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

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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

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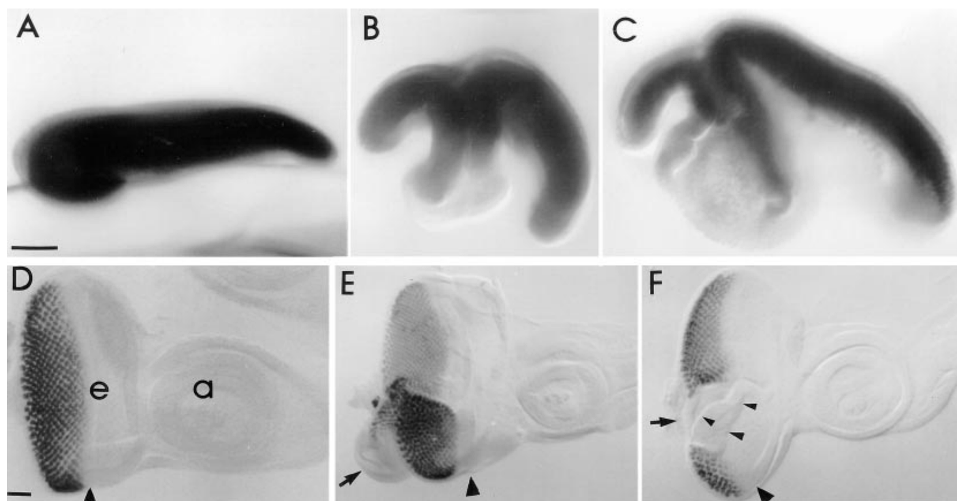

Figure 1. *so* and *eya* Control Cell Proliferation in the Eye Disc

(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

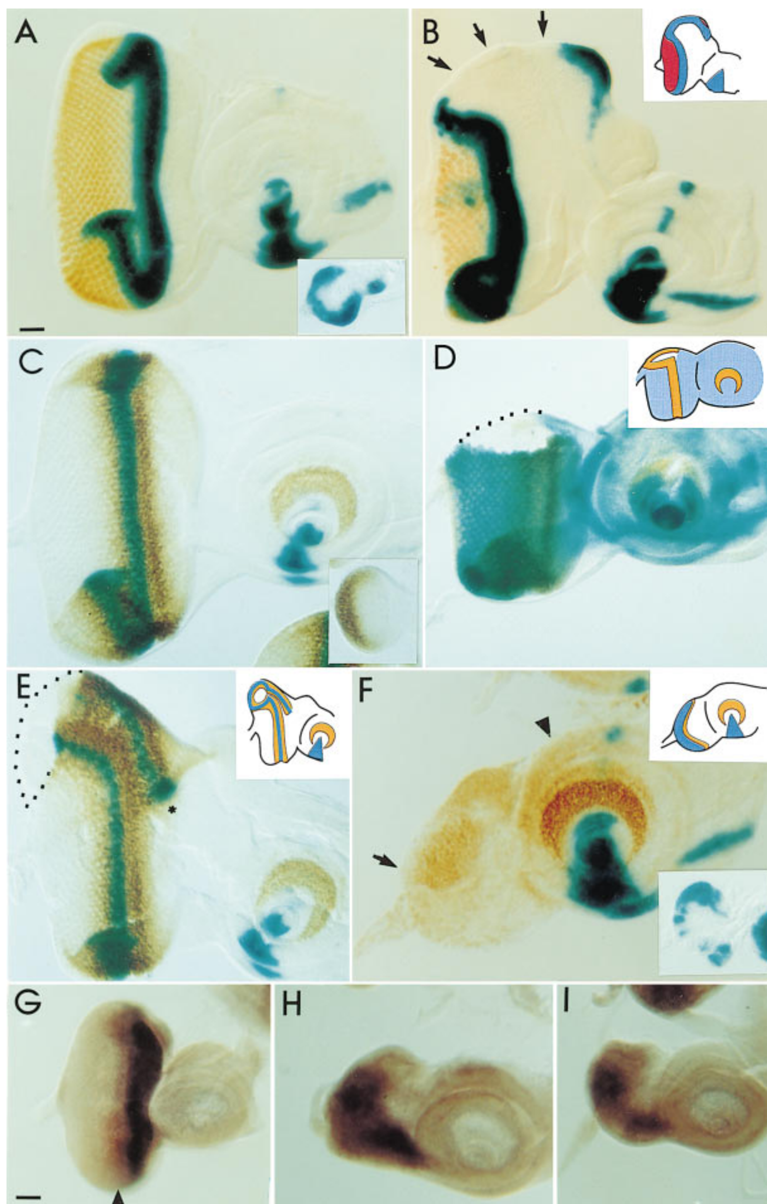

Figure 2. *so* and *eya* Are Required for the Expression of the MF-Initiation Factors *dpp* and *dac*

MF initiation does not occur in *so³* (B) and *eya^clift1* (see Figure 1F) clones encompassing the margins or in *so¹* and *eya²* 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 *so³* and *eya^clift1* mutant clones and *so¹* and *eya²* 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 *so³* 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 *so³* 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) *so¹* 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 *so¹* 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 *so¹* (H) and *eya²* (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 *so¹* 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 *so¹* and *so³* data, we assessed the phenotype of *so¹* 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 *eya²* discs show a phenotype identical to *so¹* (Bonini et al., 1993); mutant clones of *eya²* were not analyzed. Hence, the phenotypes of *so³*, *eya^clift1*, and *so¹* mutant clones suggest that the cell death in *so¹* and *eya²* 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.

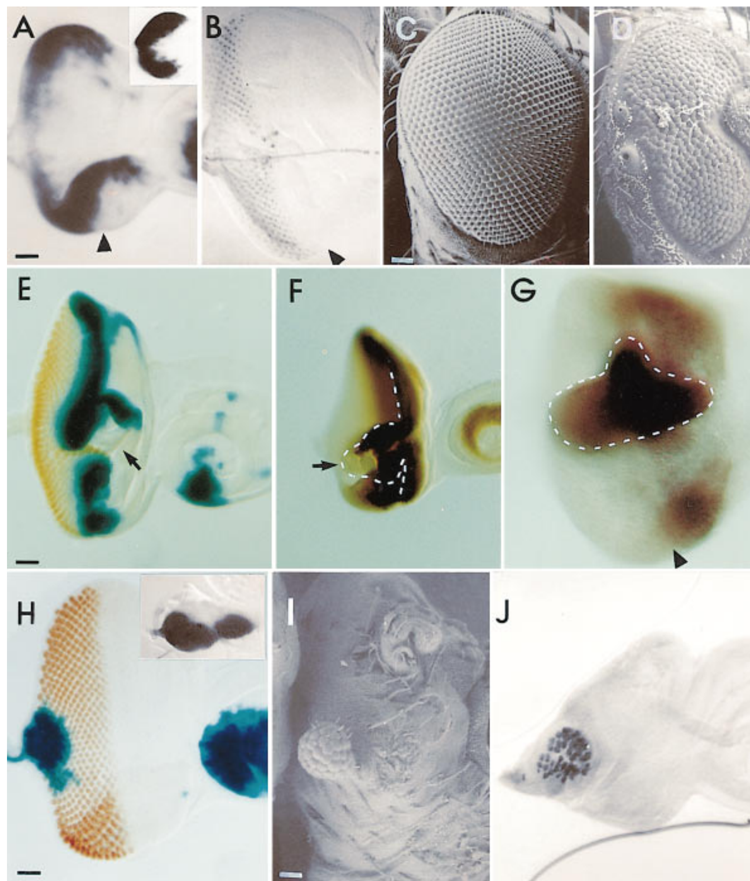

Figure 3. *so* and *eya* Are Required for MF Propagation

(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.

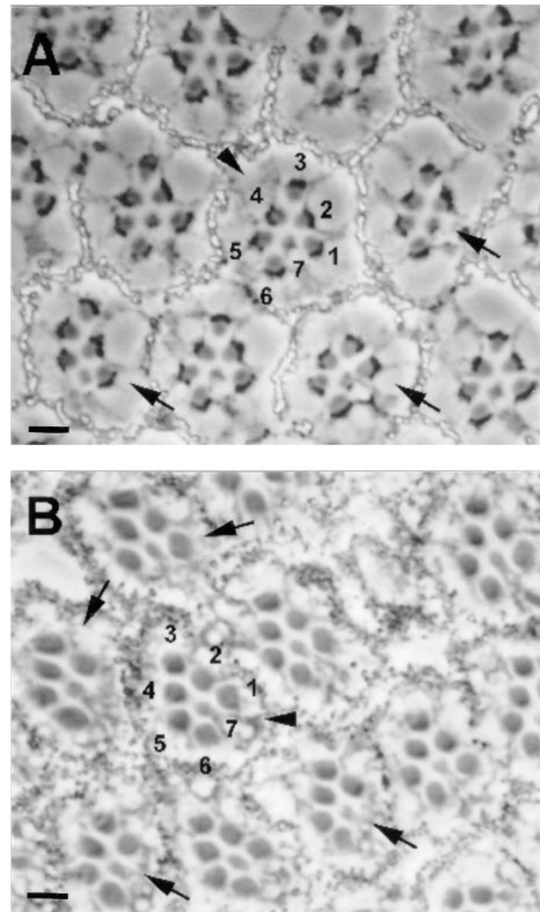

Figure 4. *so* and *eya* Are Required Posterior to the MF

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

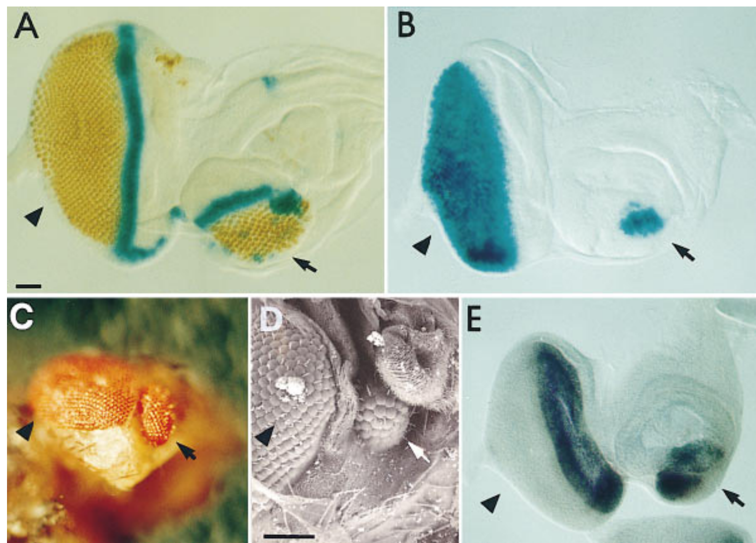

Figure 5. Coexpression of *so* and *eya* Induces Ectopic Eyes in the Antennae

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).

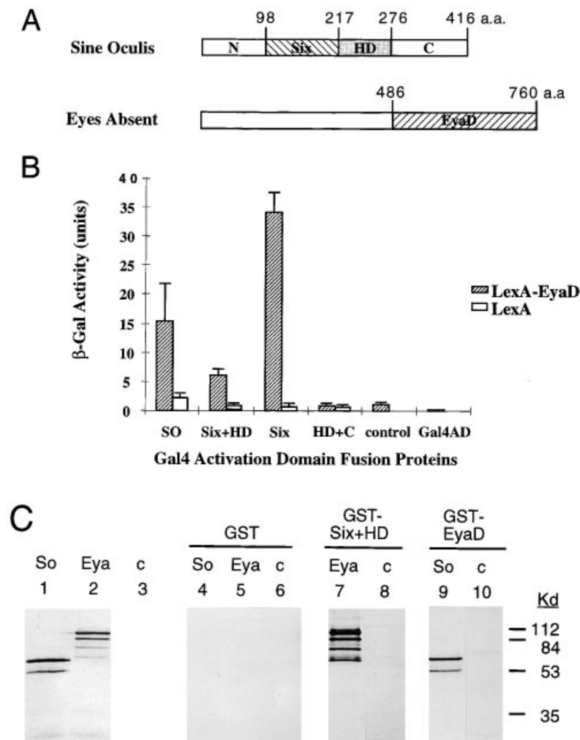

Figure 6. So and Eya Bind to Each Other in Yeast and In Vitro

(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

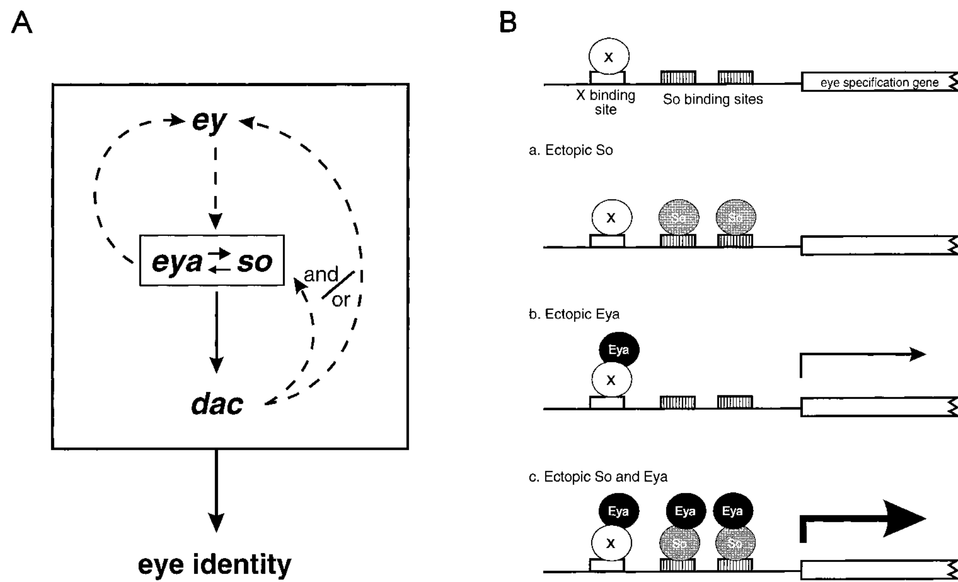

Figure 7. Regulation of Eye-Specification Genes

(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: $A420 \times 1000/OD_{600}$ of the cell culture $\times$ the culture volume (ml) $\times$ 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).

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Dachshund and Eyes Absent Proteins Form a Complex and Function Synergistically to Induce Ectopic Eye Development in Drosophila

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Summary

The *eyeless*, *dachshund*, and *eyes absent* genes encode conserved, nuclear proteins that are essential for eye development in Drosophila. Misexpression of *eyeless* or *dachshund* is also sufficient to induce the formation of ectopic compound eyes. Here we show that the *dachshund* and *eyes absent* genes act synergistically to induce ectopic retinal development and positively regulate the expression of each other. Moreover, we show that the Dachshund and Eyes Absent proteins can physically interact through conserved domains, suggesting a molecular basis for the genetic synergy observed and that a similar complex may function in mammals. We propose that a conserved regulatory network, rather than a linear hierarchy, controls retinal specification and involves multiple protein complexes that function during distinct steps of eye development.

Introduction

The molecular mechanisms controlling retinal cell fate determination are rapidly being deciphered. One of the most striking aspects of recent findings is that many of the genes controlling eye development have been highly conserved between insects and vertebrates and, perhaps, throughout much of the metazoa (reviewed in Bonini and Choi, 1995; Heberlein and Moses, 1995; Freund et al., 1996; Callaerts et al., 1997). A group of four genes, all encoding conserved, nuclear proteins, plays a prominent role during the early steps of retinal development in Drosophila. These are *eyeless* (*ey*), *dachshund* (*dac*), *eyes absent* (*eya*), and *sine oculis* (*so*). *ey* and *so* encode putative DNA-binding transcription factors, while *dac* and *eya* both encode novel proteins. Loss-of-function mutations in each of these genes cause flies to develop with no eyes (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994). Moreover, targeted expression of *ey* or *dac* is sufficient to induce ectopic retinal development in several tissues in Drosophila (Halder et al., 1995; Shen and Mardon, 1997). Strikingly, expression of a mouse homolog of *ey* is also sufficient to induce ectopic eye formation in Drosophila (Halder et al., 1995), and *ey* homologs are required for normal eye development in mammals (Hill et al., 1991; Ton et al., 1991; Glaser et al., 1992). In addition, homologs of *dac*, *eya*, and *so* are expressed in the developing vertebrate retina (G. M., unpublished data; Oliver et al., 1995; Xu et al., 1997). These results suggest that the function of these genes has been conserved for more than 500 million years, since the divergence of insects and vertebrates, and have led to the proposal that visual systems throughout the metazoa may have a single common ancestor (Beverley and Wilson, 1984; Halder et al., 1995; Glardon et al., 1997; Tomarev et al., 1997). Nevertheless, the molecular mechanisms by which these genes act remain obscure.

The adult Drosophila compound eye is a precisely organized array of about 750 repeated units called ommatidia. Each ommatidium contains eight photoreceptor cells and a set of nonneuronal accessory cells, including lens-secreting cone cells, pigment cells, and interommatidial bristles (Tomlinson and Ready, 1987a, 1987b). The adult eye is derived from a structure called the eye imaginal disc. During larval development, cells in the eye disc proliferate but remain largely undifferentiated until the beginning of the last or third instar larval stage (Wolff and Ready, 1993). Then, cells at the posterior margin of early third instar eye discs begin to organize into ommatidial precursors (Wolff and Ready, 1993). Differentiation of all cell types in the eye disc occurs progressively from posterior to anterior and is synchronized by a wave of changes termed the morphogenetic furrow (MF) (Ready et al., 1976). The MF is characterized by alterations in cell shape, cell cycle, and patterns of gene expression (Ma et al., 1993). Neuronal differentiation requires MF movement and is apparent immediately posterior to the MF as it progresses across the eye disc. Movement of the MF requires the function of the secreted signaling molecules encoded by *decapentaplegic* (*dpp*) and *hedgehog* (*hh*). *dpp* and *hh* are required for both initiation of eye morphogenesis and for progression of the MF (Heberlein et al., 1993; Ma et al., 1993; Wiersdorff et al., 1996; Chanut and Heberlein, 1997; R. Finkelstein, personal communication). However, ectopic expression of *dpp* or *hh* during larval development does not change cell fates from one disc type to another but causes patterning defects specific to each disc type (Basler and Struhl, 1994; Heberlein et al., 1995; Nellen et al., 1996; Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997). Thus, *dpp* and *hh* act as general patterning signals to control morphogenesis in all imaginal discs and are not sufficient to specify retinal cell fates. Other genes or combinations of genes more specific to eye development must control this process.

ey and *dac* are two of the key players that govern retinal specification during normal eye development; both genes are necessary and sufficient for eye development. Three types of evidence suggest that *dac* functions downstream of *ey* (Shen and Mardon, 1997). First, *dac* is not required for *ey* expression. Second, misexpression of *ey* can strongly induce *dac*. Third, *dac* is required for induction of ectopic retinal development by targeted *ey* expression. Although ectopic expression of each gene is sufficient to phenocopy initiation of the

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MF, *dac* is much less effective than *ey* in this regard (Mardon et al., 1994; Halder et al., 1995). While *ey* can induce large ectopic eyes with complete penetrance on all major appendages, *dac* induces retinal development in only a minority of animals and primarily on antennal disc-derived structures (Halder et al., 1995; Shen and Mardon, 1997). Thus, *ey* must be able to regulate other genes that control retinal cell fate specification in addition to *dac*.

The *eya* gene is a good candidate as another target of *ey* function. Like *dac*, *eya* is expressed in the eye disc prior to MF initiation and is essential for eye development, but it is not required for *ey* expression (Bonini et al., 1993; Halder et al., 1995). However, *dac* is necessary for only a subset of functions for which *eya* is essential during normal eye development. Specifically, *dac* is required for initiation of furrow movement but not for progression or photoreceptor differentiation (Mardon et al., 1994). In contrast, *eya* is required for both MF initiation and progression (Pignoni et al., 1997 [this issue of *Cell*]). In addition, while *eya* null mutant clones result in cell overproliferation and completely block photoreceptor differentiation throughout the eye disc, *dac* mutant clones present this phenotype only when they include the posterior margin of the eye disc (Mardon et al., 1994; Pignoni et al., 1997). *dpp* is also required for MF initiation and progression, and *dac* and *eya* are both likely to act downstream of *dpp* during normal eye development (Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997). Interestingly, while *eya* is necessary to maintain *dpp* expression in the eye disc, *dac* is not (Mardon et al., 1994; Pignoni et al., 1997). Thus, *eya* is required for *dpp* expression and the control of cell proliferation throughout the eye disc, initiation and progression of MF movement, and neural differentiation, while *dac* is required for only a distinct subset of these steps.

We have explored the functional and regulatory relationships among *ey*, *dac*, and *eya*. We demonstrate that, like *dac*, *eya* is a target of *ey* activity and is required for *ey* function. Moreover, *dac* and *eya* show strong genetic synergy in their ability to induce ectopic retinal development. We provide evidence that a complex forms between the Dac and Eya proteins that is mediated by highly conserved domains, suggesting a molecular basis for the genetic synergy observed. We also show that while *eya* is genetically required upstream of *dac* during normal eye development, these genes are able to positively regulate each other at the level of transcription, indicating that a positive-feedback loop is likely to exist between these genes. These results suggest that a network of conserved genes, rather than a linear hierarchy, regulates retinal development in Drosophila. Finally, we propose a mechanism whereby complex formation between Dac and Eya may provide specificity to the function of Eya during MF initiation and that such interactions are likely to be conserved in vertebrates.

Results

While *dac* and *eya* play important roles in early retinal development, the nature of the molecular and genetic association between these genes, if any, was not known. We examined the relationship between *dac* and *eya* by misexpressing these genes employing the GAL4-UAS system and using ectopic eye induction as an assay.

dac and *eya* Act Synergistically to Induce Ectopic Eye Formation

Like *ey* and *dac*, targeted expression of *eya* alone is sufficient to induce ectopic eye formation (Figures 1A and 1C). However, in contrast to *ey*, the penetrance of the ectopic eye phenotype induced by either *dac* or *eya* alone is incomplete and, when induced, such eyes are small (Figures 1B and 1C). When *dac* expression is strongly induced in all imaginal discs, ectopic eye development is observed only on the anterior surface of the fly head ventral to the antenna and in just 56% (61/109) of animals examined (Figure 1B). Although no ectopic retinal structures are induced, the morphology of the legs and wings is severely disrupted (Figures 1F and 1J). Similarly, misexpression of *eya* causes ectopic eye development ventral to the antenna in only 34% (41/119) of animals inspected (Figure 1C). Although the gross morphology of the leg and wing is not significantly disrupted by ectopic *eya* expression, a tiny spot of red pigment is usually observed (>90% of cases examined) at the joint between the coxa and the femur of the leg and on the wing blade in 26% (30/115) of animals observed (data not shown). Thus, *dac* or *eya* alone are relatively weak inducers of ectopic retinal development in Drosophila.

In contrast, coexpression of *dac* and *eya* induces substantial ectopic eyes on the head, legs, wings, and dorsal thorax of 100% of animals examined ($n > 100$). On the head, the cuticle between the normal eye field and antennae is transformed into retinal cells such that the normal retinal field is expanded (Figure 1D). Large patches of pigment are induced on the dorsal surface of the femur and tibia of all legs, which are severely truncated (Figure 1G). Ommatidial structures are observed in each case (Figure 1H). Red pigment, but no clear ommatidial morphology, is also induced on the wing blade (Figure 1K). Ectopic eyes are also formed bilaterally on the dorsal thorax (notum) of the fly (Figures 1M and 1N), a place where no ectopic pigment or ommatidia are ever induced by either *dac* or *eya* alone (data not shown). In all cases, these phenotypes are observed with 100% penetrance.

The phenotypes observed in imaginal discs are consistent with those observed in adults. Specifically, targeted *dac* or *eya* expression induces expression of the Glass protein, a visual system–specific marker (Moses et al., 1989; Moses and Rubin, 1991). Normally, *glass* is not expressed in the antennal, leg, or wing imaginal discs (Figure 2A; data not shown). *dac* or *eya* alone induces ectopic Glass protein only in a small area of the ventral side of the antennal disc with about 50% penetrance (arrows in Figures 2B and 2C), but not in the leg disc or the part of the wing disc that gives rise to the dorsal thorax or notum (data not shown). In addition, ectopic *eya* alone can induce small patches of *glass* expression in the pouch area of the wing disc with 25% penetrance (data not shown). In no case has ectopic Glass staining been observed in leg discs with either

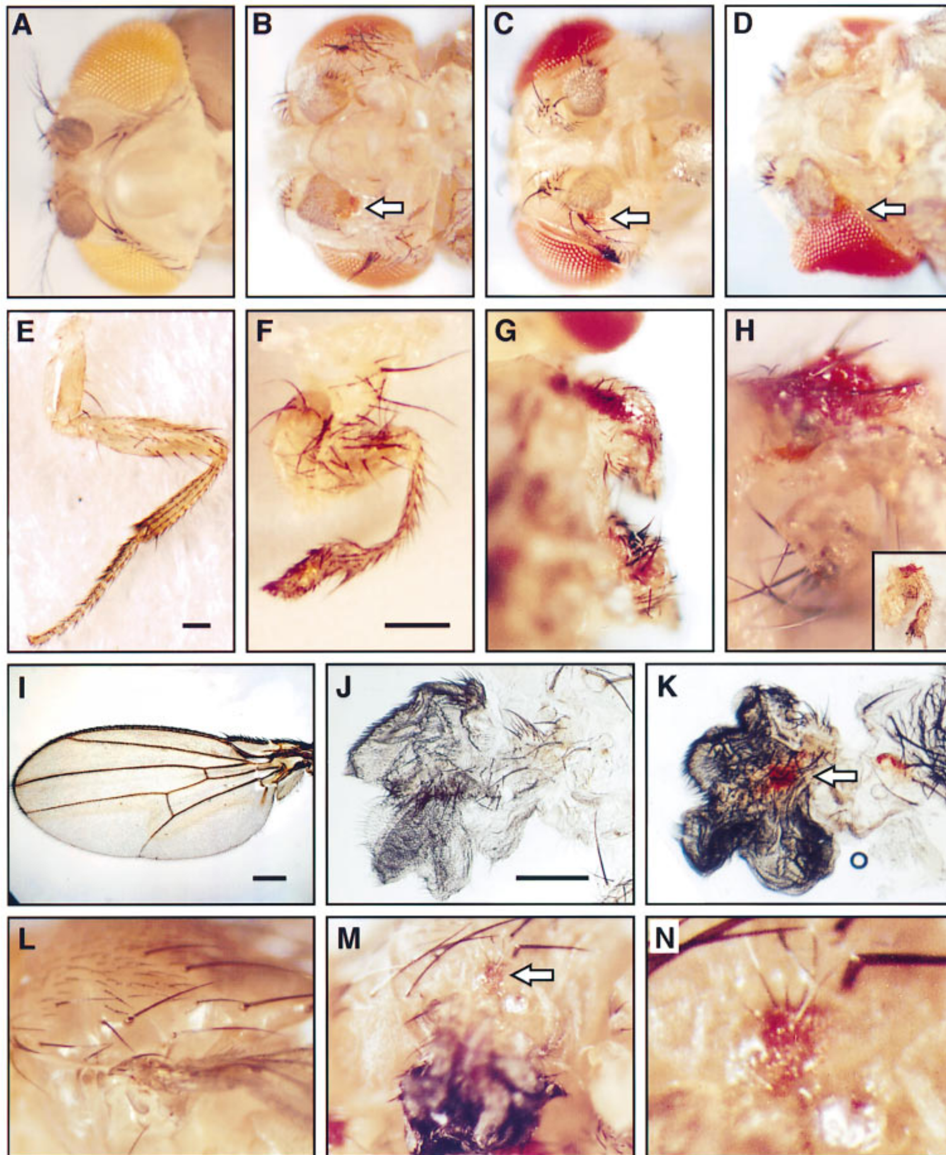


Figure 1. *dac* and *eya* Act Synergistically to Induce Ectopic Retinal Development

(A) Ventral view of the head of an adult control fly carrying the UAS-*dac*^{7c4} and UAS-*eya* transgenes but without any GAL4 driver. (B–D) Ectopic eye induction (arrows) driven by *dpp-GAL4* (Staehling-Hampton and Hoffmann, 1994). UAS-*dac*^{7c4} alone (B) or UAS-*eya* alone (C) cause very small ectopic eyes near the antennae. In contrast, UAS-*dac*^{7c4} plus UAS-*eya* (D) produces a large domain of ectopic retinal tissue that fuses with the normal eye field. (E) Wild-type leg. (F) Ectopic *dac* expression truncates the leg but does not induce eye development. Misexpression of *eya* alone has little effect on leg development (not shown). (G) Coexpression of *dac* and *eya* induces ectopic pigment on the dorsal side of all legs with complete penetrance. (H) A high-magnification view of an ectopic eye on the leg where obvious ommatidial structures are visible. The inset shows the whole leg at the same magnification as in (E). (I) Wild-type wing. (J) Ectopic *dac* expression truncates the wing but does not induce retinal development. Targeted expression of *eya* alone has little effect on normal wing morphology but can induce very small patches of pigment on the wing blade in about 25% of animals examined (not shown). (K) Coexpression of *dac* and *eya* induces ectopic pigment on the wing blade in 100% of animals examined. (L) Lateral view of a wild-type notum (dorsal thorax). Expression of either *dac* or *eya* alone never induces retinal development in the notum (not shown). (M) Ectopic pigment is induced on the notum in 100% of animals expressing *dac* and *eya* together. (N) A higher magnification view of the ectopic pigment shown in (M). Scale bars in (E) and (F) are 100 μm and in (I) and (J) are 200 μm.

dac or *eya* alone. However, when *dac* and *eya* are coexpressed, ectopic Glass staining is induced with 100% penetrance along the ventral margin of the eye-antennal disc (Figure 2D), the dorsal half of the leg disc along the anterior-posterior compartment (A/P) boundary (Figure 2K), and along the A/P boundary of the dorsal wing disc (Figure 2O). In each case, the sites of ectopic *glass* expression in discs correspond to the positions of ectopic retinal development observed in adults. Taken together, these data demonstrate that *dac* and *eya* show strong genetic synergy to induce ectopic retinal development in Drosophila.

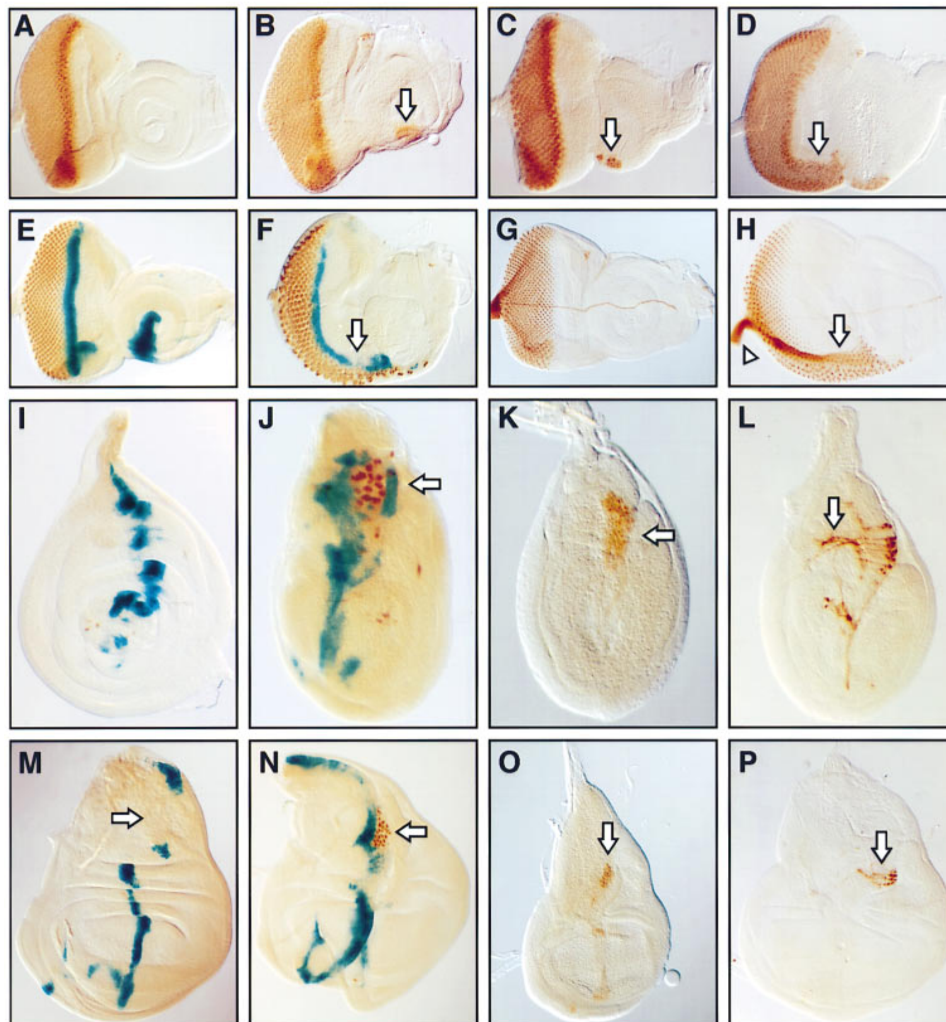


Figure 2. Developmental Analysis of Ectopic Photoreceptor Induction

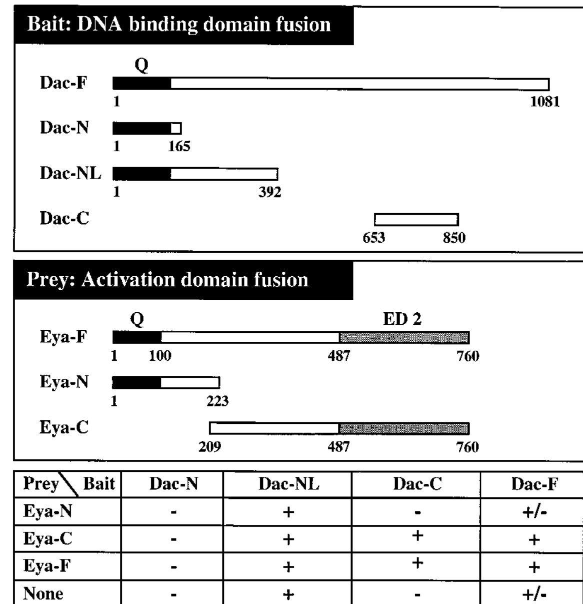
(A–D) Late larval eye-antennal discs were stained with an antibody to detect the visual system-specific Glass protein. No Glass staining is normally found in wild-type antennal discs (A). UAS-*dac*[7c4] alone (B) or UAS-*eya* alone (C) can induce small amounts of *glass* expression in the antennal disc (arrows). Coexpression of *dac* and *eya* induces ectopic Glass expression anterior to the normal retinal field on the ventral side of the eye disc (arrow in D). (E and F) Late larval eye-antennal discs were stained for the neuron-specific Elav protein in brown and the MF marker *dpp-lacZ* in blue. Compared to wild type (E), the combination of targeted *dac* and *eya* misexpression is sufficient to induce ectopic MF advancement from the ventral side of the eye and antennal discs and to cause substantial ectopic photoreceptor development (arrow in F). (G and H) The protein Neuroglian is present in all neurons and their axons and was detected using monoclonal antibody BP104 (Hortsch et al., 1990). In the wild type, the only staining seen in the antennal disc is the larval Bolwig's nerve (G). *dac* and *eya* expressed together induce ectopic neurons in the eye and antennal disc that project axons that join those of the normal retinal field (arrow in H) and are likely to exit through the optic stalk (arrowhead in H) to synapse in the larval brain. (I and J) Late larval leg discs stained for Elav protein and *dpp-lacZ* expression. (I) Only a few neurons are normally observed in the wild-type leg disc and no extra neurons are observed with *dac* or *eya* alone (not shown). (J) Targeted expression of *dac* and *eya* together induces a large cluster of Elav-positive cells in the dorsal half of the leg disc along the A/P boundary. Glass (K) and Neuroglian (L) staining reveals that ectopic neurons induced in the leg disc by coexpression of *dac* and *eya* express Glass protein and extend axons as would be expected during normal photoreceptor development. (M) A wild-type wing disc stained for Elav protein and *dpp* expression. (N–P) Wing discs from late larvae expressing both *dac* and *eya* were stained for Elav and *dpp-lacZ* (N), Glass (O), and Neuroglian (P). Ectopic photoreceptor development is indicated (arrows in N–P). Posterior is to the left and dorsal is up for all discs. For panels (A)–(H), the eye disc is to the left and the antennal disc to the right.

Developmental Analysis of Ectopic Photoreceptor Differentiation

Synergistic induction of photoreceptor differentiation resulting from *dac* and *eya* coexpression can be seen in imaginal discs using a variety of neuronal markers. The nuclear protein Elav is expressed in all neurons of Drosophila (Robinow and White, 1991). Ectopic Elav-positive cells are induced in the antennal, leg, and wing discs in response to *dac* and *eya* coexpression (Figures 2F, 2J, and 2N). These ectopic neurons must be photoreceptor cells, since the visual system-specific Glass protein is also induced in the same pattern (Figures 2D, 2K, and 2O). Moreover, ectopic eyes observed in adults corresponding to these positions contain all of the normal cell types associated with the wild-type eye, including pigment cells, lens-secreting cone cells, and interom-

matidial bristles (Figures 1D, 1H, and 1N). In addition, the ectopic neurons induced by *dac* and *eya* misexpression send out axonal projections (Figures 2H, 2L, and 2P). The axons of ectopic photoreceptors in the eye-antennal disc form a bundle that extends posteriorly into the eye imaginal disc. These axons appear to fuse with the axon tracts sent out by photoreceptors of the normal retinal field that exit through the optic stalk to synapse in the brain (arrowhead in Figure 2H). It is likely, therefore, that the fly can perceive light through ectopic photoreceptors formed in the eye-antennal disc as a result of *dac* and *eya* coexpression. In the leg and wing discs, ectopic photoreceptor axons are likely to fail to find any functional targets and retract during late larval and pupal development (Figures 2L and 2P). Ectopic neuronal marker induction is not observed in response to *dac* or *eya* alone in the eye, leg, or part of the dorsal wing disc fated to give rise to the notum (data not shown). These data demonstrate that *dac* and *eya* act synergistically to induce cells to follow the normal retinal developmental pathway and to elaborate all of the normal cell types found in the wild-type eye.

During normal retinal development, movement of the MF is required for photoreceptor differentiation (Heberlein et al., 1993; Ma et al., 1993; Heberlein and Moses, 1995). We looked for evidence of MF movement associated with ectopic eye formation using a *dpp-lacZ* reporter as an assay (Blackman et al., 1991). *dpp* expression marks the position of the MF as it crosses the eye imaginal disc and is not expressed along the dorsal or ventral margins of the eye disc anterior to the MF by the late third instar stage (Figure 2E). Specifically, *dpp* function is repressed in the anterior eye disc by *wg* (Treisman and Rubin, 1995). In addition, *dpp* is normally expressed in a wedge in the ventral half of the antennal disc (Figure 2E), along the A/P boundary in the dorsal half of the leg disc (Figure 2I), and along the entire A/P boundary of the wing disc (Figure 2M). We found that coexpression of *dac* and *eya* induces ectopic *dpp* expression in the eye-antennal disc adjacent to the field of ectopic photoreceptors (arrow in Figure 2F). In the leg disc, *dpp* expression is split by and forms a ring around the ectopic photoreceptors, again suggesting that an ectopic MF is initiated and propagates (Figure 2J). Although no obvious MF movement is observed in the wing disc, the level of *dpp* expression is significantly increased adjacent to the ectopic photoreceptor field (compare arrows in Figures 2M and 2N). These results indicate that ectopic expression of *dac* and *eya* may be sufficient to initiate MF movement along the ventral margin of the anterior eye-antennal disc, in the leg disc, and perhaps in the wing disc as well.



Prey \ Bait	Dac-N	Dac-NL	Dac-C	Dac-F
Eya-N	-	+	-	+/-
Eya-C	-	+	+	+
Eya-F	-	+	+	+
None	-	+	-	+/-

Figure 3. Dac and Eya Proteins Interact in the Yeast Two-Hybrid System

Portions of the Dac protein were fused to the GAL4 DNA-binding domain to create "bait" constructs (upper panel). Dac-F, full-length Dac; Dac-N, N-terminal 165 amino acids (aa) of Dac, containing a glutamine-rich region (Q); Dac-NL, N-terminal 392 aa of Dac; Dac-C, amino acids 653–850 from the carboxy-terminal half of the Dac protein. Portions of the Eya protein were fused to the GAL4 activation domain to create "prey" constructs (middle panel). Eya-F, full-length Eya; Eya-N, amino-terminal 223 aa of Eya containing a glutamine-rich region (Q); Eya-C, carboxy-terminal portion of Eya beginning from amino acid 209. This portion of the Eya protein contains a highly conserved domain present in all three vertebrate *eya* homologs (ED2). All combinations of bait and prey constructs were transformed into yeast and then tested for activation of a *lacZ* reporter construct (lower panel). −, no *lacZ* activity; +, strong *lacZ* activity; +/−, weak *lacZ* activity.

Dac and Eya Proteins Physically Interact

dac and *eya* both encode nuclear proteins that are expressed in similar temporal and spatial patterns in the eye imaginal disc, are required for MF initiation, and, most importantly, show strong genetic synergy in our ectopic eye induction assay. These results lead us to hypothesize that the molecular basis for the genetic synergy observed may be a physical interaction between the Dac and Eya proteins. We used two independent methods to test and confirm this hypothesis: the yeast two-hybrid system and in vitro binding studies (Fields and Song, 1989; Harper et al., 1993). First, we fused full-length and truncated portions of the Dac protein to the DNA-binding domain of the yeast transcription factor GAL4 to make "bait" constructs and full-length and truncated portions of the Eya protein to the GAL4 transcriptional activation domain to make "prey" constructs (Figure 3). These constructs were transformed into yeast that contain a transgene with GAL4 binding sites upstream of the *lacZ* gene. We found that full-length Dac is sufficient to induce weak *lacZ* expression in the absence of a prey construct (Dac-F bait with no prey, lower panel, Figure 3). This result demonstrates that some portion of the Dac protein is able to act as a transcriptional activation domain in this assay. We have mapped the position of this activation domain to an amino-terminal portion of Dac by comparing the constructs Dac-N and Dac-NL. Specifically, the amino-terminal 165 amino acid (aa) stretch of Dac, which contains a polyglutamine-rich region, does not activate transcription (Dac-N bait with no prey, Figure 3). In contrast, a construct that contains the first 392 aa of Dac is able to activate transcription even in the absence of a prey construct (Dac-NL bait and no prey, Figure 3). Thus, it is likely that an

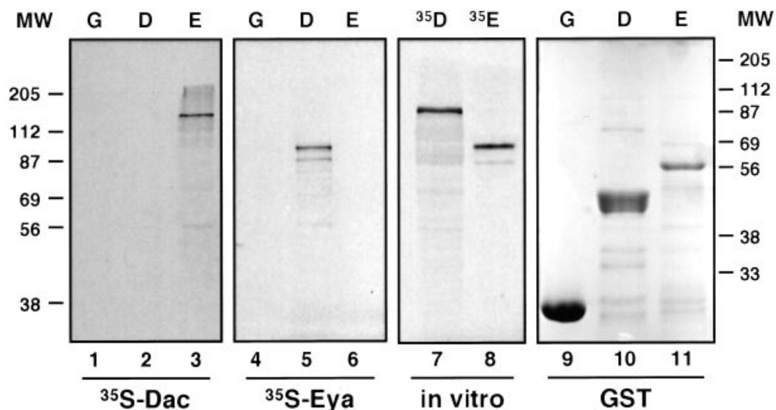


Figure 4. Dac and Eya Proteins Interact In Vitro

GST alone (G) or GST fusions to Dac (D) or Eya (E) were used to bind in vitro–transcribed and –translated, ^{35}S-labeled Dac or Eya proteins (^{35}D, ^{35}E, respectively). ^{35}S-labeled Dac binds to GST::Eya, but not to GST alone or GST::Dac (lanes 1–3). ^{35}S-labeled Eya binds to GST::Dac, but not to GST alone or GST::Eya (lanes 4–6). The in vitro–translation products (lanes 7 and 8) and the GST fusions (lanes 9–11) before binding reactions are shown. MW indicates molecular weight standard in kilodaltons. Markers for lanes 1–8 are shown to the left and for lanes 9–11 to the right.

activation domain at least partially resides within amino acids 165–392 of the Drosophila Dac protein.

We also tested a fourth bait construct in this assay that contains a domain that is highly conserved in both mouse and human *dac* homologs (G. M., unpublished data). When fused to the DNA-binding portion of GAL4, this domain is incapable of activating transcription alone (Dac-C bait and no prey, Figure 3). However, when coexpressed with prey constructs containing either full-length or C-terminal portions of the Eya protein, strong activation of *lacZ* expression is observed (Dac-C bait and either Eya-C or Eya-F prey, Figure 3). Although the full-length Dac protein activates weakly on its own, a much stronger activation of *lacZ* is observed when coexpressed with the same Eya constructs (Dac-F bait and either Eya-C or Eya-F prey, Figure 3). The C-terminal portion of Eya (Eya-C) interacts with Dac while the amino-terminal portion does not (Eya-N), suggesting that the C-terminal conserved domain of the Eya protein (ED2) is contacting a portion of the Dac protein that is also conserved (Xu et al., 1997; Zimmerman et al., 1997).

We confirmed the physical interaction between Dac and Eya using in vitro biochemistry. GST (glutathione s-transferase) fusions of the conserved portions of Dac and Eya were used to bind in vitro–translated, ^{35}S-labeled Dac and Eya full-length proteins (Figure 4). GST::Eya was immobilized on glutathione-agarose beads and then incubated with in vitro–translated, ^{35}S-labeled Dac protein. After extensive washing to remove nonspecifically adhered proteins, bound proteins were eluted, separated by SDS-PAGE, and visualized by autoradiography. While no ^{35}S-Dac bound to the control GST resin, it bound to the immobilized GST::Eya fusion protein (left panel, Figure 4). Similarly, ^{35}S-Eya can bind to immobilized GST::Dac, but it cannot be bound by GST alone. The same portions of Dac or Eya do not form homodimers in this assay (Figure 4).

Transcriptional Regulation of *dac* and *eya*

Since *dac* and *eya* are each able to induce ectopic eye development and act synergistically in this process, we investigated the regulatory relationships between these two genes and with *ey*. Determining the order of *dac* and *eya* function using traditional genetic epistasis analysis is not possible because loss-of-function mutations in each gene cause an eyeless phenotype. However, if *dac* and *eya* are acting in the same pathway, we expected that loss-of-function mutations in these genes would show dominant modification of the recessive eye phenotype of the other. Surprisingly, we have failed to observe any such interaction (data not shown). We were able to determine the regulatory relationship between *dac* and *eya* by analyzing the expression of each gene in wild-type and mutant backgrounds. We found that while *eya* expression in the eye disc does not depend on *dac* function, *dac* expression is greatly reduced in an eya^2 mutant background, demonstrating that *dac* expression requires *eya* activity (Figures 5A-5D). Similarly, *ey* induction of ectopic *dac* expression is greatly reduced in an eya^2 mutant background (Figures 5G and 5H). These results suggest that *dac* may function downstream of *eya*. Consistent with this interpretation, *eya* is unable to induce ectopic eye formation in a *dac* mutant background (data not shown).

Since *dac* expression is induced by both *ey* and *eya*, we explored the genetic and regulatory relationships among these genes. First, we found that *ey* misexpression is sufficient to induce *eya* (Figure 5E), suggesting that *eya* may be required for *ey* function. Indeed, ectopic retinal development driven by targeted *ey* expression fails to occur in an eya^2 mutant background (data not shown). We also found that induction of *eya* expression by *ey* does not depend on *dac* activity (Figure 5F), consistent with the idea that *eya* functions downstream of *ey* but upstream of *dac*. However, these genes do not act in a simple, linear pathway; targeted expression of *dac* and *eya* strongly induce the expression of each other (Figure 5I-5K), and *eya* is required for ectopic eye induction by *dac* (data not shown). In addition, misexpression of *dac* or *eya* is also sufficient to induce ectopic *ey* expression in the antennal disc (Shen and Mardon, 1997; data not shown). These results suggest that multiple positive-feedback loops exist among these genes during normal eye development and raised the possibility that *ey* may be required for ectopic retinal induction by *eya* and *dac*. Indeed, ectopic eye formation driven by coexpression of *dac* and *eya* is completely blocked in an ey^2 mutant background, indicating that induction of *ey* is essential (data not shown). Finally, these regulatory events must occur at the level of transcription because *ey*, *dac*, and *eya* all induce expression of *lacZ* reporter constructs specific for each gene (data not shown).

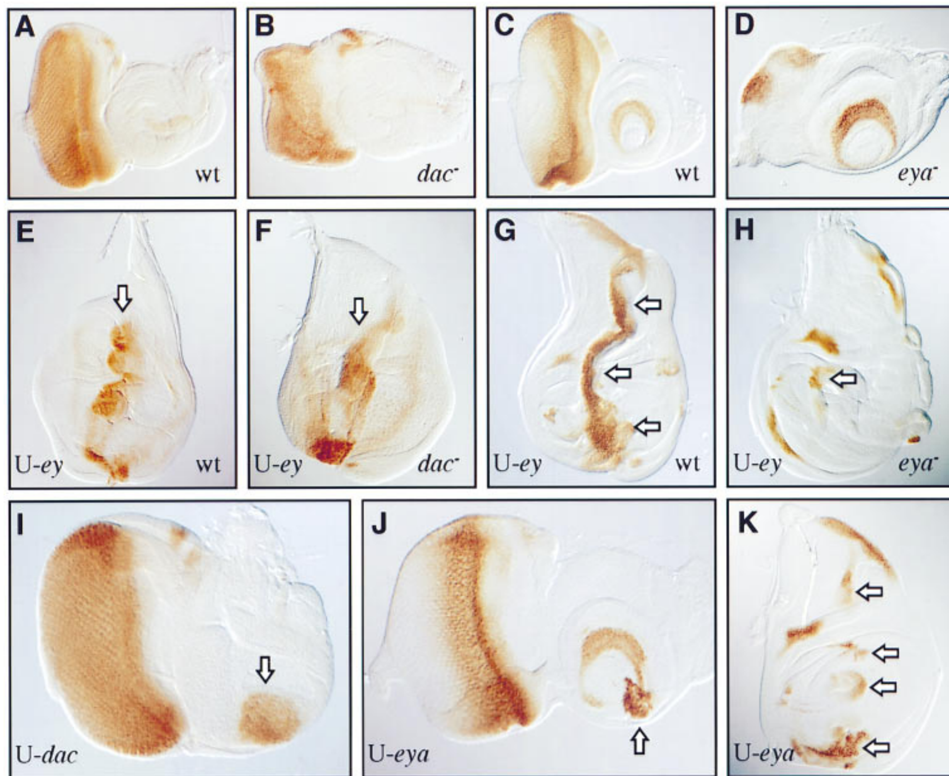


Figure 5. Regulatory Relationships between *dac* and *eya* Are Complex

(A and B) *dac* is not required for *eya* expression. Wild-type (A) and dac^3 null mutant (B) eye discs were stained with an antibody that specifically detects the Eya protein (Bonini et al., 1993). (C and D) *dac* expression is greatly reduced in eya^2 mutant eye discs. Wild-type (C) and eya^2 mutant (D) eye discs were stained with an antibody that specifically detects the Dac protein (Mardon et al., 1994). (E and F) *dac* is not required for *eyeless* to induce *eya* expression. Wing discs were dissected from late larvae carrying a UAS-*eyeless* transgene driven by *dpp-GAL4* in a wild-type (E) or in a dac^3 null mutant background (F) and stained for *eya* expression. Eya protein is induced by eyeless misexpression even in the absence of *dac* function (arrows in E and F). (G and H) Ectopic *dac* expression induced by UAS-*eyeless* is greatly reduced in an *eya* mutant background. Wing discs were prepared from UAS-*eyeless*, *dpp-GAL4* larvae in either a wild-type (G) or eya^2 mutant background (H). Dac protein induction by *eyeless* shows a strong requirement for *eya* function (arrows in G and H). (I–K) Misexpression of *dac* or *eya* can turn on the expression of each other. *eya* expression is induced in the ventral portion of the antennal disc in response to ectopic *dac* expression (arrow in I). Similarly, *dac* is induced by targeted *eya* expression (arrows) in the antennal disc (J) and the wing disc (K).

Discussion

Research concerning the genetic control of eye development in both vertebrates and invertebrates was greatly stimulated by the remarkable discovery that a single gene, *eyeless* (*ey*), was both necessary and sufficient for eye development in Drosophila (Quiring et al., 1994; Halder et al., 1995). Intriguingly, vertebrate homologs of *ey*, members of the *Pax6* gene family, are also required for normal eye development (Hill et al., 1991; Ton et al., 1991; Glaser et al., 1992). Moreover, *Pax6* from a wide range of species is sufficient to induce ectopic eye formation in Drosophila, indicating that at least some of the crucial targets of *ey* function have been conserved (Halder et al., 1995; Glardon et al., 1997; Tomarev et al., 1997). These results lead to the hypothesis that *ey* is the master control gene for eye morphogenesis, sitting alone at the top of the regulatory hierarchy of genes controlling this process (Halder et al., 1995). However, an alternate view of retinal development is suggested by the report that the *dac* gene is also sufficient to induce ectopic eye formation in Drosophila, and that *dac* and *ey* can positively regulate the expression of each other (Shen and Mardon, 1997). In particular, these results indicate that eye development may be controlled in a combinatorial manner, with multiple genes functioning together at the highest regulatory levels.

We sought to distinguish these models by determining the genetic and regulatory relationships among *dac*, *ey*, and two other genes required for early steps of eye development in Drosophila, *eya* and *so* (Bonini et al., 1993; Cheyette et al., 1994). In this paper we have focused our studies on *dac* and *eya*, while Zipursky and colleagues have analyzed *so* and *eya* function in an accompanying paper (Pignoni et al., 1997). The results reported in these papers clearly establish that retinal development in Drosophila is controlled by an interactive network of genes rather than a linear hierarchy. Moreover, we show that this network functions both at the level of gene expression and through protein–protein interactions. Finally, we propose that this combinatorial network of gene action results in the cooperative regulation of eye-specification genes and serves to lock in a retinal cell fate program once initiated.

Synergistic Induction of Ectopic Eye Development

Targeted expression studies were employed to examine the function of *ey*, *dac*, *eya*, and *so*, using ectopic eye induction as an assay. One of the most striking results of this work is that three of these four genes (*ey*, *dac*, and *eya*) are each sufficient to induce ectopic retinal development, suggesting that no single gene is the master regulator of this process (Figure 1; Halder et al., 1995; Shen and Mardon, 1997). Moreover, strong synergistic induction of ectopic retinal development is observed when *dac* and *eya* are coexpressed. The molecular basis of the genetic synergy observed between *dac* and *eya* is likely to be the physical interaction between the protein products encoded by these two genes. We found that Dac interacts specifically with Eya in two independent assays and that this interaction occurs through domains that are highly conserved in humans and mice. Since *dac* and *eya* encode novel proteins, the biochemical consequence of this interaction is not known. However, Dac and Eya are nuclear proteins that can activate the transcription of each other. In addition, these genes are each sufficient to initiate the entire cascade of gene activity required to generate the compound eye and are necessary throughout eye development. Given that 2000 to 3000 genes are involved in fly eye development (Thaker and Kankel, 1992; Halder et al., 1995), the most straightforward model to accommodate these data is that Dac and Eya are directly involved in gene regulation, most likely as coactivators of transcription. Consistent with this model, while Dac and Eya do not contain any known DNA-binding motifs, both proteins act as strong transcriptional activators in yeast (Figure 3; Pignoni et al., 1997). Thus, we propose that misexpression of *dac* or *eya* is sufficient to activate transcription of genes essential for eye development. Potential targets of *dac* and *eya*, in addition to each other, include *so*, *ey*, and presumably other genes essential for retinal development. Finally, one simple model to account for the synergy observed between Dac and Eya is that the physical association between these proteins facilitates their recruitment to the transcription complex and results in cooperative activation of downstream genes (see Pignoni et al., 1997, for a detailed description of this model).

A Network of Genes Controls Retinal Cell Fate Specification

Based on our analysis of the regulatory and functional relationships among *dac*, *eya*, *ey*, and *so*, we propose that all four genes act together as a regulatory network to control retinal cell fate specification. We have characterized these genes at three levels of analysis: transcriptional, genetic, and protein interaction. Loss- and gain-of-function experiments suggest that initiation of expression of *ey*, *dac*, *eya*, and *so* is controlled by a primarily linear pathway (Figure 6A). For example, *ey* expression does not require *dac*, *eya*, or *so* function, and *dac* is not required for expression of *eya* or *so*, but *dac* expression does require both *so* and *eya* (R. C. and G. M., unpublished data; this paper; Halder et al., 1995;

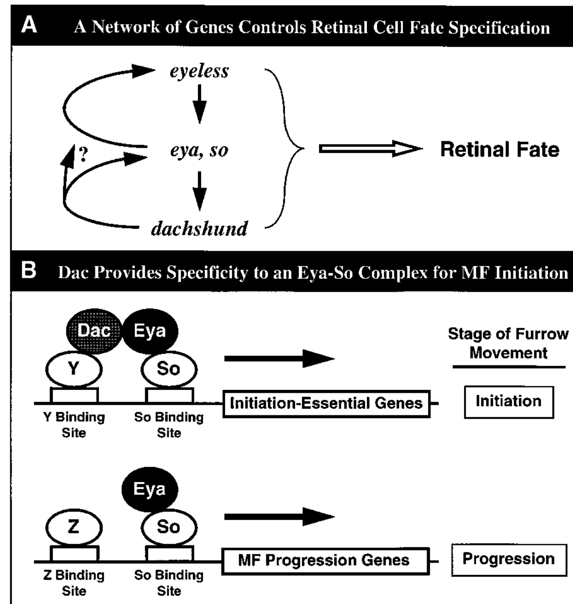


Figure 6. Models for Retinal Development in Drosophila

(A) A network of genes controls retinal cell fate specification. Solid arrows indicate positive transcriptional regulation. Whether *dac* induces *ey* directly through induction of *eya* or by some other mechanism is not known ("?"). The bracket indicates that all four gene products function together in one or more complexes to direct normal retinal cell fate specification. See text for detailed discussion.

(B) Dac provides specificity to an Eya-So complex for MF initiation. An Eya-So complex is essential to regulate genes required for both MF initiation and progression. In contrast, Dac is required for proper regulation of initiation-essential genes but not for MF progression genes. Since Dac does not contain a known DNA-binding motif, it is likely to interact with another factor(s) (Y) to be recruited to the transcription complex. In this model, interaction between Dac and Eya is required for efficient activation of genes essential for MF initiation. Other factors (Z) may provide specificity to Eya-So function during regulation of genes essential for MF progression. Cooperative binding of Dac-Y and Eya-So complexes to regulate initiation-essential genes is mediated by direct interaction between Dac and Eya and accounts for the synergistic induction of ectopic retinal development by Eya and Dac.

Shen and Mardon, 1997). However, several lines of evidence suggest that these genes function in an interactive network. First, targeted expression of *ey*, *dac*, or *eya* is sufficient to induce the expression of each other, suggesting that multiple regulatory feedback loops control the expression of these three genes. Second, *dac* and *eya* act synergistically to induce ectopic retinal development. Third, studies in vitro and in yeast indicate that the Dac and Eya proteins physically interact. Similarly, ectopic eye formation is also synergistically induced by *so* and *eya*, and their protein products physically associate (Pignoni et al., 1997). Finally, ectopic eye induction by each of these molecules, expressed either individually or in pairs, is prevented in the absence of any member of this group. Taken together, these data suggest that these proteins function together in one or more complexes to regulate each other cooperatively as well as other presumed downstream targets required

for normal eye morphogenesis. Cooperative and positive cross-regulation of genes required for eye specification may serve to lock in or firmly maintain the retinal cell fate program once it has been initiated.

dac Confers Specificity during MF Initiation

During normal eye development, *eya* and *so* are both required for MF initiation and progression and photoreceptor differentiation. Since Eya and So act synergistically and can physically associate, it is therefore likely that an Eya-So complex functions throughout retinal development (Figure 6B; Pignoni et al., 1997). In contrast, *dac* is essential for only a subset of these steps. Thus, interactions between Dac and Eya may provide specificity to an Eya-So complex during MF initiation (Figure 6B). We propose that during normal retinal development, genes essential for MF initiation are controlled by both general eye-specific factors, such as an Eya-So complex, as well as initiation-specific factors such as Dac. Dac may act as a coactivator for a basal transcription factor that is necessary but not sufficient to induce expression of initiation-essential genes ("Y", Figure 6B). Interaction between a Dac-Y complex and an Eya-So complex may be mediated by physical association of Dac and Eya. We further suggest that the physical interaction between Dac and Eya is required for efficient activation of initiation-essential genes. In addition, Dac may also be recruited directly to the transcription complex by Eya. While regulation of genes necessary for MF progression also requires an Eya-So complex, Dac is not essential for this process. Instead, other factors may provide specificity to Eya-So regulation of MF progression genes ("Z", Figure 6B).

In this model, proper assembly of these complexes is blocked and eye development arrests at an early stage in the absence of either Dac, Eya, or So. Similarly, the Eyeless protein may also function in one or more of these complexes, since *ey* is also required for ectopic eye induction by any combination of Dac, Eya, and So. For several reasons, we favor a model where these proteins function together in complexes to regulate the expression of genes required for normal retinal development. First, multiple physical interactions among these proteins have been observed in vitro and in yeast. Second, these genes act synergistically to induce ectopic retinal development. Such synergy would not be expected if each gene acts independently; simple additive effects are predicted instead. Third, this model is the simplest way to accommodate the observation that all four genes are required for ectopic eye induction by targeted expression of any combination of these genes (R. C. and G. M., unpublished results; this paper; Pignoni et al., 1997).

Finally, the interactions among Dac, Eya, and So are mediated through domains in each of the proteins that are highly conserved in mammals (G. M., unpublished data; Pignoni et al., 1997). Given that members of each of these gene families are specifically expressed in the vertebrate retina during development, it is likely that the synergistic function and protein complex formation we have observed in Drosophila also plays an important role during human retinal development. Synergistic regulation mediated by protein–protein interactions is likely to be a common mechanism to specify cell fates throughout development.

Experimental Procedures

Drosophila Genetics

All Drosophila crosses were carried out at 25°C on standard media. *dac* null mutant experiments were carried out using dac^3 and dac^4 mutant alleles (Mardon et al., 1994). The ey^2 and eya^2 mutations are eye-specific alleles that result in flies that have reduced or no eyes, respectively, but are otherwise viable and fertile (Bonini et al., 1993; Quiring et al., 1994). Previously published experiments with ectopic *dac* expression (Shen and Mardon, 1997) were carried out with a different transgene (UAS-dac^{21M5}) from that used in the present study (UAS-dac^{7c4}). Both transgenes were constructed and isolated as previously described but differ in their sites of insertion in the genome (Shen and Mardon, 1997). When induced by the same *dpp-GAL4* driver (Staehling-Hampton and Hoffmann, 1994), UAS-dac^{7c4} produces less detectable Dac protein and less severe phenotypes than UAS-dac^{21M5}. Specifically, in contrast to UAS-dac^{21M5}, UAS-dac^{7c4} is unable to induce the formation of any ectopic pigment or ommatidia on the legs or thorax of flies when crossed to *dpp-GAL4*. The UAS-dac^{7c4} line was used for all of the experiments reported in this paper. The UAS-*eya* line used in this study carries a full-length *eya* cDNA in pUAST and was a generous gift of Francesca Pignoni and Larry Zipursky. All experiments using the combination of UAS-dac^{7c4} and UAS-*eya* were conducted using a recombinant chromosome carrying both transgenes. Since these transgenes carry a white mini-gene, we were able to isolate recombinants using eye color as an assay. Putative recombinants were confirmed using single fly polymerase chain reaction (PCR) with primers specific for either *dac* or *eya*. Due to severe leg truncation resulting from UAS-*dac* misexpression, these animals fail to eclose from their pupal cases. Consequently, light microscope images of such animals were taken from dissected late pupae.

Immunohistochemistry

Imaginal discs were dissected and stained as previously described (Mardon et al., 1994). Anti-Eya (Bonini et al., 1993) stainings were performed using the same protocol, except that imaginal discs were fixed in PLP for 20 min on ice. In addition, mouse anti-Eya antiserum was first preabsorbed to compete away nonspecific staining as follows: 20 μl of serum was incubated with 30 sets of eya^2 mutant larval eye-brain complexes in 1 ml of PAXDG (Mardon et al., 1994) for 1 hr at room temperature (RT). Following preabsorption, this serum was used for staining with no further dilution. *dpp* expression was assayed using the BS3.0 *lacZ* reporter (Blackman et al., 1991). All discs were mounted in 80% glycerol in PBS.

Yeast Two-Hybrid Analysis

The yeast two-hybrid kit was a gift from Steve Elledge. An amino-terminal part of the *dac* coding region (corresponding to amino acids 1–366) was amplified by PCR so that an NcoI site was created at the AUG start codon. This PCR product was digested with NcoI to create a fragment representing amino acids 1–165 and was inserted into the unique NcoI site of the bait plasmid pAS2 (Harper et al., 1993) to create the Dac-N construct. This construct (Dac-N) was then digested with SacII (*dac* internal site) and SalI (pAS2 polylinker) and was used as a vector to clone a SacII, SalI fragment from the *dac* cDNA (Mardon et al., 1994), resulting in a full-length Dac construct (Dac-F). An NdeI fragment from Dac-F (representing amino acids 1–392) was inserted into the NdeI site of pAS2 to create the Dac-NL construct. A PCR fragment from the carboxy-terminal half of Dac (representing amino acids 653–850), flanked by artificial NcoI and BamHI sites, was cloned into pAS2 to yield the Dac-C construct. Similarly, an artificial NcoI site was introduced at the start codon of *eya* cDNA using PCR. The product was then digested with NcoI and BamHI (an internal site of the *eya* coding region) and cloned into the pACT2 prey plasmid (Durfee et al., 1993) to generate Eya-N

(amino acids 1–223). A SmaI, SalI fragment (amino acids 209–760) from the *eya* type I cDNA (Bonini et al., 1993) was inserted into pACT2 to obtain Eya-C. This same fragment was also inserted into the Eya-N construct at the SmaI and SalI sites to make the Eya-F construct. Yeast transformations and X-gal tests were carried out as previously described (Harper et al., 1993).

In Vitro Biochemistry

To prepare the GST::Dac fusion protein, a fragment of the Drosophila *dac* cDNA (representing amino acids 711–869) was amplified by PCR such that artificial BamHI and HindIII sites flanked the product. Following digestion with BamHI and HindIII, this fragment was cloned into pGEX2 (Pharmacia) to generate pGST::Dac. Similarly, the GST::Eya fusion protein was prepared from the carboxyl terminus of *eya*, using PCR to amplify a fragment encoding amino acids 487–760 and creating an artificial EcoRI at the 5′ end of the product. This fragment was then cloned into the EcoRI site of pGEX1 (Pharmacia) to generate pGST::Eya. pGST::Dac, pGST::Eya, and pGEX1 (to generate GST alone) were then introduced into E. coli (strain BL21, Novagen). Recombinant proteins were purified from induced cultures (50 μM IPTG, 2 hr, 30°C) as follows. One liter of bacterial culture was pelleted (4000 × g, 10 min, 4°C) and resuspended in 20 ml of lysis buffer (PBS, 50 mM NaCl, 5 mM EDTA, 5 mM DTT, 1% Triton X-100, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin) and sonicated for 1 min at 4°C. Lysates were pelleted again (10,000 × g, 15 min, 4°C), and the supernatant was incubated (15 min, RT) with 200 μl of 50% glutathione resin (Pharmacia) per liter of original bacterial culture. Glutathione resin with bound GST proteins was pelleted (500 × g, 3 min, 4°C) and washed three times with 10 ml of lysis buffer and resuspended in 100 μl of binding buffer (20 mM HEPES-KOH [pH 7.7], 150 mM NaCl, 0.1% NP-40, 10% glycerol, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin). ^{35}S-labeled Dac and Eya proteins were synthesized using a coupled in vitro transcription and translation kit (Promega) using *dac* or *eya* cDNAs as DNA templates (Bonini et al., 1993; Mardon et al., 1994). Translation products were separated from unincorporated label by passage over a 1 ml Sephadex G-25 column (Sigma). Labeled Dac and Eya proteins were incubated in 0.4 ml binding buffer with 10 μl of glutathione resin containing 10 μg of bound GST, GST::Dac, or GST::Eya for 2 hr at 4°C. The resin was washed three times with 1 ml of binding buffer, and labeled proteins were eluted by boiling for 3 min in 25 μl of loading buffer, fractionated by SDS-PAGE, and visualized by autoradiography (20–40 hr). About 4% of the labeled, full-length proteins were recovered.

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