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

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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 in 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 suscus, 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 gene, 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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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 spatial patterns was obtained by testing them against each other. The ectopic eye phenotypes obtained with each enhancer are illustrated in Fig. 2, A through D.

The ey gene indicates 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.

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**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.

**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. 1B 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.

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 promoter 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
were seen at one side of an ectopic photoreceptor cluster. This expression most likely corresponds to the formation of R6 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 deutocerebrum, 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 detection 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 sma 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 interac-

![Fig. 4.](image_url)

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.

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 Antp, Sex, 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 ey gene for targeted expression in Drosophila. Similar to the results obtained for the Drosophila ey gene, the mouse gene Sex 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 cascades 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.

REFERENCES AND NOTES

6. The expression pattern of ey was analyzed by staining of the different mutant eye-antennal discs with a polyclonal ras antibody and a fluorescein-labeled secondary antibody. The mutants have been de-