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

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SUMMARY

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* (*ey*) gene, ectopic expression of *eyg* induces extra eye formation, but at sites different from those induced by *ey*. Several lines of evidence suggest that *eyg* and *ey* act cooperatively: (1) *eyg* expression is not regulated by *ey*, nor does it regulate *ey* expression, (2) *eyg*-induced ectopic morphogenetic furrow formation does not require *ey*, nor does *ey*-induced ectopic eye production require *eyg*, (3) *eyg* and *ey* can partially substitute for the function of the other, and (4) coexpression of *eyg* and *ey* 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*, *ey*, *dac*, *eya* and *so* can lead to reduction or absence of the adult eye, while ectopic expression of these genes, either alone or in combination, can lead to ectopic eye formation (Halder et al., 1995; Shen and Mardon, 1997; Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997; Czerny et al., 1999; Kronhamn et al., 2002), suggesting that they act at the very early steps of eye development. Two other nuclear factor genes, *tsh* and *optix*, can also induce ectopic eye upon ectopic expression (Pan and Rubin, 1998; Seimiya and Gehring, 2000) [see also Singh et al. (Singh et al., 2002) for an additional role of *tsh*].

Epistasis analysis in loss-of-function mutants and in mis-expression situations suggests that *toy*, *ey*, *so*, *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 *ey* expression but is not regulated by *ey* (Czerny et al., 1999). Several lines of evidence suggest that *ey* acts upstream and regulates the expression of *dac*, *eya*, *so* 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 *ey* does not require *dac*, *eya* and *so*, while the normal expression of *eya* and *so* requires *ey*. (4) Ectopic *ey* expression can induce the expression of *dac*, *eya*, *so* and *tsh*. (5) Ectopic eye induction by *ey* 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 *ey* 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 *ey*). These relationships suggest a positive feedback regulation among

these genes. These feedback regulations are important for their function, because ectopic eye formation by *eya*, *so*/*eya* and *dac*/*eya* 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).

eye gone (*eyg*) is another gene required for eye development. The first mutation identified, eyg^{1}, 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.

MATERIALS AND METHODS

Fly stocks

Several *dpp-GAL4* lines (Staehling-Hampton et al., 1994) were used: *w; CyO/Sp; dpp-GAL4*$^{c40.1}$*/TM6B*, from Patrick Callaerts (University of Basel, Switzerland), *w; Sp/CyO; P[w*$^{+m}$*.hs,GAL4-dpp.blk1]*$^{40C.6}$*/TM6B*, from the Bloomington Stock Center, and *w; dpp-lacZ (BS3.0)H1-1; dpp-GAL4*$^{c40.6}$*/SM6-TM6B*, from Jessica Treisman (Skirball Inst., NY). The one from Bloomington gave a weaker phenotype in ectopic eye induction, and was used to show the synergistic effect of *eyg* and *ey*. E132-*GAL4*, *UAS-ey* and ey^{2} flies (Halder et al., 1995) were from Patrick Callaerts. Two *ey-GAL4* lines were from Uwe Walldorf (University of Hohenheim, Stuttgart, Germany). Other fly stocks were obtained from the Bloomington *Drosophila* Stock Center and the Mid-America *Drosophila* Stock Center (Bowling Green, Ohio).

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 *eyg* locus (Sun et al., 1995). The males were irradiated with γ-rays (4000R) and mated to *w; DH/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. $eyg^{M3\text{-}12}$ was generated from mobilization of P[lacW] from Eq-1. Complementation over the eyg^{1} allele gave a strong *eyg* phenotype. $eyg^{M3\text{-}12}$ homozygotes are pupal lethal. The pharate adults have a headless phenotype (see text), which is similar to that of $eyg^{M3\text{-}12}$/C1, suggesting that $eyg^{M3\text{-}12}$ is close to a functionally null allele. Similarly, $eyg^{Eq\text{-}2\text{-}d2\text{-}2}$, derived from Eq-2 by P mobilization, was defined as close to a functionally null allele. *In(3LR)gv*^{u}*/eyg*^{1} flies showed a small eye phenotype, indicating that *In(3LR)gv*^{u} (from Bloomington Stock Center) is a weak *eyg* 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. $eyg^{37\text{-}1}$, $eyg^{22\text{-}2}$ and $eyg^{94\text{-}4}$ were independently derived from mobilizing the P[GawB] in EM458. The severity of defect of *eyg* alleles was tested over null alleles (*C1* or $eyg^{M3\text{-}12}$) and over weak alleles (eyg^{1} or *In(3LR)gv*^{u}). $eyg^{M3\text{-}12}$ mutant clones were generated using the hs-FLP/FRT method (Xu and Rubin, 1993). *hs-FLP*^{22} and *FRT(w*^{hs}*)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 *Not*I site of the P[CaSpeR-hs] and P[UAST] vectors (Thummel and Pirrotta, 1992; Brand 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′-GCCAGAATTAGCGACAGTAAG-3′ respectively. The PCR products were cloned and multiple clones were sequenced.

Molecular analysis of mutants

For the analysis of $eyg^{M3\text{-}12}$ which has a P[lacW] insertion, plasmid rescue was performed from genomic DNA. A 10 kb *Sac*II 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 w^{1118} parental line. The 5′ primer read into a sequence 13 kb downstream of *eyg*. Sequence flanking the other side of the P[lacW] in $eyg^{M3\text{-}12}$, was rescued in a 12.5 kb *Bgl*II fragment. The 5′ primer read into a sequence 24 bp upstream of the *eyg* transcription start site. Thus the $eyg^{M3\text{-}12}$ 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 $eyg^{M3\text{-}12}$ homozygotes (Tb^{+} larvae and pupae selected from an $eyg^{M3\text{-}12}$*/TM6B, Tb* stock) with *Eco*RI digestion. For the $eyg^{37\text{-}1}$, $eyg^{22\text{-}2}$ and $eyg^{94\text{-}4}$

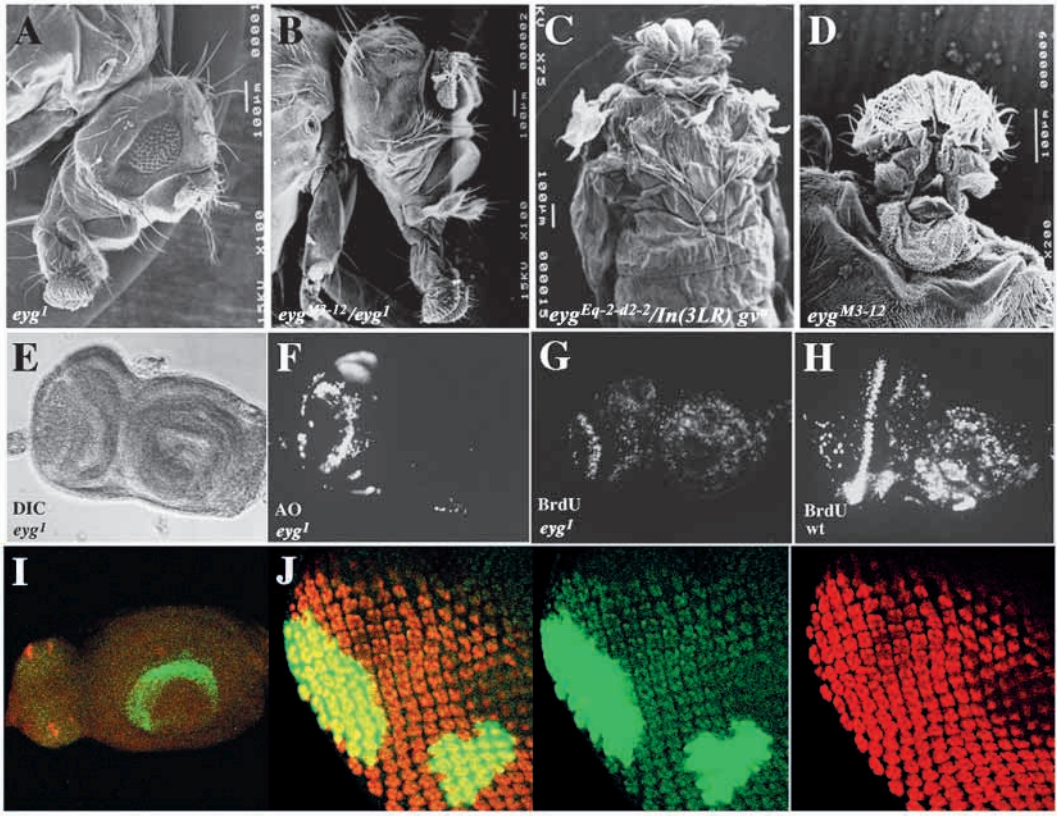

Fig. 1. *eyg* loss-of-function mutant phenotype. (A-D) Scanning electron micrographs showing a range of *eyg* mutant phenotypes. (A) eyg^{1}, (B) $eyg^{M3\text{-}12}/eyg^{1}$, (C,D) pharate adults that failed to eclose: (C) $eyg^{Eq\text{-}2\text{-}d2\text{-}2}/In(3LR)gv^{u}$, most of the head structures were lost, but the antennae were present; (D) $eyg^{M3\text{-}12}$, a headless null mutant at higher magnification. (E-H) Eye-antennal disc. (E) DIC image and (F) Acridine Orange staining of the same disc from a mid-third instar eyg^{1} larva. (G) BrdU labeling of an eyg^{1} disc and (H) a wild-type disc from a late third instar larva. (I) Expression of *P35* driven by the *ey-GAL4* (abbreviated to *ey>P35* in text) did not rescue the $eyg^{1}/eyg^{M3\text{-}12}$ phenotype. The eye disc is strongly reduced with only a few ELAV-positive cells (red), as in $eyg^{1}/eyg^{M3\text{-}12}$ mutants, while the antenna disc (with DAC expression, green) is of normal size. (J) $eyg^{M3\text{-}12}$ clones (marked by the absence of GFP staining; green) induced at 24-48 hours AEL were not detected in the eye disc. The heterozygous cells have nuclear GFP, while the wild-type twin-spots have a very strong GFP signal. ELAV (red) stains the photoreceptors.

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 *Eco*RI-linearized *toe* EST clone pOT2a-*toe* (kindly provided by Jyh-Lyh Juang, NHRI, Taiwan). The stringent hybridization condition excluded cross hybridization. w^{1118} 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 inactivation for 15 minutes at 70°C, and then by HRP-conjugated streptavidin 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 Seymor 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 γ-irradiation induced chromosomal aberrations, P-element imprecise excisions and local transpositions), some of which showed eye reduction phenotypes and failed to complement eyg^{1}.

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 eyg^{1} 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 $eyg^{M3\text{-}12}$, 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

discs. The fish-trap bristles, derived from the clypeo-labral disc, were also present (not shown). The headless phenotype is similar to those reported for *ey* and *toy* null mutants (Jiao et al., 2001; 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, no eye-antennal discs could be found in $eyg^{M3\text{-}12}$ larvae. These observations suggested that the antennal disc only requires a low EYG level or activity, so the antennal phenotype is manifested only in the null mutant, a situation similar to those of *ey* and *toy* mutants (Kronhamn et al., 2002). Alternatively, the effect of *eyg* may be specific to the eye disc, 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 either 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 $eyg^{1}/eyg^{M3\text{-}12}$ mutant (Fig. 1I and data not shown). The $eyg^{1}/eyg^{M3\text{-}12}$ 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), $eyg^{M3\text{-}12}$ 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. 1J). 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 at 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. In the early eye disc, 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 $eyg^{1}/eyg^{M3\text{-}12}$ 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

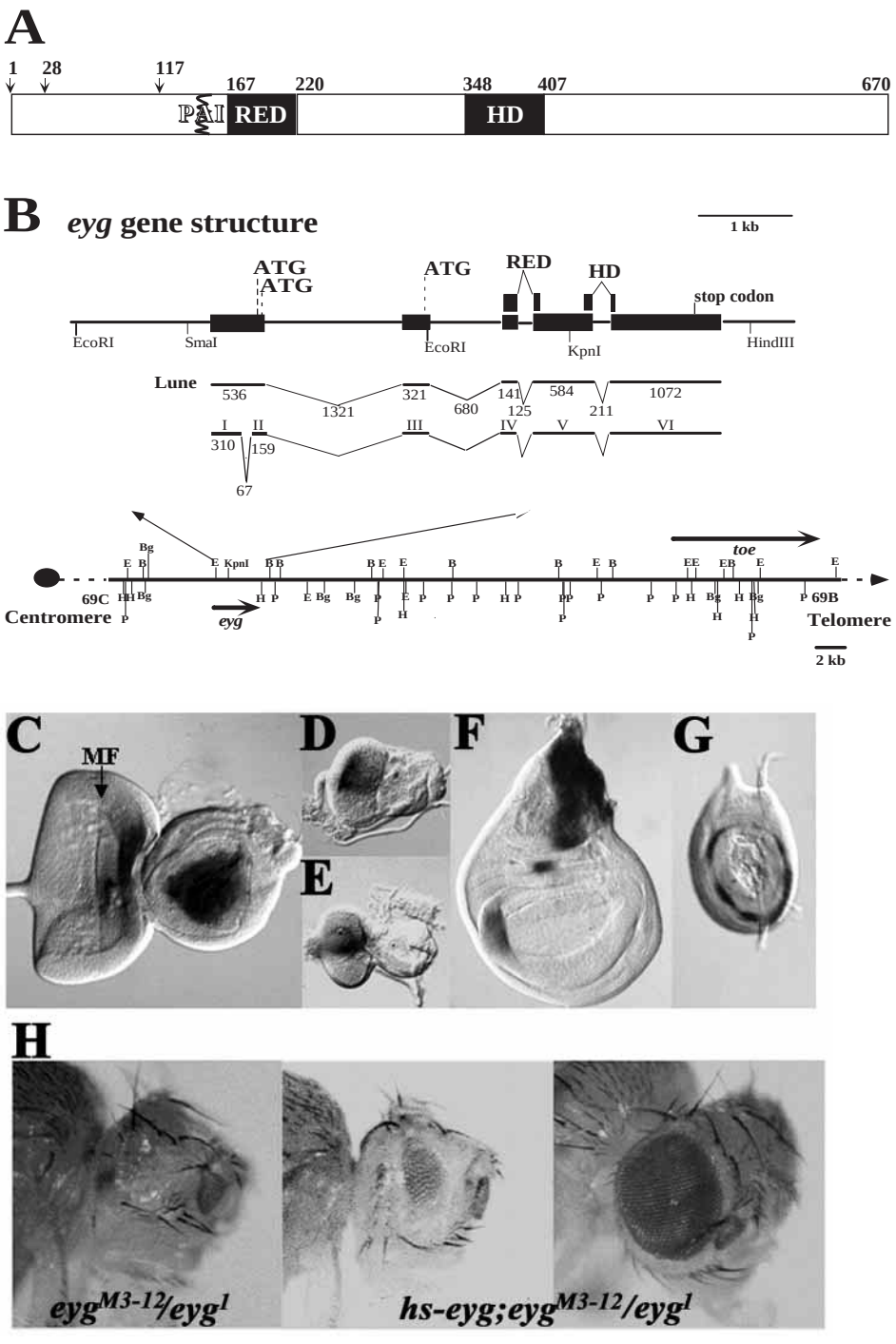

Fig. 2. *eyg* encodes a Pax protein. (A) The Lune cDNA encodes a Pax protein of 670 amino acids rather than the 523 amino acids previously reported (Jun et al., 1998). The corrected Lune sequence had been deposited into GenBank (AY099362). There are three potential start codons (indicated by arrows), 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 348-407). (B) Molecular map of the *eyg-toe* genomic region. The 5′ end of the *eyg* transcripts are defined by 5′-RACE. Two splicing isoforms were identified by 5′-RACE that have identical 5′ ends but differ in the presence or absence of a 67 intron (GenBank AY167573 and AY167574, respectively). The exon-intron structure of *eyg* is indicated. Exons are indicated by black boxes. The six exons are 310, 159, 321, 141, 584 and 1072 bp, respectively. The introns are 67, 1321, 680, 125 and 211 bp, respectively. Restriction sites indicated are: *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H) and *Pst*I (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 (C) and late second or early third instar larva (D,E), and in wing disc (F) 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 $eyg^{1}/eyg^{M3\text{-}12}$ 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 $eyg^{22\text{-}2}$ and $eyg^{94\text{-}4}$ have deletions extending into the 5′ untranslated region. $eyg^{37\text{-}1}$ is homozygous viable and results in a very weak small eye phenotype (Fig. 3B). $eyg^{22\text{-}2}$ is homozygous viable and produces a small eye (about 500 ommatidia) phenotype (Fig. 3B). The $eyg^{94\text{-}4}$ 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^{1}, 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 $eyg^{M3\text{-}12}$ 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 $eyg^{1}/eyg^{M3\text{-}12}$ mutant: the eyes were morphologically normal and often of normal size (Fig. 4B and data not shown). *ey>eyg* can rescue the eye in $eyg^{1}/eyg^{M3\text{-}12}$ 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 second 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

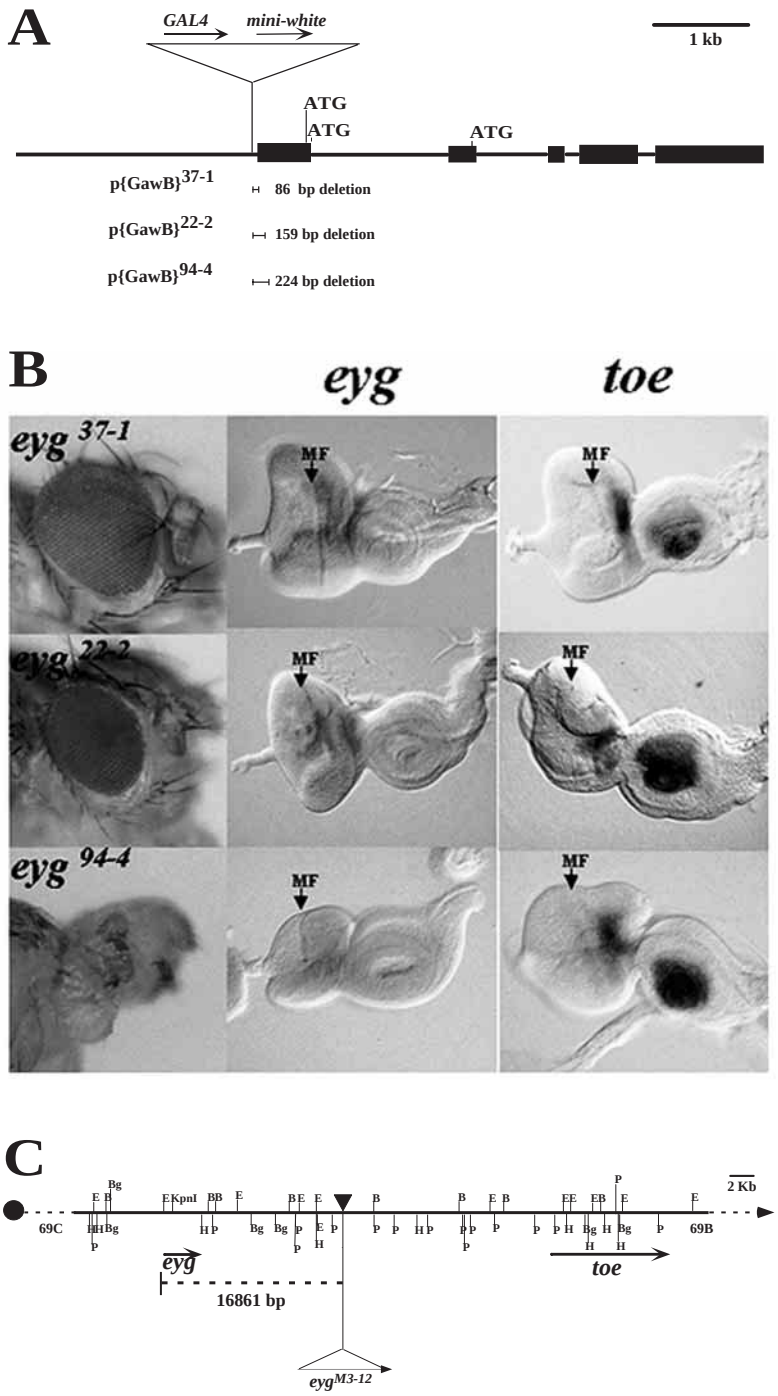

Fig. 3. Molecular analysis of *eyg* mutants. (A) $eyg^{37\text{-}1}$, $eyg^{22\text{-}2}$ and $eyg^{94\text{-}4}$ are three *eyg* mutants independently derived from mobilization of the $P[GawB]^{EM458}$ (see Materials and Methods). The three lines have the P[GawB] inserted at the same location, 124 bp 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 $eyg^{M3\text{-}12}$ 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 ($eyg^{M3\text{-}12}$) with the arrow showing the transcriptional direction of mini-*white* and *lacZ*. The black triangle indicates the presence of an opus retrotransposon.

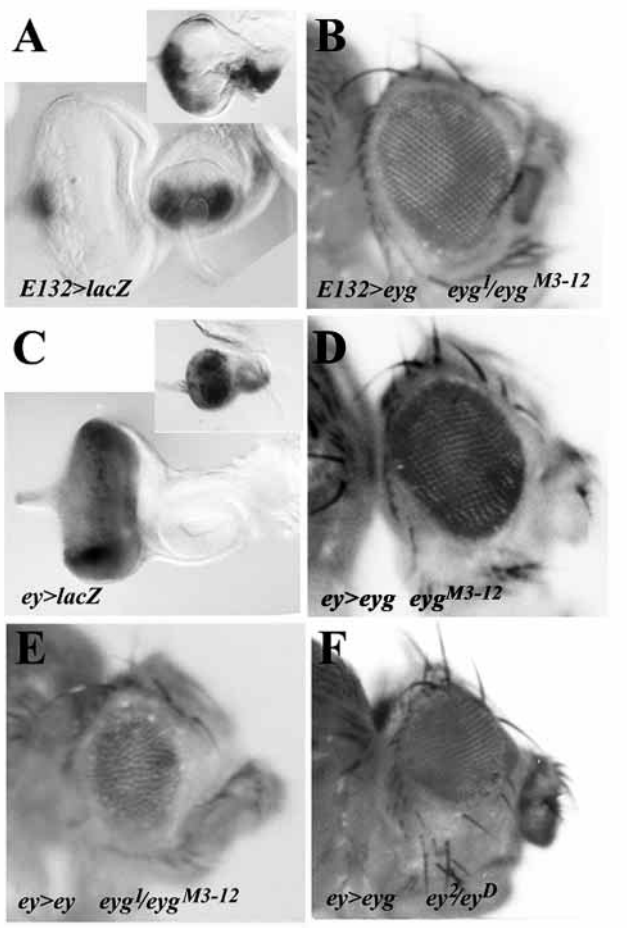

Fig. 4. Mutant rescue by targeted expression of *eyg* and *ey*. Expression pattern of E132-*GAL4* (A) and *ey-GAL4* (C) in late third instar eye-antennal disc were visualized by inducing *UAS-lacZ* expression and staining with X-gal (insets show expression in early third instar disc). (B) The $eyg^{1}/eyg^{M3\text{-}12}$ mutant phenotype (completely eyeless, see Fig. 2H) can be partially or completely rescued by targeted expression of *eyg* driven by E132-*GAL4* and by *ey-GAL4* (not shown). (D) The $eyg^{M3\text{-}12}$ mutant phenotype (headless, see Fig. 1D) can be rescued by *ey>eyg* with complete penetrance. (E) *ey>ey* can partially rescue the $eyg^{1}/eyg^{M3\text{-}12}$ mutant phenotype. (F) *ey>eyg* can partially rescue the ey^{2}/ey^{D} eyeless phenotype.

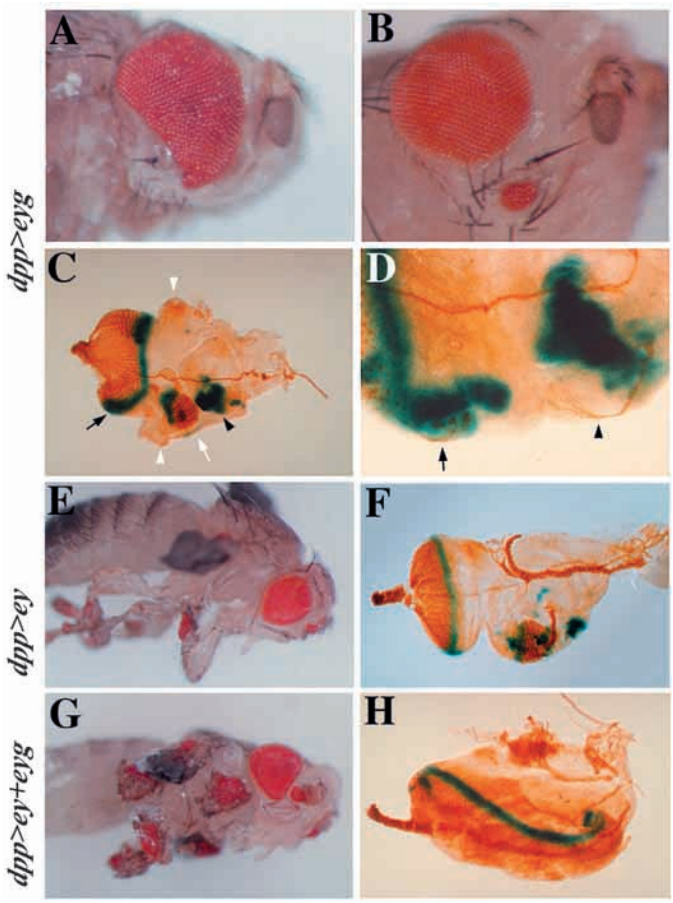

Fig. 5. Ectopic eye formation due to ectopic *eyg* and *ey* expression. (A-D) Misexpression of *eyg* can induce ectopic eye formation. (A,B) Adult phenotypes resulting from *dpp-GAL4*-induced *UAS-eyg* expression. (C,D) *dpp>eyg* eye-antenna discs double stained for *dpp-lacZ* (blue) and mAb22C10 (brown). (C) *dpp-lacZ* is expressed in the morphogenetic furrow (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,F) *dpp>ey*. (E) Ectopic eyes occurred at the base of the antennae, wings and multiple spots on the legs. (F) Ectopic MF (*dpp-lacZ*, blue) and photoreceptors (22C10, brown) can be detected in the antennal disc but not in the eye disc. (G,H) *dpp>ey+eyg* coexpression. (G) All ectopic eyes were larger than in *dpp>ey*. (H) Ectopic MF (*dpp-lacZ*, blue) and photoreceptor (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.

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 (Fig. 5C, black arrowhead). In *dpp>eyg* eye disc, ectopic *dpp-lacZ*-expressing 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 ey^2 mutant. Although ey^2 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). ey^2 eye phenotype is variable. For the ey^2 stock we used, 40% of the eyes had 300-400 ommatidia, 40% had about 200 ommatidia, and 20% had less than 100 ommatidia. The ey^2 eye disc is also variable in size. However, an ey^2 eye disc of substantial size still has no *ey* expression (Quiring et al., 1994). So we examined ey^2 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 ey^2 late third instar eye disc, the *eyg* dorsal expression was not affected while the ventral expression was reduced (Fig. 6E). In ey^2 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 ey^2 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*, *dac* and *toy*: the ectopic eyes caused by their ectopic expression cannot form in the ey^2 mutant (Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997; Czerny et al., 1999). Similarly, *dpp>ey* induced ectopic eye formation in $eyg^{M3\text{-}12}$ mutants (Fig. 7B), suggesting that *eyg* is not required for *ey* function. Again, this is in contrast to the requirement for *eya*, *so* and *dac* 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 $eyg^1/eyg^{M3\text{-}12}$ 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 ey^D/ey^2 allelic combination, which results in no eyes and is nearly completely penetrant. ey^D has a chromosomal rearrangement interrupting the *ey* gene, producing a truncated protein lacking the homeodomain

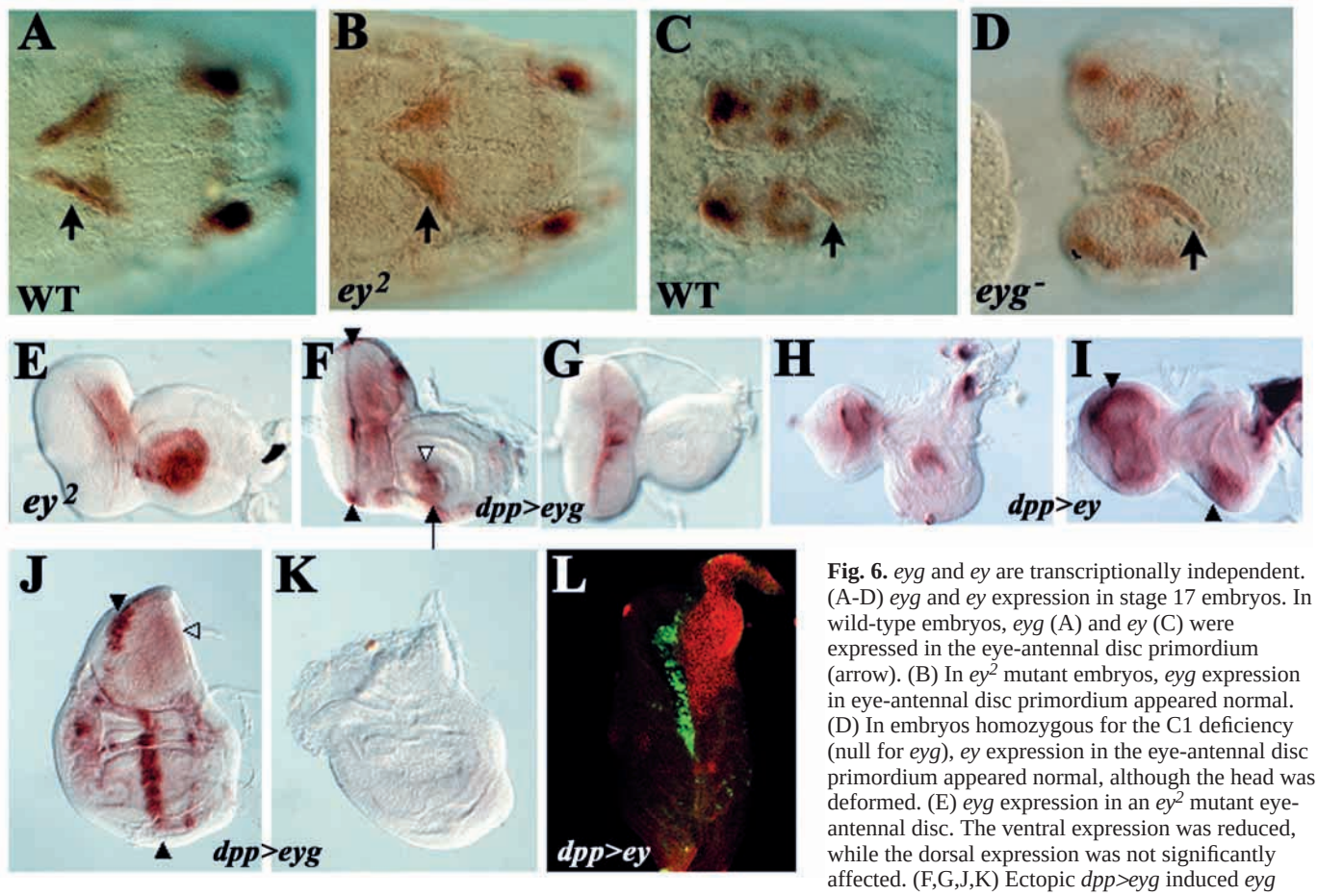

Fig. 6. *eyg* and *ey* are transcriptionally independent. (A-D) *eyg* and *ey* expression in stage 17 embryos. In wild-type embryos, *eyg* (A) and *ey* (C) were expressed in the eye-antennal disc primordium (arrow). (B) In ey^2 mutant embryos, *eyg* expression in eye-antennal disc primordium appeared 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 ey^2 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.

(Kronhamn et al., 2002). ey^2 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 ey^D/ey^2 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 eyg^1 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 eyg^1 is a hypomorphic allele and has sufficient activity in this region to suppress *wg*. Because the enhancer trap *wg-lacZ* reporter was used to monitor *wg* expression, the suppression is at the transcriptional level. In addition, ectopic expression of *eyg* (*dpp>eyg*) suppressed *wg-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).

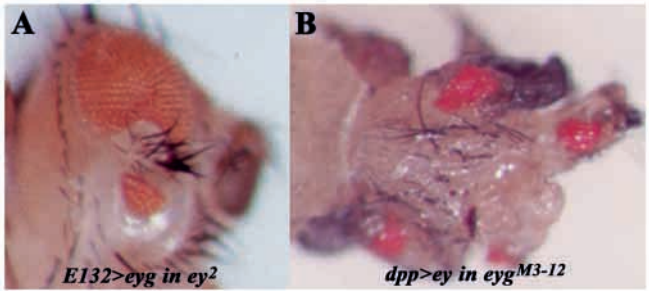

Fig. 7. *eyg* and *ey* can function independently. (A) In the ey^2 mutant, *E132>eyg* can cause an ectopic ventral eye, similar to *E132>eyg* in the wild-type background. (B) In the $eyg^{M3\text{-}12}$ mutant, *dpp>ey* can cause ectopic eye formation on wings, legs and antennae, similar to the effect of *dpp>ey* in wild-type background.

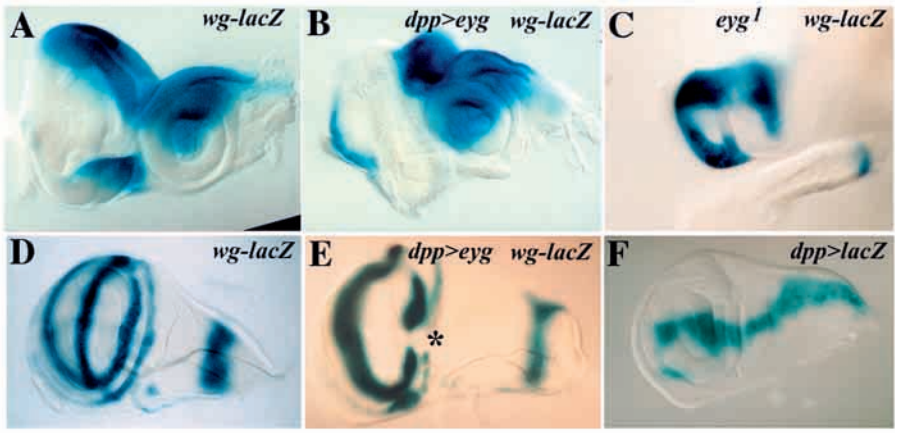

Fig. 8. *eyg* can suppress *wg* expression. (A) *wg-lacZ* expression in a wild-type eye-antennal disc. (B) The expression in the dorsal and ventral poles of the eye disc were suppressed in *dpp>eyg*, and in *dpp>ey* (not shown). Note the posterior margin and the dorsal overgrowth express *wg-lacZ*. (C) 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, $eyg^{M3\text{-}12}$, also does not complement eyg^1 and is genetically defined as being functionally null for the *eyg* eye function. $eyg^{M3\text{-}12}$ 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, $eyg^{M3\text{-}12}$ 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 *ey*/*eya*/*so*/*dac* 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 Pax6-5a 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, similarly to the Pax6-5a and Pax8(S) isoforms (Epstein et al., 1994; Kozmik et al., 1998) and distinct from the Pax6 PD. This prediction was 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 Pax6-5a 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-GAL4*), 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 eyg^1 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 eyg^1 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 eyg^1 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* (Baonza 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 *eyg*/*ey* and *wg* may be mutually antagonistic, since ectopic *ey* cannot induce *eya* and *so* expression in regions of high *wg* expression (Halder et al., 1998).

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