Two Pax genes, *eye gone* and *eyeless*, act cooperatively in promoting *Drosophila* eye development

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We report the identification of a *Drosophila* Pax gene, *eye gone* (*eyg*), which is required for eye development. Loss-of-function *eyg* mutations cause reduction or absence of the eye. Similar to the Pax6 *eyeless* (*eya*) gene, ectopic expression of *eyg* induces extra eye formation, but at sites different from those induced by *eya*. Several lines of evidence suggest that *eyg* and *eya* act cooperatively: (1) *eyg* expression is not regulated by *eya*, nor does it regulate *eya* expression, (2) *eyg*-induced ectopic morphogenetic furrow formation does not require *eya*, nor does *eya*-induced ectopic eye production require *eyg*, (3) *eyg* and *eya* can partially substitute for the function of the other, and (4) coexpression of *eyg* and *eya* has a synergistic enhancement of ectopic eye formation. Our results also show that *eyg* has two major functions: to promote cell proliferation in the eye disc and to promote eye development through suppression of *wg* transcription.

Key words: *Drosophila*, Eye development, Pax gene, *eye gone*, *eyeless*, Morphogenetic furrow

INTRODUCTION

Development of the *Drosophila* compound eye begins in the cellular blastoderm embryo, when about 6-23 cells are allocated as the eye-antennal disc primordium. These cells proliferate without differentiation to form the larval eye-antennal disc, from which most of the adult head structures will derive. At early third instar, cells at the posterior of the eye disc start to differentiate, and the differentiation progresses in a posterior-to-anterior direction (Wolff and Ready, 1993). The front of differentiation is marked by an indent called the morphogenetic furrow (MF). Cells are undifferentiated anterior to the propagating furrow, and become progressively differentiated posterior to the furrow.

In recent years, a small number of genes, all encoding nuclear proteins, have been identified as be required and sufficient (in certain contexts) for the initiation of eye development in *Drosophila* (for reviews, see Desplan, 1997; Treisman, 1999; Kumar and Moses, 2001). Loss-of-function mutations of *toy*, *eya*, *dac* and *tsh* form a complex regulatory network (Desplan, 1997; Shen and Mardon, 1997; Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997; Halder et al., 1998; Pan and Rubin, 1998; Czerny et al., 1999). *toy* acts upstream to regulate *eya* expression but is not regulated by *eya* (Czerny et al., 1999). Several lines of evidence suggest that *eya* acts upstream and regulates the expression of *dac*, *eya* and *tsh*. (1) Its ability to induce ectopic eyes is the strongest. (2) Its normal expression in the eye disc starts earlier (in the embryonic eye disc primordia) than *eya*, *so*, *dac* and *tsh*. (3) The normal expression of *eya* does not require *dac*, *eya* and *so*, while the normal expression of *eya* and *so* requires *eya*. (4) Ectopic *eya* expression can induce the expression of *dac*, *eya* and *tsh*. (5) Ectopic eye induction by *eya* requires *dac*, *eya* and *so*. However, the regulation is not a simple linear pathway, because (1) ectopic expression of *dac*, *eya*, *so* and *tsh* can also induce *eya* expression, at least in the antennal disc, (2) ectopic expression of *dac* and *eya* can induce each other, and (3) *so* and *eya* may up-regulate the expression of the other (when ectopically induced by *eya*). These relationships suggest a positive feedback regulation among...
these genes. These feedback regulations are important for their function, because ectopic eye formation by eya, soleya and dacleya also requires the upstream ey gene (Bonini et al., 1997; Pignoni et al., 1997; Chen et al., 1997).

Although ey has been called a master regulator of eye development (Halder et al., 1995), eye development does not occur in every cell induced to express ey or its downstream genes dac, eya and so (Halder et al., 1995; Shen and Mardon, 1997; Bonini et al., 1997; Pignoni et al., 1997; Chen et al., 1997; Halder et al., 1998). Other genes must collaborate with ey in the induction of eye development. For example, DPP and HH signaling collaborates with EY for ectopic eye induction (Chen et al., 1999; Kango-Singh, 2003). Therefore, the identification of genes that are required for eye development but not directly under ey regulation will lead to a better understanding of the mechanism of eye induction. The optix gene of the Six/so gene family is such a gene. It is capable of inducing ectopic eye development but its expression is not under regulation by ey (Seimiya and Gehring, 2000).

ey gone (eyg) is another gene required for eye development.

The first mutation identified, eyg1, causes the eye to become significantly smaller (FlyBase, 2003). It also shows genetic interaction with ey, as mutants doubly homozygous for hypomorphic viable eyg and ey alleles are not viable (Hunt, 1970). The lethal pharate adults have severely reduced head and complete absence of eyes. These results suggest that eyg may act in the early stages of eye development and interact with ey.

In this report, we identify eyg as a Pax gene. It is expressed in the embryonic eye disc primordium and in the larval eye imaginal disc. We characterize its function in eye development and show that ectopic eyg expression can induce ectopic eye formation. eyg is different from eya, so and dac in that its expression is not primarily regulated by ey, nor does it regulate ey expression. Its ability to induce ectopic eyes does not require ey, nor is it required for the ability of ey to induce ectopic eye. Therefore eyg acts neither upstream nor downstream of ey. In addition, coexpression of eyg and ey causes a synergistic ectopic eye formation. Thus, eyg appears to act cooperatively with ey in eye development. As both genes encode Pax proteins with a homeodomain, this is suggestive of a molecular interaction between the two gene products. We also show that the mechanism by which eyg affects eye development may be through suppressing the expression of wg, which is known to inhibit MF initiation.

**Screening and genetic characterization of eyg mutants**

Several approaches were used to generate eyg mutations. γ-irradiation was used to induce chromosomal aberrations in Eq-1, Eq-2 and Eq-3, which have P-element insertions close to the ey loci (Sun et al., 1995). The males were irradiated with γ-rays (4000R) and mated to w; DHT/TM3, ser females. The progeny were screened for loss of the Equatorial eye pigmentation pattern, i.e. loss of the P[lacW] insertion in 69C. C1 was found to be a large deficiency (69A4-5; 69D4-6; cytology determined by Adelaide Carpenter) and used as a reference for null. eyg12-12 was generated from mobilization of P[lacW] from Eq-1. Complementation over the eyg1 allele gave a strong ey phenotype. eyg12-12 homozygotes are pupal lethal. The pharate adults have a headless phenotype (see text), which is similar to that of eyg12-1/C1, suggesting that eyg12-12 is close to a functionally null allele. Similarly, eyg2-2.2, derived from Eq-2 by P mobilization, was defined as close to a functionally null allele. Int3LRgev/eyg1 flies showed a small eye phenotype, indicating that Int3LRgev (from Bloomington Stock Center) is a weak ey allele. EM458 (kindly provided by Leslie Vosshall, Columbia University) carries a P[GawB] insertion 527 bp upstream of the first ATG represented in the Lune transcript. It is homozygous viable and exhibits no apparent phenotype. eyg2-1, eyg2-2 and eyg4-4 were independently derived from mobilizing the P[GawB] in EM458. The severity of defect of eyg alleles was tested over null alleles (C1 or eyg12-12) and over weak alleles (eyg1 or Int3LRgev), eyg12-12 mutant clones were generated using the hs-FLP/FRT method (Xu and Rubin, 1993), hs-FLP22 and FRT(wh)2A (FlyBase, 2003) were used. Heat shock induction of hs-FLP was at 37°C for 1 hour at the indicated time after egg laying (AEL). The eyg mutant clones in the adult are marked by the loss of pigmentation, dependent on the mini-white marker. In eye discs, the heterozygous cells are marked by one copy of the Ubi-GFP-nls, which encodes a nuclear GFP (Davis et al., 1995). The mutant cells should have no GFP expression, while the wild-type twin-spot should have twice the GFP level. However, the twin-spots have a much stronger GFP expression.

**Transgene constructs and germline transformation**

The 2.7 kb full-length Lune cDNA was cloned into the NolI site of the P[CaSpeR-hs] and P[UAST] vectors (Thummel and Perrimon, 1993). These constructs were used in germline transformation as described previously (Rubin and Spradling, 1982).

**5′-RACE**

Embryos were collected 0-16 hours after egg laying for mRNA purification by the CLONTECH mRNA purification kit. The 5′ end of eyg transcript was amplified by the SMART™ RACE cDNA Amplification kit using two eyg-specific primers for nested PCR. The two primers were 5′-CTAGCAACTTGGAGACAGCTCC-3′ and 5′-GCCAGAATTACCGACAGTAAG-3′ respectively. The PCR products were cloned and multiple clones were sequenced.

**Molecular analysis of mutants**

For the analysis of eyg12-12 which has a P[lacW] insertion, plasmid rescue was performed from genomic DNA. A 10 kb SacII rescued plasmid was sequenced from the termini. The 3′ primer read into an opus retrotransposon [also known as nomad and yoyo (Whalen and Grigliatti, 1998; FlyBase, 2003)], which is present in the original Eq-1 fly but not in the w1158 parental line. The 5′ primer read into a sequence 13 kb downstream of eyg. Sequence flanking the other side of the P[lacW] in eyg12-12, was rescued in a 12.5 kb BglII fragment. The 5′ primer read into a sequence 24 bp upstream of the eyg transcription start site. Thus the eyg12-1/C1 is likely to have a deletion starting 23 bp upstream of eyg and extending 13 kb downstream of eyg. This was confirmed by genomic Southern analysis of eyg12-12 homozygotes (Tb+ larvae and pupae selected from an eyg12-12/TM6B, Tb stock) with EcoRI digestion. For the eyg27-1, eyg22-2 and eyg4-4
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mutants that carried a P[GawB] insert, the genomic region flanking the P insert was amplified by PCR and analyzed by sequencing.

In situ hybridization and histochemical staining

Digoxigenin-labeled antisense RNA probes were used for in situ hybridization experiments as described previously (Tautz and Pfeifle, 1989). Alkaline phosphatase histochemical staining was used to visualize in situ hybridization signals. DNA template for the ey probe was derived from ey exon 9 and was recovered by PCR from genomic DNA. The eyg probe was described previously (Jones et al., 1998). The toe probe was transcribed from an EcoRI-linearized toe EST clone pOT2a-toe (kindly provided by Jyh-Lyh Juang, NHRI, Taiwan). The stringent hybridization condition excluded cross hybridization. w1118 was used as the wild-type control. Embryos were photographed using a Leica DMRB microscope with differential interference contrast (DIC) optics. For the double-labeling RNA in situ hybridization, the fluorescein-labeled eyg antisense RNA probes and the biotin-labeled ey antisense RNA probes were transcribed by the T7, T3 promoter (Boehringer Mannheim). The detection was first with HRP-conjugated anti-fluorescein antibody (1:200) and amplified by the Cy-3-tyramides TSA (NEN Life Sciences, UK), followed by HRP-conjugated streptavidin and amplified with FITC-tyramid TSA (NEN Life Sciences, UK). The detection was first with HRP-conjugated anti-fluorescein antibody (1:200) and amplified with FITC-tyramid TSA (NEN Life Sciences, UK). X-gal staining of lacZ expression was done according to the method of Sun et al. (Sun et al., 1995). mAb22C10 (1:100) (Fujita et al., 1982; Zipursky et al., 1984) was from Seymour Benzer (Caltech). X-gal and antibody double staining was modified from the procedure of Kobayashi and Okada (Kobayashi and Okada, 1993), namely primary and secondary antibody incubation were performed first, followed by X-gal staining, then the peroxidase color development. Incorporation of 5-bromo-2′-deoxyuridine (BrdU) into dividing cells in imaginal discs was done according to the method of Baker and Rubin (Baker and Rubin, 1992). Acridine Orange staining of apoptotic cells was done according to the method of Spreij (Spreij, 1971). Confocal microscopy was performed on a Zeiss LSM510.

RESULTS

eyg is required for early eye disc development

We have identified two enhancer trap lines (Eq-1, Eq-2) (Sun et al., 1995) with the P[lacW] construct inserted near the eye gone (eyg) gene in 69C on the third chromosome. The two lines showed no eyg phenotype. Starting from these lines, a large number of derivative lines were generated (by g-irradiation induced chromosomal aberrations, P-element imprecise excisions and local transpositions), some of which showed eye reduction phenotypes and failed to complement eyg1.

Weak loss-of-function eyg mutations resulted in the reduction or absence of the adult eyes (Fig. 1A,B). In late third instar larvae of the hypomorphic eyg1 mutant, the eye discs were significantly reduced in size, while the antennal discs appeared normal (Fig. 1E). In strong loss-of-function mutants, the adults failed to emerge from the pupal case. Their heads were severely reduced in size, but the appeared normal (Fig. 1C). In a null mutant eygM3-12, the adults have a headless phenotype (Fig. 1D) and all structures derived from the eye-antennal discs were missing. The prominent remaining structure was the labellum (Fig. 1D) derived from the labial
In flies with eyg (Jun et al., 1998) and the accessories (Kammermeier et al., 2001; Kronhamn et al., 2002). In flies with eyg alleles of different strengths, the size reduction of third instar eye discs was proportional to the severity of the adult eye phenotype. Strong alleles did not affect the morphology and size of the antennal discs (Fig. 1J). However, adult eye phenotype. Strong alleles did not affect the influence of third instar eye discs was proportional to the severity of the adult eye phenotype. Strong alleles did not affect the phenotype of antennal discs (Fig. 1I). However, adult eye phenotype. Strong alleles did not affect the of antennal discs (Fig. 1J). However, adult eye phenotype. Strong alleles did not affect the phenotype of antennal discs (Fig. 1I) and the loss of antennal disc-derived structures in null mutants may be secondary effect due to the missing eye disc.

The effect of eyg is already apparent in earlier stages. Strong eyg alleles result in no eye disc or only a rudimentary stub of a disc in early third instar (not shown). Weaker allelic combinations produce a smaller eye disc than normal in early third instar (not shown), and in mid-third instar excessive cell death could be detected anterior to the morphogenetic furrow by staining with the dye Acridine Orange (Fig. 1F). Similar apoptosis anterior to the furrow has been observed in other small eye or eyeless mutants, e.g. eya, so, dac and ey (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Halder et al., 1998). In late third instar, there is no more excessive apoptosis.

The small size of early third instar mutant eye discs indicated that early cell proliferation is affected, or that there is excessive apoptosis prior to third instar, or both. Misexpression of the anti-apoptosis baculoviral protein P35 (Hay et al., 1994), driven by the dpp-GAL4 or ey-GAL4, failed to rescue the ‘no eye’ phenotype in the eyg/eygM3-12 mutant (Fig. 1I and data not shown). The eyg/eygM3-12 mutant had complete absence of the adult eyes (Fig. 2H) and had rudimentary eye discs (not shown). The adult eyes and the larval eye discs were not rescued by misexpression of P35, suggesting that apoptosis is not the major cause of the eye phenotype.

The effect on proliferation is restricted to the early eye disc, since cell proliferation in late third instar mutant eye discs seemed normal, as the two normal mitotic waves could be detected in the eye discs (Fig. 1G). A role for eyg in cell proliferation in the eye disc is supported by the finding that ectopic eyg expression can induce local overgrowth in eye discs (see below). We used the hs-FLP/FRT method (Xu and Rubin, 1993) to generate eyg homozygous mutant clones in an otherwise heterozygous eyg mutant fly. When the clones were induced at 24-48 or 48-72 hours after egg lay (AEL), eygM3-12 clones were not detected in the adult (not shown) nor in the eye discs (Fig. 1J), while the twin spots were large and appeared in different regions in the eye discs (Fig. 1I). This result suggests that eyg has a strong effect on the growth of the eye discs and the effect has no strong regional preference.

eyg encodes a Pax protein

A Pax-like cDNA (originally named Lune (Jun et al., 1998)) that also mapped to 69C was independently isolated based on the presence of a homeobox encoding a Paired-type homeodomain (HD) with a characteristic serine at position 50 (Burglin, 1994). This residue is only found when the HD is associated with a paired domain (PD). The PD is the defining character of Pax proteins (Noll, 1993). It consists of two subdomains, the N-terminal PAI and the C-terminal RED subdomains, that can both bind DNA (Czerny et al., 1993; Epstein et al., 1994; Xu et al., 1995; Jun and Desplan, 1996). Interestingly, the PD encoded by the Lune cDNA contains only a partial PAI subdomain and a complete RED subdomain (Fig. 2A) (Jun et al., 1998). eyg has an open reading frame of 670 amino acids (Fig. 2A) rather than the 523 amino acids originally reported (Jun et al., 1998). These changes involved both N-terminal and C-terminal sequences. 5′-RACE identified two splicing isoforms with their 5′ ends identical to that of the Lune cDNA, indicating that the 5′ end of the cDNA clone represents the transcription start site. Thus the Lune cDNA represents the full-length transcript, and there is no additional upstream exon to provide a functional PAI subdomain. The two isoforms differ in a 67 bp segment (intron I; Fig. 2B), which does not affect the coding region. Five introns were identified (Fig. 2B). All exon-intron junctions conform to the consensus splice site.

In situ hybridization showed that in the eye disc of late third instar larvae, Lune is expressed in the central anterior region, well ahead of the morphogenetic furrow (Fig. 2C). Expression is stronger dorsal to the equator (Fig. 2D). The expression domain is broader (Fig. 2D,E). It is also expressed in the central region of the antennal disc (Fig. 2C), in the anterior notum, dorsal hinge and in an arc at the posterior periphery of the wing pouch of the wing disc (Fig. 2F), and is weakly expressed in several arcs in the leg discs (Fig. 2G). In the embryo it is expressed in the eye-antennal disc primordium (Fig. 7B) (Jones et al., 1998), similar to ey and toy (Quiring et al., 1994; Czerny et al., 1999). In the embryo, it is also expressed in the antennal organ, salivary gland, and in a segmentally repeated lateral pattern (Jones et al., 1998).

Since the Lune cDNA mapped to the same chromosomal region as the eyg mutation, it is expressed in the developing eye, and the genes ey and toy, also Pax genes, have similar ‘eyeless’ mutant phenotype, we speculated that the Lune cDNA might correspond to the eyg gene. We tested this hypothesis using rescue experiments. The Lune cDNA was linked to the hsp70 promoter and the hs-Lune transgene was tested for its ability to rescue the eyg/eygM3-12 mutant phenotype, which is characterized by the complete absence of the eyes (Fig. 2H). Heat shock once every 12 hours throughout development resulted in a number of flies with partial or fully rescued eyes. A single heat pulse could also rescue the phenotype. The frequency of full rescue was 4% (n=50 eyes) at 12-24 hours AEL, 5% (n=20) at 24-48 hours AEL, 24% (n=50) at 60-72 hours AEL, 10% (n=60) at 84-96 hours AEL, with peak rescue efficiency at 60-72 hour AEL (about late second instar larva).

A second Pax gene was identified about 30 kb downstream of eyg, based on the fly genome sequence (Adams et al., 2000). The predicted gene is represented by an EST clone. Its encoded protein is most homologous to EYG in its paired domain and homeodomain, so we named the gene twin of eyg (toe). In the eye disc, the expression pattern of toe (Fig. 3B) is very similar to that of eyg (Fig. 2C, Fig. 3B). Three independent lines, 37-1, 22-2 and 94-4, were generated from mobilization of P[GawB]EM458, which has the insertion 124 bp upstream of the Lune transcription start site and 527 bp upstream of the first
Fig. 2. eyg encodes a Pax protein. (A) The Lune cDNA encodes a Pax protein of 670 amino acids rather than the 523 amino acids previously reported (Jun et al., 1998). The corrected Lune sequence had been deposited into GenBank (AY093962).

There are three potential start codons (indicated by arrows), translating to 670, 643 and 554 amino acids, respectively. It contains a truncated Paired domain (the N-terminal extent of the truncated PAI subdomain is indicated by a wavy line; RED subdomain, residues 167-220) and a Prd-type homeodomain (HD; residues 680-125 and 211 bp, respectively). Restriction sites indicated by EcoRI (E), HindIII (H), BamHI (Bg), bp, respectively. The introns are 67, 1321, 680, 125 and 211 bp, respectively. Restriction sites indicated are: BamHI (B), BgII (Bg), EcoRI (E), HindIII (H) and PstI (P). The three potential translation start sites are indicated. The toe gene is located about 30 kb downstream of eyg and has the same transcriptional orientation as eyg.

(C-G) eyg expression in wild type is detected by in situ hybridization in eye-antenna disc of late third instar larva (C) and leg disc (G) of late third instar larva. In this and all other figures, all eye-antenna discs are oriented with anterior to the right and dorsal to the top. (H) Heat shock induction of the hs-Lune transgene can partially (center) or fully (right) rescue the eyg\textsuperscript{M3-12} mutant phenotype, which is completely eyeless with complete penetrance (left).

ATG. The P[GawB] has transposed 8 bp downstream in all three lines, and is accompanied by an 86 bp, 159 bp and 224 bp deletion, respectively, of the flanking genomic region (Fig. 3A). Thus eyg\textsuperscript{22-2} and eyg\textsuperscript{64-4} have deletions extending into the 5' untranslated region. eyg\textsuperscript{17-1} is homozygous viable and results in a very weak small eye phenotype (Fig. 3B). eyg\textsuperscript{22-2} is homozygous viable and produces a small eye (about 500 ommatidia) phenotype (Fig. 3B). The eyg\textsuperscript{64-4} homozygote dies at the pharate adult stage. The phenotypes of pharates ranged from nearly headless (Fig. 3B; 25/57 flies=44%), to complete absence of eye (similar to Fig. 1B; 5/114 eyes=4%), to small eyes (about 300-400 ommatidia; 59/114 eyes=52%). These mutations failed to complement eyg\textsuperscript{l}, so they are eyg alleles as defined by genetic complementation. In these mutants, eyg mRNA was strongly reduced in the eye disc and in the antenna disc, while toe mRNA level was not significantly affected (Fig. 3B). The eyg\textsuperscript{M3-12} mutant has a large deletion starting at 23 bp upstream of the eyg transcription start site and extending to about 13 kb downstream of eyg (Fig. 3C). The molecular nature of these mutations, together with the rescue results, strongly suggest that the eyg gene is a Pax gene represented by the Lune cDNA.


**eyg expression in early eye disc is functionally important**

Since the eye phenotype discussed above suggests an early role for *eyg* in eye disc development, we checked the expression of *eyg* in earlier eye discs (Fig. 2D,E). Expression was detectable at least as early as late second instar eye disc, in a pattern slightly broader than in the late third instar. Expression in the antennal disc was detectable beginning in mid-third instar (data not shown).

Rescue experiments were performed by induced *eyg* expression to determine the site of functional requirement for *eyg*. The *eyg* cDNA was cloned into the pUAST vector behind a tandem array of five GAL4 binding sites (Brand and Perrimon, 1993). Expression of the *UAS-eyg* transgene is dependent on the presence of the yeast GAL4 transcription factor. Expression of *eyg* induced by the E132-GAL4 and ey-GAL4 (abbreviated E132>eyg and ey>eyg, respectively) could partially or fully rescue the strong *eygL/eygL12* mutant: the eyes were morphologically normal and often of normal size (Fig. 4B and data not shown). *eyg* can rescue the eye in *eygL/eygL12* mutants to medium to full size with complete penetrance (Fig. 4D). E132 drives GAL4 expression in the center of the posterior margin in late third instar eye discs (Fig. 4A) (Halder et al., 1995). In second instar larval eye discs E132 drives expression in a broader posterior-ventral domain (Fig. 4A inset) (Pignoni and Zipursky, 1997). ey-GAL4 drives expression from the posterior margin to a few rows of cells anterior of the MF in late third instar eye disc (Fig. 4C), and in the entire eye disc in the early third and late third instar eye disc (Fig. 4C inset). These rescue results suggest that *eyg* expression in cells common to both E132 and *ey* domain in early eye discs is important for eye development.

**Ectopic expression of *eyg* can cause ectopic eye formation**

*eyg* and *ey* are similar in that both are Pax genes and are required for eye formation. Therefore, we asked whether ectopic expression of *eyg* would be sufficient to direct the development of ectopic eyes, as is the case for *ey*. Five independent *UAS-eyg* lines were crossed with many GAL4 lines specifying a variety of expression patterns. Several phenotypes could be observed among the few GAL4 lines that gave surviving adults: missing bristles on the notum, extra scutellar bristles, shortening and malformation of the legs, and unexpanded wings. The most dramatic phenotype was the formation of extra eyes (see below), observed when ectopic expression of *UAS-eyg* was induced by *dpp-GAL4*.

In the eye disc, *dpp-GAL4* is expressed along the posterior and lateral margins (Treisman and Rubin, 1995). The *eyg*-induced extra eyes were almost always located ventral to the endogenous eyes. They often appeared linked to the endogenous eye (Fig. 5A), but occasionally were well separated and were surrounded by orbital bristles (Fig. 5B), suggesting that they represent intact eye fields. The penetrance of extra eye

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**Fig. 3.** Molecular analysis of *eyg* mutants. (A) *eyg*17-1, *eyg*22-2 and *eyg*64-4 are three *eyg* mutants independently derived from mobilization of the *P*{GawB}^{EM458} (see Materials and Methods). The three lines have the P{GawB} inserted at the same location, 124 bp upstream from the *eyg* transcription start site, but the extent of the deletion of the flanking genomic region varies. (B) *eyg* and *toe* are both expressed in the eye and antenna discs, as detected by in situ hybridization. In the three *eyg* mutants, *eyg* RNA is progressively reduced in both the eye and the antenna discs but *toe* RNA is not significantly affected. The three deletion mutants also show progressively stronger eye reduction. (C) The *eyg*64-4 mutant has a 16861 bp deletion beginning at 23 bp upstream of the *eyg* transcription start site and extending about 13 kb downstream of *eyg*. The P{lacW} insert is indicated (*eyg*64-4) with the arrow showing the transcriptional direction of mini-white and lacZ. The black triangle indicates the presence of an opus retrotransposon.
formation is low. With a strong UAS-eyg line, almost all UAS-eyg/+; dpp-GAL4/+ (abbreviated dpp>eyg) flies have some recognizable defect in the ventral head: the ventral-posterior rim of the eye was reduced and replaced by head cuticle, and/or a few extra bristles, only 16% of eyes have extra ommatidia, and only 38% of these have the extra ommatidia as an isolated extra eye.

The formation of an extra eye field could also be detected in the larval eye disc. For these experiments, we used the monoclonal antibody 22C10 (Fujita et al., 1982; Zipursky et al., 1984) to detect photoreceptor neurons and lacZ driven by a different dpp enhancer (BS3.0) (Blackman et al., 1991) to mark the morphogenetic furrow. In wild-type eye-antennal discs, dpp-lacZ is expressed in the MF (Fig. 5C, black arrow) and in a small dorsal region in the antennal disc (black arrowhead). Local overgrowth (white arrowheads) almost always occurred in the ventral and dorsal region of the eye disc adjacent to the antenna disc. These were often, but not always, accompanied by ectopic MF and photoreceptor formation (white arrow), frequently in the ventral pole and only occasionally in the dorsal pole. (D) Higher magnification showing that the axons from the ectopic photoreceptor neurons can sometimes correctly sense the direction of Bolwig’s nerve (arrow), but sometimes project in the wrong direction (arrowhead). Occasionally multiple MF formation can be seen (not shown). (E-H) Synergistic effect of eyg and ey coexpression. (E) Ectopic MF (dpp-lacZ, blue) and photoreceptors (22C10, brown) can be detected in the antennal disc but not in the eye disc. (F) Ectopic MF (dpp-lacZ, blue) and photoreceptors (22C10, brown) were enhanced in the antennal disc, and extended more anteriorly in the ventral margin of the eye disc. Multiple sites of ectopic MF formation in the eye disc and in the antenna disc sometimes led to fusion of the eye fields.

MFs could be seen (Fig. 5C,D), most often at dorsal and ventral sites between the eye and antennal discs, and at the dorsal and ventral poles of the eye disc. These were propagating toward the center of the disc and usually associated with local
overgrowth and sometimes with photoreceptor neuronal differentiation. The photoreceptor clusters were sometimes well separated from the endogenous eye (Fig. 5C), corresponding to the extra eyes found in the adults (Fig. 5B). The percentage of ectopic photoreceptors in discs is higher than the percentage of extra eyes in adults, suggesting that as the endogenous and extra eye fields expand, they often fuse. All these observations indicate that ectopic expression of eyg along the posterior and lateral margin of the eye disc can induce ectopic MF initiation. When the ectopic furrow occurs far away from the endogenous furrow, an extra eye may result.

**eyg and ey are transcriptionally independent**

Ectopic expression of eyg and ey with the same dpp-GAL4 driver produced ectopic eyes at different sites: eyg-induced eyes occurred in the ventral part of the head, while ey-induced eyes occurred at the base of antennae, wings, halteres and on leg segments (Fig. 5E) (Halder et al., 1995). In imaginal discs, dpp>ey caused overgrowth and the formation of MFs and photoreceptors in the antenna, wing, haltere and leg discs (Fig. 5F, and data not shown), consistent with the ectopic eyes seen in adults. No extra photoreceptors were detected at the ventral margin in the eye-antennal disc (Fig. 5F), in contrast to the effect of eyg expression. The preferential effect in the ventral side of the eye disc by dpp>eyg is similar to the effect of dpp>dac+eya (Chen et al., 1997). In the wing disc, dpp>eyg occasionally caused an extra dpp-lacZ-expressing spot at the anterior side of the hinge region (not shown). However, the dpp-lacZ spot does not represent an ectopic MF, as no photoreceptors were detected in the wing and leg discs. These results suggest that neither eyg nor ey activate the expression of the other, since their phenotypes are so distinct. We checked this possibility by analyzing ey and eyg expression in mutant backgrounds.

**eyg expression** was examined in the ey² mutant. Although ey² is not a null mutant (Kronhamn et al., 2002), it has no detectable ey transcript in the embryonic eye disc primordium and in the larval eye disc (Quiring et al., 1994). ey² eye phenotype is variable. For the ey² stock we used, 40% of the eyes had 300-400 ommatidia, 40% had about 200 ommatidia, and 20% had less than 100 ommatidia. The ey² eye disc is also variable in size. However, an ey² eye disc of substantial size still has no ey expression (Quiring et al., 1994). So we examined ey² eye discs that were clearly reduced in size (to be sure that it was a mutant disc) but had sufficient eye field present to check for eyg expression. In ey² late third instar eye disc, the eyg dorsal expression was not affected while the ventral expression was reduced (Fig. 6E). In ey² embryos, eyg expression was still present in the eye disc primordium (Fig. 6B). Similarly, ey expression in the eye disc primordium (Fig. 6C) was still present in eyg null mutant embryos (C1/C1) (Fig. 6D). The presence of ey expression suggests that the development of the eye disc primordium is not strictly dependent on eyg function. Halder et al. (Halder et al., 1995) indicated that ey expression is not affected in eyg mutant eye discs. These results suggested that, except for a small amount of eyg expression in the ventral part of the eye disc, the expression of neither eyg nor ey is strongly dependent on the other.

We next checked whether ectopic eyg expression could induce the expression of ey, or vice versa. In the imaginal disc, dpp>eyg induced eyg expression at the lateral margins of the eye disc (Fig. 6F, black arrowheads), in the antennal disc (Fig. 6F, black arrow) and in the wing disc (Fig. 6J, black arrowhead), but did not induce ey expression (Fig. 6G, K). dpp>ey induced ey expression in the eye-antennal disc (Fig. 6I, black arrowheads) and the wing disc (Fig. 6L, green), but did not induce eyg expression (Fig. 6H, red in L). The results with embryos were similar; neither ectopic eyg nor ectopic ey (driven in both cases by rho-GAL4) could induce expression of the other (results not shown). These results suggest that neither eyg nor ey is sufficient for the expression of the other. In summary, the two genes appeared to be transcriptionally independent.

**Functional relationship between eyg and ey**

We tested whether eyg is functionally dependent on ey, and vice versa. When E132>eyg induction occurred in an ey² mutant, ectopic ventral eyes could still form (Fig. 7A), suggesting that ey is not required for eyg function. This is in contrast to the situation with eya, so, dsc and toy: the ectopic eyes caused by their ectopic expression cannot form in the ey² mutant (Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997; Czerny et al., 1999). Similarly, dpp>eyg induced ectopic eye formation in eygM3-12 mutants (Fig. 7B), suggesting that eyg is not required for ey function. Again, this is in contrast to the requirement for eya, so and dsc in ey-induced ectopic eye formation (Bonini et al., 1997; Chen et al., 1997; Shen and Mardon, 1997; Halder et al., 1998). These results suggest that eyg and ey can function independently to induce eye formation.

While the above experiments show that eyg and ey can independently induce eye formation, their coexpression showed synergistic enhancement of ectopic eye formation. The ectopic eyes in the antenna, wing, haltere and legs are larger (Fig. 5G), similar to the effect of dpp>ey at higher temperatures (due to higher GAL4 activity). The enhancement is only evident with a weak dpp-GAL4 line. With stronger dpp-GAL4 lines, the ectopic eye phenotype is already strong with UAS-ey alone and cannot be enhanced further by adding UAS-eyg. The enhancement is more evident in the imaginal discs than in the adults. The ectopic eyes in the ventral head are not significantly enhanced in the adults, but are clearly enhanced in the eye discs (Fig. 5H). The difference between the strength of phenotypes in adults and the imaginal discs suggest that there may be some regulative mechanism in the eye field that compensates for the ectopic photoreceptors. The synergistic effect of eyg and ey coexpression was also observed when driven by the E132-GAL4 line (not shown).

Expression of ey induced by ey-GAL4 can partially rescue the eyg¹eygM3-12 mutant eye phenotype (Fig. 4E), indicating that ey can functionally substitute for eyg. Since there is endogenous ey expression, the rescue suggests that EY is required at a level higher than its endogenous expression level in order to compensate for the loss of eyg. Reciprocally, we checked whether expression of eyg could rescue ey mutant phenotype. Since even the strongest ey alleles result in a variable eye phenotype (Kronhamn et al., 2002; Benassayag et al., 2003), we used the ey⁰ey² allelic combination, which results in no eyes and is nearly completely penetrant. ey⁰ has a chromosomal rearrangement interrupting the ey gene, producing a truncated protein lacking the homeodomain
eye gone, a new Pax gene for eye development

(Kronhamn et al., 2002). ey² contains a transposon insertion in an eye-specific enhancer and has no detectable RNA and protein expression in the larval eye disc and in the embryonic eye disc primordium (Quiring et al., 1994; Halder et al., 1998). dpp>eyg can partially rescue ey² mutants (Fig. 4F), suggesting that eyg can functionally substitute for ey.

**eyg suppresses wg transcription**

The wingless (wg) gene encodes a secreted signaling protein of the Wnt family. It is expressed in the dorsal and ventral margins of the eye disc (Fig. 8A), and acts to inhibit MF initiation from these sites (Ma and Moses, 1995; Treisman and Rubin, 1995). In eyg¹ eye disc, wg expression domain expands toward the posterior margin (Fig. 8C) (Hazelett et al., 1998). However, wg expression is not derepressed in the central region (Fig. 8C) where eyg is normally expressed (Fig. 4), probably because eyg¹ is a hypomorphic allele and has sufficient activity in this region to suppress wg. Because the enhancer trap wag-lacZ reporter was used to monitor wg expression, the suppression is at the transcriptional level. In addition, ectopic expression of eyg (dpp>eyg) suppressed wag-lacZ expression in the dorsal and ventral margins of the eye disc and in part of the wing disc (compare Fig. 8B,E with 8A,D).

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Fig. 6. eyg and ey are transcriptionally independent. (A-D) eyg and ey expression in stage 17 embryos. In wild-type embryos, eyg (A) and ey (C) were expressed in the eye-antennal disc primordium (arrow). (B) In ey² mutant embryos, eyg expression in eye-antennal disc primordium appeared normal. (D) In embryos homozygous for the C1 deficiency (null for eyg), ey expression in the eye-antennal disc primordium appeared normal, although the head was deformed. (E) eyg expression in an ey² mutant eye-antennal disc. The ventral expression was reduced, while the dorsal expression was not significantly affected. (F,G,J,K) Ectopic dpp>eyg induced eyg expression in the dpp domains in eye (F, black arrowheads), antenna (F, arrow) and wing (J, black arrowheads) discs. The induced eyg expression was patchy along the eye disc lateral margins and did not occur in the posterior margin (F). The induced expression was much stronger than the endogenous eyg expression in the anterior notum in wing discs (J, white arrowhead) and in the antenna discs (F, white arrowhead), which is barely detectable with this staining condition. dpp>eyg did not induce ey expression in the eye-antennal (G) and wing (K) discs. (H,I,L) dpp>ey induced ey expression in the eye-antennal disc (I, arrowheads) and in wing disc (L, green), but did not induce eyg expression in the eye-antennal disc (H) and wing disc (L, red). Note the outgrowth of the antennal disc (H,I) that corresponds to the ectopic eye induced by dpp>ey. The expressions were detected by RNA in situ hybridization.

Fig. 7. eyg and ey can function independently. (A) In the ey² mutant, E132>eyg can cause an ectopic ventral eye, similar to E132>eyg in the wild-type background. (B) In the eyg¹ mutant, dpp>ey can cause ectopic eye formation on wings, legs and antennae, similar to the effect of dpp>ey in wild-type background.
**Fig. 8.** eyg can suppress wg expression. (A) wg-lacZ expression in a wild-type eye-antennal disc. (B) The expression in the dorsal and ventral poles of the eye disc were suppressed in dpp>eyg, and in dpp>ey (not shown). Note the posterior margin and the dorsal overgrowth express wg-lacZ. (C) The wg-lacZ expression domain expanded to the posterior margin in the eyg\(^1\) mutant eye disc. The eye disc was significantly reduced. (D) wg-lacZ expression in a wild-type wing disc. The expression in dpp>eyg (E) was suppressed in a region where the wg expression domain intersects the dpp expression domain (*). The dpp-GAL4 expression domain was visualized by dpp>lacZ (F). wg-lacZ was similarly suppressed by dpp>ey in wing disc (not shown).

**DISCUSSION**

**eyg is an important regulator of eye development**

Two Pax genes are located in the chromosomal region of the eyg mutation. One of these is represented by the previously reported Lune cDNA (Jun et al., 1998). In the eyg\(^1\) mutant, we sequenced the coding region of both Pax genes. Although a few changes were found, none affected the conserved residues (C.-C. J. and Y.H.S., unpublished). Furthermore, the expression level of both genes in eyg\(^1\) mutant eye disc appeared normal (C.-C. J. and Y.H.S., unpublished). Thus the analysis of the eyg\(^1\) mutant did not clearly indicate which of the two genes corresponds to eyg. We showed that three new eyg alleles, as defined by failure to complement the eyg\(^1\) mutation, have molecular defect in the Lune transcription unit and affected Lune RNA level, while the expression level of the adjacent Pax gene is not affected. A fourth mutant, eyg\(^{M3-12}\), also does not complement eyg\(^1\) and is genetically defined as being functionally null for the eyg eye function. eyg\(^{M3-12}\) has a deletion of the Lune transcription unit but is not affected in the downstream Pax transcription unit. In our preliminary analysis of the eyg-toe region, only one region specifying eye disc expression was identified, and it is located downstream of toe and not affected in these four mutants (S.-J. Chiou and Y.H.S., unpublished). The molecular nature of these four mutations, coupled with the rescue results, strongly suggest that the Lune cDNA corresponds to the eyg gene. We named the adjacent Pax gene twin of eyg (toe).

In the embryo eyg transcripts appear in the eye-antennal disc primordium beginning at stage 15. It continues to be expressed as the disc cells proliferate during early larval development and then is expressed in an anterior region overlapping the equator of the eye disc as photoreceptor differentiation occurs. eyg is required for eye development, as loss-of-function mutations lead to the reduction or absence of the eye. It appears to be required for the early proliferation of the eye disc cells, as the early third instar eye disc is significantly smaller. The rescue experiments (hs-eyg, ey>eyg and E132>eyg) suggested that the critical time for eyg function is in the late second instar. Excessive apoptosis occurred in the mid-third instar eye disc, but is not the major cause of the eye phenotype because blocking apoptosis did not rescue the eye phenotype. Ectopic expression of eyg can lead to ectopic MF initiation in the ventral side of the eye disc. Thus, the loss-of-function and gain-of-function phenotypes suggest that eyg acts as an important regulator of eye development.

eyg appears to have two major functions. The first is to promote cell proliferation in the eye disc. eyg loss-of-function mutants have reduced eye discs, already apparent in early third instar, before photoreceptor differentiation. In clonal analysis, eyg\(^{M3-12}\) mutant clones induced in first or second instar are undetectable in late third instar eye disc (Fig. 1). Ectopic eyg expression caused local overgrowth (Fig. 6C), a phenotype opposite of the loss-of-function phenotype. The overgrowth does not always develop into photoreceptor cells (Fig. 6C). These results indicate that eyg promotes cell proliferation independent of photoreceptor differentiation. The second function of eyg is to promote eye development or MF initiation. If the eyg-induced proliferation occurs at the ventral margin of the eye disc, ectopic MF can initiate (Fig. 6C,D). The induction of ectopic MF is probably mediated by the suppression of wg (see later), which is known to repress MF initiation along the lateral margins.

**eyg and ey act cooperatively**

Since eyg is a Pax gene that shares sequence similarity with ey and toy in the PD and HD domains (Jun et al., 1998), its relationship with ey is of particular interest. Our results indicate that eyg and ey are transcriptionally and functionally independent: (1) except for a small amount of eyg expression ventral to the equator of the eye disc, eyg and ey do not regulate each other’s expression. In this respect, eyg is different from dac, so and eya, whose expression is strongly regulated by ey (and can induce ey expression in some cases). Thus eyg transcription is neither downstream of ey, nor does eyg participate in the eylaysia locus positive feedback loop. This transcriptional independence is similar to that of optix (Seimiya and Gehring, 2000). (2) eyg and ey can each function (to induce ectopic eyes) in the absence of the other. Again, this is similar to optix, which can induce ectopic eyes in ey\(^2\) mutant (Seimiya and Gehring, 2000). Whether optix is required for ey function has not been tested, because of the lack of optix mutants.

However, other evidence indicates that the functions of eyg and ey must converge at some point in the pathway leading to eye development: (1) eyg; ey double hypomorphic mutants showed a much stronger eye-loss phenotype (Hunt, 1970), (2)
coexpression of ey and eyg caused synergistic enhancement of the ectopic eye phenotype. (3) eyg and ey are able to substitute functionally for each other. Overall, the results suggest that these two Pax genes may act cooperatively. This genetic cooperativity might mean that eyg and ey interact and cooperate as proteins in the same pathway or that they act in parallel pathways. eyg and ey are coexpressed in the eye disc primordium in the embryo (Fig. 7). Their expression domain also overlap in the eye disc, especially in the early eye disc (Fig. 4) when eyg function is critically required. So it is possible that the two Pax proteins act within the same cell, although we do not rule out the possibility they act in different cells to achieve a functional cooperativity.

If eyg and ey are both required for eye development, how could ectopic expression of either one be sufficient for ectopic eye development? One possibility is that the two Pax proteins form heterodimers, directly or indirectly via other proteins, to activate target genes. When the level of either one is low, the target genes that lead to eye formation cannot be induced. However, when either one is strongly expressed ectopically, the high level of homodimer can partially substitute for the heterodimer. Since both genes are required for normal eye formation, this model predicts that the EYG-EY heterodimer is more effective than either homodimer in inducing eye formation. As expected by this model, coexpression of eyg and ey caused enhanced ectopic eye formation.

Possible mechanisms of EYG protein function

The EYG protein has two DNA binding domains: the RED subdomain of its truncated PD and the Prd-class HD. It probably functions as a transcription factor by binding DNA targets through these domains, singly or in combination. In addition, its interaction with other proteins may affect this DNA binding.

The PD consists of two independent subdomains: the N-terminal PAI and the C-terminal RED subdomains. Based on crystal structure of the human Pax6 PD, the linker region connecting the two subdomains also contacts DNA (Xu et al., 1999). In EYG, the PAI subdomain is largely missing and most likely cannot bind DNA. One interesting possibility is that the truncated EYG PD has a dominant negative effect, competing with other PD proteins. In addition, truncation of the PAI subdomain in the Pax6-5α and Pax8(S) isoforms probably exposes the RED subdomain to recognize a distinctly different DNA sequence (Epstein et al., 1994; Kozmik et al., 1998). Thus the EYG PD may bind DNA through its RED domain, as predicted in fact proved by site-selection using the EYG RED domain (Jun et al., 1998). Through its RED domain, EYG can probably regulate different target genes than those regulated by EY. This ey-independent function of eyg is also shown by its involvement in salivary gland development (Jones et al., 1998), and in bristle formation when ectopically expressed (see Results). Vertebrate homologs of EYG have not yet been identified. It is possible that EYG plays a role equivalent to the vertebrate Pax6-5α isoform.

In addition to the PD, many Pax proteins (including EY and EYG) also contain a Prd-class homeodomain. Two Prd-type HDs can bind cooperatively to a palindromic site composed of two inverted TAAT motifs separated by 2 or 3 bps (Wilson et al., 1993). The Prd-type HD of EYG can form heterodimers with the Prd-type HD of Prd upon binding to a consensus DNA target (Wilson et al., 1993; Wilson et al., 1996). It is possible that EY and EYG also form heterodimers via their HDs. This would be consistent with our findings that they act synergistically. However, although the HD of EYG is required for its functions (J. G. Yao and Y.H.S., unpublished), the HD of EY has been shown not to be required for its function in eye development (Punzo et al., 2001). Thus the HD of EYG is required, not for direct interaction with the HD of EY, but may be for DNA binding or for interacting with other proteins.

eyg suppresses wg transcription in the eye disc

Dpp and Wg are two signaling molecules important for the initiation of eye differentiation: Dpp activates MF initiation while Wg suppresses it (Heberlein et al., 1993; Wiersdorff et al., 1996; Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997; Ma and Moses, 1995; Treisman and Rubin, 1995). Does eyg exert its effect on eye development by activating Dpp signaling or by suppressing Wg signaling?

dpp is expressed at two stages in the eye disc: an early expression along the posterior and lateral margins (represented by the dpp-GALA), and a later expression in the propagating MF (represented by the dpp-lacZ). The early expression in the margins is required for MF initiation (Burke and Basler, 1996; Wiersdorff et al., 1996). It was found that dpp expression along the lateral margins is absent in early third instar eyg1 eye disc (Hazelett et al., 1998), suggesting that dpp expression in the lateral margins is regulated by eyg. However, activating DPP signaling at the lateral margin did not rescue the eygl phenotype (Hazelett et al., 1998), suggesting that eyg has other functions in addition to activating dpp expression.

wg is expressed uniformly in the eye disc of second instar larvae (Royet and Finkelstein, 1997). In the third instar eye disc, wg is expressed in the lateral margins and acts to prevent MF initiation from the lateral margins (Ma and Moses, 1995; Treisman and Rubin, 1995). The wg-expression domain expands in eygl eye discs (Fig. 8C) (Hazelett et al., 1998). Our results further showed that ectopic eyg expression (dpp>eyg) could suppress wg expression at the transcriptional level. The suppression of wg is functionally significant, because expression of the wg-activated omb gene is similarly suppressed in dpp>eyg (J.-L. Chao and Y.H.S., unpublished). Hazelett et al. (Hazelett et al., 1998) have shown that blocking of the Wg signaling pathway can partially rescue the eyg mutant phenotype. These results indicate that the suppression of wg transcription by eyg may be a major mechanism by which eyg induces MF initiation, hence eye development. This is consistent with our finding that ectopic eyg induces ectopic eye formation primarily in the ventral margin of the eye disc, where wg expression is weaker (Fig. 8A) and most easily suppressed by dpp (Pignoni and Zipursky, 1997). wg is normally expressed in the entire eye disc during second instar (Royet and Finkelstein, 1997). It was shown that Wg signaling can suppress the expression of so and eya (Biaozza and Freeman, 2002). It is possible that in the late second instar eye disc, eyg expression in the central domain of the eye disc suppresses wg expression in the central domain, thus allowing the expression of eya and so, hence eye development.

As predicted by the eyg and ey interaction, ey also suppresses wg expression (data not shown). Suppression of wg expression
by eyg (and ey) is also seen in the wing disc (Fig. 8E). However, suppression does not occur in all cells expressing eyg, suggesting that additional factors are required for the wg suppression. The relationship of eygley and wg may be mutually antagonistic, since ectopic ey cannot induce eye and so expression in regions of high wg expression (Halder et al., 1998).

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