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RESEARCH ARTICLE

Induction of Ectopic Eyes by Targeted Expression of the *eyeless* Gene in *Drosophila*

Georg Halder,* Patrick Callaerts,* Walter J. Gehring†

The *Drosophila* gene *eyeless* (*ey*) encodes a transcription factor with both a paired domain and a homeodomain. It is homologous to the mouse *Small eye* (*Pax-6*) gene and to the *Aniridia gene* in humans. These genes share extensive sequence identity, the position of three intron splice sites is conserved, and these genes are expressed similarly in the developing nervous system and in the eye during morphogenesis. Loss-of-function mutations in both the insect and in the mammalian genes have been shown to lead to a reduction or absence of eye structures, which suggests that *ey* functions in eye morphogenesis. By targeted expression of the *ey* complementary DNA in various imaginal disc primordia of *Drosophila*, ectopic eye structures were induced on the wings, the legs, and on the antennae. The ectopic eyes appeared morphologically normal and consisted of groups of fully differentiated ommatidia with a complete set of photoreceptor cells. These results support the proposition that *ey* is the master control gene for eye morphogenesis. Because homologous genes are present in vertebrates, ascidians, insects, cephalopods, and nemerteans, *ey* may function as a master control gene throughout the metazoa.

The *eyeless* (*ey*) mutation of *Drosophila* was first described in 1915 (*1*) on the basis of its characteristic phenotype, the partial or complete absence of the compound eyes. The *ey* alleles available today are recessive hypomorphs (weak alleles) and they lead to the reduction or complete absence of the compound eyes but do not affect the ocelli (*simple eyes*) on the head of the fly. Apparent null alleles that are lethal when homozygous have also been isolated (*2*), but they have been lost, and a detailed analysis of their phenotype is not available. Cloning and sequencing of the *ey* gene (*3*) have shown that it encodes a transcription factor that contains both a paired domain and a homeodomain. The *ey* gene is homologous to *Small eye* (*Sey* = *Pax-6*) in the mouse and to *Aniridia* in humans. The proteins encoded by these genes share 94 percent sequence identity in the paired domain, and 90 percent identity in the homeodomain and they contain additional similarities in the flanking sequences. Furthermore, two out of three splice sites in the paired box and one out of two splice sites in the homeobox are conserved between the *Drosophila* and the mammalian genes, which indicates that these genes are orthologous.

Both the mouse and the *Drosophila* gene have similar expression patterns during development. In the mouse, the expression of *Sey* is observed in the spinal cord, in discrete regions of the brain, and in the developing eye. The *Sey* gene is expressed from the earliest stages until the end of eye morphogenesis: first, in the optic sulcus, and subsequently in the eye vesicle, in the lens, in the differentiating retina, and finally in the cornea (*4*). In *Drosophila*, *ey* is first expressed in the embryonic ventral nerve cord and in defined regions of the brain. Later in embryogenesis, *ey* is transcribed in the embryonic primordia of the eye as soon as these cells can be detected. In subsequent larval stages, it continues to be expressed in the developing eye imaginal discs. During the third larval stage, *ey* expression becomes largely restricted to the part of the eye disc that is anterior to the morphogenetic furrow. This region consists of undifferentiated cells whereas posterior to the furrow the differentiating ommatidia are apparent (*5*). Because mutations in the mouse and *Drosophila* genes lead to a reduction or complete absence of all eye structures, and because these genes are similar in DNA sequence and in expression pattern even at the earliest stage of eye development, it has been suggested that *ey* and *Sey* may be the master control genes involved in eye morphogenesis (*3*). Furthermore, mutations in four other *Drosophila* genes with similar phenotypes (*eyes absent*, *sine oculis*, *eye gone*, and *eyelisch*) do not affect the expression pattern of *ey*, which indicates that *ey* acts upstream of these other genes (*6*). These results are consistent with its possible role as a gene that controls eye morphogenesis, even though it may have additional functions in the developing nervous system. The cloning of the homologous genes from ascidians, cephalopods, and nemerteans (ribbon worms) suggests that this gene may be present in all metazoa (*3*).

Master control genes that act as developmental switches can be detected on the basis of their mutant phenotypes. Thus, homeotic mutations have identified master control genes that specify the body plan along the antero-posterior axis. These genes, which are characterized by a homeobox, are clustered in the Antennapedia (*Antp*) and Bithorax Complexes in *Drosophila*, and in the Hox gene clusters of the mouse (*7*). Loss- and gain-of-function mutations in these genes lead to opposite homeotic transformations. For example, in *Antp*, recessive loss-of-function mutations are lethal at the embryonic or larval stage and lead to a transformation of the second thoracic segment (T2) toward the first thoracic segment (T2→T1). Dominant gain-of-function mutations lead to a transformation in the opposite direction, that is from the anterior head and T1 segments toward T2 (H,T1→T2) (*8*). These transformations can be explained by the combinatorial interaction of several homeotic genes in order to specify a given body segment. These genes have partially overlapping expression domains in several body segments and each segment is specified by a combination of homeobox genes, that is by a Hox code (*9*). By ubiquitous (ectopic) expression of *Antp* under the control of a heat-shock promoter, we have changed the body plan of *Drosophila* and induced the formation of middle legs in place of the antennae, and

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also transformed the dorsal head capsule into structures of the second thoracic segment (H→T2). This phenotype is similar to that observed in dominant gain-of-function mutations (10). However, it proved to be difficult to transform the more posterior body segments toward T2. Data for several homeotic genes indicate that there is competition between the ectopically expressed gene and the genes normally expressed in a given segment (11). This competition frequently leads to epistasis of the posterior over the anterior genes, and to segmental transformations that are confined to the anterior body segments.

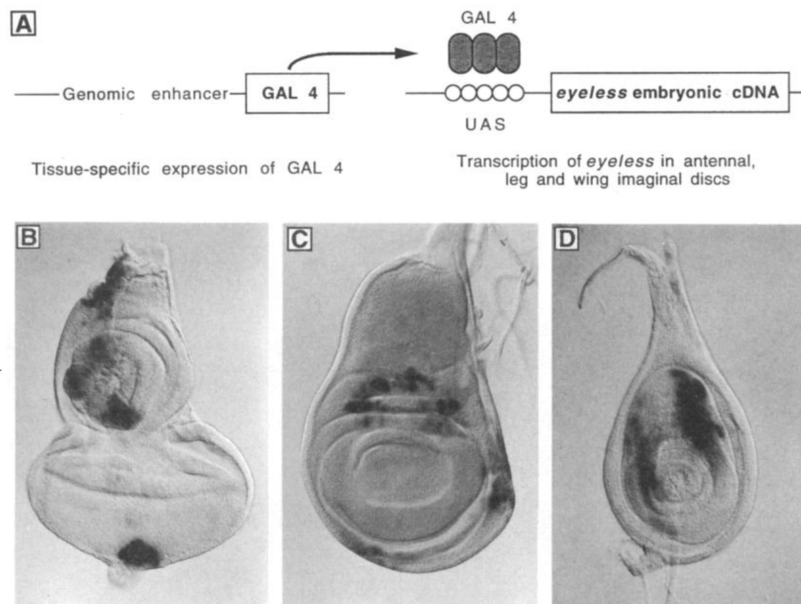

Fig. 1. Targeted expression of *ey*. (**A**) Schematic representation of the ectopic induction of *ey* by means of the GAL4 system. In (B) through (D), β-galactosidase staining of third instar imaginal discs (28) shows the activation of a UAS-*lacZ* reporter construct by the GAL4 enhancer-trap line E132. (**B**) Eye-antennal disc. The antennal portion of the disc is on the top and the eye portion is on the bottom. β-Galactosidase activity is detected in parts of the antennal disc corresponding to several antennal segments and in the periphery of the disc, which will give rise to head cuticle. The staining observed at the most posterior part of the eye disc derives from the optic nerve. (**C**) Wing imaginal disc. β-Galactosidase activity is detected in proximal regions of the future wing blade, and in portions corresponding to the hinge regions and ventral pleura. (**D**) Leg imaginal disc with *lacZ* expression in portions that correspond to the tibia and femur.

The *ey* gene, which also contains a homeobox in addition to a paired box, differs from *Antp* and the other antero-posterior homeotic genes in that the hypomorphic loss-of-function mutation leads to a loss of the corresponding eye structures rather than to their homeotic transformation. This phenotype does not necessarily imply that *ey* acts as a developmental switch; it only shows that *ey* function is required for eye development. If, however, *ey* is the master control gene for eye morphogenesis, the ectopic expression of *ey* should induce the formation of ectopic eye structures in other parts of the body similar to the transformations obtained for *Antp* (10) and the other homeotic genes (11). Therefore we used the GAL4 system (12) and a heat-inducible expression vector in order to express the *ey* gene ectopically.

Induction of ectopic eye structures. We used the GAL4 system (12) to target *ey* expression to various imaginal discs other than the eye discs in which *ey* is normally expressed. GAL4 is a yeast transcriptional activator that can activate transcription of any gene after introduction into *Drosophila* if the gene is preceded by a GAL4 upstream activating sequence (UAS) that consists of five optimized GAL4 binding sites (12). The GAL4 system is now widely used in conjunction with a method called enhancer detection (13), in which a reporter gene is provided with a weak promoter only and inserted at random sites in the genome by transposition. If the detector has inserted close to an enhancer, the reporter gene is expressed differentially. By isolating a large number of enhancer detection lines, a spectrum of different enhancers with specific temporal and

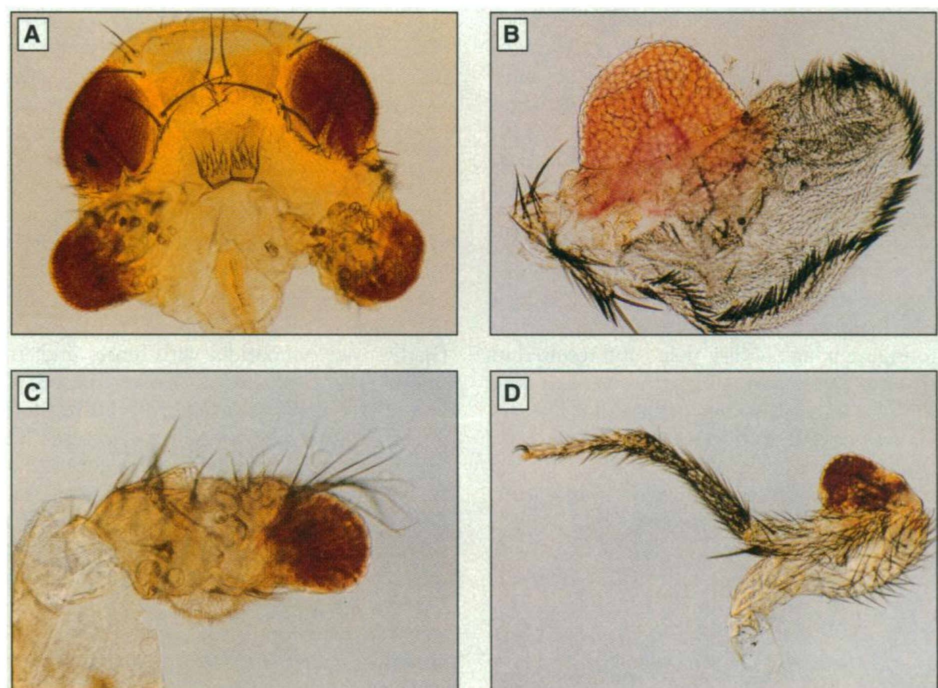

Fig. 2. GAL4 driven ectopic expression of *ey* induces the formation of eye structures in various tissues. The sites at which ectopic eyes form correspond to the regions in the imaginal discs, in which GAL4 is expressed as assayed by the activation of a *lacZ* reporter construct (Fig. 1, B, C, and D). The ectopic eye structures show ommatidial arrays, interommatidial bristles, and red pigmentation (29). (**A**) Cuticle of an adult head in which both antennae formed eye structures. (**B**) Dissected wing with a large outgrowth of eye tissue. The ectopic eye contains about 350 facets. Many interommatidial bristles are also apparent. The normal eye contains approximately 800 ommatidia. The wing is reduced in size. The anterior margin with its characteristic triple row of bristles occupies most of the circumference, whereas the more posterior structures are absent and replaced by eye tissue. The characteristic venation pattern of the wing is disturbed by the formation of the ectopic eye structures. (**C**) Dissected antenna in which most of the third antennal segment is replaced by eye structures. (**D**) Dissected middle leg with an eye-outgrowth on the base of the tibia.

spatial patterns of control can be identified. If *GAL4* is used as a reporter gene, these enhancer detection lines can be used for targeted gene expression; the enhancer drives the specific expression of GAL4, which in turn can transactivate a target gene, in our case *ey* provided with a UAS. As indicated in Fig. 1A, the GAL4 enhancer detection line was crossed to a UAS-*ey* stock to generate transheterozygous flies that express *ey* in those cells that express GAL4. We chose approximately 20 GAL4 lines, of which only 3 gave viable adult flies to analyze in more detail (*14*). The results are illustrated for the GAL4 line E132. When E132 is crossed with a stock containing a UAS-*lacZ* construct, β-galactosidase staining reveals the activation of the *lacZ* reporter gene by GAL4 and thus the expression pattern of GAL4 in the imaginal discs. E132 expresses GAL4 in discrete regions of the wing and haltere discs, all three pairs of leg discs, and in the antennal imaginal discs (Fig. 1, B through D), which are the primordia for the respective adult structures. When the GAL4 expressing line E132 is crossed with a stock carrying an *ey* embryonic complementary DNA (cDNA) (*15*) under a GAL4-UAS control element, transheterozygous flies can be generated, and the expression of *ey* can be targeted into the imaginal discs as mentioned above (Fig. 1, B, C, and D for *lacZ*). In the wild-type controls *ey* is only expressed in the eye discs.

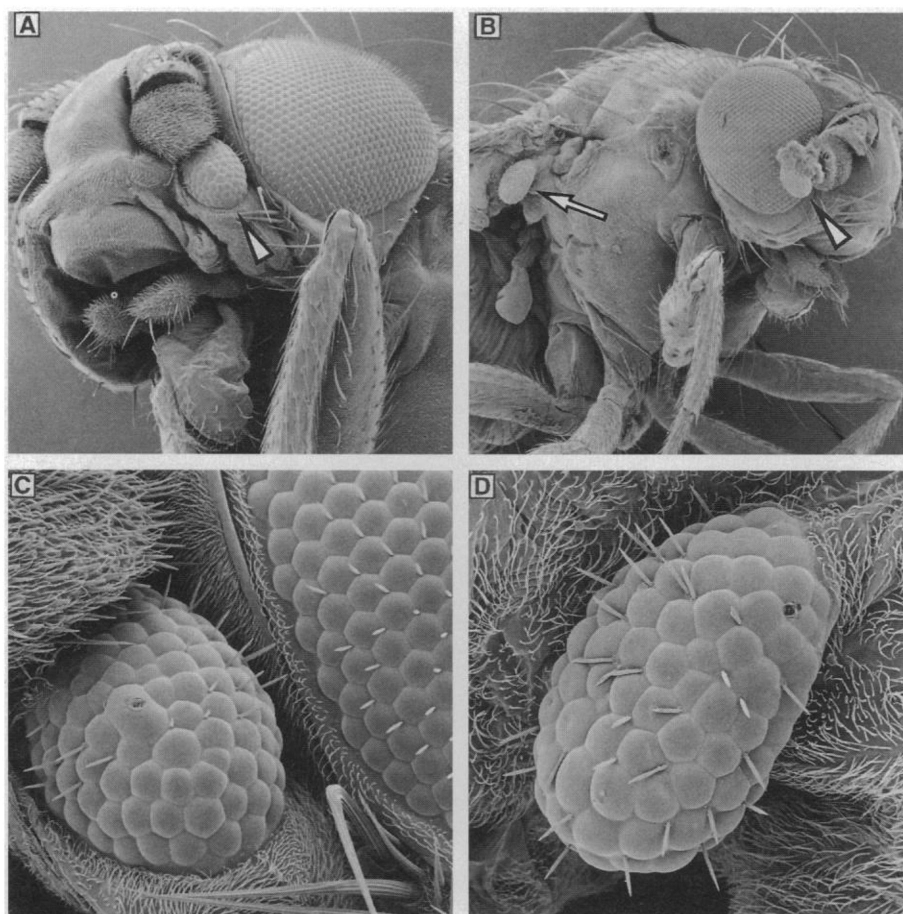

Fig. 3. Scanning electron of ectopic eyes (*30*). (**A**) Scanning electron micrograph of an ectopic eye (arrowhead) in the head region formed by the antennal disc. (**B**) Overview of a fly with an ectopic eye under the wing (arrow) and on the antenna (arrowhead). (**C**) Higher magnification of (A). The ectopic eye (to the left) contains hexagonal ommatidia and interommatidial bristles. The organization of the facets in the ectopic eye is very similar to the pattern in the normal eye (to the right). Some facets, however, are fused and some irregularities in the form of the facets are observed. (**D**) Higher magnification of the ectopic eye under the wing shown in (B) (arrow). The ectopic eye protrudes out of the thoracic body wall (ventral pleura). The organization of the facets and interommatidial bristles are similar to that of the ectopic eye shown in (C).

As a consequence of ectopic *ey* expression in line E132, ectopic eye structures were induced in the wings (Fig. 2A), all six legs (Fig. 2B, for mesothoracic legs), the antennae (Fig. 2C), and the halteres. When the flies were raised at 25°C, at which temperature the cold-sensitive GAL4 is properly active, 100 percent of the transheterozygotes produced ectopic eye structures. We observed that the eye structures in the adult cuticles bulged out of the tissue in which they were induced. This phenomenon is illustrated for the wing in scanning electron micrographs (Fig. 3, B and D), and could represent sorting out of heterotypic cells in order to minimize the contact surface between the two tissue types (*16*). In some cases, the development of the ectopic eyes interfered with pattern formation in the surrounding imaginal disc tissue and resulted in pattern duplications. In the GAL4 line MS941, all of the flies expressed *ey* in the wing discs and produced eye facets on both wings. In line p339, which expressed GAL4 in a small spot in the wings in low amounts, only red pigment was formed, but again with 100 percent penetrance. We also used a heat-inducible promotor to express *ey* ubiquitously at various times during development. However, heat shocks during embryonic and most larval stages lead to developmental arrest. To circumvent this lethality, heat-shocks were applied after 80 hours during the middle of the third larval stage. Ectopic eye structures including complete ommatidia were induced. However, targeted *ey* expression by the GAL4 system was more effective.

The fine structure of the ectopic eyes was analyzed by scanning electron microscopy. Well-developed ectopic eyes were most frequently observed on structures derived from the antennal and wing discs (Fig. 3, A and B). Distinct ommatidia with lenses and interommatidial bristles were seen (Fig. 3, C and D). The array of facets and bristles were largely normal. However, we also observed fusion of facets and irregular spacing of bristles in some cases. The eye structures induced on the legs were on average smaller than the ones on antennae or wings but nevertheless appeared to have a relatively normal organization.

Photoreceptors in the ectopic eyes. Microscopic analysis of sections of ectopic eye structures indicated that the ectopic ommatidia consisted of the full complement of the different types of cells and structures (*17*). In a longitudinal section of an antennal ectopic eye, we were able to distinguish cornea, pseudocone, cone cells, primary, secondary, and tertiary pigment cells, and photoreceptors with rhabdomeres (Fig. 4, A and B). At the base of the ommatidia, we observed the feet of secondary and tertiary pigment cells and a basal lamina that formed a structure with features characteristic of the fenestrated membrane of the retina. On a transverse section, the normal trapezoidal array of rhabdomeres was clearly visible (Fig. 4B, arrowhead).

We also analyzed the neuronal differentiation of photoreceptors by means of ELAV antibodies (*18*). Clusters of photoreceptor cells were clearly detected at ectopic sites in the imaginal discs (Fig. 4, C and D), and the sequence of neuronal differentiation observed in the normal eye disc was retained in the ectopic eye cells. A number of single cells that expressed the neuronal marker

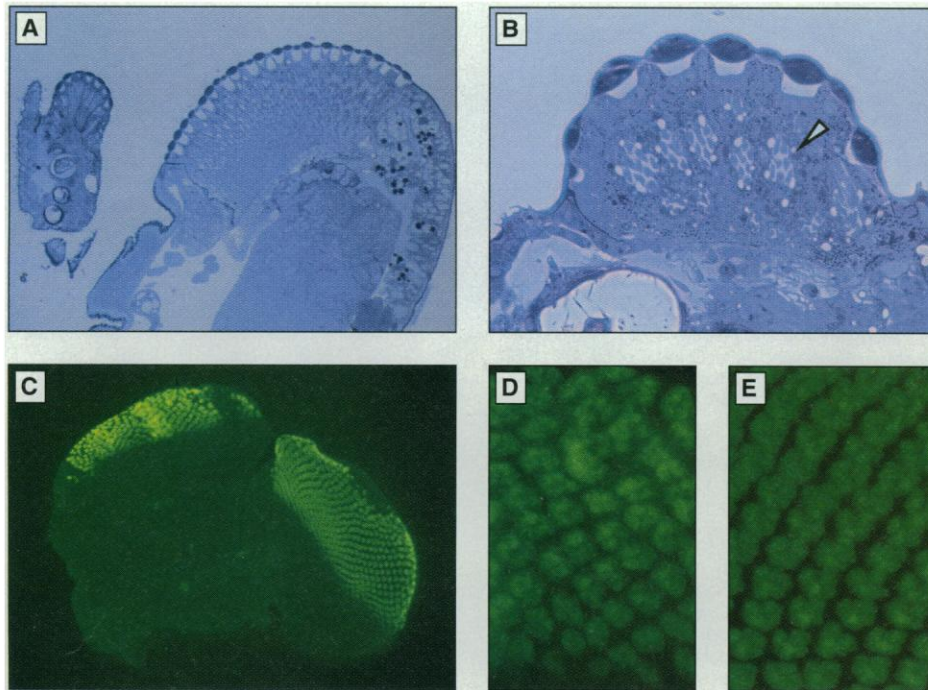

Fig. 4. Histological structure and differentiation of photoreceptors in the ectopic eye. (**A**) Micrograph of a section through an ectopic eye in the antenna (to the left) and the normal eye (to the right) stained with Azur II and methylene blue (*15*). (**B**) Phase contrast micrograph of a section through an ectopic eye on the antenna. The normal number and trapezoidal arrangement of the rhabdomeres of photoreceptors is observed in the different ommatidia (arrowhead). (**C**) Micrograph of an eye-antennal disc stained with an antibody against the neuronal marker ELAV and a secondary fluorescein-labeled antibody. In the normal eye portion (to the right), regularly spaced ommatidial clusters of differentiating photoreceptors are detected. In the antennal part of the disc (on the left), extensive cell proliferation has led to a doubling in size. In this portion, a large domain of ectopically induced photoreceptors is seen. (D) and (E) are higher magnification views of (C), which shows the photoreceptor clusters in the ectopic eye (**D**) and in the normal eye (**E**), respectively. An essentially normal cluster formation and cluster array is observed in the ectopic eye.

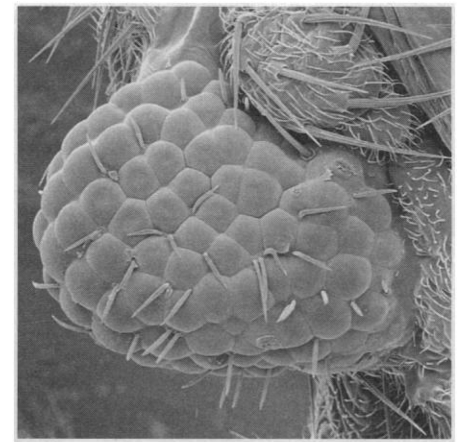

Fig 5. The ectopic expression of mouse *Pax-6* cDNA under the control of GAL4 induces the formation of ectopic eyes (*26*). The scanning electron micrograph shows a close-up of induced eye facets on a leg. Ommatidial arrays and interommatidial bristles very similar to the ectopic eye structures induced by the *Drosophila* gene (Fig. 3) were formed (*30*). In both cases the same GAL4 line E132 was used.

were seen at one side of an ectopic photoreceptor cluster. This expression most likely corresponds to the formation of R_8 photoreceptor cells. Subsequently, groups of three, five, seven, and eight cells were detected that expressed the ELAV epitope. This series of events probably corresponds to what is observed in a normal eye disc upon passage of the morphogenetic furrow. Thus, these observations suggest that morphogenesis of the ectopic eyes is normal and that it probably involves the formation of an ectopic morphogenetic furrow. In summary, the data presented above show that *ey* can induce the formation of complete and morphologically normal ectopic eyes. It is unknown whether these ectopic eyes are functional, and whether the axons of the photoreceptors innervate the correct domains of the brain, that is, the lamina and the dorsal deuterocerebrum, respectively (*19*). Initial evidence suggests that the photoreceptors in the ectopic eyes are electrically active upon illumination (*20*).

Role of *eyeless* in eye morphogenesis. The reported findings indicate that *ey* is the master control gene for eye morphogenesis, because it can induce ectopic eye structures in at least the imaginal discs of the head and thoracic segments. The expression of *ey* switches on the eye developmental pathway that involves several thousand genes. The number of genes required for eye morphogenesis can roughly be estimated on the basis of the frequency of enhancer detection lines that show reporter gene expression in the eye imaginal discs posterior to the morphogenetic furrow during eye differentiation. Because approximately 15 percent of a large sample of enhancer detector lines fall into this category (*21*), and assuming that the *Drosophila* genome contains at least 17,000 genes (*22*), we estimate that more than 2500 genes are involved in eye morphogenesis. Our results suggest that all of these genes are under the direct or indirect control of *ey*, which is at the top of the regulatory cascade or hierarchy. The *ey* gene is expressed first and controls a set of subordinate regulatory genes, including *sine oculis*, another homeobox-containing gene (*23*). Subsequently, genes that influence cell-cell interactions and signal transduction must be regulated and, finally, the structural genes like rhodopsin, crystallin, and transducin must be expressed. The lower part of this cascade, including signal transduction pathways, has been elucidated to a large extent (*24*), but the upper part, and which of these interactions are direct, remain to be determined. However, *ey* may not only control the initial steps of eye morphogenesis, but also, as suggested from the expression pattern, it may control later steps. Thus, the same transcriptional regulator may be used at consecutive steps of morphogenesis. This could be the consequence of the conservative mode of evolution whereby the same master control gene is used repeatedly to integrate new target genes into the eye developmental pathway. In addition to eye morphogenesis, *ey* controls other functions in the developing nervous system, because null mutations are lethal, and the loss of eye structures alone is not the cause of lethality.

The induction of ectopic eyes in *Drosophila* is reminiscent of the classical experiments of Spemann (*25*) in which he induced ectopic eyes by transplanting the primordia of the optic cup to ectopic sites in amphibian embryos. Our experiments extend these observations and identify the gene that is necessary and sufficient to induce ectopic eyes at least in imaginal discs. In the mouse, *Sey* is expressed at each step of the induction process; first in the optic cup, then in the lens, and finally in the cornea, which implies that *Sey* may be the master control gene in the mouse eye induction process (*4*).

The transformation of antennal, leg, and wing tissue into eye structures by *ey* induction indicates that *ey* is a homeotic gene. In contrast to the classic homeotic genes of the Antennapedia and Bithorax Complexes, hypomorphic loss-of-function mutations in *ey* do not lead to homeotic transformation, but rather, they result in the loss of eye structures. However, targeted ectopic *ey* expression induces homeotic transformations sim-

ilar to those observed in gain-of-function mutations of classic homeotic genes, like *Antp*. Therefore, *ey* represents a class of homeotic master control genes different from *Antp*. Gain-of-function mutants with phenotypes corresponding to those obtained in our targeted gene expression experiments have not been discovered previously.

The high degree of sequence conservation between the human, the mouse, and the *Drosophila* genes, the similarity of the phenotypes of *Aniridia*, *Sey*, and *ey*, and the similarity of the expression patterns suggested to us that *ey* might be a master control gene for eye morphogenesis that is shared by vertebrates and invertebrates (*3*). Because we also found homologous genes in ascidians, cephalopods, and nemerteans we propose that *ey* function is universal among metazoa. In order to test whether the mouse gene can substitute for the *Drosophila* gene, we also used the mouse *Sey* gene for targeted expression in *Drosophila*. Similar to the results obtained for the *Drosophila ey* gene, the mouse gene *Sey* can also induce the formation of ectopic eye structures (Fig. 5) (*26*). As expected, the ectopic eye structures formed contain *Drosophila*-type ommatidia and not mouse eye structures.

Previously, the function of other mouse homeobox genes has been demonstrated in *Drosophila* with the use of heat inducible vectors (*27*). In the case of HoxB6, *Drosophila* legs were induced in place of the antennae (*27*). Obviously, the responses, but not the transcriptional regulator, are species-specific.

The observation that mammals and insects, which have evolved separately for more than 500 million years, share the same master control gene for eye morphogenesis indicates that the genetic control mechanisms of development are much more universal than anticipated. It will be informative to compare the regulatory cascade required to form a *Drosophila* compound eye with that of a mouse eye, to find out what the differences are, and to determine how many new genes have been recruited into these developmental pathways in the course of evolution.

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14. GAL4-crosses were established at 25°C on standard medium. The GAL4 lines were crossed to a number of UAS-*ey* lines and most of these crosses led to embryonic or early larval lethality. This outcome is probably the result of the ectopic expression of *ey* in various tissues during embryogenesis because the GAL4 lines start to express GAL4 during embryonic stages. In crosses with the MS941, p339, and E132 lines, transheterozygote adults were recovered. For MS941, almost no lethality was observed, whereas for p339 and E132, a substantial number of dead embryos or larvae were noted. For line E132, virtually only females were obtained because most males died during the early phases of development. This result may be explained by the dependence of the lethality on the level of transactivation of *ey* by GAL4. In the E132 line, the enhancer detector construct is inserted into the X chromosome and therefore, is dosage-compensated in males. As a consequence, the transheterozygous males produce twice as much GAL4 activity as the females, and die during larval stages, whereas the females survive. Thus, all cuticles shown are derived from females.
15. The full-length embryonic cDNA was reconstructed in a Bluescript KS+ backbone from three Eco RI fragments. The full-length embryonic cDNA begins with a Hind III site [at position 45 in the published sequence (*3*)] and ends with an Xba I site (constructed by inserting Xba I linkers in the MsI I site at position 2741). The cDNA was inserted as an Xho I–Xba I fragment into the GAL UAS vector [pUAST (*11*)]. This construction results in an oriented insertion in which the cDNA is preceeded at the 5′ end by five optimized GAL4 binding sites, an hsp70 TATA box, the transcriptional start, and the cDNA is followed at the 3′ end by the SV40 intron and polyadenylation site. A *y ac w* stock was transformed as described [G. M. Rubin and A. C. Spradling, *Science* **218**, 348 (1982)]. A total of 13 independent pUAST-*ey* strains were analyzed. The heat-inducible construct was made by inserting the embryonic cDNA into the heat-shock Casper vector [V. Pirrotta, in *A Survey of Molecular Cloning Vectors and Their Uses*, R. L. Rodriguez and D. T. Denhardt (Buttesworth, Boston and London, 1988), p. 437].
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17. Wild-type and ectopic eyes were dissected and fixed for 30 minutes on ice in a cacodylate-buffered glutaraldehyde-oxmiumtetroxide fixation mixture. After postfixation in cacodylate-buffered osmiumtetroxide, the tissue was dehydrated through an ethanol series and embedded in Spurr medium. One-micrometer sections were cut and stained with staining solution (equal volumes of 2 percent Azur II and 2 percent disodiumtetraborate to 2 percent methylene blue). After drying they were mounted with Depex.
18. Staining of imaginal discs with ELAV antibodies was performed according to S. Robinow and K. White [*J. Neurobiol.* **22**, 443 (1991)]. A fluorescein-conjugated secondary rat antibody (Cappel) was used. Analysis was done on a Zeiss Axiophot microscope equipped for epifluorescence.
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26. The full-length mouse Pax-6 cDNA (a gift of C. Walther and P. Gruss) was cloned as a Not I–Xho I fragment in the GAL-UAS vector [pUAST (*11*)]. Flies were transformed as described in (*15*). To ectopically induce the mouse Pax-6 gene, the UAS–Pax-6 transformant lines were crossed to the E132 GAL4 expressing line as for the *Drosophila* gene (*14*). The figure shows an ectopic eye on a second leg of a male.
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28. β-Galactosidase staining was performed as described in M. Ashburner, Drosophila, *A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), protocol 77.
29. For cuticle preparations, adults were dissected in phosphate-buffered saline, mounted in Hoyer's or Faure's mounting medium, and immediately photographed to avoid diffusion and bleaching of eye pigments.
30. For scanning electron microscopy, freshly hatched flies were narcotized and immersed in 70 percent acetone. After critical point drying, they were mounted and coated with gold. The specimens were observed with a Hitachi S-800 field emission electron microscope at 6 kV.

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

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SUMMARY

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

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

INTRODUCTION

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

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

Several mutant alleles of *ey* were isolated (see Lindsley and Zimm, 1992), of which only few are still available today (Quiring et al., 1994). Flies homozygous for ey^2 or ey^R have reduced eyes or are completely eyeless. These two alleles have been analyzed molecularly (Quiring et al., 1994). Both mutations are caused by insertions of transposable elements into the first intron of *ey.* These insertions disrupt an eye-specific enhancer, thereby abolishing detectable *ey* expression in the embryonic eye primordia and in the developing eye discs (Quiring et al., 1994). Therefore, ey^2 and ey^R are amorphic or severely hypomorphic for *ey* function in the eye disc. Together

with the finding that *ey* can switch on the eye developmental program and induce the formation of extra eyes, these results show that *ey* acts high up in the genetic cascade regulating eye development. However, little is known about the identity of subordinate target genes that implement the eye inducing activity of *ey*.

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

EYA physically interacts with SO and DAC and ectopic expression of *eya* or *dac* alone or synergistically in combinations of *eya* with *so* or *dac* induces extra eye formation and *ey* expression (Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997; Shen and Mardon, 1997). In addition, *ey*, *eya* and *dac* are induced and required during extra eye development induced by these genes, suggesting that they act together in a positive feedback loop at some point during compound eye development (Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997; Shen and Mardon, 1997).

In this paper we show that the *ey* mutant phenotype is very similar to that of *so* and *eya*. We demonstrate that *ey* function is required for the expression of *so* and *eya* in the eye disc. On the other hand, *so* and *eya* functions are dispensable for *ey* expression. In addition, *so* and *eya* are independently induced and required during the development of *ey* induced extra eyes. Taken together, these results indicate that during normal eye development *ey* acts upstream of *so* and *eya* and either directly or indirectly induces their initial expression anterior to the furrow.

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

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

MATERIALS AND METHODS

Fly stocks

The ey^2, ey^R, so^1, so^3, so^{11}, so^{13}, eya^1 and UAS-*ey* stocks are described in Lindsley and Zimm (1992), Quiring et al. (1994), Cheyette et al. (1994), Heitzler et al. (1993), Bonini et al. (1993) and Halder et al. (1995a). Chester (1971) had noted that larval crowding reduced the expressivity of the *ey* mutation. Therefore, in order to control for larval density, we grew only 50-100 larvae per vial. The so^1 and eya^1 mutant stocks showed high penetrance and expressivity of the eyeless phenotype and required only little selection to maintain their phenotypic strength. The C(4)RM *ci* ey^2 stock was used to generate nullo 4 embryos.

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

The pEYE-lacZ (ey-enhancer-lacZ) transgene was constructed by first inserting a 3.5 kb *Eco*RI fragment derived from the first intron of the *ey* gene into pBluescript (Stratagene). This fragment contains a *Kpn*I site 100 bp from its 5′ end. A 3.5 kb *Kpn*I fragment was then excised (the 3′ *Kpn*I site is in the polylinker of pBluescript) and cloned into HZ50PL. This fragment spans about two thirds of the first intron of *ey* and contains a small portion of exon 3 at its 3′ end. The pEYE-*ey* (ey-enhancer-ey cDNA) construct contains the same 3.5 kb *ey*-enhancer fragment followed by the hsp70 minimal promoter and the full-length embryonic *ey* cDNA (Quiring et al., 1994). The SV40 polyadenylation sequence from pUAST (Brand and Perrimon, 1993) was inserted downstream of the *ey* cDNA. Detailed description of the construction of pEYE-*ey* is available on request. pHSE (Heat-shock *eyeless*): full-length embryonic *ey* cDNA was cloned into phsCaSpeR as a *Not*I-*Xba*I fragment. Flies were transformed as described by Rubin and Spradling (1982). The recipient strain was *y w*.

Rescue and quantitative determination of eye size

In order to assay for rescue activity, four stable pEYE-*ey* transformants were each independently crossed into an ey^2 mutant background. Because the expressivity of the ey^2 eye phenotype depends on the genetic background, it was necessary to compare sibling flies carrying and lacking the rescue transgene. To do so, pEYE-*ey*/*SM1* ; ey^2/ey^2 flies were crossed with ey^2/ey^2 flies and the eye sizes of the progeny were quantitated. The eye sizes of the flies carrying pEYE-*ey* were then compared to those of the flies carrying *SM1*. The *SM1* balancer itself did not affect the expressivity of ey^2.

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

Oregon R wild-type eye. Because compound eyes are not flat, the eye sizes are underestimated with that method and the differences in eye sizes thus appear slightly smaller than they actually are.

Preparation of anti-EY antibody

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

Histology

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

Antibody stainings of imaginal discs were carried out as follows. Larvae were dissected in cold PBS and fixed in PLP (McLean and Nakane, 1974) for 45 minutes on ice or in PEM (100 mM Pipes, pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA, 4% formaldehyde) for 30 minutes on ice. Disc complexes were then washed 4 times for 15 minutes in PBT (PBS with 0.3% Triton X-100) on ice and blocked in PBTB (PBS with 0.3% Triton X-100 and 3% BSA) for at least 30 minutes at 4°C. Disc complexes were incubated with the primary antibody (rat α-EY 1:600, mouse α-EYA 1:2 (Bonini et al., 1993), mouse α-SO 1:300 (Cheyette et al., 1994), mAb α-βGal (Promega) 1:1000, rabbit α-βGal (Cappel) 1:1000, rat α-ELAV 1:30 (Robinow and White, 1991)) in PBTB at 4°C overnight. Disc complexes were washed 6 × 20 minutes in PBTB at 4°C and incubated with the secondary antibody for 2 hours at room temperature or overnight at 4°C. Secondary antibodies used were from Jackson ImmunoResearch Laboratories and included the following $F(ab')_2$ fragments from donkey: DTAF α-rat (1:200-1:1000), Cy5 α-mouse (1:500-1:2000), DTAF α-rabbit (1:200-1:1000), Cy3 α-rabbit (1:500-1:2000). After secondary antibody incubation, disc complexes were washed as above and discs were dissected and mounted in Vectashield (Vector Labs).

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

Heat-shocking larvae

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

RESULTS

Rescue of the *eyeless* mutant eye phenotype

The ey^2 and ey^R stocks obtained from the stock centers were phenotypically almost indistinguishable from wild type and had apparently accumulated a significant number of phenotypic suppressors (Morgan, 1929). In order to obtain stronger phenotypes, we systematically exchanged the first, second and third chromosomes with Oregon R wild-type chromosomes and isogenized the *ey* mutant fourth chromosome. This treatment dramatically improved the penetrance and expressivity of the eyeless phenotype. Nevertheless, these stocks still showed some variability in eye size (Fig. 1A-C,E). In the ey^2 stock with the strongest phenotype, more than 50% of eyes were completely missing and more than 90% were smaller than a third of the normal size. No eyes were larger than half the normal size (Fig. 1E). We did not observe any defects in the ocelli (Fig. 1B,C, arrowheads). In general, ey^R stocks had a similar range of eye defects although with lower expressivity. In the subsequent rescue experiments and phenotypic analyses the selected ey^2 stock was used.

Before analyzing the *ey* mutant phenotype in more detail, we wanted to confirm that the eyeless phenotype is caused by mutation in *ey*. We took advantage of a 3.5 kb enhancer fragment derived from the first intron of the *ey* gene to build a rescue construct with the embryonic *ey* cDNA. This enhancer drives expression specifically in the developing eye (Fig. 1D; Quiring et al., 1994). After transformation into flies, four stable transformants were each independently crossed into an ey^2 mutant background and the eye sizes of ey^2 flies carrying or lacking the rescue construct were quantitated. For each of the four transgenes, the flies carrying the rescue constructs had significantly larger eyes than their siblings without the transgene. For example, 55% of the flies with the rescue transgene EHE6 had nearly wild-type eye sizes, while only 3% of the non-rescued flies had wild-type eyes (Fig. 1F). These experiments confirm that the cause of the ey^2 phenotype is a defect in *ey* gene expression in the eye imaginal discs.

The anlagen of the eye are formed normally in *eyeless* mutant embryos

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

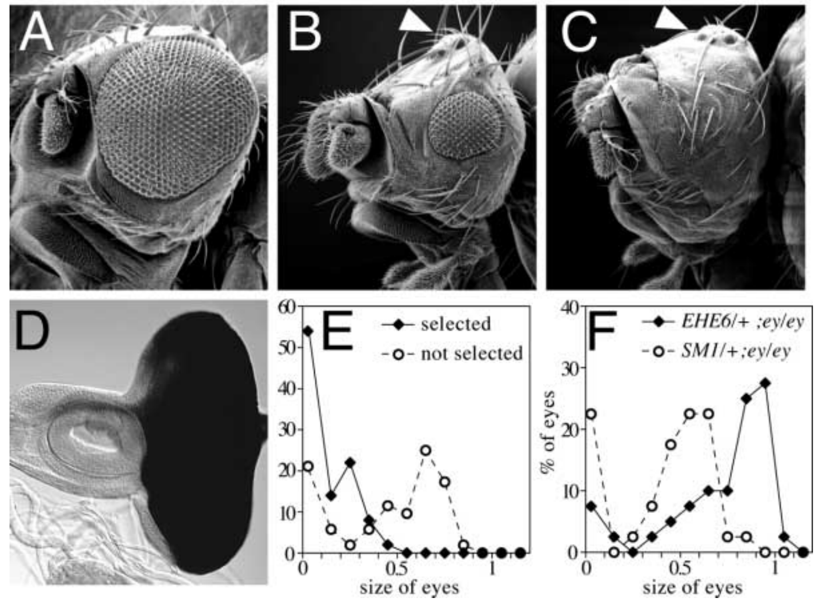

Fig. 1. The adult ey^2 phenotype and rescue by an *eyeless* minigene. (A-C) Scanning electron micrographs of heads of (A) a wild-type fly and (B,C) ey^2 flies with moderate and strong eye phenotypes, respectively. Anterior is to the left. The fly with the strong eyeless phenotype has a small head and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

type embryos (Fig. 2B). We conclude that the anlagen of the eye are formed in ey^2 mutant embryos. Therefore, defects in the first steps of eye development are not the major cause of the eyeless phenotype.

Extensive cell death in *eyeless* mutant eye discs

In contrast to the normal appearance of the eye anlagen in ey^2 embryos, the morphology of eye-antennal imaginal discs from late third instar ey^2 mutant larvae is highly abnormal with the eye portion being strongly reduced (Fig. 2C,D). The antennal part is not affected. Staining for differentiating photoreceptors failed to show any evidence of ommatidial cluster formation in most ey^2 mutant eye discs (Fig. 2D). Previous work suggested that the ey^2 phenotype was a result of cell death in third instar eye discs (Fristrom, 1969). To assess cell death we stained eye discs with the vital dye Acridine orange (Spreij, 1971). A low level of cell death is normally observed in wild-type eye discs, mainly in the region just anterior to the furrow (Fig. 2E; Fristrom, 1969; Spreij, 1971; Wolff and Ready, 1991). In contrast, eye discs from third instar ey^2 larvae displayed massive cell death in the remainder of the eye discs (Fig. 2F). Eye discs with weaker phenotypes showed ectopic cell death anterior to the furrow (not shown). This cell death phenotype is very similar to those observed in so^1 and eya^1 mutants (Bonini et al., 1993; Cheyette et al., 1994).

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

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

EYA and SO start to be expressed in eye discs later than EY. In contrast to EY, neither SO nor EYA is expressed in the eye anlagen of stage-16 embryos (Bonini et al., 1993; Cheyette et al., 1994; our own observations). Expression of EYA and SO in the eye disc starts in the late second and early third instar, respectively (Bonini et al., 1993; Cheyette et al., 1994). At these stages, both genes are expressed in a gradient with strongest expression at the posterior of the eye disc (Fig. 4, Bonini et al., 1993; Cheyette et al., 1994). Later, when the furrow moves across the eye disc, SO and EYA are expressed in a graded fashion with strongest expression just anterior to the furrow (Fig. 3F,H,I). In this region the expression pattern of EY overlaps with those of SO and EYA (Fig. 3F,I). However, in the most anterior part of the eye disc only EY is detected at high levels (Fig. 3F-I). Unlike EY, SO and EYA continue to be expressed posterior to the furrow. Both genes are also expressed in the region of the differentiating ocelli (Bonini et al., 1993; Cheyette et al., 1994). In summary, EY is expressed

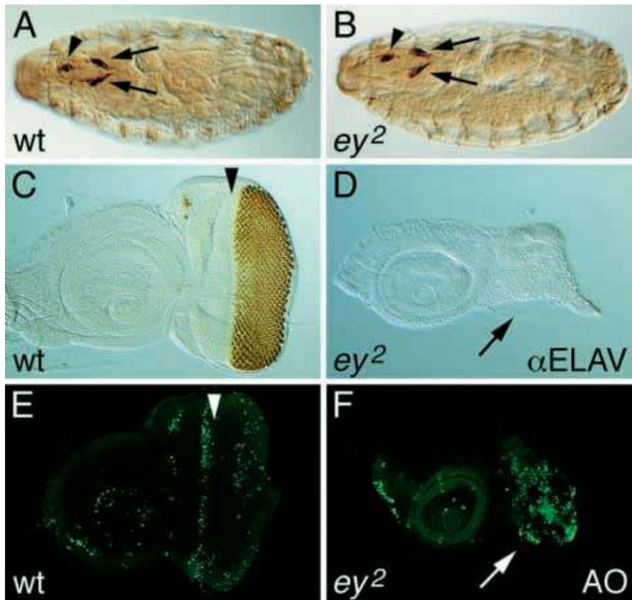

Fig. 2. The embryonic and larval eye disc phenotypes of *ey*^{2}. Left panels (A,C,E) show wild-type, right panels (B,D,F) show *ey*^{2} mutants. (A,B) Anti-β-galactosidase antibody stainings of stage-16 embryos carrying the *ey*-enhancer-lacZ reporter transgene (see also Fig. 1D). The reporter expresses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

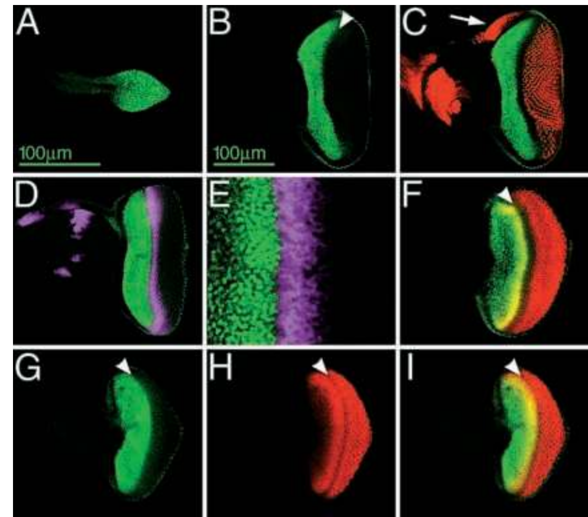

Fig. 3. Expression pattern of EY during eye imaginal disc development. In all panels anti-EY staining is green. Same orientations as in Fig. 2. (A) EY is expressed in the entire eye portion of the disc but not in the antennal part (to the left). (B) EY is expressed uniformly in the eye field anterior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

in the eye disc from embryonic stages onwards, until cells enter the furrow and start to differentiate, while SO and EYA start to be expressed later, and cells begin to express increasing levels of SO and EYA as the furrow moves across the eye disc. These results are consistent with *ey* acting upstream of *so* and *eya* during eye disc development.

To test this possibility, we analyzed gene expression in *ey*^{2}, *so*^{1} and *eya*^{1} mutant eye discs. Genetic and molecular data indicated that the *so*^{1} and *eya*^{1} alleles are amorphic or severely hypomorphic in the developing eye (Bonini et al., 1993, 1997; Cheyette et al., 1994; Leiserson et al., 1994). Because massive cell death is observed in late third instar eye discs of all three mutants, gene expression analysis at this stage is not possible. We therefore studied expression patterns in early third instar eye discs (Fig. 4). At this stage all three genes are expressed (Fig. 4A-C) and cells in the *so*^{1} and *eya*^{1} mutant eye discs are still viable. Eye discs from *ey*^{2} mutants, however, already show first signs of morphological abnormalities (Fig. 4D-F), indicating that *ey* function is required prior to this stage. In eye discs of *so*^{1} (Fig. 4G) and *eya*^{1} mutants (Fig. 4J), EY is expressed normally, indicating that the functions of *so* and *eya* are not required for EY expression. On the other hand, neither SO nor EYA expression is observed in *ey*^{2} mutant eye discs (Fig. 4E,F). This demonstrates that *ey* function is required for eye disc expression of SO and EYA. In about half of the *so*^{1} mutant eye discs weak EYA immunoreactivity was detected, suggesting that *so* may not be required for EYA expression (Fig. 4I). Expression of SO was not seen in *eya*^{1} mutant eye discs (Fig. 4K). However, because SO and EYA are expressed in nearly identical patterns and because both genes are required for cell viability, these results are not conclusive. Below we describe other experiments that address the epistatic relationships between *so* and *eya*. Finally, in neither *ey*^{2}, *so*^{1} or

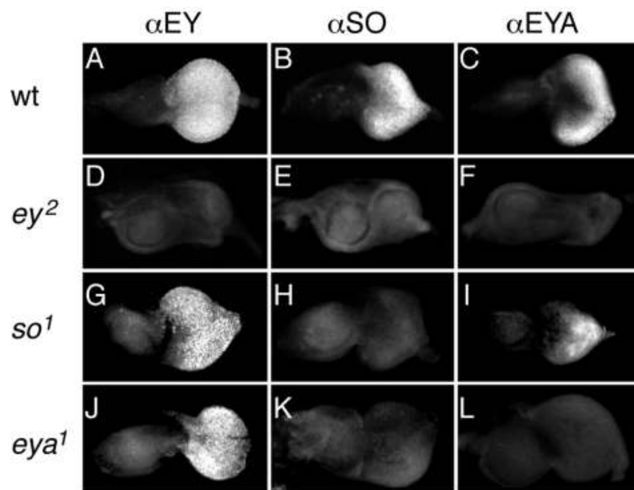

Fig. 4. Expression of EY, SO and EYA in early third instar eye discs. Same orientations as in Fig. 2. In wild-type early third instar eye-antennal discs EY expression (A) is detected in the entire eye portion of the disc and SO (B) and EYA (C) are expressed in a gradient with highest levels at the edge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

eya^1 mutants could immunoreactive material be detected with the respective antibody in the eye disc (Fig. 4D,H,L). This is consistent with *ey*^{2}, *so*^{1} and *eya*^{1} being amorphic alleles in the eye field.

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

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

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

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

The finding that *ey* positively regulates *so* and *eya* transcription raised the possibility that *so* and *eya* may be required downstream of *ey* for ectopic eye formation. Indeed, targeted expression of *ey* was unable to induce ectopic eye development in *so*^{1} and *eya*^{1} mutant backgrounds (Fig. 6A-D), although ectopic EY protein was produced (not shown) and functional as inferred from its deleterious effects (Fig. 6C,D). Consistent with the lack of ectopic eye production, no ectopic photoreceptors develop in wing discs of *so*^{1} and *eya*^{1} mutants following targeted expression of EY (Fig. 6F,G, and data not shown).

We took advantage of the ectopic induction of SO and EYA by EY to find out whether EY activates *so* and *eya* in parallel and independently of one another or whether induction of one gene depends upon the function of the other one. As discussed above, the cell death phenotypes observed in the eye discs of *so*^{1} and *eya*^{1} make such an analysis difficult in the eye discs. We reasoned that by expressing *ey* ectopically we might be able to bypass those requirements for cell viability. However, in late third instar larvae, ectopic EY expression in *so*^{1} and *eya*^{1} mutant backgrounds caused ectopic cell death in wing discs (Fig. 6E) and resulted in strongly reduced and deformed adult structures (Fig. 6C,D). Apparently, EY is able to completely reprogram wing cells into the eye developmental pathway even if that leads to cell death, as is the case in *so*^{1} and *eya*^{1} mutants. Nevertheless, we found that in early to mid third instar wing discs, EY induced ectopic expression of EYA in a *so*^{1} mutant background (Fig. 6F) and, conversely, SO was induced by EY in an *eya*^{1} mutant background (Fig. 6G). Therefore, both genes appear to be independent targets of EY. However, the ectopic expression was weaker than that induced in a wild-type background, suggesting that *so* and *eya* are required for efficient induction of each other's expression. In summary, our results show that EY acts upstream of *so* and *eya* and requires their function during ectopic eye induction.

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

In addition to its function in the developing compound eye, *so* is required for the formation of the entire visual system, including the optic lobes of the brain and the larval photoreceptor organs or Bolwig's organs. In blastoderm-stage embryos, *so* is expressed in a dorsal domain of the head region that gives rise to those structures (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Fig. 7A,B). Whether this region

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

DISCUSSION

eyeless acts anterior to the morphogenetic furrow

The loss of adult eye structures in ey^2 and ey^R results from cell death of the eye imaginal disc during larval stages. Staining with the vital dye Acridine orange revealed massive apoptosis anterior to the morphogenetic furrow in these mutants (Fristrom, 1969; this study). We found that a small proportion of early third instar eye discs from stocks with the most penetrant eyeless phenotype were already reduced in size as compared to wild type. In line with these observations Chen (1929) and Medvedev (1935) found the earliest manifestation of the eyeless phenotype in the second instar, 48 hours after egg laying. But, in contrast to what Medvedev postulated, our data suggest that *ey* is not required for the initial formation of the eye anlagen in the embryo. Nor is, as was argued by Chen, the smaller size of the eye discs (only) due to a proliferation defect, since the amount of 5-bromodeoxyuridine (BrdU) incorporation into replicating DNA is not significantly different between wild-type and ey^2 mutant eye discs (data not shown). It thus appears that the eye discs can form and grow without *ey*, but that later the cells cannot differentiate and die by apoptosis.

It is conceivable that the ey^2 allele is not totally amorphic for *ey* function in the developing eye. However, we have not detected any residual *ey* expression in the developing eyes of ey^2 mutant embryos or larvae, suggesting that ey^2 is at least a very strong hypomorphic allele. To fully answer the question of how early *ey* functions during eye development will require the isolation and characterization of null mutations. Similarly, the question of whether *ey* is required for the development of the ocelli awaits isolation and analysis of *ey* null alleles.

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

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

Our analysis showed that *so* and *eya* are ectopically induced by targeted expression of *ey* in wing and leg imaginal discs. Furthermore, *so* and *eya* are required during *ey* directed ectopic eye development. Therefore, *ey* acts upstream of *so* and *eya* during extra eye development. Several lines of evidence indicate that *ey* also acts upstream of *so* and *eya* during normal eye development. First, *ey* is expressed earlier than *so* and *eya* in the eye discs. Second, *ey* function is required for the expression of *so* and *eya* in eye discs, but not vice versa. Third, ectopic eyes appear to develop in the same way as the normal compound eyes as indicated by gene expression patterns and histology (Halder et al., 1995a; this study, and data not shown). Therefore, we conclude that *ey* acts upstream of *so* and *eya* during normal eye development and either directly or indirectly induces their expression anterior to the furrow. More recent studies in our laboratory indicate that *so* transcription is indeed directly activated by EY (T. Niimi et al., unpublished).

In our ectopic expression system, EY was able to induce EYA expression in a so^1 mutant background and SO expression in an eya^1 mutant background, indicating that *so* and *eya* are independent targets of EY. Thus, both genes may be direct targets of EY activity, rather than one being indirectly activated by EY through the other one. Loss-of-function alleles of either *so* or *eya* show massive cell death anterior to the furrow. This is very similar to the *ey* phenotype and suggests that *so* and *eya* are important mediators of *ey* function in the eye disc.

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

The expression patterns of *ey* and those of *so* and *eya* only partially overlap anterior to the furrow. While *ey* is expressed in all eye progenitor cells anterior to the furrow from embryonic stages onwards, neither *so* nor *eya* transcripts are detected in the eye discs at the end of embryogenesis and high levels of *so* and *eya* expression start later, during early third and second instars respectively. In addition, both genes are initially expressed in a gradient from posterior to anterior. Only as the furrow moves across the eye disc do all *ey*-expressing cells induce high levels of *so* and *eya* expression. Therefore, while *ey* is necessary, it is not sufficient to induce *so* and *eya*

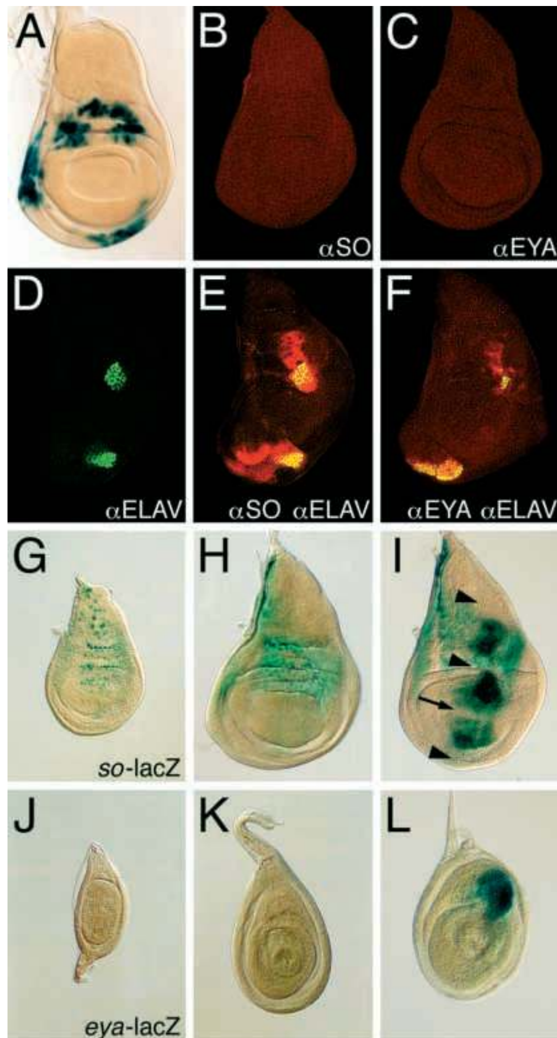

Fig. 5. EY induces ectopic expression of *so* and *eya*. (A) β-galactosidase activity staining of a late third instar wing disc expressing a UAS-lacZ reporter transgene driven by the E132 Gal4 driver (Halder et al., 1995a). (B,C) Wild-type wing discs stained for SO and EYA proteins, respectively. No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

expression. Thus a factor that acts in conjunction with *ey* may exist. Similarly, we found that ubiquitous expression of EY induced ectopic expression of *so* and *eya* preferentially along the A/P boundary in wing discs and in a dorsal domain at the A/P boundary in leg discs. This induction was not observed close to cells that secrete the WG signaling protein (Baker, 1988), consistent with the finding that WG inhibits furrow initiation and progression (Ma and Moses, 1995; Treisman and Rubin, 1995).

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

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

Insect compound eyes versus vertebrate single lens eyes

Homologs of *ey/toy* (Pax-6), *so* and *eya* are active during vertebrate eye development, suggesting that vertebrates and flies may use conserved genetic pathways during eye development (reviewed by Halder et al., 1995b; Macdonald and Wilson, 1996; Callaerts et al., 1997; Oliver and Gruss, 1997). The overall expression pattern of Pax-6 during vertebrate and *Drosophila* eye development is strikingly similar. In vertebrates, Pax-6 is expressed initially in a large area of the head neural ectoderm and the overlying surface ectoderm that gives rise to the lens and nasal placodes (Krauss et al., 1991; Walther and Gruss, 1991; Püschel et al., 1992; Li et al., 1994; reviewed in Callaerts et al., 1997). During further eye development, Pax-6 expression progressively becomes restricted to the developing optic vesicle, lens and cornea. In *Drosophila*, *toy* is initially expressed in a broad domain of the

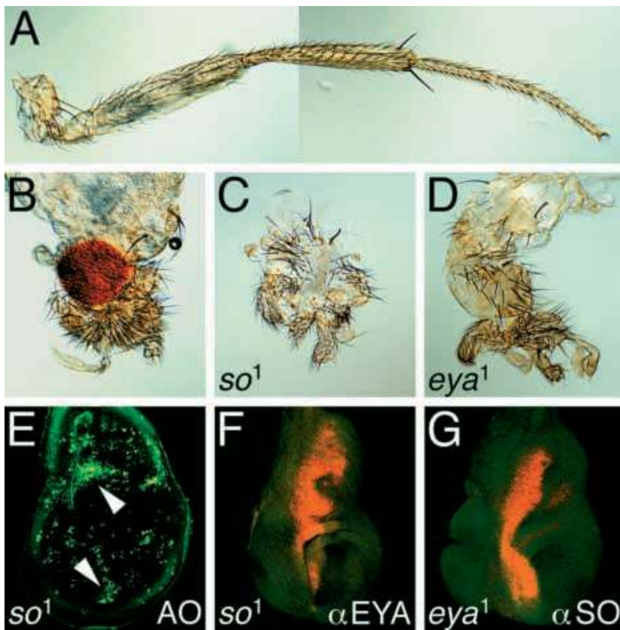

Fig. 6. SO and EYA are required for ectopic eye development and are independently induced by EY. (A) Wild-type leg. (B) Extra eye on a leg induced by dpp-Gal4 driven EY expression. The leg is abnormally short and totally deformed. Same magnification as A. dpp-Gal4 driven EY expression in a *so*^{1} (C)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

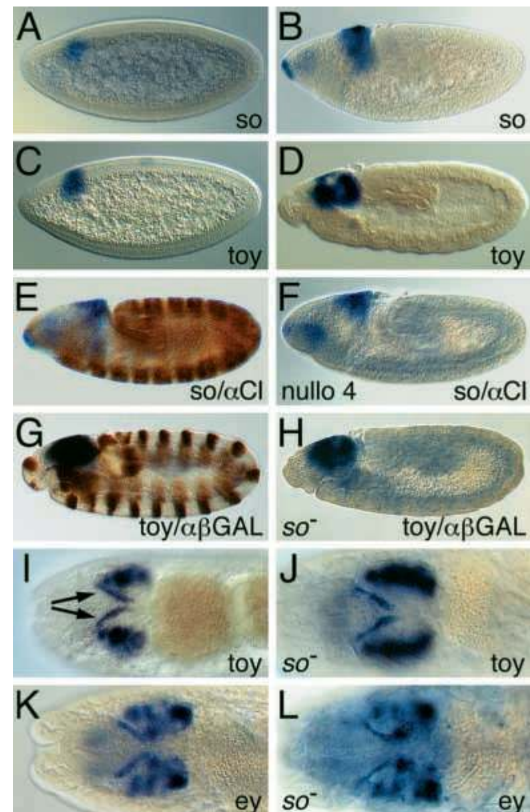

Fig. 7. *toy* and *so* act in parallel in the embryonic head. (A-D) Lateral views of wild-type embryos stained for *so* (A,B) or *toy* (C,D) transcripts. (A,C) Both genes start to be expressed at the cellular blastoderm stage in the procephalic neurogenic region (PNR) of the developing head. They continue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

embryonic head and continues to be expressed in the eye discs (Fig. 7, T. Czerny et al., unpublished data). *ey* expression in the developing eye starts later, when the eye anlagen form after gastrulation (Quiring et al., 1994). In addition, it appears that in both flies and vertebrates, Pax-6 expression in the developing eye is directly downregulated by HH signaling (this study; Ekker et al., 1995; Macdonald et al., 1995; Li et al., 1997), providing a parallel in the regulation of Pax-6 as well.

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

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

In the mouse, one of the *so* homologs, Six3, is expressed in

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

At the present time, we cannot explain why *ey* and mouse Pax-6 can induce ectopic eye development in *Drosophila*, whereas ectopic expression of Pax-6 in *Xenopus* did not induce ectopic retinal development (Hirsch and Harris, 1997) and only resulted in the induction of ectopic lenses (Altmann et al., 1997) rather than complete eyes. However, there may be different Pax-6 isoforms or cofactors required for retina and lens development. In summary, the expression patterns and hierarchical relationships between *toy*, *ey*, *so* and *eya* are comparable to a large extent to those of Pax-6, Six3 and Eya1-3, indicating a surprisingly high degree of evolutionary conservation of the eye developmental program.

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Homology of the *eyeless* Gene of *Drosophila* to the *Small eye* Gene in Mice and *Aniridia* in Humans

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A *Drosophila* gene that contains both a paired box and a homeobox and has extensive sequence homology to the mouse *Pax-6* (*Small eye*) gene was isolated and mapped to chromosome IV in a region close to the *eyeless* locus. Two spontaneous mutations, ey^2 and ey^R, contain transposable element insertions into the cloned gene and affect gene expression, particularly in the eye primordia. This indicates that the cloned gene encodes *ey*. The finding that *ey* of *Drosophila*, *Small eye* of the mouse, and human *Aniridia* are encoded by homologous genes suggests that eye morphogenesis is under similar genetic control in both vertebrates and insects, in spite of the large differences in eye morphology and mode of development.

A small multigene family of paired box–containing genes (*Pax* genes) was first identified in *Drosophila* and subsequently found in vertebrates from zebrafish to humans (*1*). *Pax* genes encode sequence-specific DNA-binding transcription factors that play an important role in embryonic development, particularly in the nervous system. Pax proteins are characterized by a 130–amino acid paired domain, which functions as a sequence-specific DNA-binding domain. In addition, some Pax proteins contain a second DNA-binding domain, a homeodomain, which in some cases is truncated, or a specific octapeptide (or both). These multiple combinations of protein domains illustrate evolutionary tinkering at the molecular level (*2*). In contrast to *Hox* genes, which in mammals are arranged in four repeated homologous clusters, the *Pax* genes are dispersed and are present in single copies. Mutants are known for at least three of the nine known *Pax* genes in mammals. *Pax-1* is mutated in *undulated* mice (*3*); *Pax-3* is affected in *Splotch* mice (*4*), which corresponds to Waardenburg's syndrome in humans; and the *Small eye* mutations in mice (*5*) and rats (*6*) and the *Aniridia* mutation in humans (*7*) affect the *Pax-6* gene. The degree of evolutionary conservation among *Pax* genes is higher than it is for *Hox* genes. For example, the coding regions of the *Pax-6* genes of mice and zebrafish (*8*) are 80% identical in their nucleotides and 97% identical in their amino acids (*8*). A single amino acid difference is found in the respective paired boxes, and the two homeodomains are identical. There is also a strong conservation of splice sites, which indicates that the two genes are homologous.

We have isolated the *Drosophila* homolog of *Pax-6*, which shows 94% amino acid sequence identity to *Pax-6* of humans, mice, and quail (*9*) and 93% identity to zebrafish in the paired domain. The homeodomains of *Pax-6* show 90% sequence identity between *Drosophila* and vertebrates. Loss-of-function mutations in *Pax-6* primarily affect eye development. A reduction in eye size and the absence of the iris are observed in heterozygous carriers of the human *Aniridia* syndrome, and the eyes are reduced in heterozygous *Small eye* mutant mice and rats, whereas homozygous mutant embryos lack eyes completely and die. We found that the *Pax-6* homolog of *Drosophila* is encoded by the *eyeless* (*ey*) gene, which is also involved in eye morphogenesis. These findings indicate that not only the amino acid sequences have been conserved in evolution but also the function of the gene in the developmental pathway leading to eye morphogenesis. Because *Pax-6* is involved in the genetic control of eye morphogenesis in both mammals and insects, the traditional view (*10*) that the vertebrate eye and the compound eye of insects evolved independently has to be reconsidered.

The *Drosophila* homolog of *Pax-6* was isolated from an expression library in a screen in which an oligonucleotide corresponding to a homeodomain binding site was used as a probe (*11*). One clone, 8321, was isolated that bound the oligonucleotide probe particularly strongly. Sequence data revealed that the clone contained both a paired box and a homeobox, and a computer search through the European Molecular Biology Laboratory database indicated that the clone had homology to mouse *Pax-6* and human *Aniridia* sequences. In situ hybridization of 8321 phage DNA to giant polytene chromosomes gave two signals, one located at 102D on chromosome IV and another located at 67B on chromosome II. A 1.1-kb Eco RI fragment containing the paired box and homeobox sequences hybridized only to 102D, a region close to the *ey* locus.

In order to isolate more complete complementary DNA (cDNA) clones, a λ gt10 library prepared from 3- to 12-hour *Drosophila* embryos (*12*) was screened by plaque hybridization at high stringency with the 1.1-kb Eco RI fragment from phage 8321. The longest cDNA clone isolated, E10, had an insert of 2.8 kb. The complete nucleotide sequence (*13*) and the deduced amino acid sequence of E10 are shown (Fig. 1). The E10 cDNA insert is 2850 base pairs (bp) long, and the open reading frame starts at position 89 and terminates at position 2603, which suggests that it encodes a protein of 838 amino acids with a predicted molecular weight of 82,490 daltons and an isoelectric point (pI) of 7.9. All three reading frames upstream of the ATG codon at position 89 are closed. The sequence preceeding this ATG, CAACTATG, corresponds to the consensus translation initiation sequence C-AAAC-AATG for *Drosophila* (*14*), except that an additional T residue is inserted in front of the ATG as it is in the zebrafish *Pax-6* (*8*) and in the *Drosophila paired* genes (*15*). This suggests that the ATG at position 89 represents the initiation codon. A termination codon TGA is found at position 839, followed by a putative polyadenylation signal (AATAAA) and a 17-nucleotide polyadenylate [poly(A)] tract (Fig. 1). Near the NH_2-terminus of the deduced protein sequence, a 130–amino acid paired domain was found, which in terms of its amino acids is 94% identical to the paired domains of *Pax-6* in mice, humans, and quail and 93% identical to that of zebrafish (Fig. 2). Amino acids differing from those found in vertebrates are indicated (Figs. 1 and 2). Two of these substitutions are in positions that so far had been found to be invariant. Position 14, occupied by asparagine in all previously known paired boxes, is replaced by glycine, whereas the invariant proline at position 78 is replaced by alanine. Twenty-four out of 28 amino acids characteristic of *Pax-6* in vertebrates are also found in *Drosophila*.

The paired box is separated from a paired-type homeobox by a linker region that is considerably larger in *Drosophila* than in vertebrates. Nevertheless, there is also scattered sequence homology in the linker region (Fig. 1). The homeodomains of *Drosophila* and of vertebrate *Pax-6* homologs differ at only 6 out of 60 positions (Fig. 2): four in α helix I, one in α helix II, and one in the turn of the helix-turn-helix motif (aspartate → glycine). A characteristic feature of the homeodomains of the paired class is an extended homology of 18 amino acids at the NH_2-terminus (*1*). However, in the *Pax-6* homologs, this extended homology is confined to six amino acids immediately preceding the homeodomain. These six amino acids, LILKRK in *Drosoph-*

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ila and LQLKRK in vertebrates (*16*), are completely conserved in at least 11 proteins, including the *Pax-6* homologs of humans, mice, rats, quail, and zebrafish and also in the *Drosophila* genes *paired* (*15*), *gooseberry*, *gooseberry-neuro* (*17*), and *aristaless* (*18*). The three basic residues in this sequence suggest that it may possibly represent a nuclear targeting signal. With the exception of *aristaless*, which contains a homeodomain only, all these proteins have a paired domain. At the COOH-terminus, another seven amino acids immediately adjacent to the homeodomain (KLRNQRR) (*16*) are completely conserved in all presently known *Pax-6* homologs. The 47 amino acids located COOH-terminally of the homeodomain show 45% sequence identity between mice and *Drosophila*. The COOH-terminal region includes 368 amino acids and is more than twice as long as the corresponding region in vertebrates. It is rich in alanine (11%) and glycine (8%), in serine (20%) and threonine (6%), and also in proline (11%). Another region of limited homology is found between amino acids 629 and 660, in which 35% identity is found between the *Drosophila* gene and mouse *Pax-6*. These sequence data indicate that the cloned *Drosophila* gene is homologous to the *Pax-6* gene in vertebrates.

The sequence of a larval transcript isolated from an imaginal disk cDNA library (*19*) is represented in the upper part of Fig. 1. The 5′ sequence of the larval cDNA differs from that of the embryonic transcript because it contains a different first exon that is spliced directly to exon 3, which contains the paired box. The proteins encoded by the two types of transcripts differ only with respect to the NH_2-terminal sequences up to the first amino acid in the paired domain.

The genomic organization of the locus was determined by the isolation and analysis of a number of overlapping clones from genomic libraries of *Drosophila melanogaster* with the 1.1-kb Eco RI fragment of phage 8321 and a 1.4-kb Eco RI fragment of the cDNA clone E10 as probes (*20*). The transcription unit spans approximately 18 kb (Fig. 3), but the transcription initiation site has not been determined. The gene encodes two transcripts, which differ with respect to their first exons, that are spliced to exon 3, which is shared. The splice junctions were determined by sequencing the genomic clones and defining the exon-intron boundaries. The paired domain is encoded by three exons. The splice site in the first codon of the paired box and the third splice site between codons 116 and 117 are at exactly the same position as reported for *Pax-6* in humans, mice, and quail, which suggests that the *Drosophila* gene is a true homolog of the vertebrate *Pax-6* gene. The second splice site in the paired box differs from that in vertebrates, which has a characteristic position between codons 44 and 45. This site serves for differential splicing, because it can accommodate an additional exon of 42 bp in some

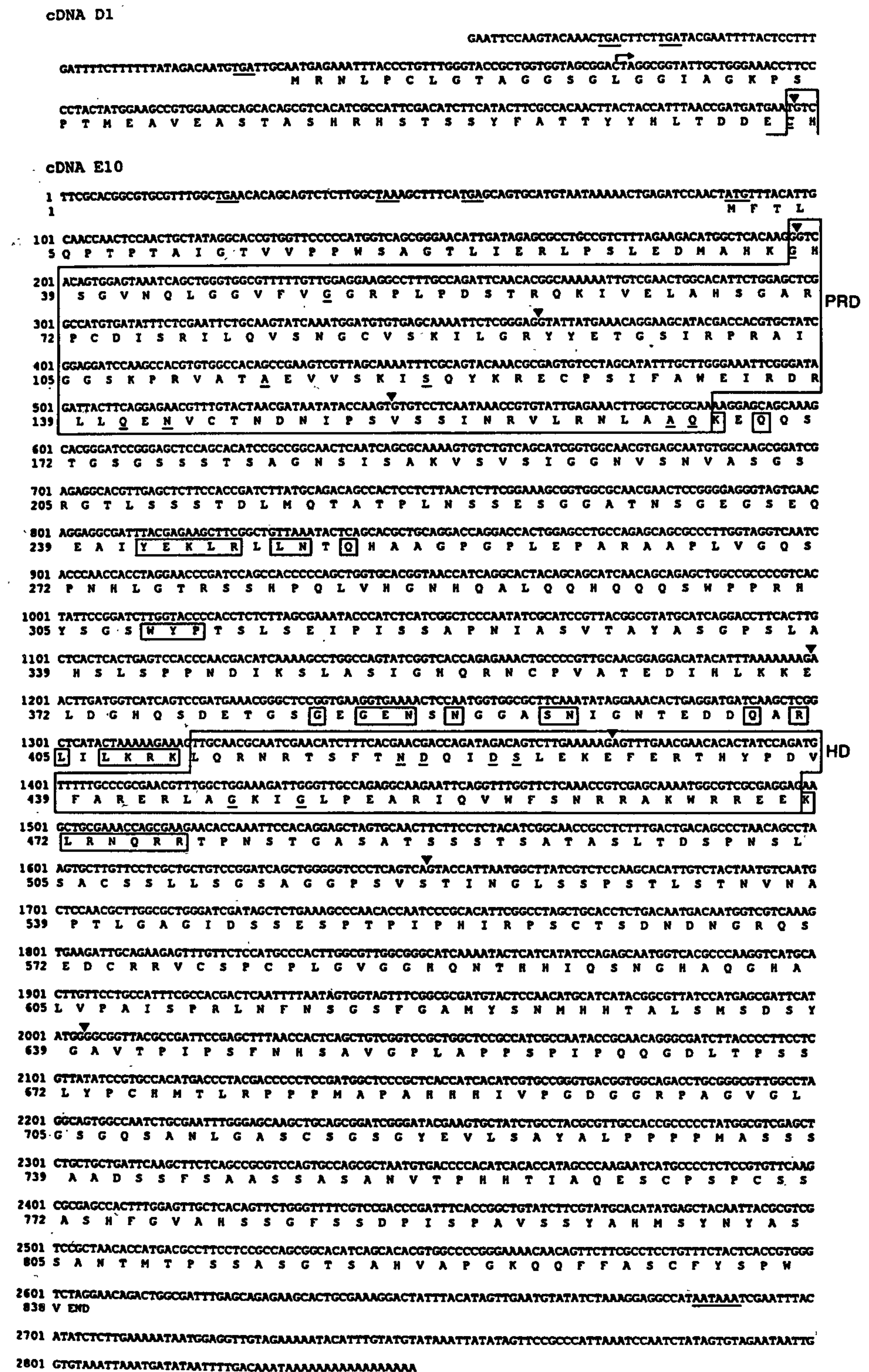

Fig. 1. Nucleotide and deduced amino acid sequences of a larval (D1) and an embryonic (E10) cDNA clone of the *Drosophila Pax-6* homolog, which corresponds to the *ey* gene (*16*). The D1 sequences differ from those of E10 with respect to the first exon preceding the paired box. The paired domain (PRD) and the homeodomain (HD) are boxed in. The stop codons preceding the putative translation initiation sites are underlined. An arrow indicates the 5′ end of D1. Amino acids differing from those found in *Pax-6* of vertebrates are underlined; identical amino acids outside of the paired domains and homeodomains are framed in. The splice sites are indicated by arrowheads. The putative polyadenylation signal is underlined.

transcripts, which encode a 14–amino acid insertion into the paired domain, that are found in humans, mice, quail, and zebrafish (*5–8*). In *Drosophila*, this site is missing, and the second splice site in the paired box is located further downstream in codon 58, which is the same position as in the *Pax* genes 2, 5, and 8 of the mouse. One of the splice sites in the homeodomain, in codon 19, is also conserved in evolution, whereas the second splice site found in *Pax-6* of humans and quail is absent in *Drosophila*.

By in situ hybridization to polytene chromosomes, the *Drosophila Pax-6* homolog was mapped to position 102D on chromosome IV, a position close to the *ey* locus (Fig. 3B). A number of different *ey* mutations have been isolated, some of which are lethal. The viable hypomorphic alleles show a characteristic reduction in the size of the compound eyes, which in some individuals may be missing completely (*21*). To find out whether *Drosophila Pax-6* corresponds to *ey*, we analyzed the available *ey* mutants by whole-genome Southern (DNA) blots. DNA from the two spontaneous alleles ey^2 and ey^R showed an alteration in the pattern of restriction fragments that hybridize to the corresponding wild-type probe. In both mutants, different restriction fragments hybridized to the 1.7-kb Sal I fragment of ey^+ (Fig. 4). This suggests that these two mutations are caused by insertions of transposable elements near the 5′ end of the cloned gene (Fig. 3), a common phenomenon in spontaneous *Drosophila* mutants. The size and restriction pattern of the insertion in ey^2 resembled the *doc* transposable element (*22*), which we confirmed by cloning a hybrid phage carrying this region from a genomic ey^2 library (*23*) and determining the DNA sequences around the point of insertion. The presence of a *doc* element in ey^2 at 102D has also been confirmed by in situ hybridization (Fig. 3C). A corresponding phage was isolated from an ey^R genomic library and analyzed (*23*). The partial DNA sequence obtained from this phage allowed us to identify the inserted element as *blastopia*, a retrotransposon identified recently by its spatially restricted expression in the prospective head region of the blastoderm-stage embryo (*24*).

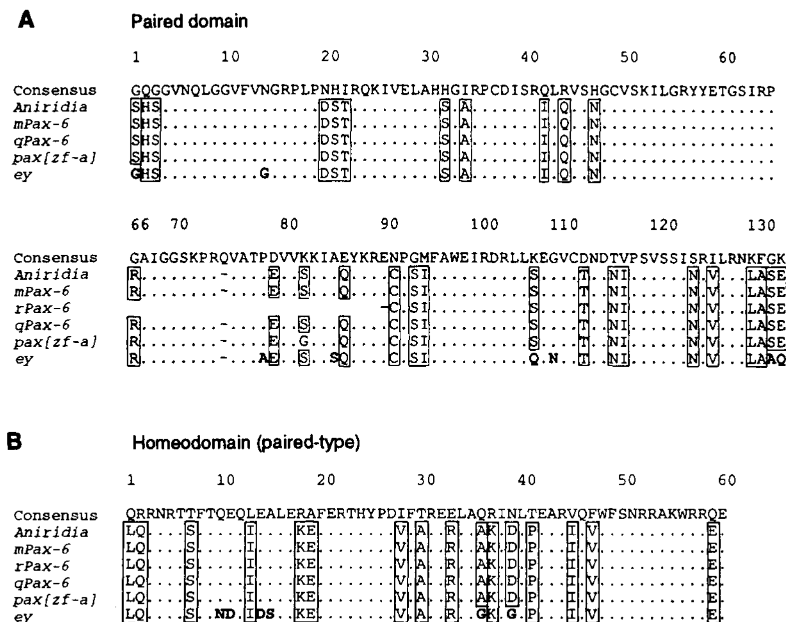

Fig. 2. Comparison of the amino acid sequences between the paired domains (**A**) and the homeodomains (**B**) of *Pax-6* homologs of vertebrates and *Drosophila* (*16*). The consensus sequences for all genes of the paired family (*33*) are compared to human *Aniridia, Pax-6* from mice (*mPax-6*), rats (*rPax-6*), quail (*qPax-6*), zebrafish [*pax*(*zf-a*)], and *Drosophila ey*. Shared amino acids (characteristic for *Pax-6*) are boxed in; amino acids differing between *Drosophila* and vertebrates are in bold.

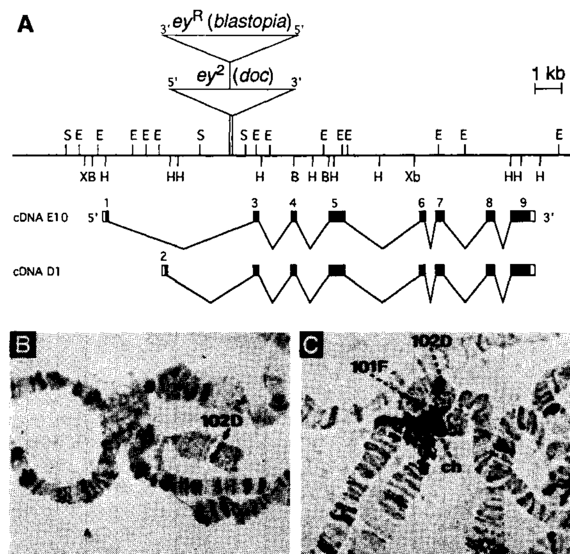

Fig. 3. Structural organization of the *ey* locus. (**A**) Genomic organization of *ey*. The restriction map of the *ey* locus is shown within the region covered by the isolated genomic phages. The two different types of cDNAs are shown below this map. Noncoding regions are indicated by white boxes, coding regions by black boxes. The exon-intron structure was determined by sequencing of the corresponding genomic regions. The positions of two transposon insertions causing mutations in the *ey* gene (ey^2 and ey^R) were determined by DNA sequence analysis (*23*) and are indicated by triangles. B, Bam HI; E, Eco RI; H, Hind III; S, Sal I; Xb, Xba I; X, Xho I. (**B**) In situ hybridization of a 1.1-kb Eco RI cDNA fragment from phage 8321 to polytene salivary gland chromosomes of ey^+ larvae (*34*). A single band of hybridization is detected at section 102D close to the *ey* locus on chromosome IV. (**C**) In situ hybridization of a *doc* transposable element (*22*) probe (pDoc) to polytene chromosomes of ey^2 larvae (*34*). Bands of hybridization are detected at 102D and 101F on chromosome IV, in the chromocenter (ch), and at several additional sites on other chromosomes.

To find out whether the two insertions affect the expression of the cloned gene, whole-mount in situ hybridizations to embryos and larvae of wild-type, ey^2, and ey^R homozygous mutants were carried out. Transcripts were detected in a bilaterally symmetrical pattern in the brain and in every segment of the ventral nervous system for ey^+ embryos at the germ-band stage (Fig. 5, A and B). Anterior to the brain, transcripts accumulated in the primordia of the eye imaginal discs (Fig. 5B) (*25*). This site of expression was not detected in ey^2 mutants (Fig. 5C). Later in embryogenesis, the transcripts became confined to the brain and the primordia of the eye disks. In the eye-antennal disks of third instar wild-type larvae, a band of expression at the anterior edge of the eye disk was detected (Fig. 5D), whereas hardly any transcripts were detectable in ey^2 (Fig. 5E) and ey^R (Fig. 5F) eye disks (*26*). The lack of expression in the eye primordia of the ey^2 and ey^R mutants indicates that the transposon insertions affect the expression of the cloned gene and that the *Drosophila Pax-6* homolog is indeed the *ey* gene. Our preliminary analysis of the distribution of *ey* transcripts showed that the expression was confined to the eye imaginal disks and was not detectable in leg or wing disks. The transcripts accumulated in the cells at the anterior

margin of the eye disk and extended posteriorly to the morphogenetic furrow and to a few rows of cells beyond the furrow. The ey^+ transcripts also accumulated in parts of the brain and the ventral ganglion, as well as in the salivary glands. The finding that both of the insertions of transposable elements in ey^2 and ey^R, which affect gene expression in the eye primordia, occurred within 75 bp suggested to us that the transposable elements might disrupt an eye-specific gene regulatory element. Therefore, the sequences flanking the points of insertion were inserted into an enhancer detection vector containing a minimal promoter and the gene encoding the lacZ reporter. Transgenic flies carrying this construct selectively express β-galactosidase in the eye primordia (*27*). This finding supports the conclusion that the eyeless phenotype in ey^2 and ey^R mutants is caused by the insertion of transposons into genomic regulatory elements required for expression in the eye primordia.

The pattern of expression of the mouse *Pax-6* gene has been studied extensively in the embryo (*5*). It is first expressed in the forebrain and the hindbrain, followed by expression in the neural tube along the entire anteroposterior axis. At day 8.5 after conception, expression is first detected in the optic sulcus, followed by expression in the optic cup and the neural retina. The overlying ectoderm, which subsequently gives rise to the lens and later the cornea, also expresses *Pax-6*, which suggests that *Pax-6* is involved in eye induction. The nasal epithelium and Rathke's pouch show expression 1 and 2 days later in embryogenesis, respectively. This pattern of expression resembles the one found in *Drosophila*: The ey^+ transcripts are detected first in the central nervous system, in the brain, and the ventral nerve cord. The first signs of expression in the developing eyes are found in the embryonic anlagen of the eye imaginal disks. During larval stages, the anteriormost part of the eye disk expresses ey^+ transcripts. This is compatible with an early determinative role of the gene in eye morphogenesis, because the differentiation of the ommatidia proceeds in a posterior to anterior direction, as reflected by the gradual movement of the morphogenetic furrow in the anterior direction (*28*). The cells immediately anterior to the furrow are still undifferentiated and express the *eyes-absent* (*eya*) gene (*29*). Because *ey* is also expressed anteriorly to *eya*, *ey* seems to control an even earlier step in eye differentiation than *eya*. These observations suggest the hypothesis that *ey* is a master control gene that initiates the eye morphogenetic pathway and is shared between vertebrates and invertebrates. We are pursuing this hypothesis further by examining more primitive metazoa with primitive eyes. By polymerase chain reaction (PCR) amplification we have detected DNA sequences similar to those of *Pax-6* in flatworms (*Dugesia tigrina*) (*30*) and in nemerteans (*31*), which are among the most primitive metazoa with eyes (*28*). If the corresponding genes in flatworms and nemerteans are also involved in eye morphogenesis, the concept that the eyes of invertebrates have evolved completely independently from the vertebrate eye has to be reexamined. Also, the hypothesis that the eye of cephalopods has evolved by convergence with the vertebrate eye (*10*) is challenged by our recent finding (by means of PCR amplification) of *Pax-6*–related sequences in the squid *Loligo vulgaris* (*32*). These findings may throw some new light on the fascinating problems of eye evolution.

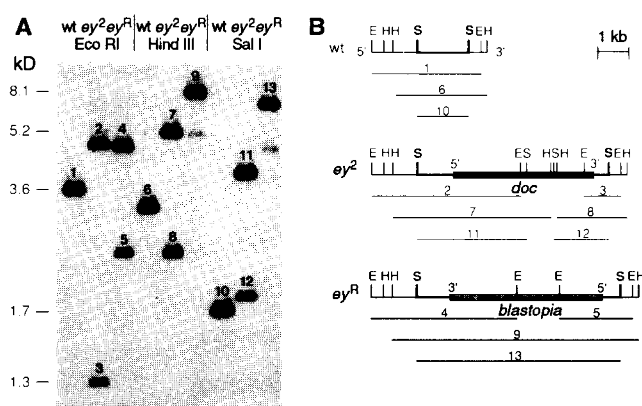

Fig. 4. Genomic Southern blots of ey^+, ey^2, and ey^R DNA. (**A**) DNA was isolated from mutant and wild-type (wt) strains, digested with the restriction enzymes indicated, run on a gel, blotted, and hybridized with the genomic 1.7-kb Sal I fragment (fragment 10 in Fig. 4B). Fragments are numbered for simplicity and are schematically shown in Fig. 4B. Some fragment sizes or marker lengths are indicated on the left. (**B**) Schematic drawings of wild-type and mutant DNA. Fragments detected in the genomic Southern blot (A) are numbered. The 1.7-kb Sal I fragment used as a probe is indicated in bold and the transposable elements with their orientation and insertion points are shown underneath.

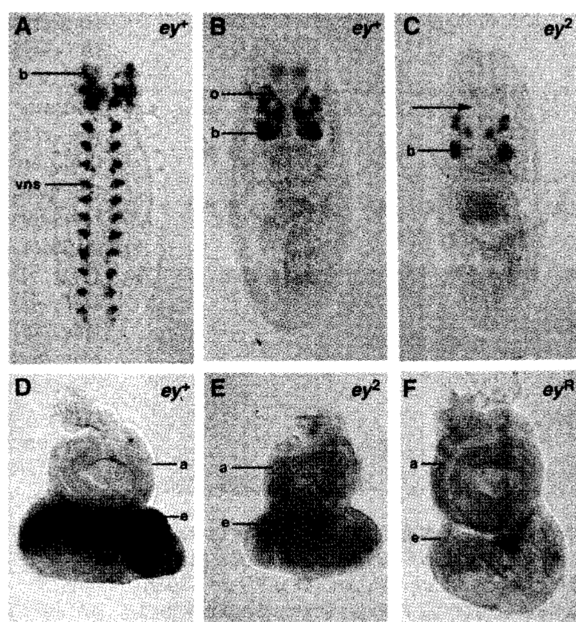

Fig. 5. Expression of *ey* transcripts in embryos and eye imaginal disks. In situ hybridization of ey^+ cDNA to RNA transcripts in embryos and larvae (*30*). (**A** and **B**) Expression pattern in ey^+ embryos. (**C**) Expression in ey^2 embryo. Note the absence of labeling of the optic primordia (arrows) in ey^2. (**D**) Wild-type (ey^+) eye-antennal disk. The anterior portion of the eye disk is labeled. In the ey^2 mutant disk (**E**) and the ey^R mutant disk (**F**) hardly any labeling is detected. Abbreviations: b, brain; vns, ventral nervous system; o, optic primordia; e, eye imaginal disk; and a, antennal disk.

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11. Approximately 10^4 phages of a random primed embryonic (0 to 24 hours) cDNA expression library in λ gt11 (provided by B. Hovemann) were screened with the oligonucleotide 5′-ACAGGAG-CAATTACAGCT-3′ as a probe, which represents a high-affinity binding site for the *fushi tarazu* homeodomain [L. Pick *et al.*, *Genes Dev.* **4**, 1224 (1990)]. The oligonucleotide was concatenated, which gave it an estimated length of approximately 200 bp. The protocols used were based on those of C. R. Vinson *et al.*, [*ibid.* **2**, 801 (1988)]; H. Singh *et al.*, [*Cell* **52**, 415 (1988)]; and M. J. Shea, D. L. King, M. J. Conboy, B. D. Mariani, and F. C. Kafatos [*Genes Dev.* **4**, 1128 (1990)]. The probe was ^{32}P-labeled with the megaprime labeling system (Amersham).
12. Embryonic cDNA clones were isolated by screening of approximately 2×10^5 phages from a 3- to 12-hour embryonic λ gt10 library of S. Poole, L. M. Kauvar, B. Dress, and T. Kornberg [*Cell* **40**, 37 (1985)] with the use of the 1.1-kb Eco RI fragment of the 8321 phage as a probe at high stringency [3× saline sodium citrate (SSC), 0.1% SDS, 1× Denhardts, and denatured herring sperm DNA (1 mg/ml) for 16 to 18 hours at 65°C, followed by two washes with 1× SSC and 0.1% SDS for 30 min at 65°C].
13. For sequencing, the inserts were subcloned into the Bluescript KS (+) plasmid vector (Stratagene). Overlapping deletions were generated with Exo III and S1 nucleases (double-stranded nested deletion kit, Pharmacia) and sequenced on both strands with the dideoxynucleotide procedure of F. Sanger, S. Nicklen, and A. R. Coulson [*Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)]. Compressed DNA regions were sequenced using the fmol DNA sequencing system (Promega). DNA sequence analysis was done on a VAX with the GCG program [J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984)]. The homology searches through the EMBL and SWISSPROT data banks were carried out with the FASTA programs [W. R. Pearson and D. J. Lipman, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2444 (1988)]. EMBL Data Library accession numbers: X79492 *D. melanogaster ey* mRNA (exon 1) and X79493 *D. melanogaster ey* mRNA (exons 2–9).
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20. Approximately 10^5 clones of an Oregon R genomic library prepared in the EMBL 3 vector (provided by C. Wilson) and in the EMBL 4 vector (gift of A. Preiss) were screened at high stringency as described for the cDNA clones (*12*).
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26. This result was confirmed by in situ hybridization of sense and antisense RNA probes to wild-type, ey^2, and ey^R larvae. In the majority of the mutant eye disks, no hybridization of antisense RNA was detected above the background that was found with sense RNA. In some of the mutant disks, a few scattered positive cells were detected, which is in agreement with the variable expression of the mutant phenotype (R. Leemans and W. Gehring, unpublished data).
27. The 3.6-kb Eco RI restriction fragment flanking the insertion sites was cloned into HZ50PL [Y. Hiromi and W. J. Gehring, *Cell* **50**, 963 (1987)], and transgenic lines were established (U. Walldorf and W. J. Gehring, unpublished data).
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31. A gene with significant homology to *Pax-6* in both the paired box and the homeobox was cloned from a genomic DNA library of the nemertean worm *Lineus sanguineus* (F. Loosli, M. T. Kmita, W. J. Gehring, unpublished data).
32. Sequences homologous to the paired box of *Pax-6* were PCR-amplified, cloned, and sequenced from DNA isolated from squid (*L. vulgaris*) sperm (P. Callaerts, J. Marthy, W. J. Gehring, unpublished data).
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Blockage of NF-κB Signaling by Selective Ablation of an mRNA Target by 2-5A Antisense Chimeras

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Activation of 2-5A–dependent ribonuclease by 5′-phosphorylated, 2′,5′-linked oligoadenylates, known as 2-5A, is one pathway of interferon action. Unaided uptake into HeLa cells of 2-5A linked to an antisense oligonucleotide resulted in the selective ablation of messenger RNA for the double-stranded RNA (dsRNA)–dependent protein kinase PKR. Similarly, purified, recombinant human 2-5A–dependent ribonuclease was induced to selectively cleave PKR messenger RNA. Cells depleted of PKR activity were unresponsive to activation of nuclear factor–κB (NF-κB) by the dsRNA poly(I):poly(C), which provides direct evidence that PKR is a transducer for the dsRNA signaling of NF-κB.

Natural defense mechanisms can be allies in the quest for therapeutic approaches to disease. One such defense, the 2-5A system (*1*), mediates certain effects of interferons, such as the inhibition of encephalomyocarditis virus replication (*2*). Key components of this system include 2-5A, short oligoadenylates with 2′,5′-phosphodiester bonds; 2-5A synthetases that generate 2-5A from adenosine triphosphate (ATP) in response to dsRNA; and the effector of the system, the 2-5A–dependent ribonuclease (RNase) (*3, 4*). This RNase, which is ubiquitous in the cells of mammalian, reptilian, and avian species (*5*), cleaves single-stranded RNA in response to 2-5A, with moderate specificity after UpNp sequences (*6*). Thus, this host defense mechanism is the basis for a strategy for the selective destruction of specific mRNA targets.

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