MOLECULAR GENETICS OF THE *DROSOPHILA EYES ABSENT* GENE

Thesis by

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Dedicated to my wife and children

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Abstract

The *Drosophila* compound eye has provided a genetic approach to understanding the specification of cell fates during differentiation. The eye is made up of some 750 repeated units or ommatidia, arranged in a lattice. The cellular composition of each ommatidium is identical. The arrangement of the lattice and the specification of cell fates in each ommatidium are thought to occur in development through cellular interactions with the local environment. Many mutations have been studied that disrupt the proper patterning and cell fating in the eye. The *eyes absent* (*eya*) mutation, the subject of this thesis, was chosen because of its eyeless phenotype. In *eya* mutants, eye progenitor cells undergo programmed cell death before the onset of patterning has occurred. The molecular genetic analysis of the gene is presented.

The eye arises from the larval eye-antennal imaginal disc. During the third larval instar, a wave of differentiation progresses across the disc, marked by a furrow. Anterior to the furrow, proliferating cells are found in apparent disarray. Posterior to the furrow, clusters of differentiating cells can be discerned, that correspond to the ommatidia of the adult eye. Analysis of an allelic series of *eya* mutants in comparison to wild type revealed the presence of a selection point: a wave of programmed cell death that normally precedes the furrow. In *eya* mutants, an excessive number of eye progenitor cells die at this selection point, suggesting the *eya* gene influences the distribution of cells between fates of death and differentiation.

In addition to its role in the eye, the *eya* gene has an embryonic function. The eye function is autonomous to the eye progenitor cells. Molecular maps of the eye and embryonic phenotypes are different. Therefore, the function of *eya* in the eye can be treated independently of the embryonic function. Cloning of the gene

reveals two cDNA's that are identical except for the use of an alternatively-spliced 5' exon. The predicted protein products differ only at the N-termini. Sequence analysis shows these two proteins to be the first of their kind to be isolated. Trangenic studies using the two cDNA's show that either gene product is able to rescue the eye phenotype of *eya* mutants.

The *eya* gene exhibits interallelic complementation. This interaction is an example of an "allelic position effect": an interaction that depends on the relative position in the genome of the two alleles, which is thought to be mediated by chromosomal pairing. The interaction at *eya* is essentially identical to a phenomenon known as transvection, which is an allelic position effect that is sensitive to certain kinds of chromosomal rearrangements. A current model for the mechanism of transvection is the *trans* action of gene regulatory regions. The *eya* locus is particularly well suited for the study of transvection because the mutant phenotypes can be quantified by scoring the size of the eye.

The molecular genetic analysis of *eya* provides a system for uncovering mechanisms underlying differentiation, developmentally regulated programmed cell death, and gene regulation.

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Chapter I

Introduction: The Genetics of *eyes absent*: a Tool with which to Study Development, Cell Death, and Gene Regulation

Development might be described as the orchestrated proliferation, differentiation, and migration of cells to form a predictable structure. While great strides have been taken toward understanding the mysteries of development, much remains to be learned. The use of *Drosophila* as a genetic system to study the mechanisms of development is paying dividends, promising to reveal genes important to vertebrate development. Two prime examples of important advances in the understanding of development are the homeotic gene clusters and the *sevenless* pathway [see (Graham, Papalopulu, & Krumlauf, 1989; Greenwald & Rubin, 1992)]. Both these systems have vertebrate homologies.

The *Drosophila* adult compound eye has emerged as a fruitful genetic system to uncover general mechanisms of development [reviewed in (Banerjee & Zipursky, 1990; Greenwald & Rubin, 1992)]. The eye is composed of approximately 750 ommatidia, arranged in a lattice. Each ommatidium contains 22 cells, distinguishable by position, morphology, and function. The eye exhibits dorso-ventral mirror image symmetry about an axis known as the equator. The ommatidia on the dorsal side of the equator are the mirror image of those located ventrally. Analysis of clones in the eye have revealed no obligatory cell lineage relationships among the different cell types, so it is thought that environmental cues play a prominent role in the specification of the eye cell fates (Lawrence & Green, 1979; Ready, Hanson, & Benzer, 1976; Wolff & Ready, 1991a). This is reminiscent of vertebrate development, where transplantation studies have shown that the cellular environment is of paramount importance for determining the cell fates assigned in the embryo.

Dozens of mutations are known that disrupt normal patterning in the eye (Lindsley & Zimm, 1992). There is a wide variety of phenotypes associated with the different mutations: roughening of the eye due to the improper arrangement of cells; a glazed or smooth appearance; and reduction in eye size. The *eyes absent* (*eya*) mutation was selected for this study because of its striking eyeless phenotype. The molecular genetic analysis of *eya*, the subject of this thesis, shows that the *eya* gene may not only contribute to the

understanding of eye development, but also to the understanding of programmed cell death and gene regulation.

The *Drosophila* eye develops from the eye-antennal imaginal disc, a neural epithelium derived from some 20 cells at the cellular blastoderm stage of embryogenesis (Garcia-Bellido & Merriam, 1969). During larval life, these cells proliferate, causing the disc to increase in size. By the third instar, the eye portion of the disc is thought to contain approximately 2,000 cells. During the mid-third instar, a pattern emerges in the disc as a wave, starting at the posterior end and progressing to the anterior (Waddington & Perry, 1960). This pattern consists of repeated units of an array that correspond to the adult lattice of ommatidia. The wave of differentiation in the disc is punctuated by a furrow, termed the morphogenetic furrow (Ready, et al., 1976). Anterior to the furrow, the disc has no obvious pattern and consists of dividing progenitor cells.

Studies, using a variety of staining techniques and analyzed by light and electron microscopy, have revealed that clusters of six or seven cells form shortly after the passage of the furrow (Tomlinson, 1985; Tomlinson & Ready, 1987). Soon thereafter, these clusters are pruned to five cells that will differentiate into the R2, R3, R4, R5, and R8 photoreceptor neurons of the adult ommatidium. The presumptive R8 cell is the first to differentiate, judged by its expression of the 22C10 neural antigen and by its being the first to send out an axon. For this reason, the presumptive R8 has been favored as being the "founder" cell of the cluster. After the five-cell cluster stage, a second wave of mitosis occurs, generating more cells that are recruited into the clusters (Ready, et al., 1976; Wolff & Ready, 1991a). Presumptive R1 and R6 are added, followed by presumptive R7 to make up the full complement of photoreceptor cells. The recruitment of the rest of the cells that make up the ommatidium is known in less detail. It is known, however, that the final stage of pattern formation involves the pruning of extraneous cells by programmed cell death (Wolff & Ready, 1991b).

A large number of mutations that affect the eye have been classified according to the stage of development at which the phenotype can be discerned (Renfranz & Benzer, 1989). Genes, such as those defined by the *Ellipse, scabrous*, and *Notch* mutations, affect the initial patterning of the lattice. They have been postulated to take part in lateral inhibitory signaling that establishes the spacing of the lattice. The *Ellipse* mutation is a gain-of-function mutation in the *Drosophila* EGF receptor gene (Baker & Rubin, 1989; Baker & Rubin, 1992). In such mutants, there are many fewer ommatidia, and they are widely spaced. It has been suggested that the normal function of the gene is to transmit an inhibitory signal intracellularly, preventing cells that are not spaced properly from adopting the founder cell fate. The *scabrous* gene encodes a secreted fibrinogen-related molecule, which may be part of the inhibitory signal (Baker, Mlodzik, & Rubin, 1990). In *scabrous* mutants, the ommatidia are not spaced normally. The spacing of clusters is also affected in the absence of *Notch* function (Cagan & Ready, 1989). Loss of function of the *Notch* gene in cells near the furrow results in all those cells adopting neuronal cell fates. It may be that loss of *Notch* completely obliterates the inhibitory signaling pathway, allowing all cells to adopt "founder" fates.

A wide variety of genes is required for patterning events that occur after the lattice is laid out. The best studied of these are those involved in the *sevenless* signal transduction pathway. Mutations, such as *sevenless*, that block this signal transduction pathway, prevent presumptive R7 from adopting its normal fate (Tomlinson & Ready, 1986). The Sevenless protein is a receptor tyrosine kinase, with homology to mammalian oncogenes (Hafen, Basler, Edstroem, & Rubin, 1987). Other genes in the pathway have homologies to mammalian oncogenes and signal transduction components [see (Greenwald & Rubin, 1992)].

Two genes are known that affect the refinement of the retinal pattern by programmed cell death: *roughest* and *echinus* (Wolff & Ready, 1991b). Mutations in these genes give rough eye phenotypes that are due to lack of programmed cell death at the late stages. The

molecular functions of these genes are unknown, although *roughest* is thought to act autonomously.

Little is known about the events that occur prior to the passage of the morphogenetic furrow. Most of our knowledge of these events stems from transplantation studies using the eye discs and hosts of different ages (Bodenstein, 1953; Gateff & Schneiderman, 1975). Eye discs can be cultured *in vivo* in the abdomens of larvae or adults. Eye discs from third instar larvae will only differentiate in a metamorphosing host. Transplants into adults survive, but do not differentiate unless subsequently transplanted into a metamorphosing larva. This suggests that hormones play an important role in the initiation of pattern formation in the eye.

Studies using heterochronic transplants have led to the notion of eye progenitor cells gaining "competence" to differentiate (Gateff & Schneiderman, 1975). Discs from 1st and early 2nd instar larvae are unable to differentiate when transplanted into metamorphosing hosts. Beginning from the middle of the 2nd instar, however, transplanted discs are able to differentiate into discernible cell types. The variety of cell types differs with the age of the donor disc before transplantation. These results suggest that a change in the state of eye progenitor cells occurs during the second instar, such that they are able to respond to the metamorphosing environment.

The *eya* mutation was selected for study because its eyeless phenotype suggested that the mutant defect might occur anterior to the furrow, before the initiation of patterning in the disc. This turns out to be so. In *eya* mutants, eye progenitor cells undergo programmed cell death anterior to the furrow. This is the subject of Chapter II. The mutant phenotype is analyzed and the molecular organization of the gene is presented. A model is presented in which the mutant cell death is due to an excessive number of progenitor cells adopting a cell death pathway that is normally adopted by very few cells.

The link between eye development, cell death, and *eya* comes at a time when a large body of evidence has accumulated demonstrating that cell death is crucial to normal

development. Cell death is found at many stages of development, and attempts have been made to classify developmental cell death according to function, morphology, and mechanism (Clarke, 1990; Glücksmann, 1951; Saunders, 1966). The most prominent form of cell death found in development is called apoptosis, or programmed cell death, which is characterized by shrinkage of cells, condensation of the nucleus, and phagocytosis by surrounding cells (Wyllie, Kerr, & Currie, 1980). Apoptosis appears to be an active, suicide pathway. The biochemical characterization of apoptotic cells frequently reveals the presence of a DNA ladder, brought about by the cleavage of the DNA by endonucleases.

Examples of programmed cell death occurring during development, for which the function is thought to be known, include the interdigital pruning in the chick limb bud (Glücksmann, 1951); the removal of cells that do not fit in the pattern of the *Drosophila* eye (Wolff & Ready, 1991b); and the sculpting of the mouse immune repertoire (Fesus, 1991; Goldstein, Ojcius, & Young, 1991) and nervous system (Hamburger & Levi-Montalcini, 1949; Oppenheim, 1991).

Cell death has been shown to play a role in the normal development of the nematode, *C. elegans*. Loss of function of either the *ced-3* or *ced-4* genes prevents all programmed cell deaths from occurring during development of the organism (Ellis & Horvitz, 1986). These two genes are thought to have a role in the mechanism of cell death. The *ced-9* gene appears to act upstream of *ced-3* and *ced-4* (Hengartner, Ellis, & Horvitz, 1992). Both loss- and gain-of-function alleles of this gene exist. Loss-of-function mutations cause ectopic cell deaths that are dependent on *ced-3*/*ced-4* function. A gain-of-function allele has a *ced-3*/*ced-4* phenotype, which is the prevention of cell deaths. Such analysis clearly demonstrates that developmental cell death in *C. elgans* is under genetic control.

The molecular mechanisms of cell death and its regulation are currently under investigation. The molecular function of the *ced-4* gene is unknown (Yuan & Horvitz, 1992). However, the *ced-3* gene encodes a protein with homology to a cysteine protease (Yuan, Shaham, Ledoux, Ellis, & Horvitz, 1993). The *ced-9* gene has homology to the

vertebrate *bcl-2* gene, which has been shown to prevent cell death when ectopically expressed in various mammalian cell lines (Korsmeyer, 1992) and in nematodes (Vaux, Weissman, & Kim, 1992). Bcl-2 has been implicated in the eradication of superoxide radicals (Hockenberry, Oltvai, Yin, Milliman, & Korsmeyer, 1993; Kane, Sarafian, Anton, Hahn, Gralla, Valentine, et al., 1993). Recently, it was shown to associate with the ras-related protein R-ras p23, suggesting the Bcl-2 molecule may be part of a signal transduction pathway (Fernandez-Sarabia & Bischoff, 1993). Despite these advances, current knowledge represents only pieces of a puzzle. Lacking is a solid framework in which the pieces fit, and an understanding of how the process is controlled.

Molecular links between mammalian cell death and cell transformation have been made, however (Williams & Smith, 1993). For example, overexpression of *c-myc in vitro* induces both proliferation and cell death (Evan, Wyllie, Gilbert, Littlewood, Land, Brooks, et al., 1992). Studies *in vitro* and *in vivo* show *c-myc* -induced cell death is sensitive to Bcl-2, suggesting that programmed cell death may be a normal mechanism to rid the body of abnormally proliferating cells (Bissonnette, Echeverri, Mahboubi, & Green, 1992; Strasser, Harris, Bath, & Cory, 1990). This idea has been extended to suggest that, in higher organisms, the default state of most cells is programmed cell death; cell survival requires proper signals (Evan, et al., 1992; Raff, 1992; Raff, Barres, Burne, Coles, Ishizaki, & Jacobson, 1993). This may be a mechanism by which the proper numbers and types of cells are selected for any given tissue.

Our improved knowledge of the function of cell death notwithstanding, there are still many examples of cell death whose function in the organism is unknown. For example, cell death is often found near invaginations and morphogenetic movements during vertebrate embryogenesis. Other cell death is associated with cell proliferation and differentiation, such as in the tadpole retina (Glücksmann, 1951). It is not known what roles such cell deaths play.

Similarly, in the *Drosophila* eye disc, the function of the wave of cell death that precedes the furrow is not well understood. Understanding the function of this cell death may contribute significantly to the understanding of developmental cell death in other organisms. Because the earliest detectable eye phenotype in *eya* mutants is progenitor cell death, the *eya* gene has emerged as a promising starting point from which to gain further knowledge of the role and regulation of cell death. To this end, we undertook the genetic and molecular characterization of the *eya* gene.

Chapter III extends the studies of the phenotypic and molecular characterization of the *eya* gene, presented in Chapter II. Loss of function of *eya* in an eye precursor cell is linked to death of that cell. Furthermore, the eye phenotype is shown to be unrelated to other functions of *eya* during development. Chapters II and III lay the foundation for the use of the *eya* system as a genetic approach to the study of early differentiation events, and to the study of programmed cell death.

The subject of Chapter IV stems from the investigation of interallelic interactions of the *eya* locus. The interaction displayed by some *eya* alleles is sensitive to chromosomal rearrangements that change the relative positions of the two alleles in the genome. Changing the location of a gene in the eukaryotic genome can have a dramatic influence on the expression of that gene. This observation, first made in *Drosophila*, is known as the position effect [for review, (Lewis, 1950)]. Position effects that have been studied over past decades generally fall into three categories. The first is exemplified in transgenic animals, where *cis*-regulatory regions near the insertion point appear to affect the expression of the transgene. This has often been found a nuisance in mouse transgenics, since it can hinder the desired transgene expression. In *Drosophila*, on the other hand, such position effects have been used to advantage for detecting interesting regulatory regions by the "enhancer-trap" method (O'Kane & Gehring, 1987).

A second phenomenon is known as position effect variegation (PEV). Its hallmark is a variegating phenotype: the mutant phenotype, rather than being equally expressed in all

cells, is differentially expressed in various patches of tissue, giving rise to a mottled appearance. Such variegation is strongly correlated with rearrangements that bring a normal allele into the vicinity of heterochromatin, a compacted form of chromatin near the centromeres. The inactivated X chromosome in mice and humans also has this character. While PEV is best characterized in *Drosophila* it may also occur in mice. Mice exhibiting a mottled phenotype have been shown to possess a translocation of the X chromosome to an autosome, which is thought to result in heterochromatin formation in the neighborhood of the affected gene (Wilson, Bellen, & Gehring, 1990).

A third category of position effect, and the subject of Chapter IV, consists of genetic interactions that are sensitive to the relative positions of alleles in the genome. I call these "allelic position effects," but they have also been referred to as proximity-dependent complementation or *trans*-sensing effects (Tartof & Henikoff, 1991). In these phenomena, the phenotype depends on the alleles being in the same position on both chromosomes. A well known example is the interaction in *Drosophila* of the $zeste^1$ mutation with the *white* gene. Flies homozygous (or hemizygous) for $zeste^1$ can have different colored eyes, depending on the state of the *white* alleles. Flies hemizygous for *white*, or with two normal *white* alleles that are in different parts of the genome, have normal eye color. Flies homozygous for normal *white* alleles that are located in homologous sites of the genome, however, have reduced *white* activity, and have pale eyes.

Perhaps the most widely studied of these phenomena is the interaction between alleles of the *bithorax* complex (BX-C), first described by Lewis, for which he coined the term "trans-vection" (Lewis, 1954). This interaction is disrupted by certain kinds of rearrangements thought to disrupt the pairing of alleles. In all cases of allelic position effects, gene expression resulting from a pair of alleles depends on how the alleles are situated in the genome.

The study of position effects holds great promise for the understanding of gene regulation, chromatin structure, and the relationship between them. The use of transgenic

systems has led to the identification of "insulator" sequences that prevent enhancers from interacting with undesired promoters (Chung, Whiteley, & Felsenfeld, 1993). Such insulators may correspond to structural elements, the scaffold attachment regions, where chromatin is thought to be anchored to the nuclear matrix. Studies in *Drosophila* have revealed a large number of mutations that can influence PEV [for review, see (Spradling & Karpen, 1990)]. Investigations of these modifiers of PEV has led to promising advances. For example, *Su(var)205* (also identified as HP1, a heterochromatin-associated protein) possesses a motif called the "chromo-domain" (Paro & Hogness, 1991). This domain is conserved in mouse and human genes, though its function is unknown. Interestingly, the chromo domain is found in the *Polycomb* gene product, known for its function to repress ectopic expression of the homeotic gene clusters. The *Polycomb* gene is the prototype of a large collection of genes (the *Polycomb* group, or PcG), that are thought to function together in chromatin [for review, see (Paro, 1990)]. Mutations in some of the PcG genes are now known to modify PEV [see (Reuter & Spierer, 1992)]. Interestingly, some of these same genes were identified as dominant modifiers of the *zeste-white* interaction (Wu, Jones, Lasko, & Gelbart, 1989). The convergence of investigations of these different position effects on related, sometimes identical, chromatin molecules suggests that the mechanisms by which chromatin structure exerts its influence on gene regulation may soon be elucidated.

Some progress has also been made in understanding the phenomenon of allelic position effects. For several loci, the interaction of alleles results from the expression of one allele that is otherwise silent when homozygous (Castelli-Gair, Micol, & Garcia-Bellido, 1990; Geyer, Green, & Corces, 1990; Kornher & Brutlag, 1986). While different mechanisms could account for the induction of otherwise unexpressed alleles, the notion that enhancers can act in *trans* is gaining support (Mueller & Schaffner, 1990; Pirrotta, 1990). In this model, alleles that are not expressed when homozygous, owing to the lack of the required enhancer sequences in *cis*, can be induced by enhancer sequences of another

allele in *trans*. The *trans* action of enhancers as a model represents a major step in our understanding of allelic position effects. However, even if it proves correct for the few interactions for which there is evidence, the model still leaves important questions unanswered.

One important question relates to differences in how various allelic position effects are manifested. All allelic position effects that have been tested are sensitive to a change in the position of one allele due to an insertional translocation to another chromosome arm. This includes the *Cbx-Ubx* interaction of the BX-C. But only interactions at the BX-C and *dpp* have been shown to be sensitive to far more subtle changes in allelic locations. The allelic position effects at these loci will be referred to here as transvection, since they are sensitive to a certain class of rearrangements, termed "transvection-disrupting rearrangements" (Gelbart, 1982), that have two common attributes: (1) one breakpoint is in what Lewis calls the "critical region," which spans much of the chromosome between the locus and the centromere; and (2) the rearrangement is such that the locus is attached to a different chromosome arm from the one on which it is normally located. Transvection is truly unique and remarkable, in that breakpoints located thousands of kilobases away from a locus can have an effect on the interaction of alleles.

The mechanism underlying transvection disrupting rearrangements is thought to be through the disruption of chromosomal pairing at the affected locus. In *Drosophila*, the polytene chromosomes of the salivary glands are usually paired with their respective homologs. Lewis observed that the rearrangements that disrupted transvection at the BX-C often resulted in loss of pairing in the polytene chromosomes in the BX-C region. Because the chromosomes of *Drosophila* are thought to be paired in somatic cells during interphase (Hiraoka, Dernburg, Marmelee, Rykowski, Agard, & Sedat, 1993; Metz, 1916), the loss of pairing could similarly impair allelic interactions in other tissues besides the salivary glands.

In the few cases where it has actually been tested, transvection-disrupting rearrangements have no detectable effect on other allelic position effects. The most in-depth study was done with the *zeste-white* interaction (Smolik-Utlaut & Gelbart, 1987). By using transpositional insertions of the *white* gene onto chromosomes bearing interacting alleles of *dpp* or BX-C, it was shown that rearrangements that disrupt transvection at these latter loci had no effect on the *zeste-white* interaction. Similarly, studies of *yellow* alleles revealed no dependence on transvection-disrupting rearrangements. However, this finding was attributed to the telomeric location of the *yellow* gene. Even with transvection disrupting rearrangements, the telomeric region might remain paired (Geyer, et al., 1990).

The allelic position effects at three loci (BX-C, *dpp*, and *yellow*) have been shown to be sensitive to the loss of function of the *zeste* gene (Gelbart & Wu, 1982; Geyer, et al., 1990; Kaufman, Tasaka, & Suzuki, 1973). (Note that the *zeste-white* interaction is dependent on a neomorphic function of *zeste*; it is not sensitive to loss-of-function alleles.) The normal function of *zeste* is not known for certain. The *zeste* gene product has been shown to bind DNA in a sequence specific manner. The Zeste protein is found in some 90 different locations in polytene chromosomes of the salivary glands. It has also been shown capable of enhancing transcription *in vitro* and *in vivo*. Nevertheless, loss of the *zeste* gene has no obvious effect on the animal. It could be a redundant transcription factor, but others argue that its role is more likely to be in chromatin structure, or to help mediate chromosome pairing. This last possibility is interesting, considering that the chromosomes of *Drosophila* are normally paired, but that loss of pairing, at least in a region of the genome, has no discernible effect. Perhaps there is some advantage conferred by the *zeste* gene and the pairing of chromosomes that is only detectable in evolutionary time. In any case, the *zeste* gene is the only gene known to be required for transvection.

The allelic position effect for *eya* is similar to the phenomenon of transvection, in which the interacting alleles are sensitive to certain kinds of chromosomal rearrangements. Since transvection at *eya* is easily quantified by the size of the eye, the study of transvection

at *eya* may hold certain advantages over the other systems. The study of the *eya* locus provides a genetic system in which to approach the understanding of the influence of chromatin structure on transcriptional regulation, in addition to furthering our knowledge of eye development and the regulation of programmed cell death.

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Chapter II

The *eyes absent* Gene: Genetic Control of Cell Survival and Differentiation in the Developing *Drosophila* eye

Introduction

In this chapter, the basis of the *eya* eyeless phenotype is explored during development, and found to be due to excessive cell death of eye precursor cells, at a time when some cell death is normally present. Cloning of the gene reveals two alternatively-spliced cDNA's, of novel sequence. The gene product is expressed in the nucleus of eye precursor cells starting in the 2nd larval instar, as detected by anitbody staining. A model is presented in which *eya* plays a role in the differentiation of eye precursor cells, repression of cell death of those cells, or both.

The results presented here stem from work performed jointly by Nancy M. Bonini and myself. Specific areas in which I made significant contributions to the experiments include: adult phenotypic analysis; genetic analysis of alleles; cytological analysis of X-ray alleles; immunohistochemistry in eye discs; genomic walking and cloning; Northern analysis; and transformation rescue of the mutant phenotype. The results were published in the following manuscript:

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

Cells become specified during development through sequential restrictions of their potential fates. This process includes mechanisms that monitor differentiation to eliminate, by programmed cell death, cells that have inappropriate specificity or developmental capacity, or that are extraneous (Glücksmann, 1951; Saunders, 1966). Many aspects of tissue development rely on cell death for the selection of proper sets of cells. In vertebrates, massive numbers of neurons generated in early development become eliminated in late stage refinement of connections (Hamburger and Levi-Montalcini, 1949; Oppenheim, 1991). The mechanism is thought primarily to occur through competition for trophic agents derived from target tissues, which may reinforce appropriate patterns of innervation. Similarly, in the immune system, progenitor cells must generate a great diversity of cell types. The differentiation process relies heavily on regulated cell death to eliminate large numbers of cells of inappropriate reactivity (Fesus, 1991; Goldstein et al., 1991).

Programmed cell death typically occurs in conjunction with critical differentiation events. Genes have been identified that function in the cell death pathway, such as the *ced* genes of C. elegans (Ellis and Horvitz, 1986; Hengartner et al., 1992). However, genes are also needed to determine when that pathway is activated during development. To coordinate differentiation and death, the activities of genes involved in select differentiation events presumably impinge on control of genes of the death pathway, to repress the suicide of appropriate cells. We describe the biological and molecular characterization of the *eyes absent* (*eya*) gene that, rather than being part of the cell death pathway, is involved in regulating whether progenitor cells differentiate or die at a specific stage early in development of the *Drosophila* compound eye. Understanding the genetic and molecular nature of genes such as *eya* may provide insight into the regulation of both normal and abnormal cellular differentiation, as well as maintenance of the differentiated state. The mechanisms have implications for oncogenesis (Williams, 1991), immune disease

(Ameisen and Capron, 1991; Meyaard et al., 1992), and conditions where excessive cell death results in tissue damage, such as neural injury (Choi, 1988).

The *Drosophila* eye is an excellent genetic system in which to approach the problem of how differentiation events and cell death interplay to achieve proper cellular development (Ready, 1989; Banerjee and Zipursky, 1990; Rubin, 1991). The adult eye is composed of some 800 repeated neural units called ommatidia, each containing cell types that include three photoreceptor classes, three kinds of pigment cells, cone cells, and a bristle cell complete with socket, neuron and glial sheath. During the third larval instar, progenitor cells commence differentiation to generate the various cell types (Waddington and Perry, 1960). Differentiation is marked by a morphogenetic furrow that moves from posterior to anterior across the field of progenitor cells in the eye portion of the eye-antennal imaginal disc (Ready et al., 1976). Anterior to the morphogenetic furrow, the progenitor cells undergo division to generate an epithelial field for the differentiation events that commence with the furrow. Thus, at a given time, the disc displays a time line of development, the earliest morphologically evident differentiation events being associated with the furrow. Progressively later events are seen toward the posterior of the disc, where a pattern emerges of developing cell clusters. Little is known about the events that occur prior to furrow formation that lead to differentiation, although cell competence, hormones and possibly inductive interactions appear to be involved (Bodenstein, 1953; White, 1961; Gateff and Schneiderman, 1975). Some cell death is a normal part of the developmental process, having been observed in the eye disc during morphogenesis (Fristrom, 1969; Spreij, 1971; Wolff and Ready, 1991).

We show that the *eya* gene is required anterior to the morphogenetic furrow for eye morphogenesis. Loss of *eya* gene function results in the elimination of progenitor cells by programmed cell death prior to this differentiation event. Instead of the small amount of cell death that normally occurs at this time during eye development, loss of *eya* activity appears to cause a massive switch in cell fate from the pathway of differentiation to that of cell

death. The gene encodes a nuclear protein that is able to block progenitor cell death and restore eye differentiation. Our data suggest that a critical survival pathway functions prior to morphogenetic differentiation of the progenitor cells; the *eya* gene is required at a specific stage for cell survival and/or an event of differentiation. *eya* mutants provide a genetic system in which to approach the molecular nature of genes that regulate cell selection at critical transitions in development.

RESULTS

The *eya*^{1} mutant lacks the compound eyes due to failure of differentiation in the eye disc.

Flies with the *eya*^{1} mutation show remarkable specificity for loss of the adult compound eyes (Fig. 1A,B; Sved, 1986; Renfranz and Benzer, 1989). All other external structures appear normal, including the adult ocelli, which develop from edges of the eye imaginal discs. In the brain, there is loss of the first optic ganglion (lamina), a reduction in size of the second optic ganglion (medulla), and the lobula and lobula plate show some disorganization (Fig. 1C,D). These brain defects are similar to those observed in other eyeless mutants, and are consistent with influence of the retinal neurons on development of the optic lobes (Power, 1943; Meyerowitz and Kankel, 1978; Fischbach, 1983; Selleck and Steller, 1991).

In normal development, eye differentiation begins during the third instar larval stage when the morphogenetic furrow sweeps from posterior to anterior across the eye portion of the eye-antennal disc, leaving clusters of differentiating photoreceptor neurons in its wake (Ready et al., 1976). The differentiating clusters can be visualized by staining with monoclonal antibodies, such as neuron-specific MAb22C10 (Zipursky et al., 1984; Fig. 2E). In *eya*^{1} eye discs, the normal expansion of the eye portion of the disc during the third instar larval stage is arrested, and no furrow forms (Fig. 2B,D,F). When stained with

antibodies that normally highlight the differentiating neurons, the mutant eye discs fail to show any evidence of cluster formation (Fig. 2F; also Renfranz and Benzer, 1989). In contrast, the antennal portion of the disc expands and differentiates normally. The larval photoreceptor organ also appears normal, and Bolwig's nerve from the larval visual organ (Bolwig, 1946) traverses the eye disc in its path into the optic stalk, as in a normal disc. The *eya*^{1} mutation thus appears specifically to affect the progenitor cells that normally form the adult compound eye.

Cell division occurs in the *eya*^{1} eye disc.

The apparent arrest in development of the eye disc epithelium in *eya*^{1} animals could result from a block in cell division to generate progenitor cells. Alternatively, the cells might be generated but fail to differentiate normally. To distinguish between these possibilities, mutant and wild-type larvae were labeled *in vivo* with pulses of 5-bromodeoxyuridine (BrdU). BrdU is incorporated into the DNA of dividing cells in S phase; these cells can subsequently be visualized by immunofluorescence using antibodies specific for BrdU (Truman and Bate, 1988). In normal eye discs, dividing cells were labeled in a scattered pattern in the region anterior to the furrow, where progenitor cells are generated. At the furrow, DNA synthesis was absent. Posterior to the furrow, cell division resumed in a restricted band, reflective of pattern formation events (see Ready et al., 1976). In *eya*^{1} mutant eye discs at the early third instar larval stage, the amount of cell division was similar to that in the region anterior to the furrow in normal eye discs of the same stage (data not shown). This result suggests that cells in the *eya*^{1} eye disc do divide; a lack of cell division to generate progenitor cells appears not to be the primary defect.

Extensive cell death in the *eya*^{1} eye disc.

Given that progenitor cells divide in the *eya*^{1} eye disc, we asked whether lack of normal differentiation results from the loss of cells by death. In normal third instar larval

eye discs relatively little cell death is present (see Fig. 4E). In contrast, eye discs of *eya*^{1} animals reveal a dramatic increase in cell death during the third instar larval stage. Cells are present in the *eya*^{1} eye disc that appear condensed and refractile by light microscopy, reminiscent of cells dying by programmed cell death in C. elegans (see Fig. 4D; Sulston and Horvitz, 1977). Dead cellular material also fluoresces brightly when stained with acridine orange (Spreij, 1971). Such staining reveals a great increase in the number of dead cells in eye discs of the *eya*^{1} mutant (see Fig. 4H). In the electron microscope, *eya*^{1} mutant eye discs show electron dense condensed fragments of cells, and many examples of these fragments engulfed within other cells (see Fig. 5). These morphological features are characteristic of cells dying by programmed cell death (Wyllie et al., 1980; Kerr et al., 1987; Clarke, 1990). The results suggest that the defect in the *eya*^{1} mutant is a loss by cell death of eye progenitor cells.

New alleles of *eya*.

Differentiation occurs in a timed manner in normal eye discs, progressing from posterior to anterior across the discs with the advance of the morphogenetic furrow. Thus, in *eya* mutants, determining when the cell death occurs relative to the furrow allows us to define the developmental stage at which the cells die. Due to the extreme phenotype of the *eya*^{1} mutant, in which no furrow occurs, it is not possible to determine this timing. We therefore generated new *eya* alleles with less extreme eye phenotypes to place the cell death relative to the critical events involved in eye differentiation.

Additional alleles of the *eya* gene were isolated by screening for mutations that failed to complement the eye phenotype of *eya*^{1}. Several mutants contributed by other laboratories were also determined to be *eya* alleles by genetic mapping and failure of complementation (see Experimental Procedures). Most of the newly-generated alleles are lethal or semi-lethal when homozygous. The lethality is embryonic (N.M.B., unpublished data), and fails to complement an independently isolated embryonic lethal mutation, *clift*

(Nüsslein-Volhard et al., 1984). Flies bearing the *clift* allele in *trans* to the viable eya^1 allele show a severe eye phenotype. In addition, some *eya* alleles and interallelic combinations show reduced or absent ocelli, abnormal morphology of the adult brain, and female sterility. These results suggest that the *eya* gene has other functions in addition to its role in eye development; the eye function appears, in part, independently mutable. The gene also displays interallelic complementation. A detailed study of these observations will be reported elsewhere (W.M.L., N.M.B., and S.B., in preparation).

The eye phenotypes of the new alleles, in *trans* to the viable eya^1 mutation, comprise a phenotypic series (Fig. 3). The mutants can be classified as: (i) mild, where the eyes are rough, but only slightly smaller than normal size (Fig. 3B,F); (ii) intermediate, where both eyes always form, but are rough and reduced in size (Fig. 3C,G); (iii) severe, where the eyes are rough, much reduced, and frequently absent from one or both sides of the head (Fig. 3D,H); and (iv) completely and consistently eyeless, like the eya^1 mutant (see Fig. 1B). In tangential sections of reduced and rough eyes of heteroallelic combinations, some ommatidia lack the full complement of photoreceptor cells (Fig. 3E-H). However, there is no obvious specific subset of cells missing with increasing allele severity, as in a mutant like *sevenless* (Harris et al., 1976). Even in severely reduced eyes, full complements of photoreceptor cells frequently form within individual ommatidia (Fig. 3H). These results suggest that *eya* mutations appear to reduce the number of progenitor cells available for recruitment into the developing eye, rather than specifically eliminating any particular cell type(s).

eya alleles with partial eye development have abnormally high cell death anterior to the furrow.

eya mutants with reduced adult eyes exhibit neural differentiation in the eye disc, as illustrated by staining with MAb22C10 (Fig. 4A-D). However, consistent with their reduced eyes in the adult, they form fewer neural clusters in the eye disc. With increasing

severity of the phenotype, the number of clusters that forms decreases. In eye discs of larvae expressing intermediate and severe allele combinations, where very reduced numbers of ommatidia form, the clusters develop in the posterior-most region of the eye disc (Fig. 4C,D). When examined with BrdU labeling, eye discs of larvae expressing intermediate allele combinations show cell division both anterior to the furrow and in a band posterior to the furrow, reflecting aspects of pattern formation seen in normal eye discs (data not shown). In eyeless allelic combinations, no furrow is seen, no clusters differentiate, and dramatic increases in cell death occur in the eye discs, as in the eya^1 mutant. The result that eye discs of the eya^1 mutant look developmentally similar to eye discs of the eya^1 allele in *trans* to lethal alleles is consistent with the eya^1 mutant being a severe hypomorph or null for a necessary eye function of the *eya* gene.

Since the eye discs of mutants showing partial eye development display a quasi-normal framework of differentiation, they allow the placement of the cell death relative to the morphogenetic furrow. Examination of eye discs of such *eya* mutant combinations revealed dramatic increases in cell death restricted to the region anterior to the furrow (Fig. 4E-H; Fig. 5). This observation can be detected by differential interference contrast optics, in which dead cells appear condensed and refractile (Fig. 4C,D), and is highlighted by fluorescence microscopy with acridine orange (Fig. 4E-H). Of several different wild-type strains of *Drosophila* melanogaster examined, we consistently find some degree of cell death just anterior to the furrow, varying from a small amount to a thin band ahead of the furrow, as in Fig. 4E (also Spreij, 1971; Wolff and Ready, 1991). In eye discs from *eya* allelic combinations that form mildly reduced eyes, the increase in cell death also occurs as a band just anterior to the furrow, in the same region where the low level of cell death normally takes place (compare Fig. 4E,F). This result suggests the possibility that loss of *eya* function shunts cells into a normally occurring cell death pathway anterior to the furrow. In eye discs from intermediate and severe allele combinations that form more severely reduced eyes, the amount of cell death is greater, covering a broader region

anterior to the furrow (Fig. 4G,H). Normal discs also show some cell death in the differentiating region of the disc posterior to the furrow during the third instar (Spreij, 1971; Wolff and Ready, 1991); that cell death is not increased by *eya* mutations (Figs. 4E-H).

We examined the ultrastructure of the dying cells in the region anterior to the furrow by transmission electron microscopy (TEM). In both normal (data not shown) and mutant discs (Fig. 5), the morphological changes appear characteristic of programmed cell death (Wyllie et al., 1980; Kerr et al., 1987; Clarke, 1990). Dead cells condense into electron dense bodies containing well-preserved cellular organelles. These bodies appear to become engulfed by surrounding cells, such that the debris is cleared by the time the furrow passes. Consistent with observations using acridine orange, the elevated level of cell death is restricted to the region of the disc anterior to the furrow (Fig. 5A). In eye discs of larvae bearing mild allele combinations, the cell death appears rapid: since the furrow advances at a rate of about one column of clusters per two hours, we estimate that dead cells fragment and are cleared within 2-4 hours.

The earliest defect that we can observe in mutant discs is an increase in the number of cells undergoing cell death prior to furrow formation. By TEM, we were unable to find any obvious morphological or structural abnormalities in progenitor cells anterior to the furrow, other than changes characteristic of programmed cell death in dying cells. Furthermore, in alleles which make reduced eyes, progenitor cells that survive anterior to the furrow appear to enter into the normal progression of events marked by the furrow. Together, these results suggest that the primary phenotype of loss of *eya* gene function in the eye is the loss of progenitor cells through programmed cell death prior to furrow formation.

Cloning the *eya* gene.

To approach the molecular function of the *eya* gene in the differentiation vs. death process, we cloned the gene. Of sixteen X-ray-induced alleles, six with cytologically visible rearrangements in the polytene chromosomes of the larval salivary glands had a common breakpoint on the left arm of chromosome 2, in cytological region 26E. This location is consistent with the position of the *eya* gene determined by meiotic recombination, which placed both the eye phenotype (mapped for all cytologically normal alleles) and the lethality (mapped for eya^{E4} and eya^{P1}) between *dp* and *spd* on the left arm of the second chromosome. The region was cloned in a 60 kb genomic walk (see Experimental Procedures). Restriction fragments were tested against *eya* breakpoint alleles by *in situ* hybridization to the salivary chromosomes and by Southern blots. The chromosomal breaks of the *eya* mutants fell within a 25 kb region (Fig. 6A).

To identify transcripts derived from this region, we probed blots of poly(A)$^+$ RNA from adult heads with restriction fragments spanning the 25 kb. A transcript of 3.5 kb was identified that is disrupted by at least five of the breakpoint alleles; it is present in wild-type adult heads, and is less abundant in heads of eya^1 flies (Fig. 6B). It is also present in embryos.

cDNA libraries, constructed using poly(A)$^+$ RNA purified from adult heads (Itoh et al., 1986; Zinsmaier et al., 1990), were screened. Two types of cDNAs were obtained that correspond to alternatively-spliced products identical at the 3'-end, but differing in the use of 5' exons (Fig. 6A). Both cDNA classes recognize transcripts of 3.5 kb on Northern blots of poly(A)$^+$ RNA from wild-type adult heads. Using the cDNAs as probes, we also detected transcripts of 3.5 kb on Northern blots of poly(A)$^+$ RNA isolated from third instar larval eye disc preparations (Fig. 6C). These transcripts are reduced in intensity in eye discs from eya^1 larvae. To determine whether additional alleles demonstrated transcripts of altered size, we used the cDNAs to probe poly(A)$^+$ RNA prepared from heads of *eya* mutants. Of 12 alleles that exhibit no cytologically visible chromosomal rearrangements;

one showed an altered transcript of 4.7 kb (*eya*^{X2}). Taken together, these results suggest that the transcripts are products of the *eya* gene.

Sequence of the *eya* gene.

Sequence comparison of the type I and II cDNAs showed that the 5'-most sequences differ, while the approximately 2800 bp of 3'-sequence are shared. Conceptual translation of the type I cDNA, starting from the first potential initiation codon, revealed a single large open reading frame of 2280 nucleotides (Fig. 7). The predicted protein has 760 residues, with a predicted molecular weight of 80 kD. The open reading frame of the alternatively-spliced type II cDNA is 2298 bp. As a result of the alternative splicing, the proteins predicted for the two classes of cDNA differ in their extreme amino terminal sequences (Fig. 7).

Comparison to proteins in the Genbank and EMBL databases revealed that the *eya* protein is novel, with no striking homology to previously cloned genes. Study of the amino acid sequence suggests that the protein may be divided into two domains. The amino terminal half, corresponding roughly to amino acids 1-436 (numbers in reference to the type I cDNA class) is rich in alanine, glycine and serine, and shows several single amino acid repeats, including a polyglutamine-rich stretch corresponding to an *opa* repeat (Wharton et al., 1985). The carboxy terminal half of the protein has fewer amino acid repeats, and contains three charged stretches that are arranged as basic-acidic-basic domains, although the protein as a whole is predicted to be relatively neutral (predicted pI = 6.8). In addition to these features, the protein is predicted to have 5 hydrophobic a-helical stretches (Fig. 7 legend; Eisenberg et al., 1984). Given the nuclear localization of the gene product (below), these are unlikely to represent transmembrane domains.

eya transcript and protein are expressed in progenitor cells anterior to the furrow.

To study the temporal and spatial expression pattern of transcription of the gene in normal eye discs, we used whole-mount tissue *in situ* hybridization (see Experimental Procedures). The probe used included sequences common to the type I and II cDNAs. Strong *eya* RNA expression occurs in the region of the disc just anterior to the furrow (Fig. 8F,G). The transcript is also present posterior to the furrow, primarily in the basal region of the disc. In addition, two sites of expression are present on the edge of the eye disc, far anterior to the furrow, near the antennal disc (Fig. 8G). These regions probably correspond to the progenitors of the ocelli, which are derived from this area (Bryant, 1978). The ocelli are, in fact, missing in some heteroallelic combinations of *eya* mutants (W.M.L., N.M.B., and S.B., in preparation).

To determine the protein expression pattern, we raised a mouse polyclonal antiserum against a fusion protein (see Experimental Procedures). The pattern of antiserum staining revealed that the *eya* protein becomes first detectable in cells of the eye portion of the eye-antennal disc during the second larval instar; the expression appears graded, being stronger in cells in the posterior and edges of the eye portion of the disc, than in cells in the anterior and central region (Fig. 8A). This staining pattern persists to the third larval instar (Fig. 8B). As the morphogenetic furrow forms, the protein remains on in a graded manner anterior to the furrow, with strong expression just anterior to the furrow (Fig. 8C,D). Protein expression persists in the cells as they enter and pass through the furrow (Fig. 8D,E). Posterior to the furrow, the expression appears patterned, reflecting the array of developing neural clusters. Cells with nuclei in the basal region of the epithelium, which are presumably cells not yet recruited into developing neural clusters, show expression of the protein; in the apical region of the disc, expression appears strong in some cells of the differentiating neural clusters (Fig. 8D,E). The protein appears localized to the nucleus; it is not present in the nucleolus (Fig. 8E). In larvae homozygous for the eya^1 allele, which

produces normal ocelli but is eyeless (see Fig. 1B), *eya* protein expression occurs in the eye discs only in the ocellar progenitors (data not shown). The lack of detectable protein expression in the eye progenitor cells of the mutant is consistent with its being null or a severe hypomorph for *eya* gene activity in these cells. The onset of expression of both transcript and protein in the progenitor cells anterior to the morphogenetic furrow, where the increase in cell death occurs in *eya* mutants, is consistent with critical functioning of the gene in events that precede furrow formation.

We also examined the expression pattern of the gene elsewhere in normal animals (N.M.B., W.M.L., and S.B., in preparation). This analysis revealed a specific and dynamic expression pattern in the embryo, beginning with the onset of zygotic gene expression in the cellular blastoderm, and continuing in regions of the developing head and in segments. The gene does not appear to be expressed in the embryonic eye primordia or in eye discs during the first larval instar. The gene is also expressed in select other tissues in patterns that may be related to the embryonic lethal and adult phenotypes of select *eya* alleles. Thus, far from being ubiquitously expressed, the *eya* gene shows select expression in specific regions of the developing and adult organism.

Rescue with *eya* cDNA restores development of the eye.

To show definitively that the biological activity of the *eya* gene is encoded in the transcripts identified, we undertook transformation rescue of the eyeless phenotype. Type I cDNA was subcloned into the pHT4 vector (Schneuwly et al., 1987), downstream of the *Drosophila hsp70* heat shock promoter, and injected into embryos (see Experimental Procedures). Stable inserts were crossed into eya^1 and eya^2 mutant flies, which express viable eyeless phenotypes. Mutant larvae, harboring an *hsp-eya* mini-gene insert, were heat pulsed at 37°C for 1 hr every 6-8 hr, from the first instar larval stage to pupation to determine whether expression of the normal cDNA could restore eye development.

Mutant lines bearing the mini-gene, raised without heat shock, were eyeless (Fig. 9B). Heat pulsing of *eya*^{1} or *eya*^{2} mutant larvae, lacking an insert, had no effect on the mutant phenotype. However, heat pulsing of *eya*^{1} or *eya*^{2} mutant larvae with an *hsp-eya* mini-gene insertion restored the adult compound eye (Fig. 9C). Tangential sections through rescued eyes showed that the restored ommatidia appear normal (Fig. 9E). No discernible dominant effects of heat-shock induced expression of the *hsp-eya* mini-gene were evident in normal flies or in *eya* mutants, other than rescue of the eye in the latter.

The effective period of heat-shock induced rescue of the *eya* mutant phenotype was further narrowed down by heat pulsing during the second and third instar larval stages. Heat pulses during only the second instar larval stage were ineffective. However, eyes of small size could be restored with three 30 min heat pulses every 6 hr starting from the third instar larval molt (data not shown), the stage during which the morphogenetic furrow begins. With 4 or 5 heat pulses, eyes of intermediate size could be restored, and to a greater percentage of transformants. These data suggest that *eya* activity is required during the same stage of development at which some cell death normally occurs anterior to the furrow.

Rescue of the *eya* mutant phenotype with the heat shock cDNA construct shows that the protein encoded by the type I cDNA can provide the biological activity required to rescue the progenitor cells from death and restore the sequence of patterning events that generate an eye.

DISCUSSION

Since the demonstration that cell-cell interactions, rather than lineage, are involved in specification of the various cell types that comprise the ommatidia (Ready et al., 1976; Lawrence and Green, 1979), there has been much interest in events of pattern formation and cell specification that begin with the morphogenetic furrow (reviewed in Ready, 1989; Banerjee and Zipursky, 1990; Rubin, 1991). The *eya* gene is of special interest because it functions upstream of this landmark event. The nuclear protein encoded by the *eya* gene

appears to be required at a critical stage in eye morphogenesis, prior to the furrow, to promote the survival and/or the differentiation of the eye progenitor cells. In *eya* mutants, reduction in gene function results in an increase in the amount of cell death that normally occurs anterior to the furrow. The *eya* mRNA and protein expression begin prior to furrow formation, and restoring gene activity with an *hsp-eya* mini-gene during the third larval instar, when the furrow normally progresses, rescues progenitor cells from death and restores the pattern formation events that generate an eye.

eya mutants reveal a selection process in eye development prior to the furrow.

Since some *eya* alleles are lethal when homozygous, we have used *trans*-heterozygous mutant combinations with viable *eya* alleles to observe a function of the gene in early eye development. Alleles such as eya^1 and eya^2 appear to specifically affect eye development; while these eyeless alleles are severely hypomorphic or null for *eya* activity in the eye progenitor cells, they complement the vital function(s) of the gene. Analysis of heteroallelic mutant combinations that produce reduced eyes, together with molecular data indicating the ability of the *hsp-eya* mini-gene to rescue progenitor cells from death in the third larval instar, have revealed an important function of the *eya* gene in eye progenitor cells prior to furrow formation. Whether the *eya* gene provides other functions in the eye progenitor cells has not been revealed by these mutations.

Analysis of *eya* mutants indicates that loss of gene activity in the eye results in progenitor cell death. The morphology resembles programmed cell death rather than necrosis. Necrotic cells swell, losing membrane integrity (Wyllie et al., 1980; Kerr et al., 1987). Dying cells in *eya* mutant discs, on the other hand, appear to condense, becoming refractile by light microscopy. By TEM, we see condensed bodies containing intact cellular organelles; these fragments are seen engulfed within healthy cells, as is typical of

programmed cell death in other systems where the debris is rapidly removed by phagocytosis (Wyllie et al., 1980; Kerr et al., 1987; Clarke, 1990).

Cell death in the mutant discs overlaps a stage during which some progenitor cell death normally occurs prior to the furrow in eye morphogenesis (see Fig. 4E; Spreij, 1971; Wolff and Ready, 1991). This observation suggests that loss of *eya* activity skews the distribution of cells into a normally occurring cell death pathway. In eye discs of larvae bearing mild to severe alleles, only a fraction of the progenitor cells undergoes cell death; the remaining cells appear to proceed normally to form a furrow with clusters differentiating behind it. The incidence of cell death anterior to the furrow appears to correlate inversely with the final number of ommatidia. Since mutation of the *eya* gene does not appear to affect division of the progenitor cells, the data are consistent with lack of *eya* activity resulting in a switch in cell fate from the pathway of differentiation to that of cell death. Moreover, the increased cell death in the mutant discs appears highly restricted to the region anterior to the furrow: dying cells are not observed within the furrow, and no increase in cell death is seen posterior to it. These observations suggest the existence of a regulated mechanism, acting prior to furrow formation, that allows some cells rather than others to undergo pattern formation events. The data suggest that a selection point may normally occur prior to the furrow when some cells, presumably inappropriate or extraneous, are eliminated; the *eya* gene appears to function critically in this selection process.

This phenomenon may be analogous to other early cell death events, such as occur in neural and immune system development. In insects, some cell death is observed early, during neuroblast delamination in formation of the ventral nerve cord (Doe and Goodman, 1985; Jiménez and Campos-Ortega, 1990). In vertebrates, cell death which is not associated with target innervation is observed in the spinal ganglia (Hamburger and Levi-Montalcini, 1949; Panese, 1976; Hamburger et al., 1981; Carr and Simpson, 1982). Administration *in vivo* of nerve growth factor prevents that cell death (Hamburger et al.,

1981), suggesting that competition for factors may participate even at early developmental stages in the selection of neural cells. Progenitor cells of the oligodendrocyte lineage also appear to require sufficient levels of survival factors during early development (Barres et al., 1992). The types of factors that can influence cell survival can change as the cells mature (Barres et al., 1992), suggesting different signals may be involved in the selection of cells at different developmental stages. In the immune system, the elimination of cells through cell death functions at multiple stages. T-cell maturation appears to involve two types of selection processes. Cells lacking appropriate receptors fail to be positively selected for further differentiation, and are eliminated (Sha et al., 1988; Teh et al., 1988). Cells that do develop receptors, but are self-reactive, are also eliminated (Kappler et al., 1988; MacDonald et al., 1988; Smith et al., 1989). Regulated cell death thus appears to function together with selection to sculpt an appropriate repertoire of cells.

eya mutants present a genetic model in which to study how cell death functions together with differentiation to mediate proper development. The *eya* gene is the first defined component of *Drosophila* eye development that functions prior to the furrow. Two other genes, *string* (Alphey et al., 1992) and *hairy* (Brown et al., 1991), also show expression anterior to the furrow, but their functions in the developmental process are currently unclear. A biological change is known to occur in the eye progenitor cells during the second instar larval stage, when they acquire the competence to differentiate into eye cells if implanted into mature host larvae (Gateff and Schneiderman, 1975). *eya* expression in the cells may reflect a molecular difference related to this biological event. However, competence is acquired at different times for different cell subtypes of the eye, whereas *eya* mutations appear not to discriminate among the cell subtypes (see Fig. 3). To achieve eye differentiation, cell-intrinsic mechanisms, factors from the local environment (Bodenstein, 1953), and previous interactions among the progenitor cells (White, 1961) may all participate. The molecular mechanisms by which these processes function may contribute to cell selection.

Role of the *eya* gene in the progenitor cells.

Features of the *eya* mutant phenotype are consistent with *eya* function being required within the entire population of progenitor cells prior to the furrow. In flies with allelic combinations that make reduced eyes, greater cell death goes *pari passu* with reduction in eye size. Of the cells that survive to form an eye, there appears to be no specific cell type missing with increasing allele severity. These observations suggest a function for the *eya* gene in every progenitor cell, rather than in any particular differentiating cell type. This observation is consistent with the pattern of *eya* protein expression in all the eye progenitor cells anterior to the furrow. Also, genetic mosaic analysis reveals that loss of *eya* activity in progenitor cells anterior to the furrow is cell lethal in a cell-autonomous way (W.M.L., N.M.B. and S.B., in preparation).

The dynamic and specific expression pattern of the *eya* gene during development refutes the idea that the *eya* gene may be required in all cells for maintenance. The pattern is also inconsistent with a role of the gene in repression of all programmed cell deaths of the organism; for example, expression is restricted to specific regions embryonically, arguing against a survival function in every cell. In the eye, the activity of the *eya* gene appears to be specifically required anterior to the furrow to promote the differentiation or survival of eye progenitor cells.

The choice of differentiation or death for an individual cell in an *eya* mutant disc appears to be all-or-none. In eye discs of mutants having allelic combinations that make partial eyes, a cell either dies and is rapidly eliminated anterior to the furrow, or else survives that critical point and continues normally in patterning of the neural structure. These data are consistent with the repression of a suicide program in progenitor cells expressing a sufficient level of *eya* activity. The *eya* protein is strongly expressed in the cells in the region anterior to the furrow, overlapping the region of increased cell death that occurs with reduction of gene function in mutant eye discs. Together, these data suggest

that a critical level of *eya* function may be required within all progenitor cells at a time closely coupled to the cell death event in the mutants. In eye discs of mutants carrying mild allele combinations, a band of cell death precedes the furrow; in severe allele combinations, the region of cell death anterior to the furrow is broad. This may reflect a greater demand on the phagocytosis system to remove dead cell debris, or may indicate a broad window of time within which *eya* function is required prior to furrow formation.

Currently, we have no evidence for a role of the gene in the cell-cell inductive events that occur posterior to the furrow, although the RNA and protein continue to be expressed there. Temperature-shift experiments using conditional alleles, and rescue experiments with the *hsp-eya* construct indicate that *eya* activity appears not to be needed for further developmental events, once the furrow has passed. Ubiquitous expression of the *hsp-eya* mini-gene by heat shock, while sufficient for rescue of *eya* mutants, did not induce any evident phenotypic abnormalities. In the normal fly, there is a later, pupal stage of programmed cell death which removes supernumerary pigment cells (Wolff and Ready, 1991). Expression of the *hsp-eya* mini-gene appears unable to interfere with that process (N.M.B., W.M.L., and S.B., unpublished data). Thus, overexpression of this nuclear gene product by heat shock does not appear able to activate a program of excessive eye differentiation, nor does it prevent late stage cell death in the eye. As one possibility, the *eya* protein may require activation, enabling the progenitor cells to receive or interpret a restricted developmental signal. This characteristic is reminiscent of genes thought to function as receptors for restricted signals, like *sevenless* and *patched*, that similarly do not display dominant effects when ubiquitously expressed from heat shock promoters (Basler and Hafen, 1989; Bowtell et al., 1989; Ingham et al., 1991; Sampedro and Guerrero, 1991).

The sequence of the *eya* gene reveals several potential domains of interest regarding protein function. Multiple charge clusters such as occur in the *eya* protein are uncommon, and are generally associated with regulatory proteins, including proto-oncogenes,

transcription factors, and various types of receptors (Brendel and Karlin, 1989; Karlin and Brendel, 1990). The hydrophobic moment plot of the predicted a-helical regions is consistent with potential involvement in protein-protein interactions (Eisenberg et al., 1984). The two identified *eya* transcripts may represent proteins of partially different function, given that the extreme amino-terminal sequences differ. These transcripts may also reflect tissue-specific regulation of the gene. Identification of the molecular defects of mutant alleles, correlated with their phenotypes, may define such regulatory regions and identify specific functional domains of the protein.

Models for the function of the *eya* gene.

The function of the *eya* gene in eye morphogenesis impinges on initiation of the cell death pathway. The *eya* gene appears different from the *ced* cell death genes described in C. elegans (Ellis and Horvitz, 1986; Hengartner et al., 1992). The expression pattern suggests that *eya* does not function in all cell deaths of the organism, and we have no current evidence that gene expression can dominantly block cell death at stages in development other than in eye progenitor cells prior to furrow formation. Thus, the *eya* gene displays a specificity of action suggesting that its activity in the eye impinges on control of activation of genes of the cell death pathway, rather than being part of that pathway itself. The *eya* gene appears to function in the coupling of a differentiation event to initiation of a cell death pathway during development.

We present three possible models for the function of the gene (Fig. 10). These models incorporate the emerging concept that programmed cell death is a default fate that will occur unless actively inhibited (Barres et al., 1992; Raff, 1992). Differentiation and cell death are represented as separate pathways within the cell. Although these events are typically associated, studies with recessive mutants in C. elegans indicate that the two pathways can be uncoupled genetically. In mutants that lack the normal programmed cell death scenario, the cells that survive can differentiate and function (Ellis and Horvitz, 1986;

Hengartner et al., 1992). Thus, blocking cell death enables the surviving cells to respond to signals for differentiation. The *eya* gene might function to repress cell death, it might be involved in a differentiation event that is linked to cell death, or it may function as a molecular switch that couples the two pathways.

The first model proposes that *eya* activity is required at a critical stage anterior to the furrow to repress cell death (Fig. 10A). An insufficient level of *eya* activity would therefore subvert progenitor cell differentiation by allowing the default fate, cell suicide. Progenitor cells for different cell and tissue types presumably have mechanisms to regulate genes of the cell death pathway in precise ways during critical selection periods, as part of their specific developmental processes. Although the *ced* genes of C. elegans affect cell death in many lineages (Ellis and Horvitz, 1986; Hengartner et al., 1992), mutations have been identified in other genes that appear involved in the programmed deaths of individual cells (Trent et al., 1983; Ellis and Horvitz, 1991). In *Drosophila*, which consists of an enormously larger number of cells, the eye progenitor cells as a group might be thought of as analogous to a small cell group in the nematode lineage. The time anterior to the furrow when the *eya* gene functions defines a critical survival stage for progenitor cells in eye morphogenesis; the *eya* gene may be required to prevent activation of the cell death pathway at this specific stage in early eye development.

A second model is that the *eya* gene is required to promote a differentiation step; that process itself would generate a signal that represses activation of the alternative fate of cell death (Fig. 10B). With loss of *eya* activity, the differentiation process would not be promoted, and the default pathway of cell death would become activated. Such a model links differentiation to cell death to assure that initiation of the death pathway is tightly controlled. Cells that acquire the necessary differentiation signals continue in development; those that fail are eliminated.

A third model postulates that the *eya* gene has a dual function, at once promoting differentiation and repressing cell death (Fig. 10C). In other systems, it is possible to direct

cells down such alternative pathways by manipulating components within and outside the cell. In the immune system, certain extracellular signals are hypothesized to promote either proliferation or death, depending upon the combination of signals presented to the cell (McConkey et al., 1990). The products of the proto-oncogene *c-myc* (Askew et al., 1991; Evan et al., 1992; Shi et al., 1992) and the tumor suppressor gene *p53* (Yonish-Rouach et al., 1991), which are generally thought to function in proliferation or differentiation, can induce cell death, depending upon the state of the cell and available signals. The *eya* gene product may, when active, promote differentiation in response to certain signals; when inactive, the same combination of signals instead activates a cell death program. The *eya* gene product thus might function pivotally in the coupling between differentiation and the repression of cell death.

The *eya* gene is a molecular handle into the events that occur and signals that function prior to furrow formation. Study of the *eya* gene, and the genetic pathway in which it functions, should provide new insights into the mechanisms by which differentiation events and cell death function to achieve proper development. The study of genes that function in the fly eye has provided insight into the developmental roles of many proteins homologous to human genes (Greenwald and Rubin, 1992). Anopthalmia occurs in many organisms, including humans (Apple and Rabb, 1991). In a striking example, congenital aniridia in man resembles the *Small eye* mutation of mouse (Glaser et al., 1990; van der Meer-de Jong et al., 1990), and both result from mutations of genes that are homologous (Hill et al., 1991; Ton et al., 1991; Jordan et al., 1992). With the *eya* gene in hand we are able to address questions regarding its function in regulating cell differentiation and cell survival during development. Such studies, as well as the isolation of vertebrate homologs, may ultimately lead to a greater understanding of the differentiated state, and potentially to the design of effective therapies for syndromes involving abnormal regulation of cellular death pathways.

EXPERIMENTAL PROCEDURES

Fly Strains

Flies were cultured on standard cornmeal medium at 25°C. Mutant strains are listed in Lindsley and Zimm (1992). The wild-type strain normally used was Canton-S (C-S). Additional wild-type strains used to examine cell death were Urbana-S, Lausanne-S, Oregon-R, and Oregon-R-C, kindly provided by E.B. Lewis (California Institute of Technology).

Alleles of the *eya* gene

Four *eya* alleles were generated in other laboratories, and kindly shared with us. These are eya^{1} (Sved, 1986), eya^{2} (also eya^{ph} or $eya^{pinhead}$; T. McQuirre, Rutgers Univ.), eya^{3} (D. Mohler, Univ. of Iowa, Iowa City) and eya^{4} (*ey*-2 of Eisenberg and Ryerse, 1991). Once we had mapped the *eya* gene cytologically, we tested other mutations in the region for complementation. This analysis revealed that T(2;3)DTD46 has a breakpoint in the *eya* gene (Gelbart, 1982), and that the mutation *clift* is allelic (Nüsslein-Volhard et al., 1984). We generated 35 new alleles using ethyl methanesulfonate (EMS), X-rays, and P-element hybrid dysgenesis as mutagens. All were isolated by failure to complement the eye phenotype of the eya^{1} mutation. Putative alleles were recovered over a second-chromosome balancer chromosome.

For the EMS mutagenesis, 2051 spd^{fg} male flies were mutagenized with EMS (Lewis and Bacher, 1968), mated to eya^{1} virgin females, and progeny raised at 29°C. Of 90,000 flies screened, twelve independent alleles, eya^{E1} to eya^{E12}, were isolated, one of which displayed temperature sensitivity. For the X-ray screen, about 1000 spd^{fg} males were subjected to 4500 rads, then mated to eya^{1} virgin females. Of 57,000 total progeny screened, 16 alleles were isolated. Eight of these have cytologically visible rearrangements: eya^{X1} with In(2L)26E;36-37; eya^{X3} with T(2;3)26E;86C; eya^{X8} with In(2L)26E;39;

eya^{X10} with T(2;3)26E;67A; *eya*^{X15} with T(2;3)26E;70A; *eya*^{X16} with T(2;3)26E;3L;heterochromatin; and *eya*^{X9} and *eya*^{X11} with complex breakpoints that were not determined.

Seven alleles (*eya*^{P1} to *eya*^{P7}) were isolated in two independent screens using P element hybrid dysgenesis, after Robertson et al. (1988). In the first screen, a *Sp* chromosome was used as the parental chromosome; in the second screen, the alleles were generated on an isogenic wild-type second chromosome, or on the Birmingham second chromosome. In both screens, progeny from the Birmingham 2 by P[*ry*$^{+}$ Δ2-3](99B) (Laski et al., 1986) cross were mated to virgin *eya*^{1} females, and screened for failure of complementation. In screen one, one allele (*eya*^{P1}) was isolated of 200,000 F2 flies scored. In the second screen, 70,000 F2 flies were scored, and six additional alleles recovered. None of these alleles was associated with a P element at the chromosomal location of *eya*, analyzed by chromosomal *in situ* hybridization.

The eye disc phenotypes of *eya* alleles were studied in *trans* to the *eya*^{1} chromosome marked with *Cy*. *Cy* was recombined onto the *eya*^{1} chromosome by R. Hackett from the *CyO* second chromosome balancer. This was possible since the *eya*^{1} mutation arose on an inversion (Sved, 1986) with breakpoints on 2L similar to those of the balancer. We refer to this chromosome as *Cy,eya*^{1}.

Isolation of genomic and cDNA clones

Standard molecular techniques were from Sambrook et al. (1989). Flies mutant for various *eya* alleles were mated to C-S, and polytene chromosomes of the larval salivary glands were dissected, stained with orcein, and analyzed for visible cytological rearrangements. A DNA probe for the 26E cytological region was obtained from *Drosophila* YAC DY817 which spans into the region (kindly provided by I. Duncan, Washington Univ. at St. Louis; Garza et al., 1989). DNA was isolated from a 5 ml culture of DY817. This was digested with Eco RV and Hinc II, ligated, and amplified with primers

to the YAC vector, to generate by inverse PCR *Drosophila* DNA probes for the ends of the YAC, as described by Ochman et al. (1990). Primers used were 5'-GCGATGCTGTCGGAATGGAC-3' and 5'-GTTGGTTTAAGGCGCAAGACT-3' for the Eco RV side, and 5'-CGAGTCGAACGCCCGATCTC-3' and 5'-AGGAGTCGCATAA GGGAGAG-3' for the Hinc II side. The PCR reactions were run on a 1.5% low melt agarose gel, and the major bands isolated. These were labeled by nick translation with bio-16-dUTP (ENZO Biochemicals), and used to probe C-S larval salivary gland chromosome squashes to determine the cytological site of hybridization. The signal was detected by the streptavidin-peroxidase complex (Detek-1-HRP kit of ENZO Biochemicals). A 740 bp probe that hybridized in cytological region 26E was labeled by random primer reaction and used to screen a cosmid library from an isogenic strain (kindly provided by J. Tamkun, Univ. California, Santa Cruz), and a *Drosophila* genomic phage library (Stratagene). Overlapping clones of the walk were oriented by chromosomal *in situ* analysis. The location of the breakpoint fragments in the alleles were determined by DNA Southern analysis and chromosomal *in situ* analysis. cDNAs were identified and isolated from *Drosophila* head cDNA libraries (Itoh et al., 1986; Zinsmaier et al., 1990). cDNAs and genomic fragments in the region were subcloned into the pBluescript vector (Stratagene), and transformed or electroporated into XL-1 blue cells (Stratagene). Sequencing of cDNA and genomic clones was performed on CsCl-banded DNA preparations or mini-preparations of plasmid DNA, using 5-deaza-dGTP (USBiochemicals) and making sequential primers. Sequence comparison to the EMBL and Genbank databases was performed according to Pearson and Lipman (1988) and Altschul et al. (1990). Sequence analysis was performed using the GCG Sequence Analysis Software Package (Devereux et al., 1984), the SAPS (statistical analysis of protein sequences) program (Brendel et al., 1992), the PEST-FIND program (Rogers et al., 1986; Rechsteiner, 1988), and the algorithm of Eisenberg et al. (1984) for potential a-helical transmembrane regions.

RNA northern analysis and tissue *in situ* expression

Nucleic acid was extracted using a modification of the procedure of Sargent et al. (1986). Adult flies and embryos were collected and stored frozen at -70°C. Eye-antennal disc complexes, with mouthhooks attached, were dissected and immediately frozen on dry ice. Eye disc poly(A)$^+$ RNA was prepared using the Micro-FastTrack System (Invitrogen Corp.). For adult tissue, body parts ground to powder in liquid nitrogen were mixed with 4.2M guanidine isothiocyanate, 0.5% sarkosyl, 25 mM Tris, pH 8.0, 0.7% 2-mercaptoethanol, at a ratio of 1 gm tissue per 30 ml. Samples were Dounce-homogenized. Tubes were prepared with one volume phenol extraction buffer (100 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS) layered over two volumes of phenol-chloroform. One volume of tissue suspension was added, mixed and centrifuged. The suspension was extracted twice more with an equal volume of phenol-chloroform, then once with chloroform. The nucleic acid was precipitated with an equal volume of isopropanol.

Poly(A)$^+$ RNA was isolated over oligo dT columns by the Fast Track mRNA isolation protocol (Invitrogen Corporation). The RNA was then separated on 1% agarose-formaldehyde gels, and blotted onto reinforced nitrocellulose paper (Schleicher and Schuell). Probes were made by random primer labeling or by making single stranded RNA probes using the T7 and T3 promoters of the pBluescript vector. The direction of transcription determined by single strand probes agreed with the structure of the cDNAs by sequence analysis.

To examine the tissue expression of the cDNAs in eye discs, a modification of the protocol of Tautz and Pfeifle (1989) was used. Probes were made by random prime labeling, using digoxigenin-11-dUTP (Boehringer Mannheim). After hybridization and detection of the signal (Boehringer Mannheim Genius Kit), discs were mounted in Aquamount (Lerner Laboratories). Some discs were post-fixed 30 min in 1% glutaraldehyde, 1% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, dehydrated and

embedded in Epon. Serial sections of 0.8 μm were cut, lightly stained with toluidine blue and mounted under Permount for photography.

Transformation rescue

Type I cDNA was subcloned into the Kpn I site of the vector pHT4 (Schneuwly et al., 1987), to make an *hsp-eya* mini-gene for transformation. The cDNA was first subcloned into the Eco RI site of a modified pBluescript vector with a Kpn I linker added to the Sma I site (Van Vactor et al., 1991). Partial digests were used, since the cDNAs have an internal Kpn I site. The pHT4 vector contains the *Drosophila hsp70* promoter, a polyadenylation site from SV40, and ry^+ gene as an eye color marker. The plasmid for transformation was CsCl-banded, then mixed at a 5:1 ratio with a transposase source, phsπ (Steller and Pirrotta, 1985). This was injected into ry^{506} eggs (Rubin and Spradling, 1982). Lines were established from adults harboring an *hsp-eya* mini-gene insert, as assessed by ry^+ eye color, then crossed into *eya* mutant backgrounds. Two independent transformant lines, A23.4 and A67.1, were used for rescue of the eye phenotype. To express the cDNA during development, eggs were laid in vials, then after 24 hours, heat pulsed in a 37°C water bath 1 hr every 6-8 hr during larval development, which was slowed to a period of about 7 days. For staged larvae, eggs were collected over 3 hr intervals, and larvae were collected within 5 hr of the 2nd or 3rd instar larval molts. Larvae were transferred to 0.5 ml tubes with cornmeal medium, and were heat pulsed in a PCR machine for 30 min every 6 hr for the desired number of pulses. For heat shocks during the pupal stage, white pre-pupae were collected every 1-2 hr over a 9 hr period from ry^{506}, and transformant lines A67.1 and B58.1 in a ry^{506} background. Pupae were aged 15-24 hr, heat shocked 30 min every 6 hr for 48 hr. Although this treatment caused much death of both control and experimental animals, eyes could be scored on emerged flies, and with dissection on dead pupae. The period of heat shock covered the phase of pigment cell death, which normally occurs 35-50 hr after pupation (Wolff and Ready, 1991).

Antisera to the *eya* protein

A 2.3 kb Sma I fragment of type I cDNA was subcloned into the Sma I site of expression vector pGEX-2 (Smith and Johnson, 1988). Sequence analysis confirmed that the insert was in frame. The fusion protein was of predicted size of 86 kD, of which 60 kD corresponded to the carboxy-terminal 551 amino acids of the cDNA and 26 kD to glutathione *S*-transferase. The protein sequence produced is common to both type I and II cDNAs. A 500 ml culture was grown 1.5 hr at a 1:10 dilution in LB plus ampicillin, then the fusion protein was induced by addition of 1 mM IPTG. The cells were collected by centrifugation at 4,000xg for 5 min, then resuspended in 7.5 ml of 50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1 mM PMSF. Lysozyme was added to 0.3 mg/ml, and the sample incubated at room temperature for 20 min. DNAase I was then added to 3 mg/ml for another 30 min. The sample was spun at 5,000xg for 10 min, and resuspended in 9 ml of 50 mM Tris, pH 8.0, 10 mM EDTA, 100 mM NaCl, 1 mM PMSF, 1% Triton X-100, and allowed to sit 10 min on ice. This was spun at 10,000xg for 15 min, resuspended in 4 ml Laemmli sample buffer and boiled. One ml of the sample was run on a preparative 0.1%SDS-7.5% polyacrylamide gel, stained 10 min in 0.05% Coomassie-blue in water, destained, and the region of the gel with the fusion protein sliced out. Mice were immunized 10 times with 50 µg of fusion protein per injection, and tail sera collected. The antigen recognized by the polyclonal antiserum is expressed ectopically in heat-shocked transformant larvae carrying the *hsp-eya* mini-gene construct.

Histology and immunocytochemistry

Scanning electron microscopy was performed on unfixed flies, or on flies stored in 70% ethanol, dehydrated to 100% ethanol and critical point dried. In both cases, flies were coated with gold-palladium 80:20. For tangential eye sections, fly heads were fixed in 1% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, dehydrated and

embedded in Epon (Polysciences, Inc.). Sections of 0.7 μm thickness were cut, lightly stained with 1% toluidine blue, 1% borax solution in water, and mounted in Permount (Fisher Scientific). To examine eye discs by transmission electron microscopy (TEM), discs were dissected in 0.1 M phosphate buffer, pH 7.4, fixed for 1 hr in 1% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Tissue was stained 1 hr in 1% osmium, 0.5% uranyl acetate, dehydrated through ethanol, embedded in Epon, and serial 0.08 μm sections cut.

Silver staining of adult heads was performed according to the procedure of Meyerowitz and Kankel (1978), as modified by Harte and Kankel (1982) and by K. Stark (Yale University), who kindly shared many helpful suggestions.

Labeling and detection of cells in S phase in wild type and *eya*^{1} mutant eye discs was performed as described by Truman and Bate (1988), with the following modifications. Staged larvae were labeled *in vivo* for 2 hr with BrdU at 1 mg/ml. Primary anti-BrdU antibody (Becton Dickinson) incubation was overnight at 4°C with slow shaking, and the secondary antibody was fluorescein-conjugated (Cappel).

Eye discs were stained with acridine orange after the protocol of Spreij (1971). After dissection in Ringer's solution, discs were incubated 5 min in 1.6×10^{-6} M acridine orange in Ringer's solution, rinsed, and viewed with fluorescence optics.

Eye discs were dissected, fixed and stained with MAb22C10 overnight, as described by Van Vactor et al. (1991), with some modifications. The horseradish peroxidase-conjugated secondary antibody (BioRad Laboratories) was used at a 1:50 dilution, and substrate was detected with 0.5 mg/ml diaminobenzidine plus 2 mg/ml NiCl. For visualization of the *eya* protein with the polyclonal antisera on whole-mount preparations of eye discs, a modification of the protocol of Renfranz and Benzer (1989) was used. Eye discs were dissected in TBS (100 mM Tris, pH 7.5, 130 mM NaCl, 5 mM KCl, 5 mM NaN_3, 1 mM EGTA), fixed 30 min in 2% paraformaldehyde in TBS, then permeabilized 30 min in 0.5% NP-40 in TBS. After rinsing in TBS, discs were incubated

30-60 min in TBS plus 5% normal goat serum (NGS; Vector Laboratories). Primary antibody staining was at 1:500-1:1000 in TBS plus 5% NGS for 60 min, discs were rinsed 30 min in TBS plus 5% NGS, then incubated in fluorescence-conjugated secondary antibody at 1:500 (Cappel) diluted in TBS plus 5% NGS. After washing 30 min in TBS plus 5% NGS, discs were mounted in 90% glycerol plus 0.1% phenylenediamine, and viewed by fluorescence microscopy. Localization by light microscopy of the *eya* protein with the mouse polyclonal antiserum was by the protocol above for MAb22C10, with some modifications. The antiserum was used at a 1:500 dilution. Following substrate detection, discs were post-fixed 20 min in 1% paraformaldehyde plus 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and embedded in Epon. Longitudinal sections of 0.7 μm thickness were cut, and tissue was lightly stained with 1% toluidine blue, 1% borax solution in water, prior to mounting in Permount.

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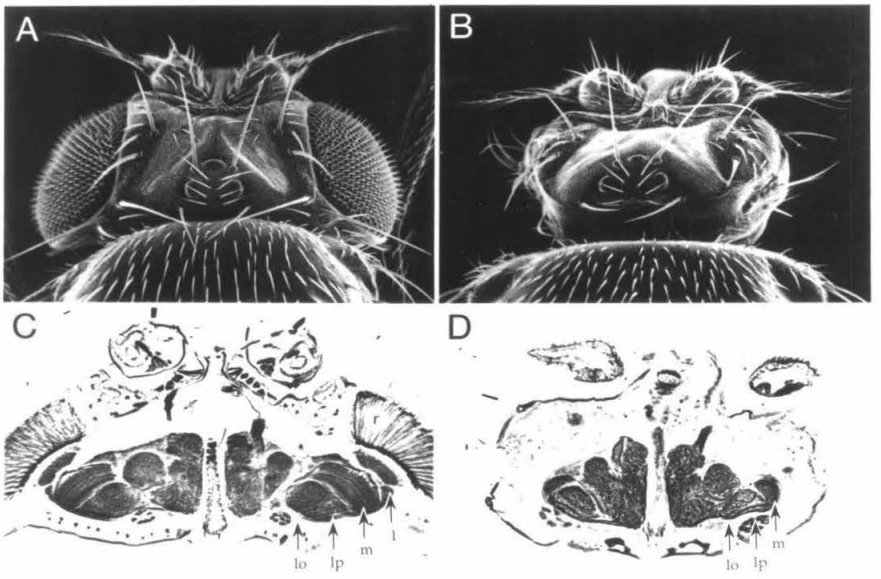
Fig. 1. Adult phenotype of *eya*^{1} flies.

(A,B) Scanning electron micrographs of normal (A) and *eya*^{1} mutant (B) heads. The compound eyes are missing entirely in *eya*^{1} flies. The three dorsal ocelli and other external head structures are present.

(C,D) Silver stained horizontal sections of a normal fly brain (C) and *eya*^{1} mutant fly brain (D). In the mutant, the retina (r) and lamina (l) are completely missing, the medulla (m) appears reduced in size, and the lobula (lo) and lobula plate (lp) are less organized than normal.


A
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C
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m

Fig. 2. The phenotype of *eya*^{1} in the developing eye disc.

Eye-antennal discs from wild type larvae (left) and *eya*^{1} mutant larvae (right).

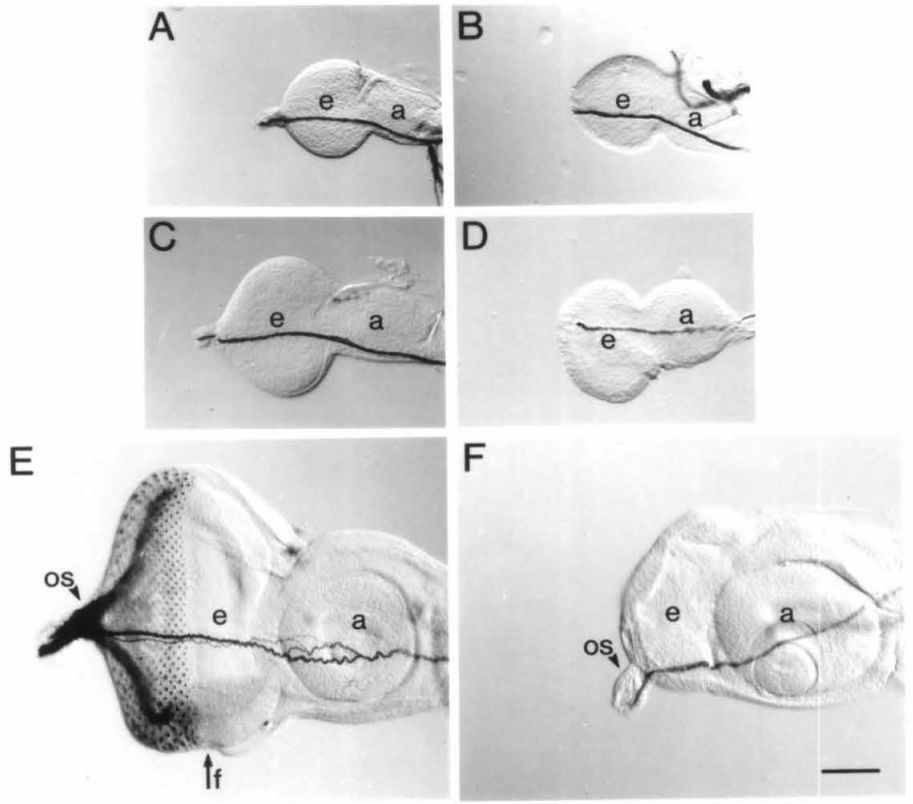
(A,B) Eye-antennal discs from early third instar larvae, stained with neuron-specific monoclonal antibody MAb22C10. The furrow is not yet present; no clusters have formed. The eye portion (e) of the *eya*^{1} disc appears normal in size at this stage. Bolwig's nerve, which stains with MAb22C10, traverses the discs.

(C,D) Slightly older discs from early third instar larval stage. The eye portion (e) of the *eya*^{1} disc is still expanding.

(E) Wild type eye-antennal disc from a late (crawling) third instar larva stained with MAb22C10. The morphogenetic furrow (f) is halfway across the eye portion (e) of the disc. The developing neuronal clusters posterior to the furrow are highlighted by the antibody staining.

(F) *eya*^{1} mutant eye-antennal disc from a late (crawling) third instar larva stained with MAb22C10. The antennal portion (a) of the disc has expanded normally, but the eye portion (e) of the disc is much smaller than normal. No furrow is formed; neural clusters fail to develop, illustrated by the lack of staining with MAb22C10. Bolwig's nerve is present.

Bar = 50 μm. e = eye portion of the disc; a = antennal portion of the disc. os = optic stalk. Anterior to the right.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a
os

Fig. 3. Compound eye phenotypes of flies expressing various alleles of *eya*.

(A-D) Scanning electron micrographs. (E-H) Tangential sections. Anterior to the right.

(A,E) Wild-type. Note the regular ommatidial pattern (white arrow in E). The rhabdomeres of seven photoreceptors cells are visible: six outer, and the inner seventh. The rhabdomere of the eighth photoreceptor is beneath that of the seventh. The photoreceptor cells are surrounded by a pigmented lattice.

(B,F) Mild allele eya^{E3} heterozygous with eya^{1}. The eye is slightly reduced and rough. Photoreceptor cells are occasionally missing (black arrow in F) and the pigmented lattice is less regular than in wild type. Many ommatidia appear normal (white arrow).

(C,G) Intermediate allele eya^{E4} heterozygous with eya^{1}. The eye is reduced and rough. Many ommatidia contain the full complement of photoreceptor cells (white arrow in (G)), although cells are sometimes missing (black arrow) and the pigmented lattice is less organized than wild type.

(D,H) Severe allele eya^{E1} heterozygous with eya^{1}. In flies expressing this allelic combination, the eye is greatly reduced and rough (D), or missing altogether. Although the ommatidial pattern of such severely reduced eyes shows disorganization (H), many ommatidia contain all the photoreceptor cell types (white arrows) and the pigmented lattice is present.

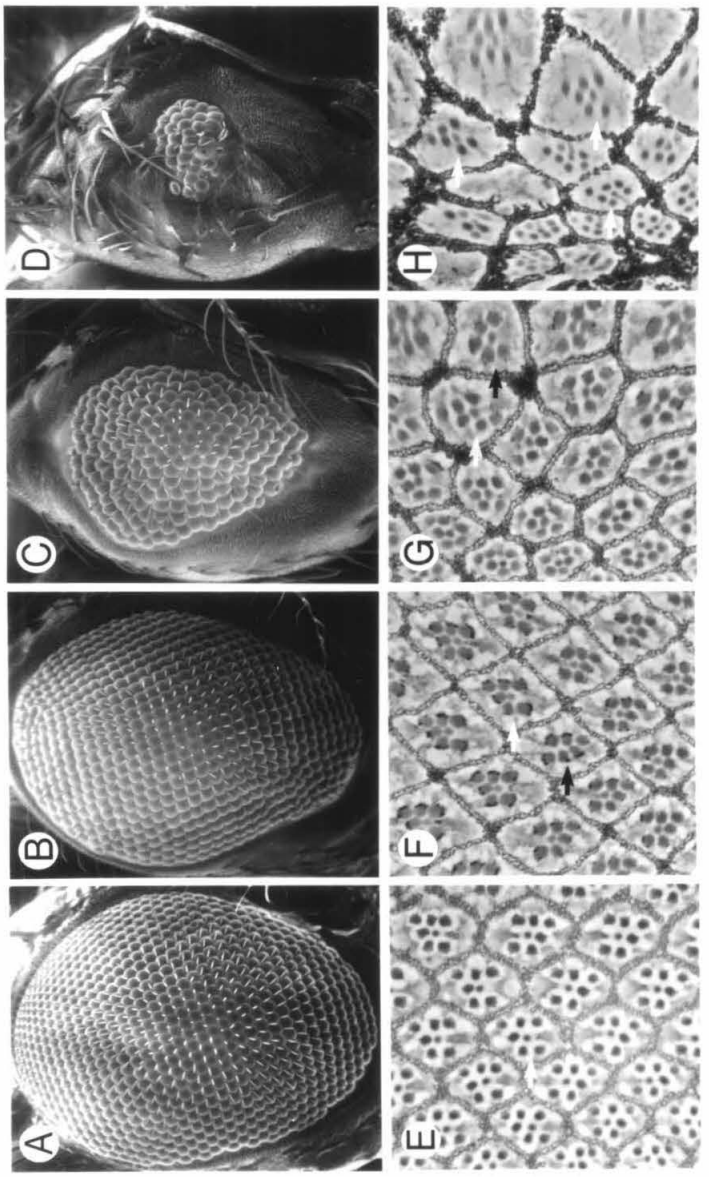
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Fig. 4. Eye disc phenotypes of larvae expressing various alleles of *eya*.

Eye portions of eye-antennal discs from crawling third instar larvae. (A-D) MAb22C10 staining, which highlights neural clusters. (E-H) Acridine orange staining, which highlights dead cells. Arrows mark the morphogenetic furrow.

(A,E) Wild-type. (A) Posterior to the furrow (arrow) clusters are developing. (E) Dead cells appear as bright dots of fluorescence. A small amount of cell death occurs in a band just anterior to the furrow.

(B,F) Mild allele eya^{E3} heterozygous with eya^{1}. (B) Many neural clusters form, consistent with the only slightly reduced eye in the adult (see Fig. 3B). (F) Anterior to the furrow (arrow), an increase in the amount of cell death occurs, highlighted by acridine orange staining. The increased cell death occurs in the same region of the disc just anterior to the furrow where normally some cell death occurs (compare Fig. 4E).

(C,G) Intermediate allele eya^{E4} heterozygous with eya^{1}. (C) Fewer clusters form. Anterior to the furrow, condensed and refractile dying cells are visible. (G) Acridine orange highlights the increased degeneration that occurs in a broad band anterior to the furrow (arrows).

(D,H) Severe allele eya^{E1} heterozygous with eya^{1}. (D) Many fewer clusters form in the eye disc. Anterior to the furrow (arrow), condensed dead cells are visible. Inset: Higher magnification view of condensed and refractile bodies anterior to the furrow. Arrows highlight a few examples. (H) Acridine orange staining highlights the great increase in cell death that occurs anterior to the furrow in the disc.

Bar in (D) = 50 μm for (A-D). Bars in (E-H) are 50 μm. Anterior to the right.

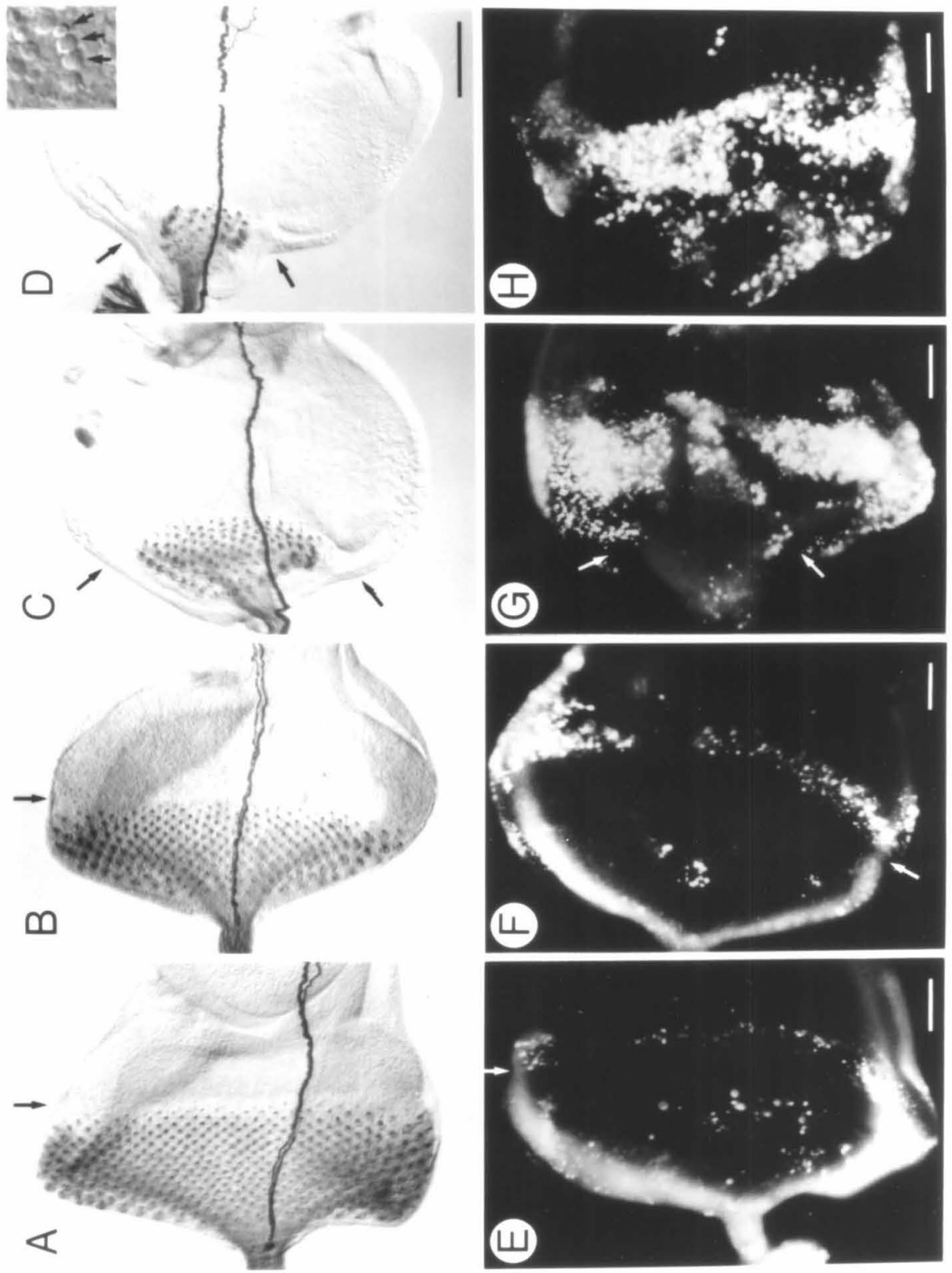
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Fig. 5. Cell death in an eye disc of an *eya* mutant that forms a reduced eye.

(A) Eye portion of an eye-antennal disc from a third instar larva expressing an intermediate allele eya^{E9} heterozygous with eya^{1}. Anterior to the furrow (arrow), cell death is seen in the basal region of the disc (boxed area). Dead cells fragment into electron dense bodies; these appear to become engulfed by surrounding cells. Posterior to the furrow where the clusters are differentiating, no cell death is seen.

(B) Higher magnification view from the region boxed in (A). Within dead cell fragments, intact cellular organelles may be seen (arrow points toward what appears to be a nucleus). Also visible are examples of fragments of dead cells within other cells (arrowhead highlights one example).

Anterior to the right. Bar in (A) = 10 μm, bar in (B) = 2 μm. pm = peripodial membrane.

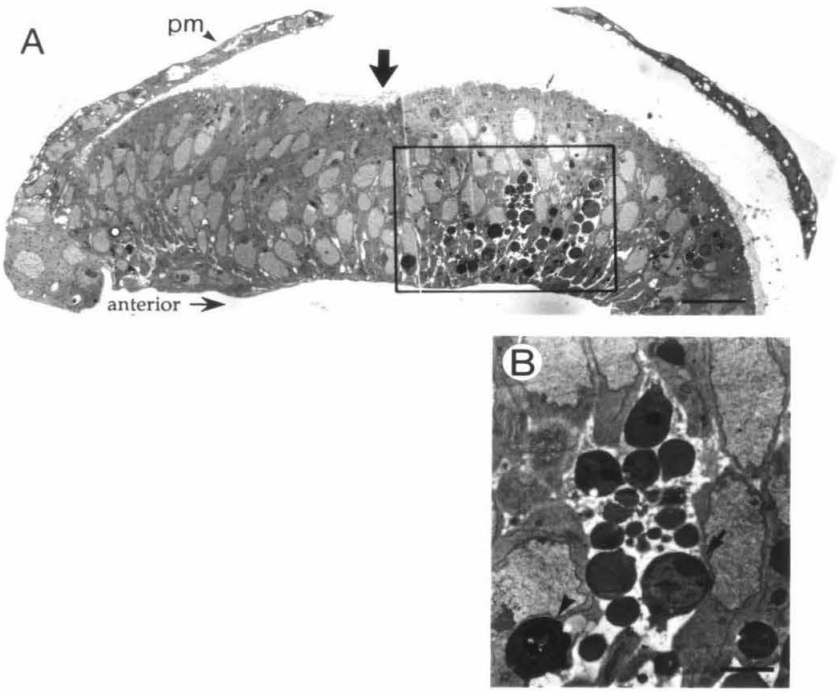
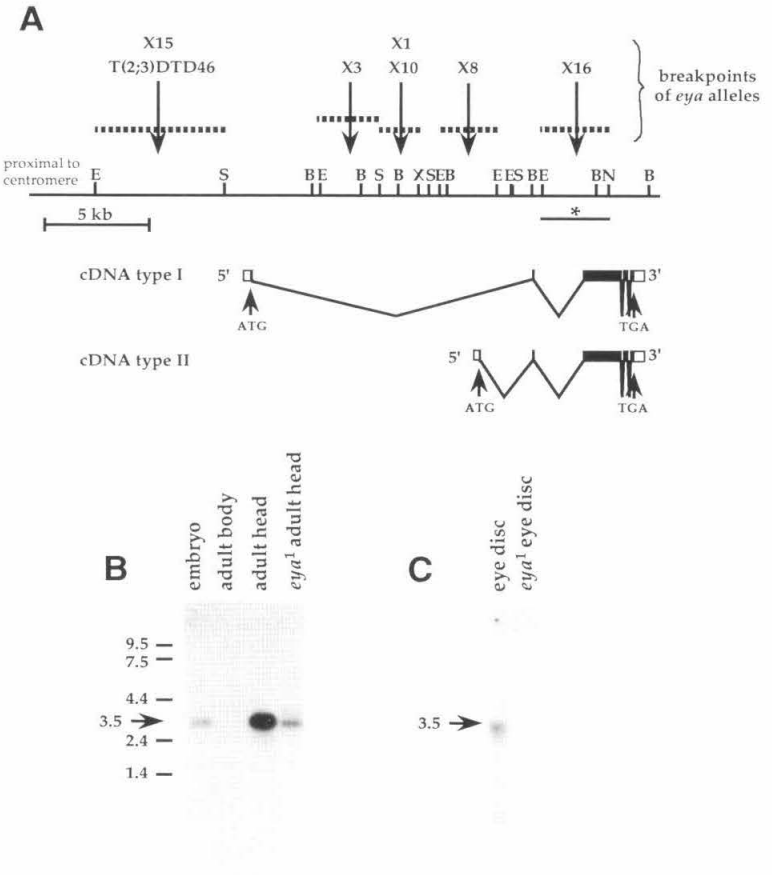

A
pm
anterior
B

Fig. 6. Molecular analysis of the *eya* chromosomal region.

(A) Molecular organization of the *eya* region. The restriction map from overlapping cosmid and phage clones shows Not I (N), Bam HI (B), Eco RI (E), Sal I (S), and Xba I (X) sites. This region resides in cytological region 26E on chromosome 2. Above the restriction map are indicated the DNA restriction fragments within which fall the breakpoints of six X-ray induced alleles. Also shown is the location of the breakpoint of an X-ray allele of *dpp*, T(2;3)DTD46, that has one breakpoint in 26E (Gelbart, 1982) and that fails to complement *eya* mutations. Illustrated below the restriction map are the intron/exon structures of prototypes of the two classes of cDNA that span through the region. The start (ATG) and stop (TGA) codons correspond to those of the longest potential ORFs. The initiation sites of transcription have not been determined.

(B) Northern blot analysis. Northern blot of poly(A)$^+$ RNA isolated from wild-type embryos (0-24 hr), adult bodies and heads, and from adult heads of the *eya*^{1} mutant. Each lane has 10 μg of RNA. The blot was probed with the Not I/Eco RI restriction fragment indicated by (∗) on the restriction map in (A). A transcription unit of 3.5 kb is detectable in embryos and adult heads; it is reduced in intensity in heads of the *eya*^{1} mutant.

(C) Northern blot of eye disc poly(A)$^+$ RNA. Northern blot of poly(A)$^+$ RNA isolated from 200 mid-third instar larval eye-antennal discs from wild type or from the *eya*^{1} mutant, probed with the entire type I cDNA. A transcript of 3.5 kb is detectable in wild type, which is missing in the *eya*^{1} mutant.


A
X15
T(2;3)DTD46
X1
X3
X10
X8
X16
breakpoints
of eya alleles
proximal to
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E
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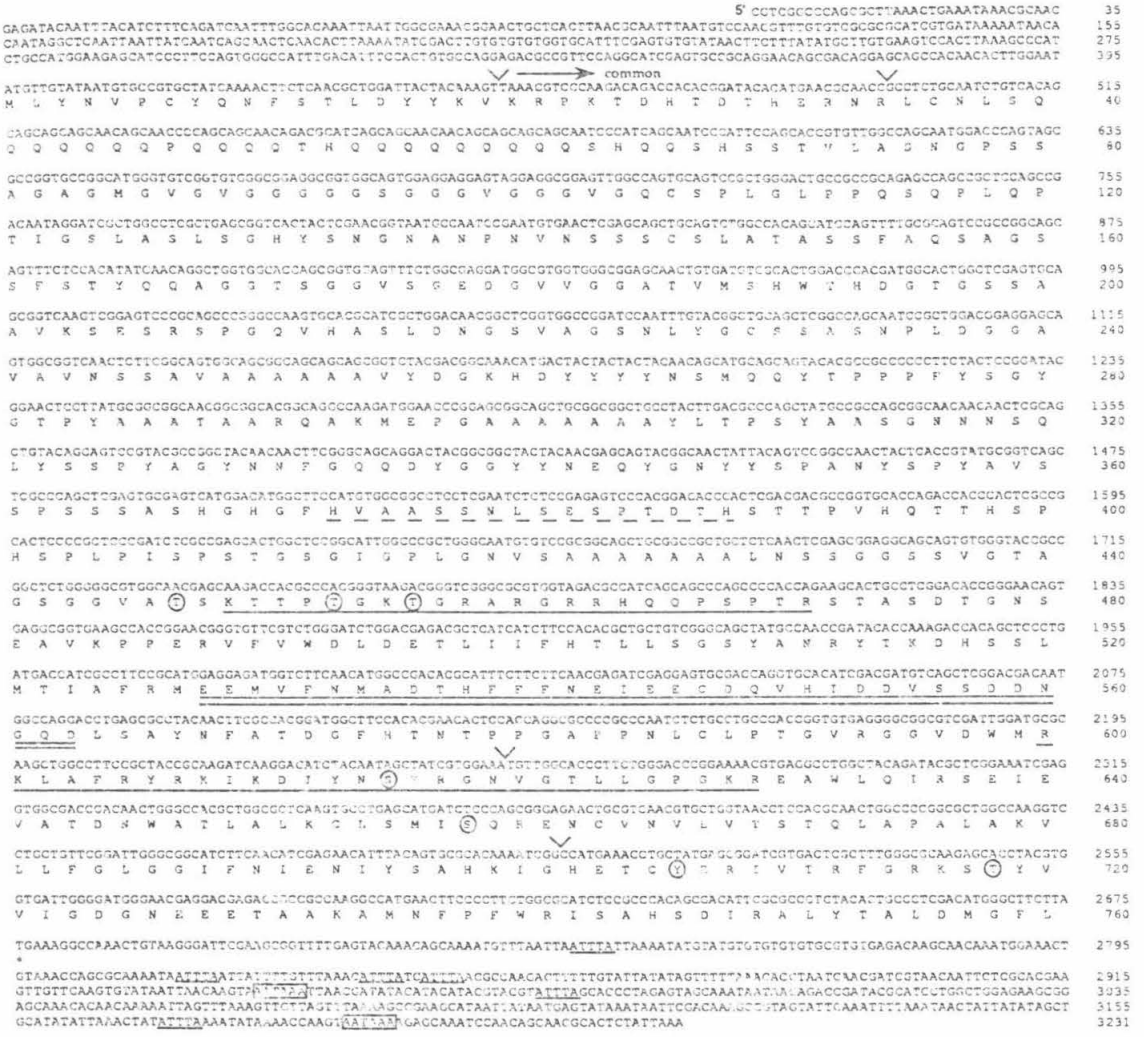
B S B X SEB
EES BE
BN
B
5 kb
*
cDNA type I
5'
3'
ATG
TGA
cDNA type II
5'
3'
ATG
TGA
B
embryo
adult body
adult head
eya1 adult head
9.5
7.5
4.4
3.5
2.4
1.4
C
eye disc
eya1 eye disc
3.5

Fig. 7. Sequence of the *eya* cDNAs.

The nucleotide sequences represent a consensus of genomic and cDNA sequences. The deduced amino acid sequences correspond to those of the longest potential ORFs. For the type I cDNA, the sequence at the proposed start site has a 2/4 bp match with the *Drosophila* translation initiation consensus (Cavener, 1987). For the type II cDNA, the match is 3/4 bp. The first 19 amino acids of the type I cDNA and the first 25 amino acids of the type II cDNA are generated by alternative splicing. Within the amino acid sequence common to both cDNA classes, three charged clusters (Brendel et al., 1992) are arranged as a basic stretch (solid underline), an acidic stretch (double underline), then a second basic stretch (solid underline). Five regions (amino acids 83-103, 236-256, 409-429, 489-509, and 671-691) are predicted to be hydrophobic a-helical regions by the algorithm of Eisenberg et al. (1984). The *opa* repeat spans amino acids 40-62. A possible PEST protein degradation sequence (Rogers et al., 1986; Rechsteiner, 1990) is underlined with a dashed line, and potential cyclic nucleotide-dependent, protein kinase C and tyrosine kinase phosphorylation sites are circled. Amino acids 18-23 are a candidate for a nuclear localization signal (Chelsky et al., 1989). It is interesting to note that these amino acids span the beginning of the common sequence region. The 3' untranslated region is approximately 500 nucleotides, and has two AATAAA sites (boxed) that could serve as polyadenylation signal sequences. Within the 3' tail region are six ATTTA repeats (underlined), which are found in the 3' untranslated regions of dynamically expressed genes and are implicated in rapid message turnover (Shaw and Kamen, 1986). The intron sites (carats) were determined by comparing the genomic sequence to that of the cDNAs.

type I cDNA



type II cDNA

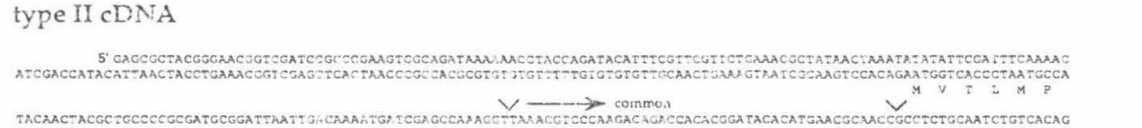


Fig. 8. Protein and transcript expression of the *eya* gene in eye discs.

(A-E) Eye discs stained with a mouse polyclonal antiserum directed against the *eya* protein.

(F,G) Transcript expression using digoxigenin-labeling of the type I cDNA.

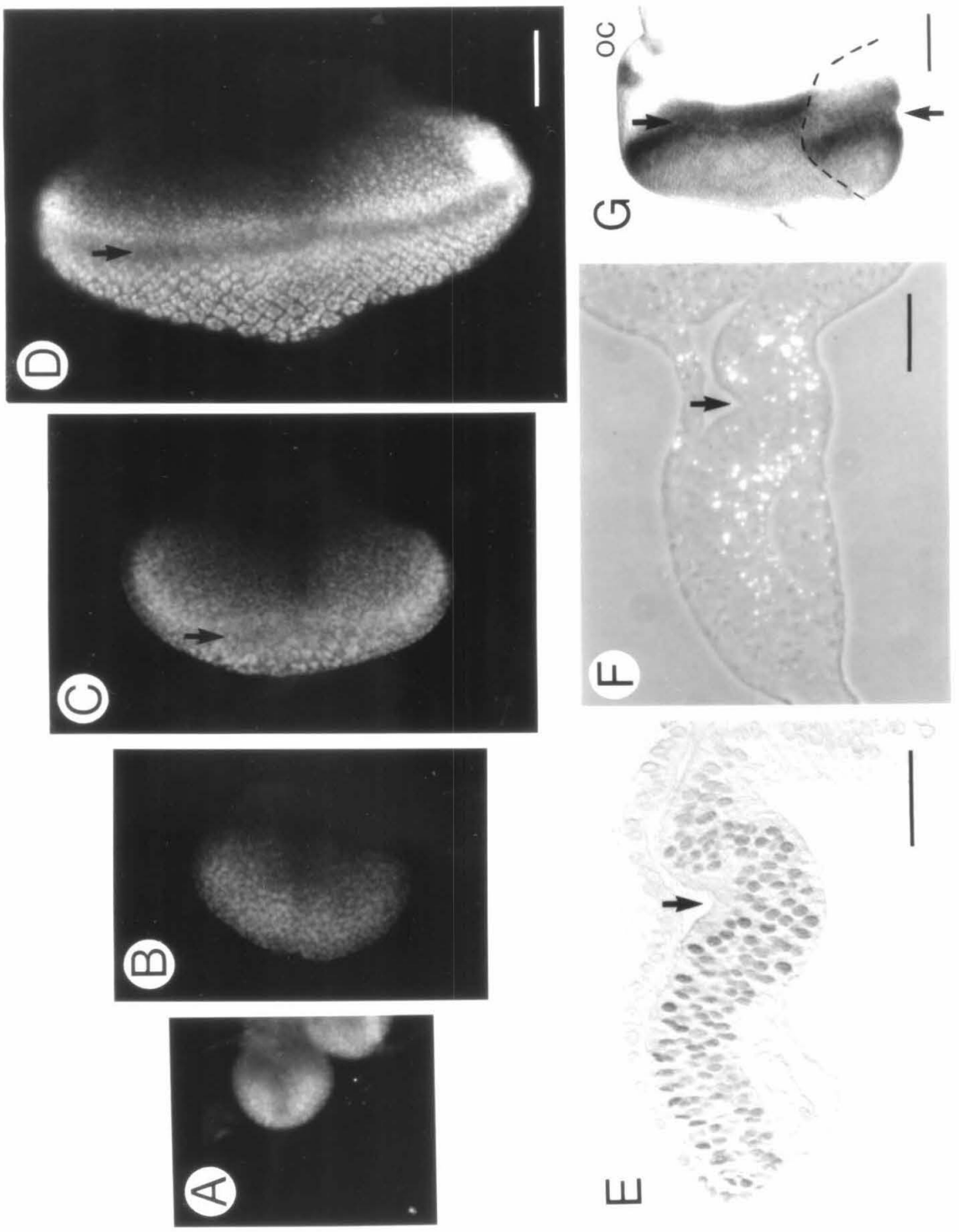
(A-D) Fluorescence of wild-type eye discs from (A) second instar and (B-D) third instar larvae, increasing in age from (A) to (D). (A,B) Protein expression begins during second instar, remains on prior to furrow formation in third instar, and appears graded with stronger expression in the posterior than anterior region of the disc. Two eye discs are visible in (A). (C,D) As the furrow forms and progresses across the disc, expression remains strong in the region just anterior to the furrow (arrows). The protein is expressed more weakly to the anterior of the eye portion of the disc. Posterior to the furrow, expression continues.

(E) Longitudinal section of an eye disc stained with the polyclonal antiserum, detected with a secondary antibody conjugated to horseradish peroxidase. Onset of expression is anterior to the furrow (arrow). The protein is localized to nuclei.

(F) Longitudinal section of an eye disc labeled by *in situ* hybridization. Dark-field image superimposed on bright field image. Onset of transcript signal (white dots) is in the region of the disc anterior to the furrow (arrow), and continues posterior to the furrow, where it appears to be expressed primarily in the basal region of the disc.

(G) *In situ* expression in a whole-mount preparation of an eye disc from a wild-type crawling third instar larva. One flap of the disc is curled over at the bottom (outlined with dashes). Transcript expression begins anterior to the furrow (arrow), and is weaker in the posterior region of the disc. oc = presumptive ocellar primordia.

Anterior to the right. Bar in (D) = 50 μm for (A-D). Bars in (E) and (F) = 20 μm. Bar in (G) = 50 μm.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oc

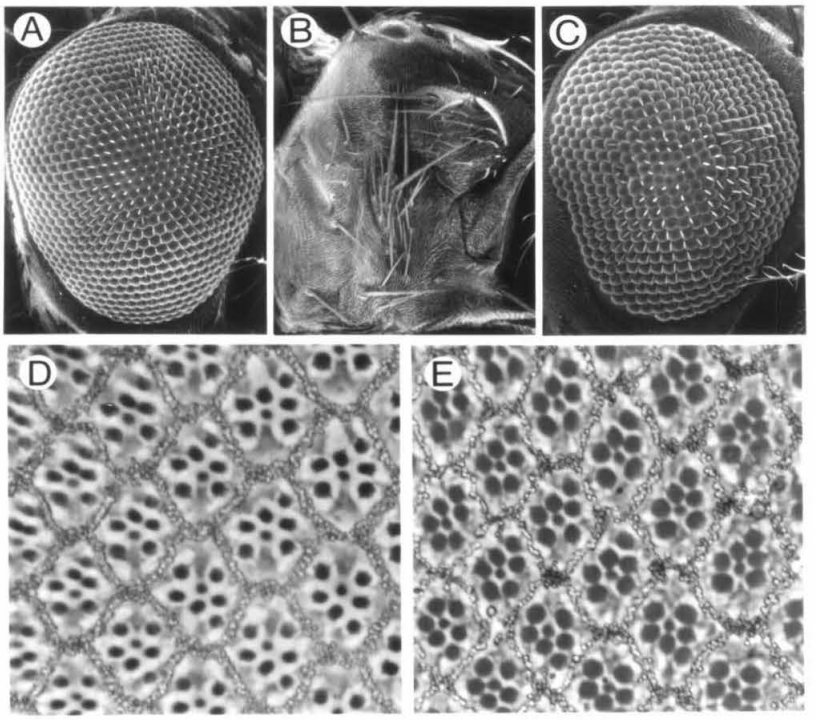
Fig. 9. Expression of the *hsp-eya* mini-gene by heat shock restores development of the eye to *eya* mutants.

(A-C) Scanning electron micrographs. (D-E) Tangential sections of the eye.

(A,D) Wild-type eye and ommatidial pattern.

(B) *eya*^{2} mutant harboring an *hsp-eya* mini-gene insert, raised without heat shock. The mutant is completely eyeless.

(C,E) *eya*^{2} mutant bearing an *hsp-eya* mini-gene insert, heat pulsed during the larval stages of development. Both the external eye morphology and the internal ommatidia are restored. Anterior to the right.


A
B
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E

Fig. 10. Models for the role of the *eya* gene in the regulation of cell survival and differentiation in eye progenitor cells.

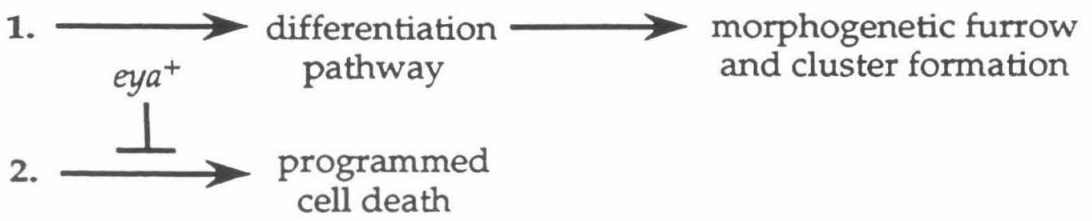
Within a progenitor cell, two pathways are postulated. Pathway 1 represents the pathway of differentiation. Pathway 2 is the default pathway of programmed cell death. In the absence of signals to differentiate, the default pathway occurs and the cells die. Moreover, for differentiation to proceed, the cell death pathway must be repressed. Signals for differentiation and those for repression of cell death may be distinct or in common. The horizontal axis represents time. The events in which the *eya* gene functions occur prior to the morphogenetic furrow.

(A) Model I. The function of the normal *eya* gene is to repress cell death, thus allowing differentiation to occur.

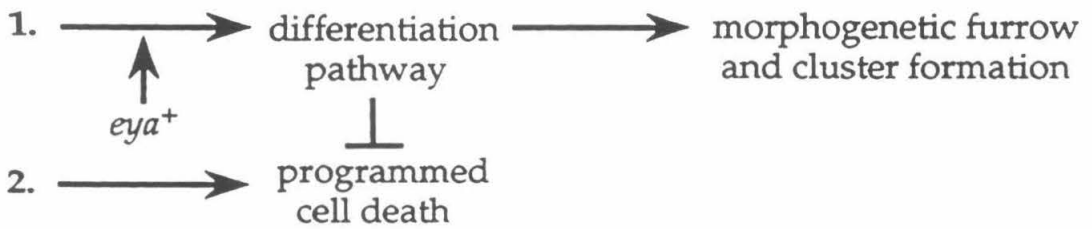
(B) Model II. The function of the *eya* gene is to promote a differentiation step. Once initiated, the differentiation process generates a signal that represses cell death.

(C) Model III. The *eya* gene has dual functions, being required both to promote differentiation and to repress cell death. Without *eya* gene activity, differentiation fails and the default pathway of cell death is activated.

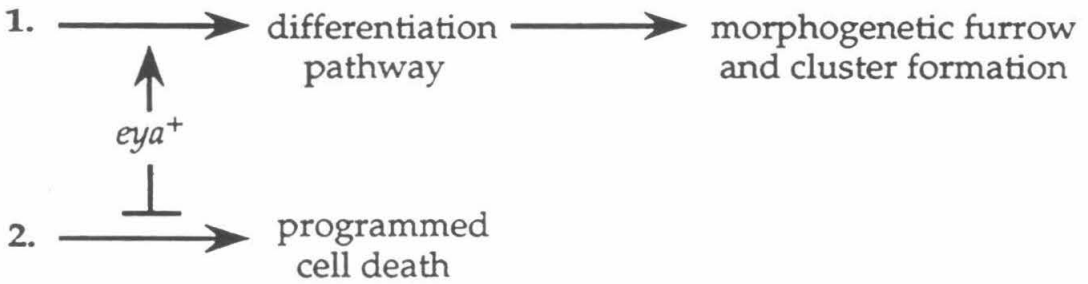
A. Model I: *eya* represses cell death



B. Model II: *eya* promotes differentiation, which represses cell death



C. Model III: *eya* promotes differentiation and represses cell death



Chapter III

Molecular Genetic Analysis of the Requirement for *eyes absent* in the Eye Progenitor Cells

Introduction

The *eya* gene plays an essential role in the events that lead to pattern formation in the eye disc. Without *eya* function, excessive numbers of progenitor cells adopt a cell death fate, when normally only a small number do so. This suggests the existence of a normal selection point ahead of the furrow. Cells mutant for *eya* are unable to survive this normal selection. Cloning of the gene revealed two alternately-spliced cDNA's that code for novel proteins that differ at their N-termini.

In addition to its role in the eye, the *eya* gene provides a vital function to the organism, since many alleles of *eya* are homozygous lethal. Here we use molecular and genetic analysis to show that the eye phenotype is a direct consequence of loss of function of *eya* in the eye progenitor cells. The eye and vital functions of the gene are mapped at the molecular level, and the relationship between the alternatively-spliced transcripts and the eye function is explored. These results demonstrate that the eye function can be studied independently of other functions of the gene.

Results

Alleles of *eya* can be classified into two groups according to whether or not they reduce organismal viability

Although the first isolated mutation of *eya* is remarkable for its specificity to the eye (Fig 1B), subsequently isolated induced alleles reduce organismal viability when homozygous. Mutants bearing these alleles in *trans* to any of four spontaneous alleles survive normally to adulthood. Mutants bearing any of these alleles in *trans* to other induced alleles or a deficiency die. With some induced allele combinations, a small proportion of progeny survive to adulthood, and have reduced eyes (Fig 1C). In such mutants, both the eye and vital functions are compromised. In contrast, mutants homozygous for either of two spontaneous alleles have an eyeless phenotype, yet are

viable. This suggests that different mutations in the *eya* gene can have different effects on the eye and vital phenotypes.

To place the earliest time at which *eya* is required for viability, we observed the phenotypes of severe hypomorphs. Several different allele combinations were used yielding similar results. Animals die as embryos, exhibiting anterior defects consistent with a failure of proper head involution (data not shown). Since head involution normally occurs by approximately 12 hr after fertilization, we infer that *eya* is required in the embryo prior to that time, in order for the organism to survive. The earliest defect detectable in the eye occurs some 60 hours later, providing evidence that the eye phenotype is not a secondary consequence of the embryonic defect. To address this question more directly, mosaic analysis was performed.

In order for the eye to develop, the *eya* gene must function postembryonically in the eye progenitor cells.

Somatic mosaics were used to determine where proper *eya* function is required for normal eye development. A second chromosome bearing a semi-dominant *white* gene was used to allow us to determine, by scoring the color of patches, the genotypes of the three possible chromosome combinations that arise in the eyes of somatic mosaics: w^+/w^- (light) ; w^-/w^- (white); and w^+/w^+ (dark); (see Materials and Methods). Somatic recombination was induced by X-irradiation of 1st instar larvae. The larvae were allowed to develop into adults, which were scored for mosaic patches in the eye. The results are tabulated in Table I. Mosaic patches made in eya^+ animals often consisted of twin spots, as shown in Figure 2A. In contrast, mosaic patches induced in eya^-/eya^+ animals never consisted of twin spots (Fig. 2B). The frequency of homozygous *eya* mutant patches (as judged by patch color in the adult) was drastically reduced as compared to controls, indicating that eye cells mutant for *eya* are unable to survive to adulthood.

Since the homozygous mutant cells were induced postembryonically, the mosaic analysis shows that the requirement in the eye is not only intrinsic to the eye progenitor cells but occurs well after the embryonic requirement. This inference is consistent with our previous finding that the Eya protein begins expression in the eye disc during the 2nd instar and is located in the nucleus. Together, these results indicate that the Eya protein is required in eye progenitor cells at some time between the onset of expression during the 2nd instar and the onset of excessive mutant cell death during the 3rd instar. In contrast, the earliest vital function of the *eya* gene occurs during the first half of embryogenesis. Therefore, the eye and lethal phenotypes are due to loss of gene function at distinct times and places in the organism.

The eye and lethal phenotypes correlate with the molecular map of the alternate transcripts of *eya*.

To see if the pleiotropy is reflected in the structure of the *eya* gene, we mapped the lethal and eye phenotypes at the molecular level. This fine map was generated using six breakpoint alleles. The vital and eye functions of these breakpoint alleles were quantified in crosses to various hypomorphic alleles, by observing both the proportion of escapers among the progeny and their eye sizes (Figs. 3 and 4).

While the eye size of these escapers does not vary with the position of the breakpoint (Fig. 4), the strength of the lethal phenotype varies dramatically with the position of the breakpoint (Fig 3). This analysis shows more vital function in the 5' region than in the 3' region of the gene. The two alleles with breakpoints in the 3' end of the region (X9 and X17) are 100% lethal in combination with the alleles shown, and with every other non-viable allele of *eya*. The alleles with breakpoints in the 5' end of the region can provide enough *eya* function, in combination with certain hypomorphs, to allow some animals to survive to adulthood. Since all adult survivors have necessarily survived embryogeneis,

these results suggest that the embryonic function of the gene maps to the 3' half of the region.

To assay the embryonic function directly, the hatching rate was measured of eggs from crosses of X1 and X9 by a deficiency. In these crosses, one quarter of the progeny would be expected to be mutant for *eya*. For the X9 allele, roughly 25% of the embryos died. For the X1 allele, however, fewer than 7% died, suggesting that the embryonic function maps to the right (3') of X1. It was not possible to use this assay for the other breakpoint alleles, since they are associated with translocations that cause many embryos to die from chromosomal aneuploidy.

The ability to map the eye and embryonic functions to different (albeit overlapping) regions of the gene supports the view that the eye function is distinct from the embryonic one. Interestingly, this map corresponds to the map of alternately-spliced cDNA's that were previously isolated and mapped to the region (see Chapter 2, Figure 6). These cDNA's encode proteins that are identical except at their N-termini, raising the possibility that the N-terminus is a critical part of the *eya* gene products, that confers tissue-specific activity.

Both types of cDNA's are capable of functioning in the eye progenitor cells

The type I cDNA has already been shown to rescue the eye phenotype of *eya* mutants. This was done by transforming flies with a transgene, consisting of the type I cDNA under the control of a heat shock promoter. To test the ability of the type II cDNA to function in the eye disc, we performed a similar experiment using trangenic flies with type II cDNA. Induction of the type II cDNA in larvae homozygous for both the *eya* mutation and the transgene is sufficient to restore *eya* function and allow the formation of the eye (for an example of a rescued eye, see Chapter II, Fig. 9). This suggests that the two alternate transcripts are equally capable of providing *eya* function in the eye.

Rescue experiments with either cDNA alone does not rule out an important role for the N-terminus when both *eya* gene products are present in the same tissue. If such were the case, then the expression of the two cDNA's together could be expected to rescue the eye phenotype better than either one alone. To test for such synergism, we performed phenotypic rescue with one copy of each cDNA construct. If the two cDNA's synergize, then transgenics bearing one copy of each would rescue better than two copies of either alone. The heat-shock conditions used were such that flies bearing either the type I or type II construct alone gave partial rescue, with smaller than normal eyes. While both constructs could be expressed in these experiments, no significant difference was observed between the three groups (Type I, Type II, and Type I + Type II). Thus, there is no evidence for a synergistic effect of the two transcripts for the eye development function.

Discussion

The *eya* gene has been shown to have an important role for the developing eye. Loss of function of the gene is associated with excessive cell death of eye progenitors. Here, we show that the link between loss of function of the gene and the eye phenotype is direct, and distinct from a separate function in the embryo. These two tissue-specific requirements for the *eya* gene are differentially mutable and map to distinct, but overlapping, regions of the gene.

The mosaic experiments show that the requirement for the eye function can be provided after embryogenesis is complete, and is intrinsic to the eye progenitor cells. Loss of this function cannot be rescued by surrounding cells possessing normal *eya* function. Coupled with previous studies showing that Eya protein expression in the eye disc starts during the 2nd instar, this result suggests a direct role in eye progenitor cells. This is also consistent with the nuclear localization of the protein. For whatever process the *eya* gene is required, that process is most likely located in the nucleus of each progenitor cell.

The tissue-specific functions of the *eya* gene are reflected in the dichotomy of the viable and lethal classes of alleles. Three of the four viable alleles give eyeless adults when homozygous. In contrast, many different combinations of lethal alleles that severely reduce viability give escapers that have eyes. These eyes are much smaller than wild type, but show that for lethal alleles, reduction of viability and eye function go hand in hand. The genetic evidence suggests, then, that the viable alleles have lost the eye function without concomitant loss of the embryonic function. The lethal alleles, on the other hand, show reductions in both eye and embryonic functions.

Phenotypic analysis of the breakpoint alleles shows a similar dichotomy. Alleles with breakpoints that severely reduce or eliminate the eye function while leaving the embryonic function largely intact map to the 5' end of the gene. The other breakpoint alleles disrupt both eye and embryonic function, and map in the 3' region. Thus, the breakpoint alleles follow a similar pattern as the other alleles with respect to the loss of eye and embryonic function.

While this may be true, it is important to remember that all the breakpoint alleles cause a reduction in organismal viability as well as a reduction in eye function. Therefore, these breakpoints are unable to dissociate the two phenotypes as completely as seen in the viable alleles. In this light, one might hypothesize that the viable alleles have more subtle lesions in the 5' part of the gene that reduce *eya* function in the eye, without affecting the vital function. Rejection or affirmation of this hypothesis awaits the elucidation of the molecular nature of the viable allele mutations.

The phenotypic analysis of the breakpoint alleles points with more certainty to the type II transcript as the source of embryonic function. This transcript is located entirely in the 3' half of the region. Two alleles have breakpoints that interrupt this transcription unit and they both behave like null alleles. Three alleles have breakpoints located 5' of the presumed start of the type II transcript, but are in the middle of the type I transcription unit. These alleles can provide substantial embryonic function, presumably in the absence of the

(broken) type I transcript. Thus, it appears that the embryonic function is normally provided by the type II transcript.

If the type II transcript provides the embryonic function, it still does not explain why even the 5'-most breakpoint allele shows a significant reduction in organismal viability (see Fig 4). One possibility is that the 5' breakpoints separate from the type II promoter, regulatory regions which are necessary to provide full embryonic function. Without them, the organism can survive embryogenesis but, crippled, does not survive well to adulthood. Another possibility is that there are other tissue-specific vital functions of the gene that are disrupted by the 5' breaks. Depending on whether such putative vital functions are provided by the type I or type II transcripts, these disruptions either could be caused by the separation of regulatory regions from the type II promoter or by the disruption of the type I transcription unit.

The phenotypic analysis of the breakpoint alleles reveals that the entire region spanned by the breaks is important for *eya* eye function. This indicates that either type I, or type II, or both transcripts could be important for the eye. Both are able to rescue the eye mutant phenotype. Nevertheless, when co-expressed in developing eye discs, no synergistic effect is detected. Because the two types of gene products are predicted to differ by the N-terminal 16-20 amino acids, these results suggest that the N-termini either have no functional role in the eye, or they are functionally equivalent.

One way to find out which *eya* gene product(s) normally functions in the eye and embryo is to look at gene expression. Preliminary studies using Northern analysis in eye discs and tissue *in situ* hybridization to embryos suggest that the type I transcript is expressed in the eye disc, type II in the embryo (data not shown). It is not known at this time, however, whether or not other *eya* transcripts are coexpressed in these tissues.

Regardless of the expression pattern of the *eya* gene products, the results presented here demonstrate that the *eya* gene has at least two tissue-specific functions, one in the eye and one in the embryo. The function in the eye is autonomous to the eye progenitor cells,

consistent with the nuclear localization of the *eya* protein. Therefore, the cell death phenotype is likely to be a direct result of the loss of *eya* eye function, and not a secondary consequence or epigenetic phenomenon.

Experimental Procedures

Fly strains. The alleles of *eya* used in this study are described in Bonini et al. (1993). The *whs* strain is of genotype, w^{1118}; P[>whs>]17A, and was generously provided by Kent Golic and Susan Lindquist (Golic and Lindquist 1989). It has a P-element insert, located in polytene division 33, bearing a *white* gene under the control of a heat shock promoter. When reared under normal (non-heat shock) conditions, this *white* gene is semi-dominant.

Culture conditions. Flies were cultured on standard cornmeal medium at 25°C.

Crosses. Five males and five virgin females of the appropriate genotypes were mated in vials for approximately one day, then transferred to bottles. Flies were allowed to lay eggs for three to five days, then transferred to fresh bottles, or discarded. In this way, the bottles were not allowed to get overcrowded. For the analysis of lethality, the complete F1 generation from at least one bottle was scored.

Mosaic analysis. Males and virgin females of the appropriate genotype were mated in vials, then transferred to egg laying chambers fitted with petri dishes half-filled with standard cornmeal medium. Embryos were collected every 24 hours, and allowed to age for an additional 24 hours. The plates bearing these larvae were irradiated with 1000R to induce somatic recombination. The rate of somatic mosaicism detected in the eye was approximately 3%. For the controls, the cross consisted of w^{1118};whs^{+} eya^{+} x w^{1118};whs^{-} $eya^{+}$and produced white and dark patches on a light background. The

experimental mosaics were generated in two different crosses, all involving the allele eya^2. The cross, w^{1118};whs^+ eya^- x w^{1118}; whs^-eya^+, produced white *eya+/eya+* patches. The other cross, w^{1118};whs^+ eya^+ x w^{1118}; whs^-eya^-, produced dark patches of the genotype eya^+/eya^+.

Transgenes and transformation. Standard molecular techniques were used for these manipulations (Sambrook, Fritsch et al. 1989). EcoRV-XbaI restriction fragments bearing either the type I and type II cDNA's were directionally subcloned into pCaSpeR-hs, via the HpaI and XbaI sites. After purification on CsCl gradients, the plasmids were mixed in a ratio between 5 and 10 to 1 with a transposase source, phsπ (Steller and Pirotta 1985). The mixture of DNA was ethanol precipitated, then resuspended in injection buffer at a concentration of 500-1000 µg/ml. The injection procedure was identical to that used in Bonini et al. (1993) except that w^{1118} embryos were injected. The K58.1 and other lines were established and balanced. To test its ability to rescue the mutant *eya* phenotype, the strain w^{1118}; eya^2; K58.1 was synthesized. Flies were allowed to lay in vials for 12 hours, then the vials were aged for an additional 48 hours. The vials were subjected to heat-shock in a 37°C water bath in the following approximate daily regime, starting in the morning: 1 hr at 37°C; 6.5 hr at 25°C; 0.5 hr at 37°C; 6.5 hr at 25°C; 1 hr at 37°C; then overnight at 25°C. The vials were heat-shocked until a substantial number of animals had pupated (around seven days).

References

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Sambrook, J., E. F. Fritsch, et al. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, Cold Spring Harbor Laboratory Press.

Steller, H. and V. Pirotta (1985). Expression of the *Drosophila white* gene under the control of the *hsp70* heat shock promoter. *The EMBO Journal* **4**: 3765-3772.

Figure 1. Adult phenotype of *eya* viable and lethal (escaper) mutants. (A-C) Scanning electron micrographs

(A) CS (wild type)

(B) eya^{2}/eya^{2}, a viable allele

(C) eya^{E11}/eya^{E11}, a lethal (reduced viability) allele

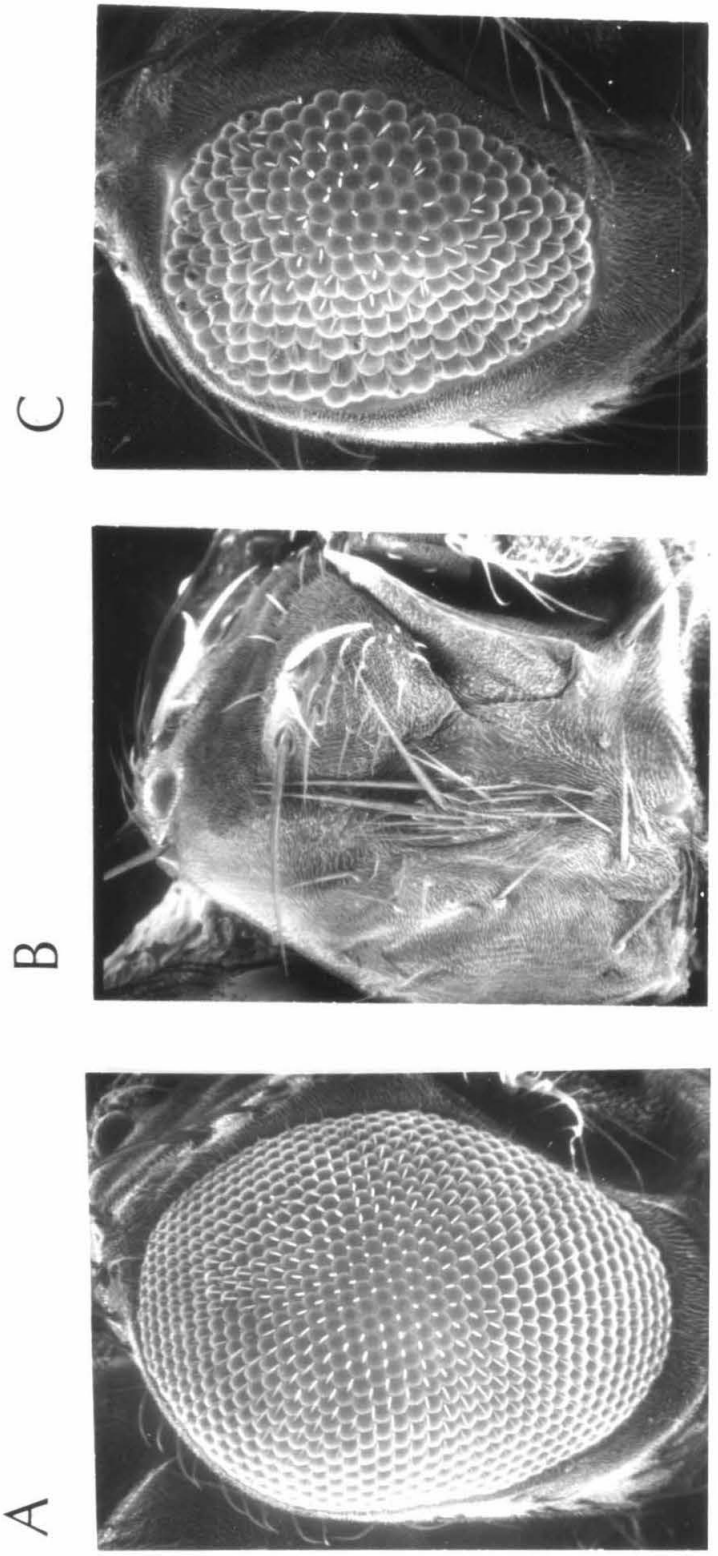
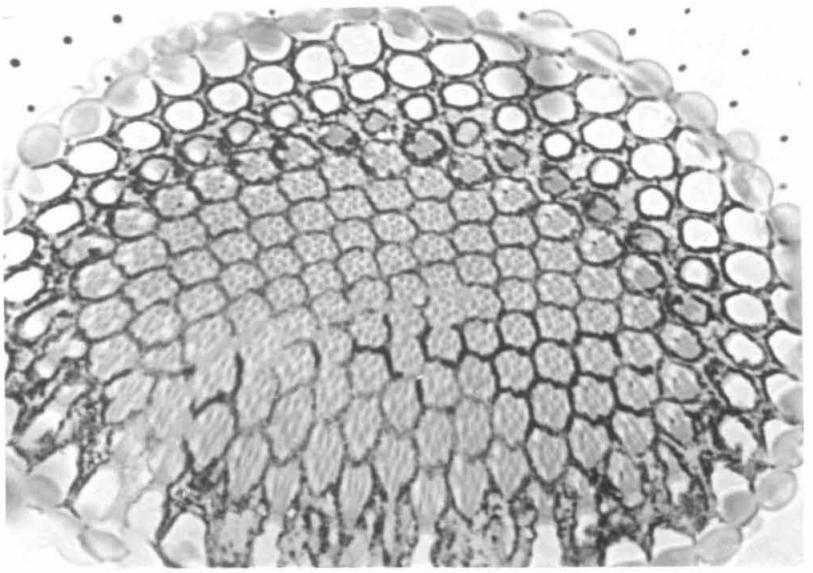

A
B
C

Figure 2. Clonal analysis of the *eya* eye phenotype. (A,B) Tangential sections showing mosaic patches in the adult eye.

(A) Control (eya^+) showing twin spot: dark and white patches of tissue, corresponding to two or no copies of the *white* insert, respectively.

(B) Mosaic patch derived from w^- eya^2/w^+ eya^+ heterozygote. The dark patch is homozygous w^+ eya^+. The corresponding white patch of mutant cells of the genotype, w^- eya^2/w^- eya^2, is missing.

A



B

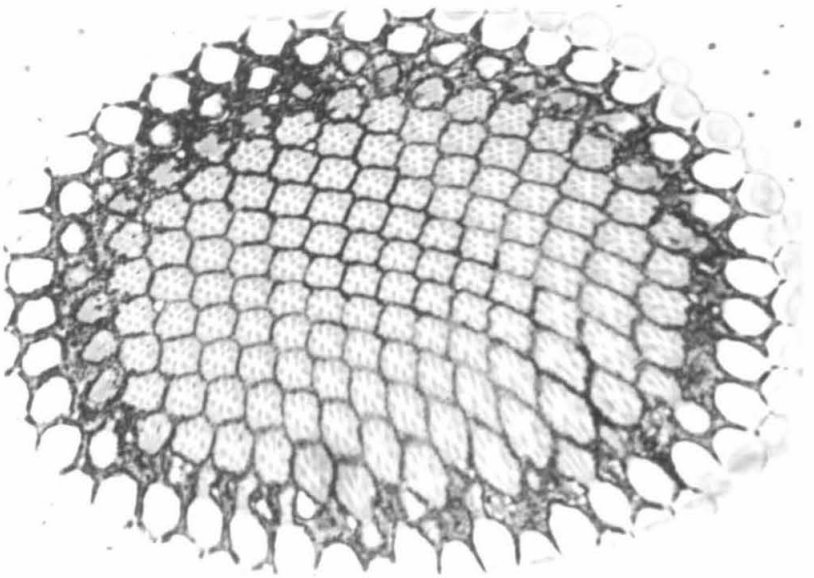


Figure 3. Analysis of the vital function of *eya* breakpoint alleles. The X-axis represents relative map position of a breakpoint. Plotted on the Y-axis is the fraction of adult survivors (expressed as percent of the expected number that would be found using the wild type *eya* allele). The results are shown of selected *eya* hypomorphic alleles over alleles having various breakpoints. All the breakpoint alleles, themselves, are completely lethal in *trans* to a deficiency. (Note: the relative positions of X1 and X11 are inferred from the genetic data; they have not been resolved at the molecular level.)

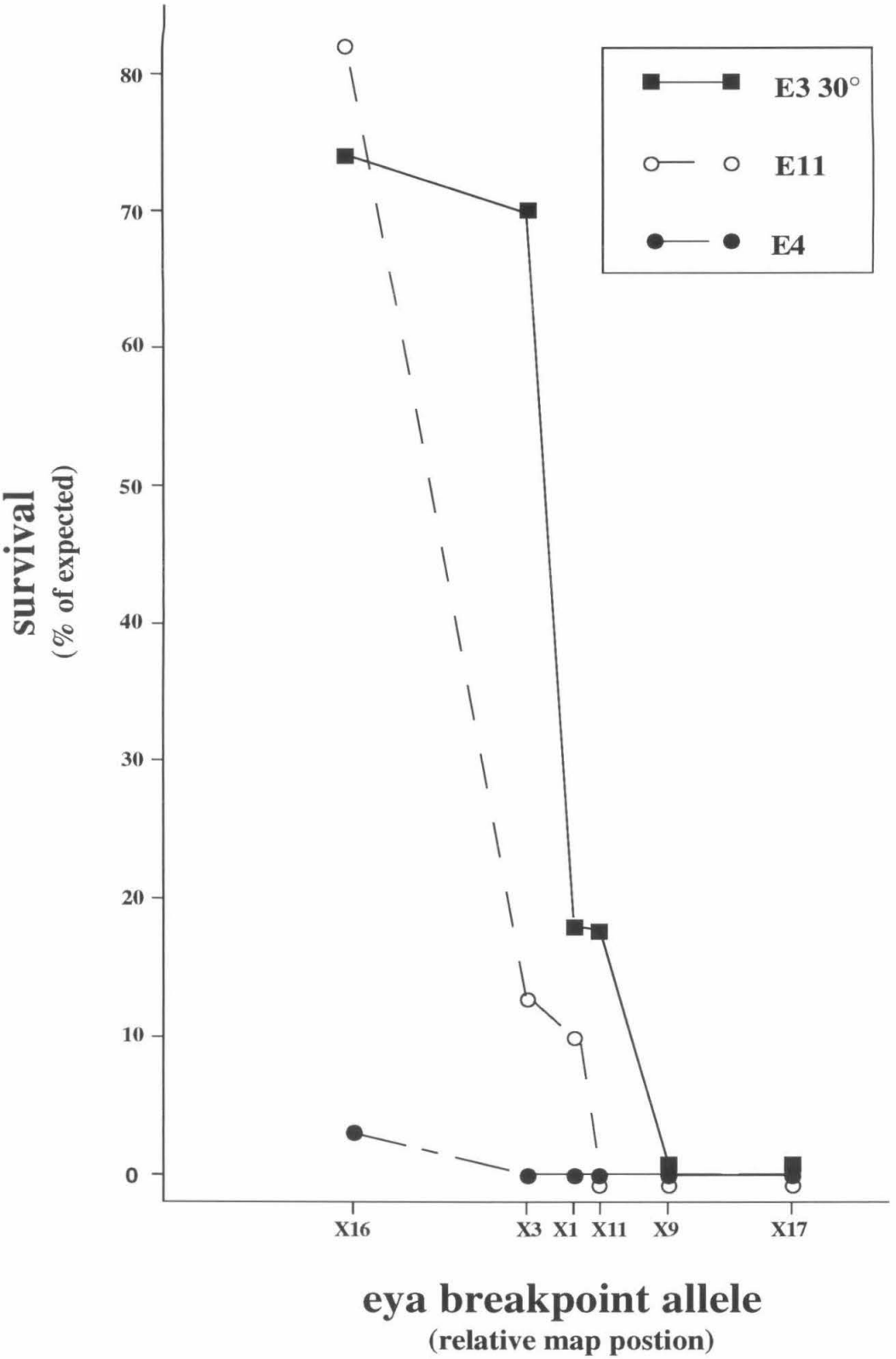

E3 30°
E11
E4
survival
(% of expected)
80
70
60
50
40
30
20
10
0
X16
X3
X1
X11
X9
X17
eya breakpoint allele
(relative map postion)

Figure 4. Analysis of the eye function of *eya* breakpoint alleles. The X-axis represents relative map postion of a breakpoint. Plotted on the Y-axis is the eye phenotype of adult survivors, expressed as eye area relative to wild type, as judged through the stereomicroscope. These are the same crosses as in Figure 3. In addition, the results of putting the breakpoint alleles in *trans* to eya^{1} and eya^{3} are shown. For all alleles (except eya^{1} and eya^{3}), the eye phenotype of some crosses could not be determined because the genotype was completely lethal.

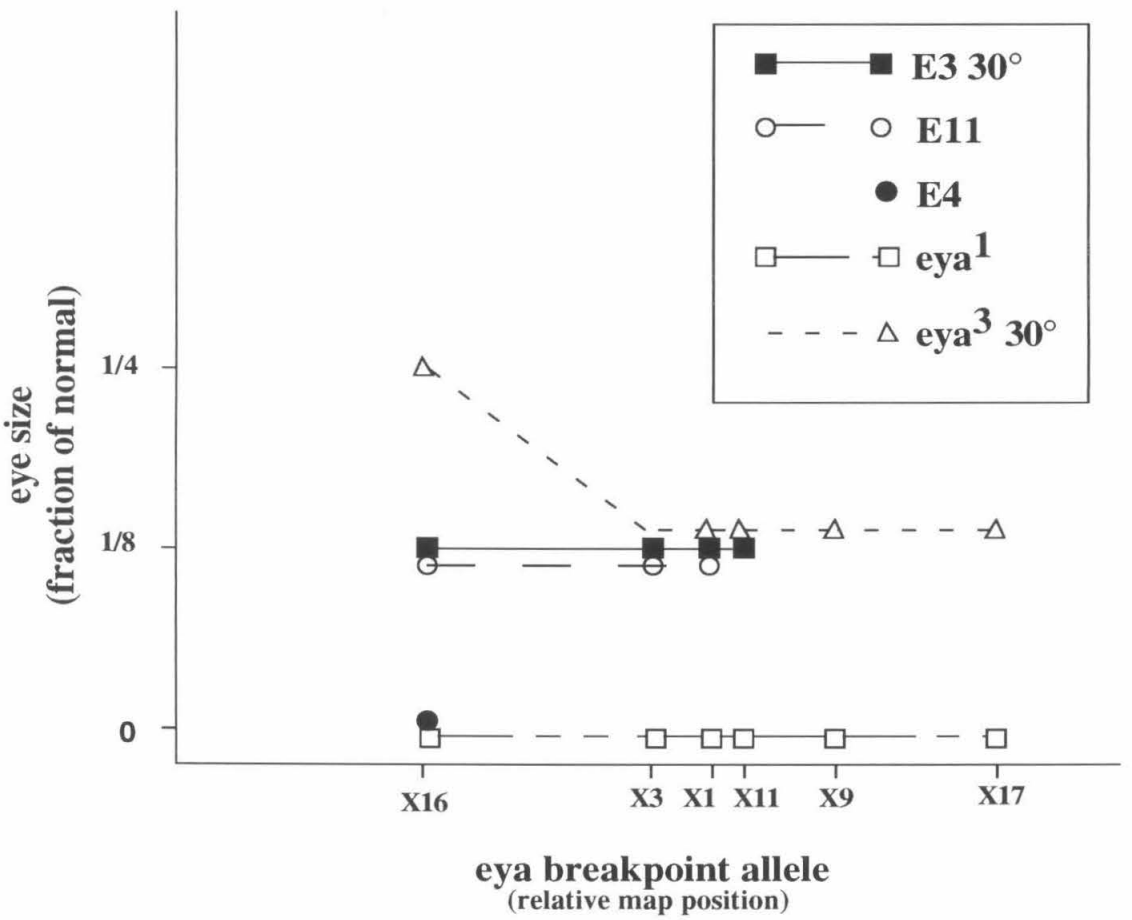

E3 30°
E11
E4
eya1
eya3 30°
eye size
(fraction of normal)
1/4
1/8
0
X16
X3
X1
X11
X9
X17
eya breakpoint allele
(relative map position)

Table I. Quantitation of *eya* clonal analysis

	genotype	mutant tissue color	twin spots	white only patches	dark only patches	total patches
Control	$\frac{+ \quad whs}{+ \quad +}$		44	51	22	117
Exp. 1	$\frac{eya \quad whs}{+ \quad +}$	dark	0	50	0	50
Exp. 2	$\frac{eya \quad +}{+ \quad whs}$	white	0	(5)[1]	59	64

Quantitation of *eya* clonal analysis. The results of induced control and *eya* mosaics are shown. The genotype is given of the animal in which somatic recombination was induced. For the two experimental classes, the color of the patch that corresponds to *eya* mutant tissue is shown. The number of eyes are tabulated that had a twin spot (both white and dark patches), only a white patch, and only a dark patch. No twin spots were found in mosaics made in heterozygotes for *eya*.

[1]This figure is shown in parentheses because the five flies were all found in one bottle. Subsequent bottles showed no such flies.

Chapter IV

Transvection at the *eyes absent* Locus

Introduction

Nearly four decades have passed since E. B. Lewis published his account of an unusual position effect in *Drosophila* for which he coined the term "transvection." He showed that certain kinds of chromosomal rearrangements were able to reduce complementation of alleles of the Bithorax Complex: *bithorax* (*bx*) and *Ultrabithorax.* (*Ubx*). It is now generally accepted that the important attribute common to transvection-disrupting rearrangements is that they disrupt the normal chromosomal pairing of the interacting alleles (Lewis, 1954). With this proximity-dependent allelic complementation in mind, many investigators have since used transvection to refer to any allelic position effect. But with the exception of the *decapentaplegic* (*dpp*) gene (Gelbart, 1982), no pairing-sensitive interaction has been demonstrated to be affected by the transvection-disrupting rearrangements identified by Lewis.

Perhaps because this brand of transvection seemed the exception rather than the rule, the contrast between the BX-C/*dpp* interactions and all other allelic position effects has been largely ignored. This, despite the fact that the BX-C/*dpp* interactions are remarkable since they can respond to rearrangements involving breaks that are megabases away from their respective loci.

Understanding the mechanism of transvection may contribute to the understanding of the relationship between genomic structure and gene expression. Investigations of position effects in *Drosophila* have uncovered gene products that appear to be important for both proper chromatin structure and gene regulation. Mutations that modify the *zeste-white* interaction have revealed a number of genes, the *Polycomb* group, now thought to be linked to chromatin structure (Jones & Gelbart, 1990; Wu, Jones, Lasko, & Gelbart, 1989) The link was uncovered by the discovery of the "chromo domain," a region of homology between Pc and HP1, a heterochromatin-associated protein (Paro & Hogness, 1991). Mutations in HP1, also known as *Su(var)205*, can suppress position-effect variegation (Eissenberg, James, Foster-Hartnett, Hartnett, Ngan, & Elgin, 1990). This connection is

made even more intriguing by the phenotype of the Pc group mutants: the massive derepression of the homeotic gene clusters.

While second-site modifiers of position effects have linked gene regulation to chromatin structure, the mechanism(s) underlying *trans*-sensing phenomena have been investigated by other means. Because the *zeste* gene is required for a number of these interactions, a large number of investigations have focused on the molecular function of this gene (Wu, 1993; Wu & Goldberg, 1989). Although there is no obvious mutant phenotype of *zeste* null alleles, Zeste binding sites are found throughout the genome. The Zeste protein must aggregate in order to function, and is capable of enhancing transcription (Pirrotta, 1990). Whether it is a transcription factor, mediates chromosomal pairing, or facilitates some other nuclear process remains a mystery, partly because *zeste* function is apparently redundant.

In this chapter, we unveil a striking interaction among certain alleles of *eyes absent*. We show that this interaction is an allelic position effect, that is sensitive to the same kinds of transvection-disrupting rearrangements as the BX-C and *dpp*, and that it is dependent on normal *zeste* function. These results indicate that the BX-C/*dpp* class of transvection may be more general than previously thought.

Results

The *eya* gene has been characterized for its function in eye development. Loss of function of the gene in the eye primordium, the eye portion of the eye-antennal imaginal disc, results in eye precursor cell death. The amount of cell death in the eye disc is correlated with the adult phenotype: the size of the compound eyes. The eye phenotype can be quantified by counting the number of ommatidia in the eye. For most allele combinations, the phenotype is similar from individual to individual, so differences in different genotypes can be observed in the stereo microscope.

***Interallelic* complementation at the *eya* locus**

The *eya* gene has embryonic, ocellar, and possibly other functions. Here we focus on the eye phenotype, where a dramatic interaction between alleles can be observed (Figure 1). This interaction is demonstrated with two spontaneous alleles, eya^2 and eya^4. The homozygous phenotype of eya^2 is eyelessness (Figure 1A); of eya^4, severely reduced eyes (Figure 1B). The penetrance of these phenotypes is complete: eya^2 homozygotes are always eyeless; eya^4 homozygotes always have fewer than 50 ommatidia. In the eya^2/eya^4 *trans*-heterozygote, however, a large eye is observed, roughly 3/4 normal size (Figure 1C and1D). A simple interpretation of this effect is that these two alleles are able to partially complement each other.

Two lines of evidence suggested that this effect was not due to genetic background effects involving other genes: (1) recombinant lines in which parts of the second chromosome were replaced and the other chromosomes were outbred still show the effect; (2) other allele combinations show the same effect. By testing a large number of alleles against each other, we were able to place eya^1 and eya^2 into one intragenic complementation group and eya^3, eya^4, and many induced alleles into a second group. Thus, it seemed unlikely that these interallelic interactions are due to genetic background effects.

We also considered the possibility that the complementation groups represent different genes and that eya^1 and eya^2 are not allelic to the other mutations. All 40 mutations tested, that fail to complement eya^1 and eya^2, map to the *dumpy-spade* interval of chromosome 2L. Six breakpoint alleles have been mapped at the molecular level to a 25kb span of the genome, located in polytene bands 26EF. All six breakpoint alleles fail to complement each class equally well. This suggests that, if there are two genes involved, they both map within the 25kb span of the breakpoints. Interestingly, we have detected two alternately-spliced transcripts in this region, which could correspond to the two complementation groups. Because cDNA's of either type can rescue members of either

group, it seems unlikely that the alternate splicing can account for the interallelic interactions.

Transvection-disrupting rearrangements reduce *eya* allelic interactions

We considered transvection as one of several hypotheses to account for the genetic interactions. If transvection underlies the interaction, then we should be able to isolate, in a suitable X-ray screen, rearrangements that disrupt the effect (i.e., give smaller eyes). In similar screens done by Lewis and Gelbart, over 1% of the progeny of X-irradiated males had transvection disrupting rearrangements (Gelbart, 1982; Lewis, 1954). The expected frequency of progeny bearing such rearrangements (1/100) is much larger than expected for X-ray induced lesions in the *eya* region (1/3,000). This difference in expected frequency between the two types of events made it possible to test whether the *eya* alleles undergo transvection by doing a simple F1 X-ray screen.

The screen was done by irradiating eya^2 or eya^4 males and crossing them to females homozygous for the interacting allele. Whether or not the interaction is by transvection, the vast majority of the progeny should have eyes that are 3/4 the size of wild type, which is the phenotype normally seen in eya^2/eya^4 heterozygotes. If the alleles can interact by transvection, then about 1% of the progeny would be expected to have reduced eyes, and most of these reduced-eye progeny would be expected to bear transvection-disrupting rearrangements. If, however, the allelic interaction is not a case of transvection, then fewer than 0.1% of the progeny would be expected to have reduced eyes, and most of those would be expected to have lesions in the *eya* gene.

Of course, these outcomes are not the only ones possible. For example, many progeny could have reduced eyes due to dominant modifier mutations. In that case, this screen might not be useful in determining whether transvection is the basis of the allelic interactions. However, we did not expect to find many dominant modifier mutations of *eya* because none were turned up in previous screens designed to isolate additional alleles that

fail to complement eya^1. As shown below, neither did we find dominant modifier mutations in this screen.

Approximately 5,000 progeny were scored from the two reciprocal screens. A broad spectrum of eye sizes was observed, ranging from 3/4 wild type (the normal eya^2/eya^4 phenotype) to eyeless. Over 30 flies had eyes that were less than half the size of the normal eya^2/eya^4 phenotype. Twenty of these flies were bred for further analysis, from which 11 independent lines were successfully established (see Table I).

Each of these lines was tested genetically by crossing to a panel of *eya* mutants to determine if any chromosomes had suffered a lesion in the *eya* gene itself. From the many screens done in our laboratory, the expected phenotype of induced lesions in the *eya* gene is lethality. Since both eya^2 and eya^4 are viable alleles, spontaneous in origin, testing lethality was a convenient assay for secondary lesions in these alleles. Of the eleven lines, only one (ETD4.6) failed to complement the lethality of *eya*, and thus had an induced lesion affecting the *eya* gene. By this criterion, the other ten lines had normal eya^2 or eya^4 alleles.

The lines were also analyzed for chromosomal rearrangements, by examining squashes of the salivary gland polytene chromosomes. The results are listed in Table I. The ETD4.6 line, which failed to complement the lethality of *eya*, has a deficiency spanning the *eya* region, confirming that the lesion involves the *eya* gene itself. Of the remaining ten lines (listed in Table I), nine have rearrangements with a breakpoint on 2L that is proximal to *eya*, such that the distal portion of 2L (bearing the *eya* locus) is rearranged to another chromosome arm. Thus, as explained above, they are transvection-disrupting rearrangements. The remaining line (ETD4.4) has a complex rearrangement with one breakpoint just distal to *eya*. This line does not conform to the previously identified class of rearrangements that disrupt transvection. Like the other ten lines, however, ETD4.4 could be observed to actually disrupt chromosomal synapsis in the

salivary gland nuclei. Thus, this rearrangement still conforms to the notion that disruption of pairing correlates with a reduction in transvection mediated interactions.

The distribution of rearrangements is plotted in Figure 2. Judging from the locations of breakpoints on chromosome 2L, the so-called critical region of *eya* extends at least to polytene division 35. Transvection-disrupting rearrangements of the BX-C and *dpp* have been analyzed to see if there is some pattern that would dictate where the non-2L break could occur. At the BX-C, transvection-disrupting rearrangements that have breaks near the locus, were found to have second breaks in euchromatin. Breakpoints located more proximally from the BX-C were found to have second breaks that could occur anywhere. At *dpp*, a different pattern was found. Breaks near *dpp* could rearrange anywhere, while breaks more proximal rearranged only to heterochromatin. For *eya*, breaks near the locus can be found that are rearranged to either euchromatin or heterochromatin.

The reduction in eye size resulting from the reduction in chromosomal pairing can be seen in Figure 3B and 3C. The eye size corresponding to genotypes shown in Figures 3 and 4 have been quantified by counting the number of ommatidia and are presented in Table II. The eyes shown in Figure 3B and 3C are typical for most of the lines obtained from the screen, ranging from 1/8 to 1/4 the normal size. Three lines (ETD4.4, ETD2.1, and ETD2.3) show a slightly larger eye, ranging from 1/4 to 1/2 normal.

We attempted to restore chromosomal pairing in heterozygotes bearing rearranged alleles by putting two rearrangements with similar breakpoints in *trans*. If chromosomal pairing is the determining factor in the allelic interaction, then restoring the pairing between rearranged chromosomes should restore the interaction, resulting in a larger eye than when either allele is over a rearrangement of the other. Of the rearrangements at our disposal, the ones involving heterochromatin are the most likely to be able to pair (E. B. Lewis, pers. comm.). The ETD4.3 line was crossed to all the lines bearing rearranged eya^2 chromosomes. The three lines with rearrangements involving heterochromatin all gave progeny with eyes larger than controls of the genotype R(eya^2)/eya^4. One such example is

shown in figure 3D (compare with figure 3B,C). In contrast, the two lines with rearrangements that do not involve heterochromatin gave progeny with smaller eyes than the controls (data not shown).

Thus, the disruption of allelic interaction was reduced in combinations of rearrangements where the chromosomes were expected to pair, while it was enhanced in combinations that would reduce pairing further. This suggested that chromosomal pairing is the determining factor behind the allelic interaction, rather than other mechanisms, such as the effects of dominant modifiers.

Normal *zeste* function is required for *eya* transvection

Some allelic position effects have been shown to be sensitive to loss-of-function *zeste* mutations. To test this possibility for *eya*, we used the $z^{ae(bx)}$ mutation, which has been shown to reduce the interactions of transvecting alleles at the BX-C and *dpp*. Flies of the genotype $z^{ae(bx)}$; eya^2/eya^4 (figure 4B) have eyes that are roughly half the size of flies of the genotype +;eya^2/eya^4 (figure 4A). Thus, in addition to being sensitive to disruptiion by rearrangements, the interaction at *eya* depends on *zeste* function.

Discussion

The allelic position effect detailed here shares many properties with transvection at BX-C and *dpp*. Interaction between *eya* alleles responds to rearrangements that disrupt chromosomal pairing in the *eya* region. Three lines of evidence support this notion. First, in a screen for suppression of the interaction, we pulled out transvection-disrupting rearrangements in 9 of 10 lines examined (the 11th does not count since it is a hit in the *eya* gene). The probability of obtaining these rearrangements at random is vanishingly small. Second, a pairing of rearrangements, to restore chromosomal synapsis, also restores the interaction. Third, normal *zeste* gene function, the only gene known to help mediate transvection, is required for the *eya* interaction. By these three criteria, the interaction at *eya* is identical to transvection effects described for the BX-C and *dpp*.

One rearrangement deserves special mention; it does not fit into the standard pattern of transvection disruption. Unlike the others, ETD4.4 has a breakpoint distal to *eya*, and therefore might not be expected to disrupt transvection. One possibility is that this line has a second site mutation that is a dominant modifier of *eya*. While it is difficult to completely rule such modifiers out, analysis with non-interacting *eya* alleles revealed no differences between ETD4.4 and its parent chromosome eya^4. If there is a dominant modifier in ETD4.4, it appears to specifically modify the transvection interaction.

Actually, there is a precedent for transvection-disrupting rearrangements having breaks distal to the locus. Gelbart reported twelve such cases (Gelbart, 1982). Eleven were insertional translocations of the region spanning *dpp*. The remaining one was a paracentric inversion, In(2L)21B;40F (*dpp* is located at 22F). Gelbart noted that this rearrangement disrupts pairing of the polytene chromosomes. The rearrangement of ETD4.4 is quite complex, involving a pericentric inversion of chromosome 2 and a reciprocal translocation between 2R and 3R. Like the exceptional *dpp* rearrangement, however, the ETD4.4 rearrangement disrupts pairing of the relevant region in polytene chromosomes.

That pairing of alleles is the crucial element required for transvection effects follows from the examination of pairing in the polytene chromosomes (Gelbart, 1982; Lewis, 1954). Pairing in polytene chromosomes is considered to be relevant to other cells because of evidence that, in Diptera, the chromosomes of somatic cells are paired during interphase. This stems from Metz' observation that when condensing chromosomes become visible in early metaphase, they are already paired with their respective homologs (Metz, 1916). Recently, sophisticated imaging of *in situ* hybridization to alleles in embryonic nuclei has provided support for this view: the loci were shown to be paired (Hiraoka, Dernburg, Parmelee, Rykowski, Agard, & Sedat, 1993). Interestingly, the phenomenon of transvection itself has long provided strong evidence for pairing of interphase chromosomes. For example, in addition to transvection-disrupting rearrangements,

insertional translocations of the BX-C to other parts of the genome suppress transvection. An expected consequence of such insertional translocations would be loss of pairing of the BX-C alleles.

The use of transpositions has lead to the demonstration of other allelic position effects, notably the *zeste-white* interaction (Wu & Goldberg, 1989) and interactions between *yellow* alleles (Geyer, Green, & Corces, 1990). In both systems, transpositions that would prevent pairing of alleles have been shown to reduce their interaction. Still, efforts to disrupt these interactions using transvection-disrupting rearrangements have failed, suggesting there may be a qualitative difference between these interactions and transvection at BX-C, *dpp* and *eya*. For the case of *zeste-white*, it has been demonstrated that the interaction is not sensitive to transvection-disrupting rearrangements by testing the effects of rearrangements on autosomal transpositions of the *white* gene (Smolik-Utlaut & Gelbart, 1987). For the *yellow* locus, however, the lack of sensitivity to rearrangements has been ascribed to the telomeric position of the gene, which may remain paired despite the presence of rearrangements that would be expected to disrupt pairing of large segments of the X chromosome (Geyer, et al., 1990). Nevertheless, it still remains to be shown that the interaction of *yellow* alleles can be sensitive to transvection disrupting rearrangements. The qualitative difference between transvection at BX-C, *dpp*, and *eya* on the one hand, and *zeste-white* and *yellow* on the other, may reflect important differences in the underlying mechanisms of these interactions.

Unfortunately, the other loci which exhibit allelic position effects have not been tested for their sensitvity to transvection-disrupting rearrangements. We therefore do not know how many transvecting loci there are. The results presented here suggest that they may be more prevalent than previously thought.

The molecular mechanism of transvection

The molecular mechanism underlying allelic position effects is in the process of being uncovered. For *Cbx* (of the BX-C) and *Sgs-4* (a glue gene), it has been shown that

expression of one allele is dependent on its being paired with the other (Castelli-Gair, Micol, & Garcia-Bellido, 1990; Kornher & Brutlag, 1986). While a number of models have been proposed to account for this, the *trans* effect of enhancers is the only one that has substantial experimental support. It has been proposed that enhancers can interact with the transcriptional machinery at the promoter by looping out intervening DNA (Ptashne, 1986). This view is bolstered by experiments demonstrating that enhancers need not reside on the same DNA molecule from which transcription is initiated (Dunaway & Dröge, 1989; Mueller & Schaffner, 1990; Müller, Sogo, & Schaffner, 1989; Wedel, Weiss, Popham, Dröge, & Kustu, 1990). *Cbx* is a regulatory mutation that misexpresses *Ubx* in the wing disc (White & Akam, 1985), and it has been demonstrated that *Cbx* can misregulate *Ubx* in *trans* (Castelli-Gair, et al., 1990). Regulation in *trans* is also consistent with the analysis of mutations of *yellow*, in which defects in transcriptional regulation appear to be complemented by a *trans* allele that has a defective transcription unit but an intact enhancer.

Our present knowledge of the *eya* gene supports this view of *trans* action of enhancers. Both eya^1 and eya^2 appear to be specifically defective in their ability to express the *eya* gene in the eye disc, as judged by their phenotype and antibody staining, while the gene functions normally in other tissues (data not shown). A simple interpretation of these data is that the eya^1 and eya^2 alleles have defects that eliminate activity of an eye disc enhancer, while most of the alleles that interact with the enhancer-defective class are likely to have lesions in the *eya* coding region. These alleles do not show the exquisite tissue specificity of eya^1 and *eya*2. Rather they affect more than one tissue during development. Many of these alleles were induced with the mutagen ethylmethane sulfonate, which tends to generate point or small lesions. The vast majority of such lesions, that have detectable effects on gene function, is found in the coding regions of genes. Included in this class are two spontaneous *eya* alleles. One of these alleles is sensitive to temperature, a property often associated with a defect in the protein product. Thus, from the data available at this time, it seems likely that the interacting groups of *eya* alleles represent enhancer mutations

on the one hand, and mutations affecting the coding sequence on the other. As the molecular basis of the mutations is determined, the basis of the interacting classes may be revealed.

Two classes of allelic position effects

The *eya*, BX-C and *dpp* are three loci that exhibit allelic interactions that are sensitive to transvection-disrupting rearrangements. The proximity dependent interactions at most other loci, such as *zeste-white* and *yellow*, have only been demonstrated to be sensitive to insertional translocations of alleles. Indeed for the *zeste-white* and *yellow* interactions, it has been shown that they are insensitive to transvection-disrupting rearrangements. Insertional translocations would be expected to eliminate pairing between alleles, while transvection disrupting rearrangements would reduce, but not eliminate, pairing. Thus, allelic position effects can be classified into two groups which may reflect a significant difference in the mechanisms responsible for these two kinds of allelic position effects.

One possibility to explain this dichotomy is that the *eya*, *dpp*, and BX-C phenotypes respond proportionately to the amounts of their respective gene products, while even a small amount of product from the other *trans*-sensing loci (which would be available with reduced pairing) is sufficient to rescue their respective mutant phenotypes. In this model, the amount of gene product made in response to intermediate levels of pairing would be similar for both groups. The difference between the groups would be in the phenotypic response to these intermediate levels of gene expression. Thus, the BX-C, *dpp*, and *eya* phenotypes would be intermediate at intermediate levels of gene expression, while the *zeste-white* and *yellow* phenotypes would be wild type at intermediate levels. This hypothesis seems unlikely since the *white* gene, at least, can produce many intermediate phenotypes that correspond to different levels of gene function.

A different and more interesting interpretation is that the dichotomy is due to a significant difference in how the genes from each group are regulated. This hypothesis follows from considering what effect loss of chromosomal pairing would have in

interphase nuclei. The "amount of pairing" in the interphase nucleus may actually translate into "amount of time spent being paired." Evidence is emerging for this dynamic view of the chromosome synapsis in the nucleus. For example, the histone loci of *Drosophila*, shortly after fertilization, are initially unpaired, then become paired near the onset of zygotic transcription (Hiraoka, et al., 1993). This dynamic view is quite different from the static impression derived from examining synapsis in squashes of polytene chromosomes.

Interactions that respond quantitatively to reduction in chromosomal synapsis (BX-C, *dpp*, and *eya*) may require constant *trans* interaction of alleles in order to maintain gene expression. Any change in pairing would have an immediate effect on the transcription of genes of this class, which would then be reflected in the phenotype. The other class, represented by *zeste-white*, would only need a short period of *trans* interaction, after which the transcriptional state of the gene would somehow remain imprinted at the locus. With only a short period of interaction required to effect a lasting change in transcription, the interaction would be insensitive to partial reduction in pairing. These alternative mechanisms, constant interaction of enhancer with promoter versus imprinting a state of transcriptional regulation, are common notions in current work on chromatin structure and gene regulation (Paro, 1990; Pirrotta, 1990; Shaffer, Wallrath, & Elgin, 1993). Understanding the mechanisms underlying these different allelic position effects may further our knowledge of chromatin structure, gene regulation and the relationship between the two.

Implications of transvection

It is striking that the known examples of transvection operate in large genes (over 30kb) that have complex expression patterns, with correspondingly large transcriptional regulatory regions, and that function at multiple times and places in development. All three genes, BX-C, *dpp* and *eya*, have vital functions. In all three cases, the transvection effects observed involve one allele that is defective for a specific adult tissue, but that still provides the vital function.

Two of the three loci (the BX-C and *dpp*) have known mammalian homologs (the Hox genes and TGF-ß gene, respectively; the *eya* gene was recently cloned and has a novel sequence; no mammalian homolog is yet known). One may be led to wonder, then, whether transvection is also found in mammals. Many facets of gene regulation are conserved across species: transcription factors, silencers, and non-histone chromatin proteins that can influence gene expression. Transgenic studies have shown that many gene regulatory elements can function in heterologous species [e.g., "insulators" in mice and *Drosophila* (Chung, Whiteley, & Felsenfeld, 1993)]. With so many similarities, therefore, it seems plausible that transvection effects could occur in mammals, as well.

For many years, it has been assumed that transvection does not occur in mammals because the strong association of mitotic chromosomes observed in *Drosophila* is not observed in mammals. Evidence is emerging, however, that mammalian chromosomes can pair in interphase. Mitotic recombination has been demonstrated with Bloom's syndrome, a recessive autosomal disorder, suggesting that homologous chromosomes in humans can pair. *In situ* hybridization to interphase chromosomes suggests that certain regions of some chromosomes may be paired in certain tissues and unpaired in others (Tartof & Henikoff, 1991). So, although chromosomal pairing may occur in humans, it is likely to be spatially and temporally restricted.

Somatic chromosome pairing in mammals might be important for DNA repair as it is in cells undergoing meisosis. Furthermore, pairing may conceivably be important for the proper expression of many genes, since the pairing of loci appears to be under developmental control. The study of transvection may shed light on this possibility. Even more than this, the study of transvection should provide insights into the mechanism of chromosome pairing itself. Genes that exhibit transvection effects could thus serve as assay systems to probe chromosomal pairing mechanisms, which is of fundamental importance for the proper assortment of chromosomes in all eukaryotic organisms, including humans.

Experimental Procedures

Fly strains: The alleles of *eya* used in this study are described in Chapter 2. The *zeste* mutation, $z^{ae(bx)}$, obtained from E. B. Lewis, is described in Lindsley and Zimm (Lindsley & Zimm, 1992).

Culture conditions: Flies were cultured on standard cornmeal medium at 25°C. For cytological analysis of polytene chromosomes, larvae were grown at 19°C, in a humidified atmosphere.

Screen for transvection disrupting rearrangements: Males (either eya^2 or eya^4) were exposed to 4000R, then mated to females mutant for the interacting allele. Approximately 2500 progeny from each cross were scored for reduced eyes. Twenty of the reduced-eye progeny were crossed to homozygotes of the interacting allele, to make sure the reduced-eye phenotype bred true. Of the twenty lines, 16 produced progeny and all of these had more severely reduced eyes than unirradiated eya2/eya4 heterozygotes. From these 16, 11 lines were successfully established and analyzed, with the irradiated second chromosome balanced over SM6a, *al* dp^{lv2} *Cy pr sp*.

Cytology: For cytological analysis of the salivary gland chromosomes, the second chromosome was balanced over In(2LR) Gla, *Gla Bc*. Males of this genotype were mated to CS or OR females. Salivary glands from Bc^+ crawling 3rd instar larvae were dissected, fixed in 45% acetic acid for 1-5 min., then treated in 1-2-3 solution (1 volume lactic acid, 2 volumes H_20, and 3 volumes acetic acid) for 1-2 min., after which they were placed on a cover slip in a drop of lacto-acetic-orcein solution and allowed to stain for several minutes then squashed using standard techniques.

Scanning electron microscopy: Flies stored in 70% ethanol were dehydrated by incubating for a minimum of six hours in 85%, 95%, then three times in 100% ethanol. Next, the specimen were critical-point dried, mounted, and coated with gold-palladium 80:20. The microscopy was performed using 5kV.

Quantitation of eye size: Ommatidia were counted from scanning electron micrographs of randomly selected specimens of each genotype.

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Figure 1. Interaction of two alleles of *eya*. (A-D)Scanning electron micrographs showing the adult eye phenotype.

(A) eya^{2}/eya^{2}

(B) eya^{4}/eya^{4}

(C) eya^{2}/eya^{4}

(D) Canton-S (wild type)

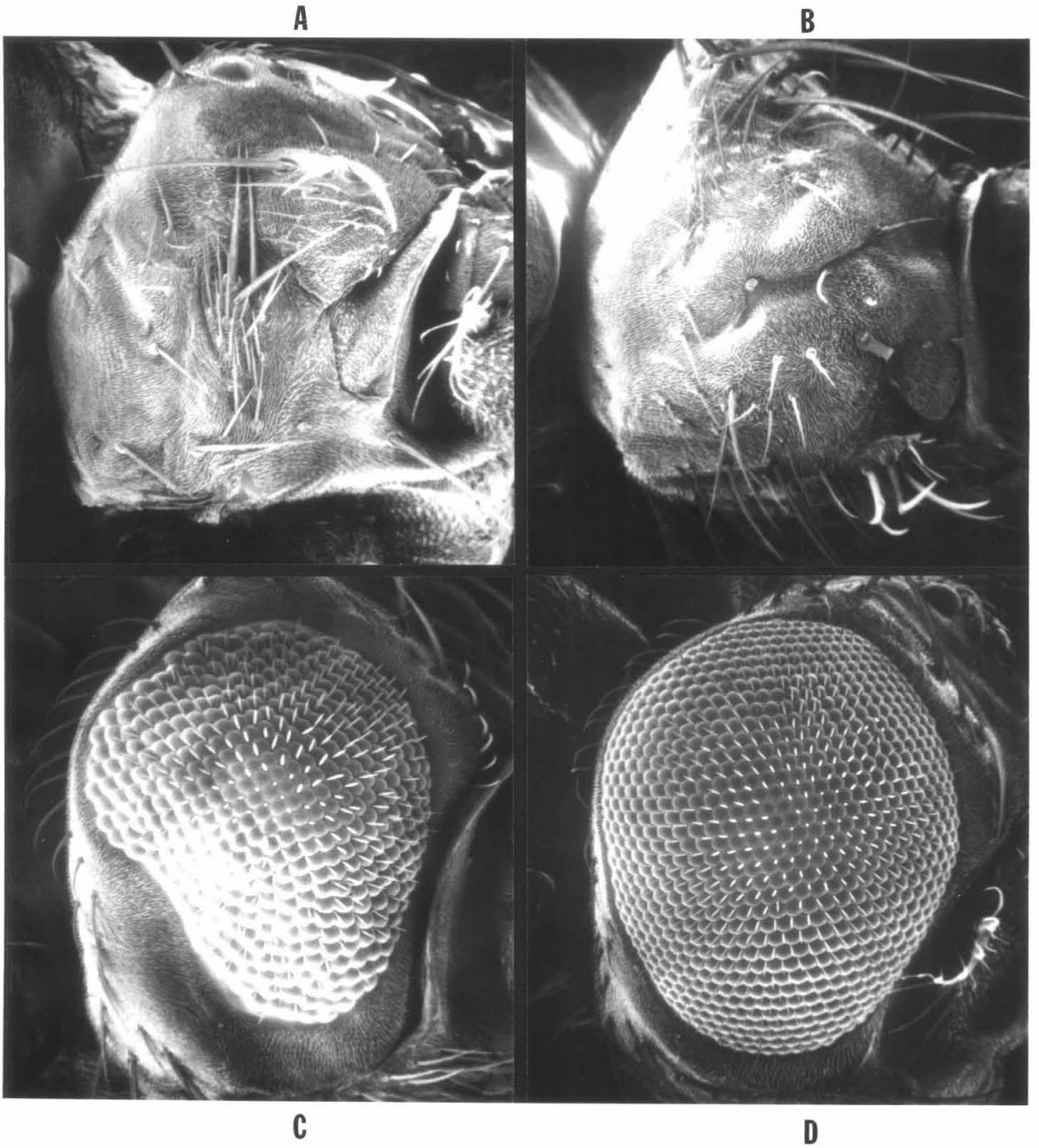
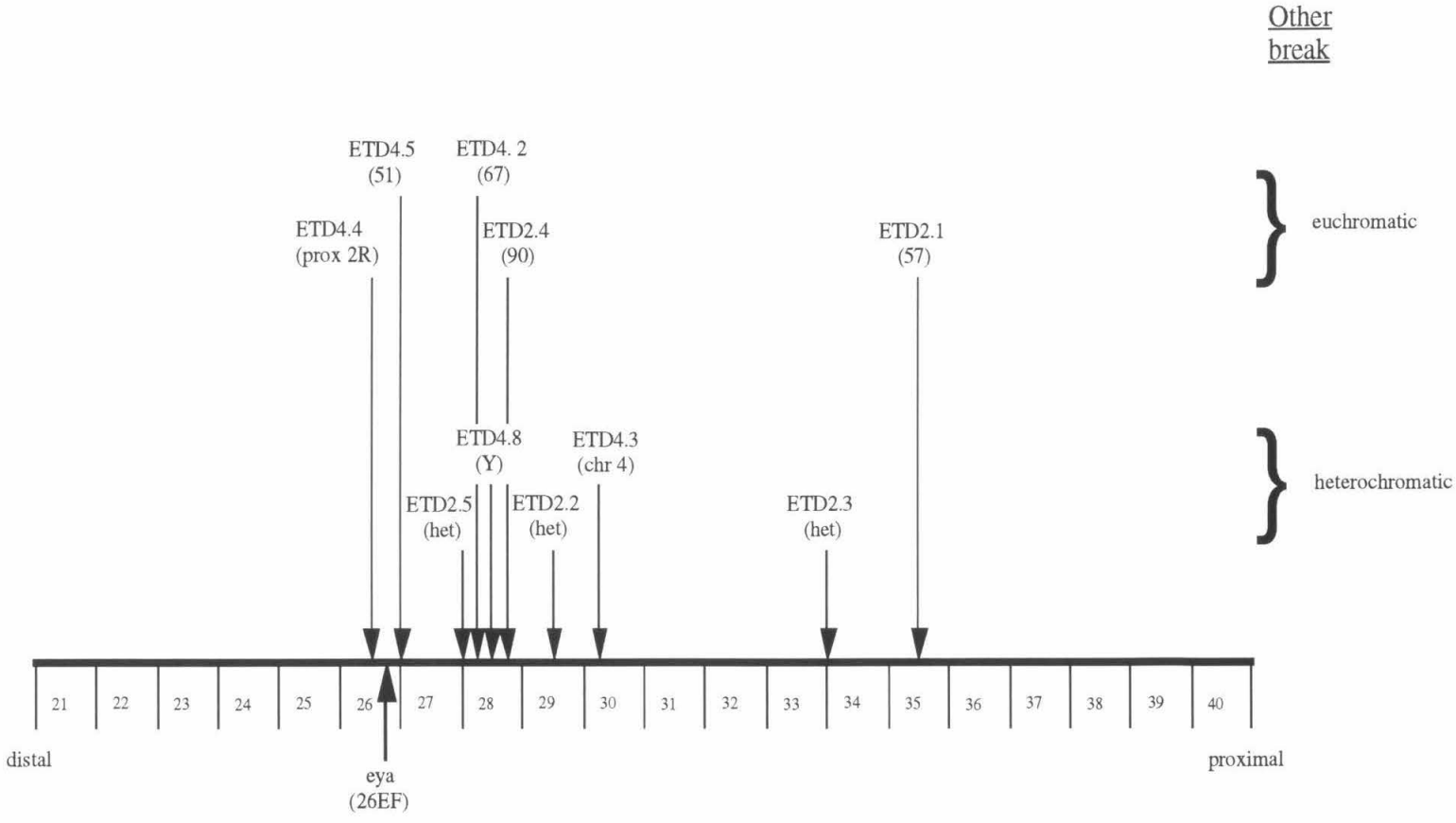

A
B
C
D

Figure 2. Distribution of *eya* transvection-disrupting breakpoints. The X axis represents the segments of polytene chromosome 2L. The breakpoint on 2L of each line is indicated. In parentheses is shown the location of the other breakpoint to which the distal portion of 2L has rearranged. The upper level corresponds to rearrangements having their second breakpoints in euchromatic regions, the lower level to ones in heterochromatic regions.



Break on chromosome 2L
(polytene division)

Figure 3. Transvection-disrupting lines of *eya*. (A-D) Scanning electron micrographs showing the eye phenotype of mutants bearing different combinations of *eya* transvection-disrupting rearrangement

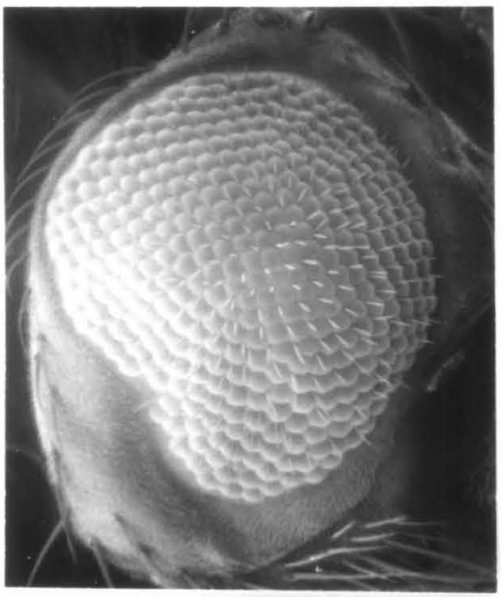
(A) eya^{2}/eya^{4}

(B) ETD2.5,eya^{2}/eya^{4}

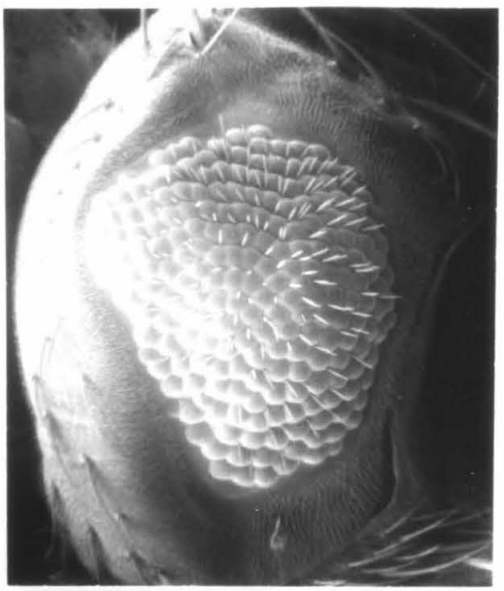
(C) ETD4.3,eya^{4}/eya^{2}

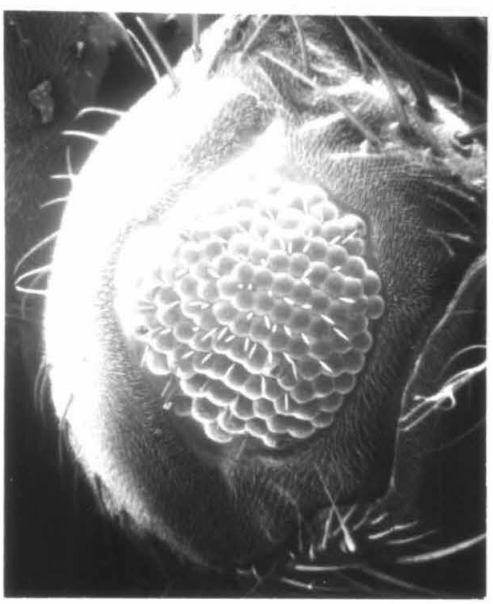
(D) ETD2.5,eya^{2}/ETD4.3,eya^{4}

A

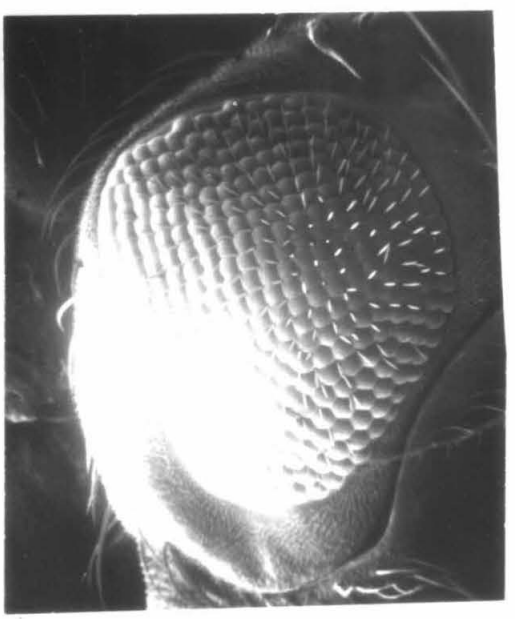


B





C



D

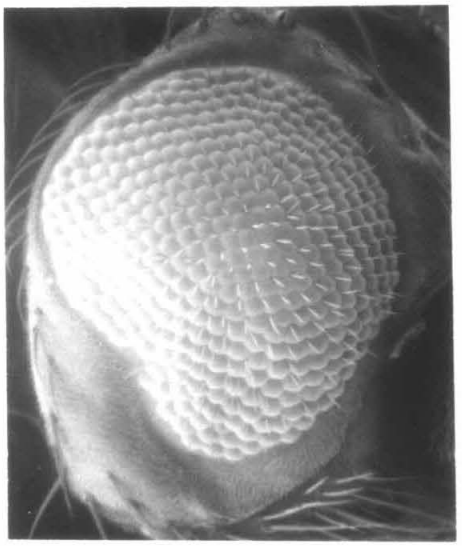
Figure 4. A loss-of-function *zeste* mutation suppresses transvection at *eya*.

(A,B) Scanning electron micrographs showing eye phenotypes

(A) z^{+};eya^{2}/eya^{4}

(B) $z^{ae(bx)}$;eya^{2}/eya^{4}

A



B

Table I. Analysis of *eya* transvection disrupting lines

Line[1]	Eye Phenotype[2]	Cytology	Remarks
ETD4.2	1/4 to 1/2	T(2L;3L)28;67A & In(3L)61A;65E	
ETD4.3	1/8 to 1/4	T(2L;3R;4) 30A;101-102;96-97	cyclical translocation: tip 2L-4; tip 4 to 3R; tip 3R to 2L
ETD4.4	1/4 to 1/2	2L (26D); 2R (58 and proximal); 3R (81)	complex: tip of 2R rearranged to base of 3R; tip of 3R to inversion of 2
ETD4.5	1/8 to 1/4	In(2LR) 26EF;51CD	
ETD4.6	eyeless	Df(2L)25E-26F	
ETD4.8	1/8 to 1/4	T(2;Y)28C-29A	
ETD2.1	1/4 to 1/2	3 breaks: 35;57;3R?	
ETD2.2	1/8 to 1/4	29;het[3]	
ETD2.3	1/4 to 1/2	33E34A;het	
ETD2.4	1/8 to 1/4	T(2L;3R)28E;90C	
ETD2.5	1/8 to 1/4	T(2L;3L)27E-28A;80	

[1]Line nomenclature: ETD (*eya* transvection disruptor); ETD2 lines were derived from eya^2 chromosomes; ETD4 lines were derived from eya^4 chromosomes

[2]Eye phenotype is given of the chromosome in *trans* to the interacting allele (eya^2 or eya^4, as appropriate). The entry is given as fraction of normal eye size, as judged through the stereomicroscope. The eya^2/eya^4 mutant eye is 3/4 the size of a normal eye.

[3]Abbreviation: het=heterochromatin of unknown chromosomal origin

Table II. Quantitation of transvection disruption results

Figure	Genotype	number of ommatidia
3A, 4A	+;al dp eya^2/al dp eya^4	381 ± 28 (n=5)
3B	+; ETD2.5,eya^2/eya^4	207 ± 30 (n=4)
3C	+; ETD4.3,eya^4/eya^2	114 ± 41 (n=4)
3D	+; ETD2.5,eya^2/ETD4.3,eya^4	283 ± 25 (n=4)
4B	$z^{ae(bx)}$;al dp eya^2/al dp eya^4	221 ± 30 (n=4)

The number of ommatidia were counted on scanning electron micrographs of eyes corresponding to the genotypes shown in figures 3 and 4.

Chapter V

Discussion and Future Prospects

Loss of function of the *eya* gene in the eye disc results in excessive numbers of progenitor cells undergoing cell death

The reduced eye and eyeless phenotypes associated with *eya* mutants are the direct result of loss of function of the gene in eye progenitor cells. The gene has multiple developmental functions: in addition to the eye, there is an embryonic phenotype. Mosaic analysis and *eya* expression studies indicate that there is a tissue-specific requirement of the gene in the eye, and that the eye phenotype is not the secondary or epigenetic consequence of failure of the gene to function in the embryo. Analysis of cell death in the eye discs of these mutants reveals that progenitor cells undergo cell death prior to the passage of the furrow. Thus, rather than differentiate, mutant eye progenitor cells die.

Morphologically, this cell death looks identical to apoptosis or programmed cell death. By the same criteria, the mutant cell death is indistinguishable from a small amount of cell death observable ahead of the furrow in normal eye discs. This normal cell death appears as a wave that precedes the passage of the furrow. The analysis of an *eya* allelic series indicates that cells that lack the *eya* gene appear to die at the same stage as the normal wave of cell death.

Structure of the *eya* gene and its relationship to transvection

Reflecting the duality of the embryonic and eye phenotypes, the structure of the *eya* gene reveals two transcripts and two different maps of these tissue-specific functions. The embryonic function maps to the 3' half of the *eya* region, while the eye function maps across the entire region. The type II transcript co-maps to the embryonic function and is predicted to normally encode the embryonic function. Preliminary results support this prediction.

The structure of the gene may also be reflected in the allelic interaction by transvection. The *eya* gene shares similarities with the other two loci at which transvection has been demonstrated, BX-C and *dpp*. All three genes are large, have multiple functions

in development, and appear to have (or have been demonstrated to have) one interacting class of alleles that have lesions in transciptional regulatory regions. For all three loci, this class of alleles produces a visible adult phenotype, while maintaining a separate vital function. In all three loci, these alleles are able to interact with alleles exhibiting a more widespread loss of function, that have been shown in some cases to involve mutations affecting the transcription unit. Thus, observations at *eya* lend support to the model of transvection as the *trans*-action of transcriptional regulatory regions. The identification of the molecular lesions in the various interacting alleles will help test this model.

Molecular function of the *eya* gene

The cloning and sequencing of the gene revealed two alternatively-spliced products whose predicted protein sequences differ at their N-termini. The *eya* sequence is the first of its kind to be discovered, and so provides little insight into the function of the protein. But there are expression and genetic data that speak to this issue.

The *eya* protein appears to function in a permissive way: ectopic expression of the gene (either cDNA) leads to no detectable phenotype, except the rescue of the *eya* mutant phenotype. This suggests that the gene product is either incapable of functioning without being "activated," or that other factors are required for the gene to exert its effect. For example, in order to function the Eya protein might require modification (e.g., phosphorylation), or interaction(s) with other proteins. This is a situation similar to the Sevenless receptor tyrosine kinase, for which ectopic expression of the wild type gene has no effect on development (Basler & Hafen, 1989; Bowtell, Simon, & Rubin, 1989). Ectopic expression of a Sevenless protein with a constitutively-active regulatory domain, however, leads to a dominant phenotype (Dickson, Sprenger, & Hafen, 1992). Perhaps Eya has a similar regulatory domain, which, when removed, could lead to detectable dominant phenotypes. It is interesting to note that the sequence of the gene appears to define two regions of the molecule: the N-terminal half with strings of amino acid repeats

and the C-terminal half with three regions of alternating charge. Perhaps these regions correspond to functional domains of the protein.

The N-termini of the two Eya proteins appear to provide the same function to the molecule in the eye, since the two proteins are indistinguishable in their ability to rescue *eya* mutants. This could mean that these are non-essential parts of the protein. On the other hand, they could be polypeptides of divergent sequence that nontheless provide the same function in the eye, e.g., to mediate interactions with other proteins. In any case, our results make it unlikely that the presence of the two alternatively-spliced transcripts arose because of functional differences in the protein products. Instead, it seems likely that they are under different transcriptional control.

The Eya protein is primarily found in the nucleus, suggesting a nuclear function for the gene product. Examples of such functions are transcription and its regulation, post-transcriptional processing, management of the genome (e.g., DNA repair, packaging, replication), and nuclear structure. As more is learned about the molecular function of the Eya protein, increasingly incisive experiments that address its role in the eye can be designed.

The role of *eya* in eye development

A wave of cell death precedes the furrow, in both wild type and *eya* mutant eye discs, suggesting that there may be a selection point ahead of the furrow. From the analysis of cell death in an allelic series of *eya* mutants, it appears that mutant eye progenitor cells are adopting a normal cell fate, at a normal time of development, but in excessive numbers. The function of this cell death, like the cell death observed in many places of vertebrate development, remains uncertain. However, based on the *eya* allelic series, this is the stage in *Drosophila* eye development when the total number of ommatidia that will comprise the adult eye is determined. The programmed cell death that occurs later during the pupal stage prunes the number of cells associated with each ommatidium, but does not alter the total

number of ommatidia (Wolff & Ready, 1991). Thus, the cell death in the eye disc may be functionally akin to the cell death in the vertebrate nervous system (to determine the total number of neurons), except that it occurs at an earlier developmental stage. This is only one possibility for the cell death that occurs ahead of the furrow in *Drosophila*. Naturally, there are many other possible explanations. Exploring the basis of the selection in the *Drosophila* eye may help reveal the function of developmental cell death in other organisms.

Little is known about the events that precede the furrow, which marks the first obvious signs of cell fating and pattern formation in the eye disc. Because of this, understanding the role of *eya* in this process may take some time. Nevertheless, one study revealed an important change in the ability of different stage eye discs to undergo morphogenesis (Gateff & Schneiderman, 1975). The analysis of heterochronic transplants of larval eye discs of different stages into metamorphosing hosts showed that, starting in the middle of the second instar, cells could be induced to differentiate precociously into eye tissue. This suggests there is an important cell fating event that starts during the second instar, and has been referred to as the gaining of competence.

The relationship between *eya* function and competence is unknown. It is interesting to note that *eya* expression in the eye disc is initiated during the same stage as competence is acquired. The relationship between these two events may be a coincidence, since different cell types were shown to gain competence at different stages, while *eya* mutations seems to affect all cell types equally. This could be explained if weak *eya* hypomorphs affect eye progenitor cells in a stochastic manner; one might not observe any pattern in the loss of cell types.

There is a second piece of evidence that weighs against the *eya* product conferring competence on its own. Ectopic expression of the *eya* gene does not produce a phenotype, suggesting that the *eya* product alone can not bring about a change in the developmental state of cells. In any case, the acquisition of competence may not be the result of the

expression of one gene. Instead, it may be a state of the cell that is acquired through the proper expression of many genes. This may explain why different cell types acquire competence at different stages of development. Thus, it is not known whether the *eya* gene product is necessary, let alone sufficient, to confer competence on immature eye progenitor cells.

If *eya* gene expression were necessary for the gaining of competence, then one might envision the wave of cell death ahead of the furrow as a selection against incompetence. In normal development, cells that failed to acquire the proper state would be induced to die. In *eya* mutants, cells that fail to express enough *eya* gene product would be selected against in the same way. One way to test whether the selection point is a test for non-competence would be to transplant non-competent eye discs into metamorphosing hosts, then see if the cells in the transplant undergo programmed cell death. It is known that such transplants into adult hosts survive and proliferate. If the selection ahead of the furrow is against non-competent cells and can be triggered by a metamorphosing environment, then non-competent transplants would be expected to die in the face of such selection. In this case, *eya* might be considered necessary for the acquisition of competence.

To test whether *eya* is sufficient to impart competence on eye progenitor cells, one could precociously express the gene in non-competent eye discs, then transplant them into metamorphosing hosts. If such discs were able to differentiate into adult structures, then one could conclude that *eya* is sufficient to make eye discs competent.

The role of cell death in development

Selection criteria are thought to be known for some developmental cell death, such as the late refinement of patterning in the *Drosophila* eye (Wolff & Ready, 1991) and the selection of the immune repertoire in vertebrates (Fesus, 1991; Goldstein, Ojcius, & Young, 1991). The idea that cell death is important in vertebrates for the eradication of potential neoplasms has even been proposed (Williams, 1991). Furthermore, it has been

suggested that the default for all mammalian cells may be cell death, as a method of eliminating misplaced, extraneous, or inappropriate cells (Raff, 1992).

In contrast, in most systems for early development, the selection criteria remain largely undefined at this time. Even in the nematode, *C. elegans*, where mutants that prevent the occurrence of most cell deaths in the organism exist, the lack of cell death has no major impact on the adult, except that it has extra cells (Horvitz, Ellis, & Sternberg, 1982). In *Drosophila*, a genomic region has been identified which, when deficient, prevents embryonic cell deaths (H. Steller, pers. comm.). Phenotypic analysis of such embryos may shed light on the role of cell death in development. The study of *eya* and cell death in the eye provides a genetic system to identify genes that function in such an event.

It would be of great interest to know whether or not elimination of the wave of cell death ahead of the furrow has a detectable effect on development. With our current assays for cell death, it would be hard to know whether certain mutations or treatments repress the small amount of cell death ahead of the furrow, and therefore to see the effect of its loss. It is possible that lack of cell death would have no detectable phenotype in the eye, although this would not rule out a selection point there. The wave of cell death found in *eya* mutants strongly supports the existence of a selection point; if the lack of cell death produced no visible phenotype, it could be because there are other mechanisms that correct the deficit.

Prospects for the future

The study of cell death in the eye disc will likely follow a different path from studies of cell death in *C. elegans* and the *Drosophila* embryo. The discovery of a gene such as *eya*, whose earliest mutant phenotype in the eye disc is cell death, allows for the study of the regulation of cell death and the linking of the cell death pathway to differentiation. Finding out where *eya* functions with respect to other genes involved in the pathways of cellular differentiation and cell death will help this cause. This goal might be achieved by biochemical and genetic approaches to identify proteins that interact with the *eya* gene

product. These future studies may also provide a dividend, shedding light on the early events in eye development, prior to the passage of the furrow.

The *eya* gene, with so many possibilities to help unravel the mysteries of death and differentiation, may also be important to the study of another fundamental aspect of development: gene regulation. The discovery of transvection at *eya* makes the phenomenon initially described by Lewis (Lewis, 1954), more likely to be a general property of genes. If the study of other position effects is any indication, the study of transvection may advance the understanding of gene regulation, chromatin structure, and the relationship between the two. Furthermore, the ability of transvecting alleles to sense even modest changes in chromosome pairing may help elucidate the mechanisms underlying this event. The use of transvecting *eya* alleles may prove beneficial in this regard, since the effects of chromosomal pairing can be quantified by observing the size of the eye. Truly, the prospects are bright for *eya* to further our knowledge of mechanisms of development at multiple levels.

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