennal discs stained for *ey* expression using in situ hybridization. Robust expression is seen in the region anterior to the MF (arrowhead) in wild-type (G) and throughout so^1 (H) and eya^2 (I) mutant discs.

Scale bars, 25 μm (A–F, H, I) and 35 μm (G).

a consequence of a change in identity since mutant cells in the undifferentiated region of the epithelium express *ey*, a marker for eye disc identity (see Figure 3G).

We previously reported that in discs entirely mutant for the eye-specific so^1 allele, neuronal differentiation was blocked and massive cell death was observed. Overproliferation was not seen (Cheyette et al., 1994). To address the discrepancy between the so^1 and so^3 data, we assessed the phenotype of so^1 homozygous clones (Figure 1C). As with the null mutant clones, massive overgrowth was observed, which was followed by cell death. Similarly, eya^{clift1} mutant clones overgrow (Figure 1F), whereas eya^2 discs show a phenotype identical to so^1 (Bonini et al., 1993); mutant clones of eya^2 were not analyzed. Hence, the phenotypes of so^3, eya^{clift1}, and so^1 mutant clones suggest that the cell death in so^1 and eya^2 is a secondary consequence of defects in development rather than reflecting a direct role for these genes in controlling cell death. In conclusion, both *so* and *eya* play a role in controlling proliferation in the eye primordium and may therefore contribute to regulating the size of the disc.

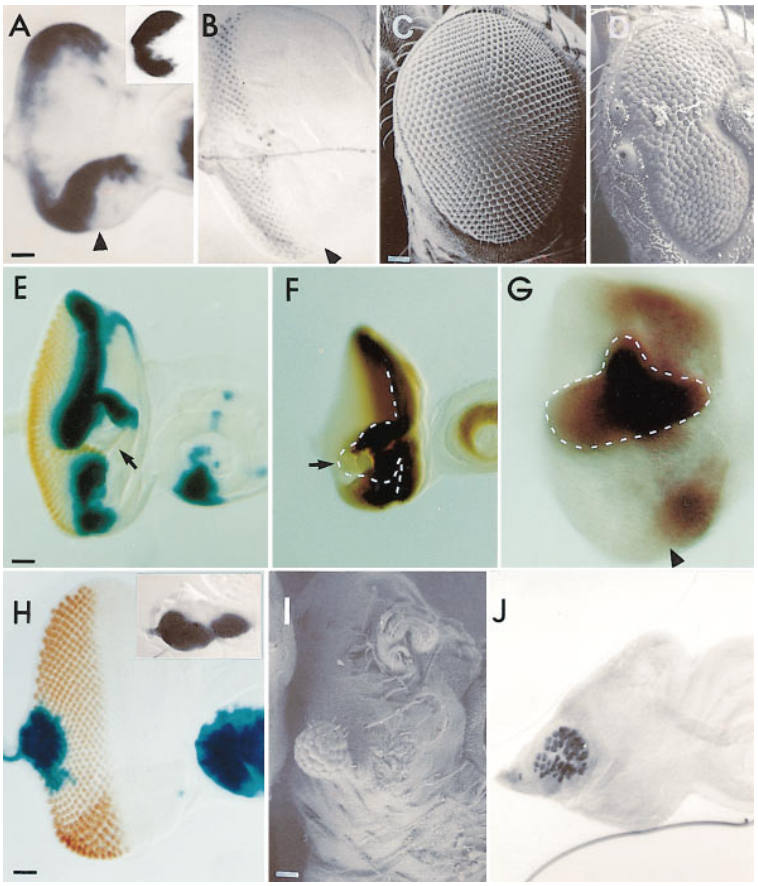

Figure 3. *so* and *eya* Are Required for MF Propagation

(A–D) Posterior expression of *so* and *eya* does not rescue anterior eye development in so^1 and eya^2 mutants. Scale bars, 25 μm (A and B) and 40 μm (C and D).

(A) *UAS-lacZ* expression driven by *dpp-GAL4* in early (inset) and mid-third instar discs. The position of the MF is marked by an arrowhead. Note that, in contrast to *dpp-lacZ* (Figure 2A), *dpp-GAL4* expression remains in the posterior and lateral regions during MF propagation. The reason for this discrepancy is not known (Chanut and Heberlein, 1997).

(B) Development of the neuronal array is rescued along the posterior and lateral but not the anterior regions of an so^1 mutant disc by *dpp-GAL4*-driven *so* expression. Ommatidial differentiation was assessed by the neuron-specific MAb22C10 staining. The arrowhead indicates the position of the MF. Differentiation is seen in less than 5% of so^1 discs without the *so* transgene (Cheyette et al., 1994; data not shown).

(C) Wild-type adult eye as seen using scanning electron microscopy (SEM). Thirty-two to 34 vertical columns of ommatidia can be counted across the eye from posterior to anterior (Wolff and Ready, 1993).

(D) A *UAS-eya* transgene under the control of *dpp-GAL4* rescues the posterior and lateral regions of an adult eya^2 mutant eye as seen using SEM. Nine vertical columns of ommatidia can be counted across the center of the eye from posterior to anterior. eya^2 flies without the *eya* transgene are completely eyeless.

(E–G) Propagation of the MF does not occur in so^1, so^3, and eya^{clift1} mutant clones as shown by lack of *dpp-lacZ* staining, lower Dac expression, and persistence of *ey* mRNA. A single focal plane of the region spanning the MF is shown for each preparation. Analyses through multiple focal planes reveal the highly irregular folding of the mutant tissue. See Experimental Procedure for analysis of mutant clones. Scale bar, 25 μm.

(E) An so^3 clone spanning the MF does not express *dpp-lacZ* (arrow).

(F) eya^{clift1} clone spanning the MF (white broken line) does not express Dac (arrow).

(G) An so^1 clone (outlined by white broken line) spanning the MF expresses *ey* as detected by in situ hybridization.

(H–J) So and Eya function are required during MF propagation. Scale bars, 25 μm (H and J) and 40 μm (I).

(H) The enhancer trap line *E132* drives *UAS-lacZ* expression (blue) in the posterior-most region of the late third instar eye disc. The neuronal array is visualized by anti-Elav staining (brown). Inset shows that *E132* drives expression through most of the early third instar eye disc. The transition from the early pattern to the late pattern occurs gradually.

(I) A *UAS-so* transgene under the control of *E132* rescues the most posterior region of an adult so^1 mutant eye as seen using SEM. Greater than 95% of so^1 flies without the *UAS-so* transgene are completely eyeless.

(J) Development of the neuronal array, detected by anti-Elav staining, is rescued in the most posterior region of eya^2 disc by expression of a *UAS-eya* transgene under the control of the *E132* driver. No anti-Elav staining was seen in eya^2 discs lacking the *UAS-eya* transgene.

so *and* eya *Are Required for MF Initiation*

MF initiation does not occur in so^3 and eya^{clift1} mutant clones (Figure 2B and 1F) or in so^1 and eya^2 mutant discs (Bonini et al., 1993; Cheyette et al., 1994). To examine further the role of *so* and *eya* in MF initiation, we assessed the expression of *dpp*, *dac*, and *ey* in mutant discs. In wild-type discs, *dpp* and *dac* are expressed along the posterior and lateral edges of the disc prior to MF initiation (Blackman et al., 1991; Mardon et al., 1994). During MF propagation, these genes are expressed in the advancing MF. Dac expression was assessed by antibody staining (Figure 2C), and *dpp* expression was visualized using a *dpp-lacZ* reporter (Figure 2A) that reproduces the in situ hybridization pattern (Blackman et al., 1991). Weak Dac expression was detected in so^1 and eya^2 discs (Figure 2F; data not shown) and along the posterior margin encompassed by so^3 and eya^{clift1} clones (Figures 2D and 2E). *dpp* expression was not detected in mutant clones or in mutant third instar discs (Figures 2B, 2E, and 2F; data not shown). A low level of *dpp* expression, however, was detected in second instar so^1 and eya^2 discs (see lower right inset in Figure 2F; data not shown). In contrast to Dac and *dpp*, *ey* mRNA is expressed at high levels in third instar so^1 and eya^2 discs (Figures 2H and 2I). In conclusion, both *so* and *eya* are required for MF initiation and play similar roles in this process.

so *and* eya *Are Required for MF Propagation*

To assess whether *so* and *eya* are required for MF propagation, it was necessary to examine mutant discs in which MF initiation occurs normally. This was accomplished using *dpp-GAL4* to drive expression of *UAS:so*

and *UAS:eya* transgenes in the posterior and lateral regions of so^1 and eya^2 mutant discs, respectively. *dpp-GAL4* drives expression in the posterior and lateral regions of the eye disc continuously from early second to late third instar (Figure 3A). Note that in contrast to *dpp-lacZ*, *dpp-GAL4* is not expressed in the MF (compare Figure 3A to Figure 2A; Chanut and Heberlein, 1997). Development of eye tissue in the posterior and lateral regions but not the anterior region of so^1 and eya^2 discs was rescued by *UAS:so* and *UAS:eya*, respectively (Figures 3B and 3D). The region rescued correlates well with the domain of *dpp-GAL4* expression. Consistent with these findings, so^1, so^3, and eya^{clift1} mutant clones spanning the MF also fail to differentiate. These clones express *ey*, overproliferate, and do not express MF associated markers (Figure 3E–G; data not shown). Hence, both *so* and *eya* are required for MF propagation.

Since *so* and *eya* mutant clones generated in first instar exhibit an overgrowth phenotype, the lack of development in third instar may reflect this early role rather than a requirement for these genes *during* MF propagation. To assess the timing requirement for *so* and *eya* activity, these genes were transiently expressed in the anterior region of the so^1 and eya^2 mutant discs during second and early third instar. This was accomplished by using the *E132-GAL4* driver (Halder et al., 1995; Pignoni and Zipursky, 1997). In *E132*, GAL4 is expressed through most of the eye disc during the late-second and early-third instar stage (see inset in Figure 3H). During third instar, Gal4 expression gradually becomes restricted to the region adjacent to the optic stalk (Figure 3H). Rescue was restricted to this most posterior region of the mutant discs (Figures 3I and 3J). Hence, expression anteriorly in early third instar is not sufficient to support MF propagation. In conclusion, *eya* and *so* are required *during* MF propagation.

so *and* eya *Are Required for Neuronal Development*

so and *eya* are also expressed in most, if not all, cells posterior to the MF (Bonini et al., 1993; Cheyette et al., 1994). To assess whether *so* and *eya* are required in this region, homozygous mutant cells were produced selectively posterior to the MF. This was accomplished by inducing mitotic recombination posterior to the MF using a variation of the FLP/FRT method (Xu and Rubin, 1993). FLP recombinase was placed under the control of GMR, an eye-specific enhancer that turns on only after cells enter the MF (Hay et al., 1994). This restricts FLP expression and, hence, mitotic recombination between FRT-containing chromosomes to regions posterior to the MF. The patterns of mitosis in the developing eye disc are highly regulated, with the precursors of R2, R3, R4, R5, and R8 undergoing their final mitosis anterior to the MF, while the precursors of R1, R6, and R7 undergo their final mitosis posterior to the MF (reviewed by Wolff and Ready, 1993). Thus, only precursors to R1, R6, and R7 divide after the onset of FLP expression and hence are susceptible to mitotic recombination. Control experiments established that GMR-FLP drives mitotic recombination in only those cells dividing posterior to the MF; GMR-FLP-induced recombinants mutant for a gene required in R1, R6, and R7 (i.e., *phyllopod,*; Chang et al., 1995; Dickson et al., 1995) displayed the mutant phenotype, whereas GMR-FLP-induced recombinants mutant for a gene required in R8 (i.e., *bride of sevenless*; Reinke and Zipursky, 1988) did not.

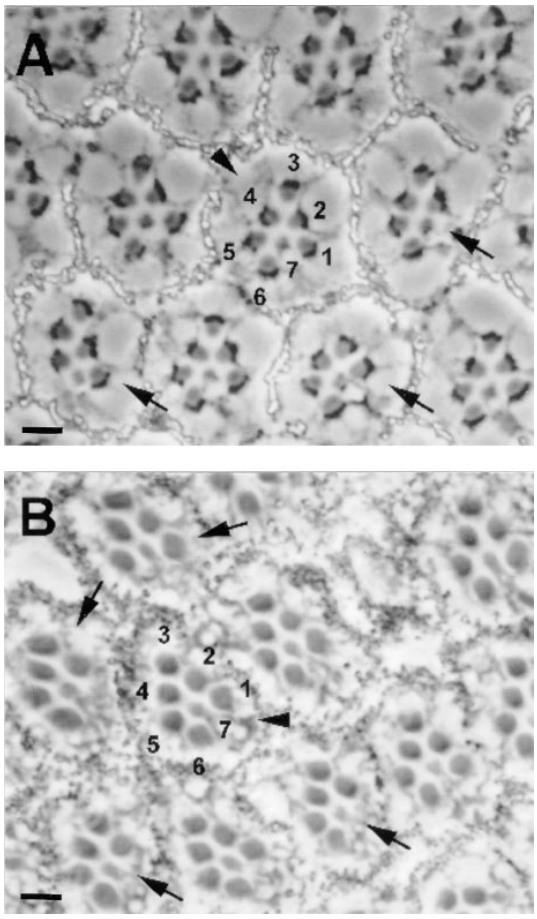

Figure 4. *so* and *eya* Are Required Posterior to the MF

R cell development is disrupted in so^3 or eya^{clift1} mutant cells. Plastic sections of adult eyes from flies carrying *GMR-FLP* and *FRT-*so^3 (A) or *FRT-*eya^{clift1} (B). Several ommatidia lack a single R cell (i.e., R1, R6, or R7; arrows). *GMR-FLP* mediates recombination in only a fraction of the precursor cells to R1, R6, and R7. It does not mediate recombination in the precursors to R2, R3, R4, R5, and R8. Examples of wild-type ommatidia are marked by arrowheads; R cell are indicated by numbers. See text for quantification, controls, and explanation of method used to generate mutant cells. Scale bars, 3 μm.

GMR-FLP-induced recombinants of so^3 and eya^{clift1} were analyzed in sections of adult eyes. If every dividing cell posterior to the MF were to undergo mitotic recombination, about 25% of the R1, R6, and R7 cells would be homozygous mutant. For *phyllopod*, 7% (111/1542) of the R1, R6, and R7 cells were missing. Similarly, for eya^{clift1} and *so3* some 9% (49/555) and 3% (45/1473) of these cells were missing, respectively (Figure 4). Variation in the fraction of mutant cells affected by these mutations may reflect differences in recombination efficiency of different FRT chromosomes and/or in the perdurance of the wild-type gene products. These findings are consistent with inhibition of R cell differentiation induced by selective expression of a dominant negative form of *so* posterior to the MF (data not shown). In

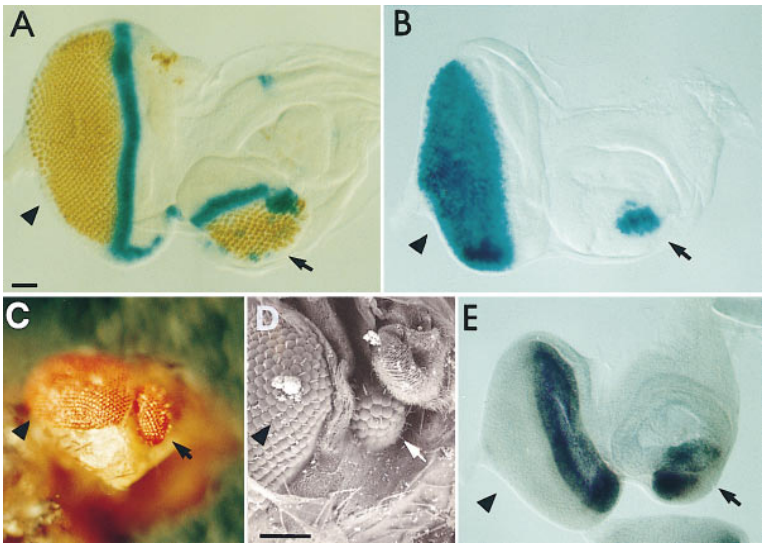

Figure 5. Coexpression of *so* and *eya* Induces Ectopic Eyes in the Antennae

In all panels the normal eye is marked by an arrowhead and the ectopic eye by an arrow.
(A and B) Coexpression of *so* and *eya* in the antennal disc under the control of the *dpp-GAL4* driver induces an ectopic eye field as detected by staining for Elav (A) and the eye-specific marker *GMR-lacZ* (B). An MF is induced as shown by the *dpp-lacZ* staining observed in (A). *dpp-GAL4* drives expression of UAS responder genes in the ventral region of the antennal disc. This corresponds to the position of the ectopic eyes induced by *so/eya*.
(C) Ectopic eye on the antenna of an adult fly as seen using light microscopy.
(D) Ectopic eye on the ventral side of the antenna as seen using SEM.
(E) Ectopic expression of *ey*, detected by in situ hybridization, is induced by coexpression of *so* and *eya* in the antennal disc.
Scale bars, 25 μm.

conclusion, both *so* and *eya* are required for neuronal development posterior to the MF.

In summary, detailed phenotypic analysis of *so* and *eya* establishes that these genes have indistinguishable mutant phenotypes in the developing eye (see Discussion) and are required at multiple steps in eye development.

Ectopic Eye Development Is Induced by Coexpression of *so* and *eya*

The identical mutant phenotypes and expression patterns of *so* and *eya* raised the possibility that these proteins may function together. To test this hypothesis, we assessed genetic interactions between them. Whereas loss-of-function studies were uninformative (see Experimental Procedures), ectopic expression studies revealed a striking synergy. *UAS:eya* and *UAS:so* were expressed alone or in combination under the control of *dpp-GAL4* (Figure 5). This driver promotes *ey*- or *dac*-induced ectopic eyes in other imaginal discs (Shen and Mardon, 1997). Ectopic *so* expression had little or no effect on antennal (0/63), wing, or leg disc development, whereas ectopic *eya* expression often caused mild growth alterations resulting in extra folds in the epithelium and, rarely, formation of small ectopic ommatidial arrays in the antennal disc (2%; 2/89). In adult flies, ectopic *eya* often induced very small patches of red pigment cells on the antennae, wings, and legs. We have observed considerable variation in the efficiency of ectopic eye induction with different *UAS* responder lines expressing the same gene (e.g., *ey*, *eya*, or *dac*) and different *dpp-GAL4* lines. The conditions used in these experiments induce eye development at a low frequency, allowing us to better assess synergy between genes.

Coexpression of *so* and *eya* led to a dramatic increase in the development of ectopic eye tissue in antennal discs (76%; 58/76) as assessed with the neuron-specific anti-Elav antibody and the eye-specific reporter *GMR-lacZ* (Figures 5A and 5B). That these ommatidial arrays lead to adult eye structures is shown in Figures 5C and 5D. Extensive growth alterations and scattered cells expressing both Elav and *GMR-lacZ* were seen in the wing and leg discs (data not shown). In the adult, an increase in the frequency and size of red pigmented patches on wings and legs was observed. The striking synergy detected between So and Eya provides strong genetic evidence for a functional interaction between them.

Ectopic *so/eya* induced *ey* expression in the antennal disc (Figure 5E). To assess whether *ey* was required for ectopic eyes, *so* and *eya* were coexpressed in an *ey* mutant background. Although growth alterations were still seen, ectopic eyes were not observed. Furthermore, ectopic eye formation was sensitive to the dosage of *ey* showing a reduction in an *ey* heterozygous background. This is consistent with the identification of loss-of-function alleles of *ey* as dominant suppressors of a weak *so* phenotype (F. P. and S. L. Z., unpublished data). These and other cross-regulatory interactions (see Discussion) reported among early eye genes may be necessary for both ectopic and normal eye induction.

So and Eya Interact through Evolutionarily Conserved Domains

The simplest explanation for the genetic results presented in the previous sections is that So and Eya function requires their physical interaction. Interactions between So and Eya were tested in yeast and in vitro. Various combinations of LexA DNA-binding domains and GAL4 activation domains fused to So and Eya protein fragments were tested for interactions in a yeast two-hybrid system (Figure 6A and 6B). Full-length Eya fused to LexA showed strong transcriptional activation of the *lacZ* reporter gene on its own. The sequences responsible for this activation were localized to the N-terminal domain of Eya (amino acids 1–483; data not shown; Xu et al., 1997b). The C-terminal Eya domain fused to the LexA DNA-binding domain (LexA-EyaD) did not activate transcription on its own. However, it did activate transcription through its interaction with full-length So fused to the Gal4 activation domain. Further deletion studies of So localized the interacting region to the Six domain (Figure 6B).

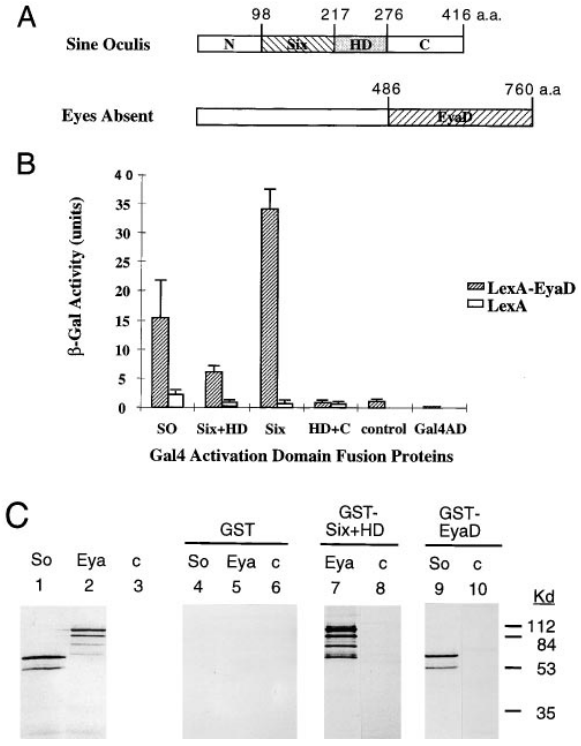

Figure 6. So and Eya Bind to Each Other in Yeast and In Vitro

(A) Schematic diagram of the domain structures of So and Eya. Abbreviations: N, N-terminal; Six, domain conserved between mammalian So homologs (*Six* genes) and So; HD, homeodomain; C, C-terminal; EyaD, domain conserved between mammalian homologs and Eya.

(B) Interactions between the indicated regions of So fused to the transcription activation domain of Gal4 and EyaD fused to the LexA DNA-binding domain (LexA-EyaD) or LexA DNA-binding domain alone (LexA) were assessed using a yeast two-hybrid assay. Interactions between fusion proteins in yeast result in the expression of β-galactosidase (β-galactosidase activity is expressed as Miller Units, see Experimental Procedures). The Gal4 activation domain, alone (Gal4AD) or fused to the SH2-SH3 adapter protein Dock (control), is shown as controls.

(C) So and Eya interact in vitro. Aliquots (20 μl) of in vitro–translated [^{35}S]methionine-labeled So (lanes 4 and 9), Eya (lanes 5 and 7), or a control protein (c, Cyclin A; lanes 6, 8, and 10) were incubated with glutathione-agarose beads containing bound GST (lanes 4–6), GST-Six+HD (lanes 7 and 8) or GST-EyaD (lanes 9 and 10). Bound proteins were fractionated by SDS-PAGE and visualized by autoradiography. An aliquot (2 μl) of in vitro translation products is shown in lanes 1–3. In this experiment, GST-Six+HD bound 55% of the input ^{35}S-Eya protein, whereas GST-EyaD bound 7% of the input ^{35}S-So protein.

To assess whether the yeast interaction data reflected direct binding between So and Eya, the ability of these proteins to interact in vitro was tested. Eya and So were labeled with ^{35}S-methionine using an in vitro transcription/translation reaction. Labeled products were then absorbed to either GST fused to a fragment of So containing the Six and homeobox domains (GST-Six+HD) or to the Eya domain (GST-EyaD) immobilized on glutathione agarose. Following extensive washing, bound proteins were eluted and analyzed by SDS-PAGE, followed by autoradiography. GST-Six+HD bound Eya and GST-EyaD bound So (Figure 6C). As negative controls, GST-Six+HD and GST-EyaD did not interact with other labeled proteins (Figure 6C; see Experimental Procedures). Hence, Eya and So interact directly through the evolutionarily conserved Eya and Six domains.

Discussion

In this paper we have presented a detailed study of *so* and *eya* function during eye development. We demonstrated that: (1) *so* and *eya* regulate multiple steps in eye development and display indistinguishable mutant phenotypes; (2) *so* and *eya* show marked synergy in inducing ectopic eye tissue; (3) ectopic eye induction by *so* and *eya* is *ey*-dependent; (4) So and Eya physically interact in yeast and in vitro; and (5) eye-specification genes are linked by multiple cross-regulatory interactions.

These results and those of Mardon and coworkers (Shen and Mardon, 1997; Chen et al., 1997) suggest an alternative view of eye specification from that proposed by Gehring and colleagues (Halder et al., 1995). In their model, *ey* is the master control gene for eye morphogenesis and functions as a genetic switch to specify eye tissue: *ey* occupies a position at the top of a genetic cascade inducing the expression of a subordinate set of regulatory genes controlling different aspects of eye morphogenesis. This model was based on the ability of *ey* to induce ectopic eyes in other imaginal discs, its expression in the undifferentiated region of the developing eye primordium, and its "eyeless" loss-of-function phenotype. However, since *ey* is expressed and required in other tissues, it cannot specify eye tissue alone but must do so in combination with other factors. The studies described in this paper and by Mardon's group have led to the identification of a set of eye-specification genes that when misexpressed, alone or in combination, induce ectopic eyes. Like *ey*, these genes all display an "eyeless" loss-of-function phenotype, are expressed in the undifferentiated eye epithelium, and are required for the development of other tissues. Genetic and molecular studies revealed that these genes are all required for eye specification and are interconnected through extensive cross-regulatory interactions at the levels of gene expression and direct protein–protein interactions. We propose that eye specification is controlled by a network of interacting genes, including *ey*, *so*, *eya*, and *dac*, rather than by *ey* as the master regulator. That homologs of all these genes are expressed in the developing mouse eye raises the possibility that they all contribute to an evolutionarily conserved eye-specification program as originally proposed for *ey/Pax-6* by Gehring and colleagues.

So and Eya Form a Complex and Function at Multiple Steps in Eye Development

Based on genetic and molecular studies, we propose that So and Eya form a transcription factor complex; So binds to specific *cis*-acting regulatory sites through the Six- and homeo-domains (Kawakami et al. 1996a) and the N terminus of Eya provides a transcriptional activation function. The domains mediating the interaction between these two proteins map to the evolutionarily conserved Six and Eya domains. That this interaction

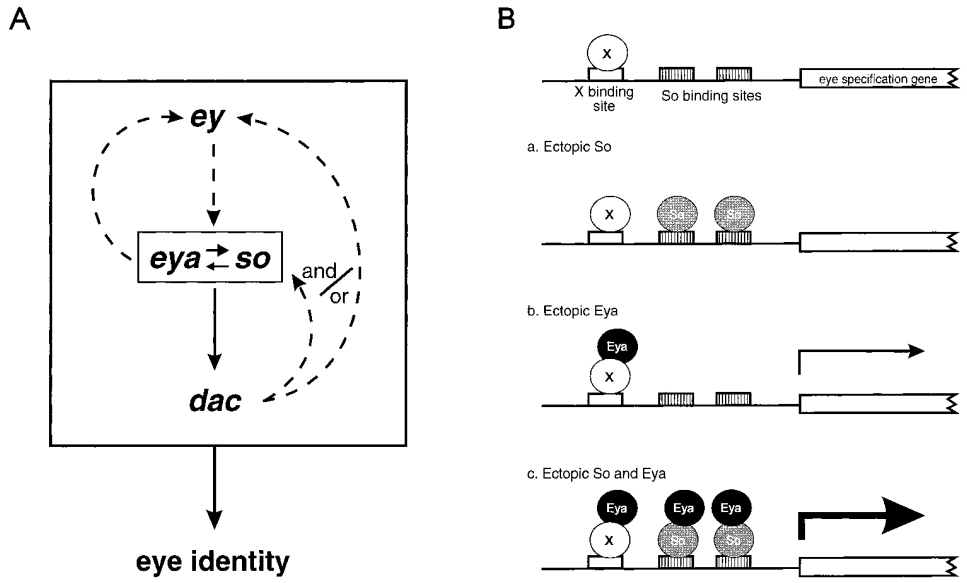

Figure 7. Regulation of Eye-Specification Genes

(A) A network of genes specifies eye tissue identity. The solid and dashed arrows indicate genetic interactions inferred from loss- and gain-of-function studies, respectively (this paper; Halder et al., 1995; Chen et al., 1997; Shen and Mardon, 1997; and G. Mardon, personal communication). The relationships between these genes are based on the following evidence: (1) *ey* is expressed in *so*, *eya*, or *dac* mutant tissue; (2) normal levels of *dac* require both *so* and *eya*, whereas *dac* is not required for *so* or *eya* expression; (3) although *eya* is required for initiation of *so* expression and *so* is not required for *eya*, both *so* and *eya* cross-regulate to maintain their expression (Pignoni and Zipursky, unpublished data); (4) ectopic expression of *eya*, *so/eya*, or *dac* leads to induction of all other genes including *ey*; and (5) induction of ectopic eye tissue requires the activities of all four genes.

(B) A model for ectopic eye induction by Eya and So. We consider the simplified view of a control region of a hypothetical eye-specification gene in the antennal disc. In a normal antennal disc, one site is occupied by factor X (either a specific or general transcription factor), which on its own does not support transcription. The ectopic expression of Eya and So leads to the following results: (a) Binding of So alone is not sufficient to induce expression. (b) X can recruit ectopic Eya. This provides relatively weak gene induction through the Eya activation domain. (c) Eya is recruited both by X and So bound to multiple sites. The multiplicity of Eya binding sites leads to synergistic transcriptional activation. This model can be modified to incorporate the ability of Ey and Dac expression to induce ectopic eyes and for Eya to synergize with Dac (see text and Chen et al., 1997). For simplicity, the model emphasizes synergy at the level of transcriptional activation. Other mechanisms such as cooperative binding of proteins and protein complexes to promoters may also contribute to synergy between eye-specification factors. A model for the function of So/Eya and Dac in regulating the expression of genes during normal development is presented by Chen et al. (1997).

may be of functional consequence in mammals is suggested by the extensive overlap in expression pattern of the *Six* and *Eya* genes during mouse embryogenesis (Xu et al., 1997a). This has led to the view that different combinations of *Six* and *Eya* genes control the development of diverse tissues (Xu et al., 1997a). Our data raise the intriguing notion that functional diversity may reflect distinct activities of different Six/Eya complexes.

So and Eya regulate multiple steps in eye development. They may be required continuously to control the expression of a set of genes which, in turn, regulate different aspects of eye development. However, we favor an alternative view wherein the So/Eya complex directly controls different steps in the developmental program in combination with different transcriptional regulators. Two proteins that may function in combination with So/Eya are Mad and Dac. *Mad* encodes a DNA-binding transcription factor in the *dpp* pathway and *Mad* mutations exhibit a similar MF-initiation phenotype to *so* and *eya* (Kim et al., 1997; Wiersdorff et al., 1996). In contrast to *eya* and *so*, however, *Mad* is not required for either MF propagation or neuronal development. Mad and So/Eya may directly bind to different sequences in the regulatory regions of genes required for MF initiation and act in concert to control their expression. Dac may function in a different way. It directly binds to Eya but does not have a known DNA-binding domain. *dac* displays a subset of *so* and *eya* mutant phenotypes in both initiation and neuronal development, but is not required for MF propagation. Studies of Chen et al. (1997) suggest a model in which Dac is recruited to regulatory regions of MF-initiation-specific promoters through interactions with other protein complexes and modulates the activity of So/Eya complexes bound to different sequences within the promoter/enhancer region. Dac may also function in this manner in a subset of steps in which Eya and So function during subsequent neuronal development. Thus, we propose that the function of So/Eya bound to specific DNA sequences is modulated at different stages of eye development by combinations of proteins bound to other regulatory sequences, the interactions between them and the So/Eya complex, or both.

Although we propose that Eya and So function as a complex during eye development, So and Eya are not obligate partners during development of other tissues. They are expressed in different patterns in the embryo and have different embryonic mutant phenotypes (Cheyette et al., 1994; V. Hartenstein, personal communication). Hence, Eya and So also function on their own or in other complexes during development. Indeed, we cannot exclude the possibility that So and Eya may also

function independently of each other in the developing eye, in addition to the activities associated with the So/Eya complex.

A Network of Genes Regulates Eye Specification

How does ectopic expression of different eye-specification genes (i.e., *ey*, *dac*, *eya*, *eya/dac*, *eya/so*) lead to ectopic eye induction? This reflects the extensive cross-regulatory interactions between eye-specification genes (Figure 7A). These genes, alone or in combination, induce transcription of the other eye-specification genes, all of which are then required for ectopic eye development (this paper; data not shown; Chen et al., 1997). Although we do not know how this occurs at the molecular level, the structures and properties of the encoded proteins suggest that they may directly regulate each other's transcription. Both Ey and So are DNA-binding proteins. Eya and Dac are nuclear proteins, which, though lacking recognizable DNA-binding motifs, contain strong transcriptional activation domains. We propose a simple model to account for the ability of these genes to activate expression of eye-specification genes in other imaginal discs (Figure 7B). Ey or a So/Eya complex would directly bind to specific DNA sequences and activate transcription. In contrast, high levels of Eya or Dac would drive their association with factors already bound to eye-specification genes in other tissues, thereby promoting transcription. Binding of Eya to Dac (Chen et al., 1997) would promote more efficient assembly of transcriptional activation complexes resulting in the observed synergy of ectopic eye induction.

The data presented in this paper and those of Mardon and colleagues (Chen et al., 1997; Shen and Mardon, 1997) establish that, whereas initiation of eye-specification gene expression occurs in a largely linear fashion (see Figure 7A), all eye-specification genes are linked in a regulatory network encompassing controls at the levels of transcription and protein–protein interactions. We propose that it is this network that "locks in" the eye-specification program. Since all eye-specification genes identified so far also function in other tissues, it is the unique combination of eye-specification genes that confers eye identity.

Experimental Procedures

Genetics

For description of *so* and *eya* mutants see Cheyette et al. (1994) and Bonini et al. (1993), respectively. For other mutations see Lindsley and Zimm (1992). In genetic interaction crosses, the adult eye phenotype of so^1 $eya^2/+$ eya^{clift1}, so^3 $eya^2/+$ eya^{clift1}, and so^3 eya^2/so^9+ flies did not differ significantly from eya^2/eya^{clift1} and so^3/so^9. For the misexpression experiments, *so* (Cheyette et al., 1994) and type II *eya* (Bonini, et al., 1993) cDNAs were cloned into the pUAST transformation vectors. The *dpp-GAL4* line used was obtained from the Bloomington Stock Center. *dpp-GAL4/UAS:so-UAS:eya* flies were unable to eclose and were dissected out of the pupal case. To test the induction of ectopic eyes in a mutant background, we used the ey^2 and so^1 alleles. Patches of red pigment cells could be observed on the legs of ey^2/ey^2; *dpp-GAL4/UAS:so-UAS:eya* flies.

Generation and Analysis of Mutant Tissue

Homozygous mutant clones of so^3 and eya^{clift1} were generated during first instar by *hsp70-FLP*-mediated *FRT* recombination (Xu and Rubin, 1993). FRT-containing chromosomes (Xu and Rubin, 1993) carrying *dpp-lacZ*, so^3, so^3 and *dpp-lacZ*, or eya^{clift1} were generated by meiotic recombination. Third instar discs containing so^3 and eya^{clift1} mutant clones were singly stained for the *lacZ* marker "construct D" (Tio and Moses, 1997) on 2L (for eya^{clift1}) or 2R (for so^3), for the *dpp-lacZ* BS3.0 construct (Blackman et al., 1991), the neuronal antigen Elav (Robinow and White, 1991) or the Dac protein (Mardon et al., 1994). In assessing the expression of Elav or Dac, we also relied on double staining with construct D (so^3) or *dpp-lacZ* (so^3 and eya^{clift1}) to locate the mutant tissue within the disc. Due to the highly folded configuration of the mutant clones, scoring for *dpp-lacZ* or Dac expression in double- or single-stained tissue was carried out on discs suspended in a droplet of mountant prior to placing of the coverslip. In most cases, the precise clonal boundaries were difficult to follow in these preparations. Hence, while we can conclude that mutant tissue fails to develop, we cannot exclude the possibility that surrounding wild-type tissue may also be affected in a nonautonomous fashion. Mutant cells posterior to the MF were generated by *GMR-FLP*-mediated recombination. so^1 mutant clones were generated by transposase-induced loss of a *hsp70-so* transgene in an so^1/so^1 mutant background. In addition to basal expression of the *hsp70-so* transgene, heat shock–induced expression (during third instar) was also used to rescue the mutant phenotype in cells retaining the transgene. The partial rescue of so^1 and eya^2 by *dpp-GAL4*- or *E132*-driven expression of *UAS:so* and *UAS:eya* transgenes resulted in very similar but not identical phenotypes. Whereas rescue of eya^2 with the *UAS:eya* transgene gave a uniform amount of rescue in both larvae and adults, rescue of so^1 with the *UAS:so* transgene was more variable and frequently more robust than rescue of eya^2 by *UAS:eya*. This difference is most likely due to the nature of the so^1 allele: about 5% of so^1/so^1 adult flies have eyes, though of reduced size; and the so^1 allele disrupts initiation but not maintenance of *so* transcription (Cheyette et al., 1994).

Histology and Scanning Electron Microscopy

Antibody, β-galactosidase, and in situ hybridization staining procedures were as previously described (Pignoni and Zipursky, 1997). The following antibodies were used: anti-Elav MAb (Robinow and White, 1991), anti-Eya MAb (Bonini et al., 1993), anti-Dac MAb (Mardon et al., 1994), and 22C10 MAb (Fujita et al., 1982). *so* (Cheyette et al., 1994) and *eya* type I (Bonini et al., 1993) cDNAs were used as templates to produce digoxygenin-labeled RNA probes. SEM and plastic eye sections were carried out as previously described (Cheyette et al., 1994).

Yeast Two-Hybrid Assay

A variation of the yeast two-hybrid assay was used in which one protein was fused to the DNA-binding domain of E. coli LexA (in vector pBTM116) and the other protein to the transcription activation domain of yeast Gal4 (in vector pGAD424) (Bartel and Fields, 1995). Yeast strain L40 (MATa his3Δ200, trp1–901 leu2–3,112 ade2 LYS::$(LexAop)_4$-HIS3 URA::$(LexAop)_8$-LacZ) (Hollenberg et al., 1995) was used in all experiments. Cotransformants with the two fusion plasmids were selected on trp^-, leu^-, ura^-, and lys^- plates. Interaction between fusion proteins activates *lacZ* expression. Liquid β-galactosidase assays were performed as described (Bartel and Fields, 1995). β-galactosidase activity (in Miller units) was calculated as follows: A420 × $1000/OD_{600}$ of the cell culture × the culture volume (ml) × reaction time (min). Values shown are averaged from assays on cultures of at least three independent transformants.

In Vitro GST-Fusion Protein Binding Assay

For in vitro binding, 20 μl of reticulocyte translate (Promega) was added to 0.5 ml binding buffer (20 mM HEPES-KOH [pH 7.7], 100 mM NaCl, 0.05% NP-40, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 100 μg/ml BSA, 10% glycerol) with 30 μl glutathione-agarose containing 1–2 μg of bound GST, GST-Six+H, or GST-EyaD, and rotated for 2 hr at 4°C. The beads were washed five times with 0.5 ml of PBS before electrophoresis and autoradiography. GST-fusion proteins were prepared as described in Smith (1983).

Acknowledgments

We thank U. Banerjee, J. A. Lengyel, L. Leyns, and G. Mardon for comments on the manuscript; L. Ballard for sectioning of adult eye sections; G. Mardon, G. M. Rubin, N. Bonini, and S. Benzer for antibodies and cDNA clones; and the Bloomington Stock Center

for fly stocks. We also thank G. Mardon and G. Oliver for helpful discussions and for communicating results prior to publication. This work was supported by a Helen Hay Whitney foundation fellowship (P. A. G.), a Leukemia Society of America fellowship (K. H. Z.), and a grant from the NIH (S. L. Z.). S. L. Z. is an Investigator of the Howard Hughes Medical Institute.

Received September 22, 1997; revised November 10, 1997.

References

Bartel, P.L., and Fields, S. (1995). Analyzing protein-protein interactions using two-hybrid systems. Methods Enzymol. *254*, 241–263.

Blackman, R.K., Sanicola, M., Raftery, L.A., Gillevet, T., and Gelbart, W. (1991). An extensive 3′ *cis*-regulatory region directs the imaginal disc expression of *decapentaplegic*, a member of the TGF-β family in Drosophila. Development *111*, 657–666.

Bonini, N.M., Leiserson, W.M., and Benzer, S. (1993). The eyes absent gene: genetic control of cell survival and differentiation in the developing Drosophila eye. Cell *72*, 379–395.

Chang, H.C., Solomon, N., Wassarman, D.A., Karim, F.D., Therrien, M., Rubin, G.M., and Wolff, T. (1995). Phyllopod functions in the fate determination of a subset of photoreceptors in Drosophila. Cell *80*, 463–472.

Chanut, F., and Heberlein, U. (1997). Role of decapentaplegic in initiation and progression of the morphogenetic furrow in the developing Drosophila retina. Development *124*, 559–567.

Chen, R., Amoui, M., Zhang, Z., and Mardon, G. (1997). Dachshund and Eyes Absent proteins form a complex and function synergistically to induce ectopic eye development in Drosophila. Cell *91*, this issue, 893–903.

Cheyette, B.N.R., Green, P.J., Martin, K., Garren, H., Hartenstein, V., and Zipursky, S.L. (1994). The Drosophila sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system. Neuron *12*, 977–996.

Dickson, B., Dominguez, M., van der Straten, A., and Hafen, E. (1995). Control of Drosophila photoreceptor cell fates by phyllopod, a novel nuclear protein acting downstream of the Raf kinase. Cell *80*, 453–462.

Fujita, S.C., Zipursky, S.L., Benzer, S., Ferrus, A., and Shotwell, S.L. (1982). Monoclonal antibodies against the Drosophila nervous system. Proc. Natl. Acad. Sci. USA *79*, 7929–7933.

Glaser, T., Jepeal, L., Edward, J.G., Young, S.R., Favor, J., and Maas, R.L. (1994). PAX6 gene dosage effect in a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects. Nature Genet. *7*, 463–471.

Halder, G., Callaerts, P., and Gehring, W.J. (1995). Induction of ectopic eyes by targeted expression of the *eyeless* gene in Drosophila. Science *267*, 1788–1792.

Hammerschmidt, M., Brook, A., and McMahon, A.P. (1997). The world according to hedgehog. Trends Genet. *13*, 14–21.

Hay, B.A., Wolff, T., and Rubin, G.M. (1994). Expression of baculovirus P35 prevents cell death in Drosophila. Development *120*, 2121–2129.

Heberlein, U., Wolff, T., and Rubin, G.M. (1993). The TGFβ homolog *dpp* and the segment polarity gene *hedgehog* are required for propagation of the morphogenetic wave in the Drosophila retina. Cell *75*, 913–926.

Heberlein, U., Singh, C.M., Luk, A.Y., and Donohue, T.J. (1995). Growth and differentiation in the Drosophila eye coordinated by hedgehog. Nature *373*, 709–711.

Hill, R.E., Favor, J., Hogan, B.L.M., Ton, C.C.T., Saunders, G.F., Hanson, I.M., Prosser, J., Jordan, T., Hastie, N.D., and Van Heyningen, V. (1991). Mouse small eye results from mutations in a paired-like homeobox-containing gene. Nature *354*, 522–525.

Hollenberg, S.M., Sternglanz, R., Cheng, P.F., and Weintraub, H. (1995). Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. Mol. Cell. Biol., *15*, 3813–3822.

Kawakami, K., Ohto, H., Ikeda, K., and Roeder, R.G. (1996a). Structure, function and expression of a murine homeobox protein AREC3, a homologue of Drosophila sine oculis gene product, and implication in development. Nucleic Acids Res. *24*, 303–310.

Kawakami, K., Ohto, H., Takizawa, T., and Saito, T. (1996b). Identification and expression of six family genes in mouse retina. FEBS Lett. *393*, 259–263.

Kim, J., Johnson, K., Chen, H.J., Carroll, S., and Laughon, A. (1997). Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. Nature *388*, 304–308.

Kingsley, D.M. (1994). The TGF-β superfamily: new members, new receptors, and genetic tests of function in different organisms. Genes Dev. *8*, 133–146.

Ma, C., Zhou, Y., Beachy, P.A., and Moses, K. (1993). The segment polarity gene *hedgehog* is required for progression of the morphogenetic furrow in the developing Drosophila eye. Cell *75*, 927–938.

Mardon, G., Solomon, N., and Rubin, G.M. (1994). dachshund encodes a nuclear protein required for normal eye and leg development in Drosophila. Development *120*, 3473–3486.

Oliver, G., Wehr, R., Jenkins, N.A., Copeland, N.G., Cheyette, B.N., Hartenstein, V., Zipursky, S.L., and Gruss, P. (1995a). Homeobox genes and connective tissue patterning. Development *121*, 693–705.

Oliver, G., Mailhos, A., Wehr, R., Copeland, N., Jenkins, N.A., and Gruss, P. (1995b). *Six3*, a murine homologue of the *sine oculis* gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. Development *121*, 4045–4055.

Pignoni, F., and Zipursky, S.L. (1997). Induction of Drosophila eye development by decapentaplegic. Development *124*, 271–278.

Quiring, R., Walldorf, U., Kloter, U., and Gehring, W.J. (1994). Homology of the *eyeless* gene of Drosophila to the *small eye* gene in mice and *aniridia* in humans. Science *265*, 785–789.

Reinke, R., and Zipursky, S.L. (1988). Cell-cell interaction in the Drosophila retina: the *bride-of-sevenless* gene is required in photoreceptor cell R8 for R7 cell development. Cell *55*, 321–330.

Robinow, S., and White, K. (1991). Characterization and spatial distribution of the ELAV protein during Drosophila melanogaster development. J. Neurobiol. *22*, 443–461.

Serikaku, M.A., and O'Tousa, J.E. (1994). Sine oculis is a homeobox gene required for Drosophila visual system development. Genetics *138*, 1137–1150.

Shen, W., and Mardon, G. (1997). Ectopic eye development in Drosophila induced by directed dachshund expression. Development *124*, 45–52.

Smith, D.B. (1983). Purification of glutathione-S-transferase fusion proteins. Methods Mol. Cell Biol. *4*, 220–229.

Tio, M., and Moses, K. (1997). The Drosophila TGF alpha homolog Spitz acts in photoreceptor recruitment in the developing retina. Development *124*, 343–351.

Ton, C.C.T., Hirvonen, H., Miwa, H., Weil, M.M., Monaghan, P., Jordan, T., Van Heyningen, V., Hastie, N.D., Meijers-Heijboer, H., and Drechsler, M. (1991). Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. Cell *67*, 1059–1074.

Wiersdorff, V., Lecuit, T., Cohen, S.M., and Mlodzik, M. (1996). *Mad* acts downstream of Dpp receptors, revealing a differential requirement for *dpp* signaling in initiation and propagation of morphogenesis in the Drosophila eye. Development *122*, 2153–2162.

Wolff, T., and Ready, D.F. (1993). Pattern formation in the Drosophila retina. In The Development of Drosophila melanogaster, A. Martinez Arias and M. Bate, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), pp. 1277–1325.

Xu, T., and Rubin, G.M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. Development *117*, 1223–1237.

Xu, P.-X., Woo, I., Her, H., Beier, D.R., and Maas, R.L. (1997a). Mouse Eya homologues of the Drosophila eyes absent gene require Pax6 for expression in lens and nasal placode. Development *124*, 219–231.

Xu, P.-X., Cheng, J., Epstein, J.A., and Maas, R.L. (1997b). Mouse Eya genes are expressed during limbs tendon development and encode a transcriptional activation function. Proc. Natl. Acad. Sci. USA *94*, 11974–11979.

Zimmerman, J.E., Bui, Q.T., Steingrimsson, E., Nagle, D.L., Fu, W., Genin, A., Spinner, N.B., Copeland, N.G., Jenkins, N.A., Bucan, M., and Bonini, N.M. (1997). Cloning and characterization of two vertebrate homologs of the Drosophila eyes absent gene. Genome Res. *7*, 128–141.