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Differential requirements for the Pax6(5a) genes *eyegone* and *twin of eyegone* during eye development in Drosophila

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Abstract

In eye development the tasks of tissue specification and cell proliferation are regulated, in part, by the Pax6 and Pax6(5a) proteins respectively. In vertebrates, Pax6(5a) is generated as an alternately spliced isoform of Pax6. This stands in contrast to the fruit fly, *Drosophila melanogaster*, which has two Pax6(5a) homologs that are encoded by the *eyegone* and *twin of eyegone* genes. In this report we set out to determine the respective contributions that each gene makes to the development of the fly retina. Here we demonstrate that both *eyg* and *toe* encode transcriptional repressors, are expressed in identical patterns but at significantly different levels. We further show, through a molecular dissection of both proteins, that Eyg makes differential use of several domains when compared to Toe and that the number of repressor domains also differs between the two Pax6(5a) homologs. We predict that these results will have implications for elucidating the functional differences between closely related members of other Pax subclasses.

Keywords: Pax6(5a); Eyegone; Twin of eyegone; Retina; Eye; Drosophila

Introduction

Pax6 genes play an indispensable role in the development of wide range of retinal systems. Mutations within Pax6 orthologs lead to severe retinal abnormalities in humans, mice and flies (Ton et al., 1991; Hill et al., 1991; Quiring et al., 1994). In contrast forced expression of Pax6 is sufficient to rewrite the developmental program of non-retinal tissues thereby producing an ectopically situated eye (Halder et al., 1995a). Furthermore, the universal presence of Pax6 in all seeing animals examined so far has underscored its importance in eye development and sparked a rethinking of the evolutionary origins of the eye (Halder et al., 1995b; Gehring, 2002, 2005). As a consequence Pax6 has turned into one of the best-studied members of the paired box (Pax) family of transcription factors (Gehring, 1996; Gehring and Ikeo, 1999; Pichaud and Desplan, 2002).

Pax6, like all other Pax proteins, contains a PAIRED DNA binding domain (PD) which itself is comprised of two functionally separable helix–turn–helix motifs, the PAI and the RED domains (Noll, 1993; Jun and Desplan, 1996). In addition Pax6 contains a third nucleic acid recognition motif, the homeodomain (HD). The composition and structure of Pax6 provides for considerable flexibility in its interactions with

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nucleic acids thereby allowing for the combinatorial use of three functionally independent DNA recognition domains. While vertebrates have only a single Pax6 gene, the fruit fly, *Drosophila melanogaster*, contains two Pax6 orthologs *eyeless (ey)* and *twin of eyeless (toy)*. Both play central roles in the specification of the retina (Quiring et al., 1994; Halder et al., 1995a,b; Czerny et al., 1999).

Alternate splicing of vertebrate Pax6 leads to the production of a second isoform, Pax6(5a). This isoform (1) lacks a functional PAI domain; (2) binds to DNA through its RED and HD; and (3) has a different PD binding specificity than canonical Pax6 (Walther et al., 1991; Jaworski et al., 1997). In vertebrates Pax6 and Pax6(5a) appear to play different roles in retinal development. Pax6(5a) loss-of-function mutants have different phenotypes than those of Pax6. Likewise, overexpression of Pax6(5a) induces different developmental defects and patterns of gene expression than Pax6 (Duncan et al., 2000a,b; Chauhan et al., 2002a,b,c; Singh et al., 2002; Haubst et al., 2004).

Pax6(5a) is also found in *Drosophila* but, unlike vertebrates, does not result from alternate splicing of Pax6 but rather is encoded by two separate genes, *eyegone (eyg)* and *twin of eyegone (toe)*. These genes arose from a relatively recent duplication event and together with vertebrate Pax6(5a) represent a novel subclass of Pax genes (Jun et al., 1998; Aldaz et al., 2003). Similar to vertebrates, *Drosophila* Pax6 and Pax6(5a) appear to play different roles in eye development. While *ey* and *toy* act primarily in retinal specification, the main function of *eyg* is to promote cell proliferation (Dominguez et al., 2004; Chao et al., 2004). Each isoform exerts its influence on development through different transcriptional mechanisms: Ey acts as an activator while Eyg has the unique property of acting as a dedicated repressor (Punzo et al., 2001, 2004; Yao and Sun, 2005).

The Pax6 genes in *Drosophila* do not play completely redundant roles in development. There are some differences in the expression patterns of the two genes (Quiring et al., 1994; Czerny et al., 1999; Kammermeier et al., 2001). As a result *ey* and *toy* loss and gain-of-function mutants have some significant phenotypic differences (Kammermeier et al., 2001). Even within the eye specification hierarchy *toy* appears to sit atop *ey* (Czerny et al., 1999; Kronhamn et al., 2002). Interestingly, there are also disparities between the abilities of the two genes to direct eye formation in non-retinal tissues (Halder et al., 1995a; Czerny et al., 1999; C. Salzer and J. Kumar unpublished data). Differences in the activities of individual DNA recognition domains and protein–protein interaction motifs account for these many distinctions (B.M. Weasner and J. Kumar unpublished data).

Since the *Drosophila* genome encodes two Pax6(5a) genes we set out to determine if there are disparities between the roles that *eyg* and *toe* play in eye development. We will show that *eyg* and *toe* are expressed in identical patterns in the eye but *eyg* mRNAs account for the vast majority of Pax6(5a) transcripts. A comparison of the effects that loss of each gene has on eye development demonstrates that *eyg* and *toe* are differentially required in the retina. We have gone on to show that while Toe, like Eyg, is a transcriptional repressor, the number of repressor domains is different. And finally, we demonstrate that each Pax(5a) protein makes use of a unique combination of domains during normal eye development and extra eye field induction. Together, these results suggest that although *eyg* and *toe* arose through a recent duplication event, the two Pax6(5a) proteins likely play non-redundant roles in the eye and exert their influence on retinal development through differential use of combinations of protein domains.

Materials and methods

Fly stocks, reagents and microscopy

The following stocks were used in this study: $eyg^{[1]}$, $eyg^{[22\text{-}2]}$, $eyg^{[M3\text{-}12]}$, $wg^{[W11]}$-GAL4, eyg-$GAL4^{22\text{-}2}$, tub-GAL4, ey-GAL4, GMR-GAL4, dpp-GAL4, CD-Gal4, UAS-ey, UAS-toy, UAS-so, UAS-optix, UAS-eya, UAS-GFP, wg-lacZ and an additional 220 GAL4 lines from the Bloomington *Drosophila* Stock Center (details of these stocks are available upon request). The eyg-$Gal4^{22\text{-}2}$ (also referred to as EM458) carries a P[GawB] insertion 527 bp upstream of the eyg transcript. It is homozygous viable and has no visible phenotype on its own (Jang et al., 2003). CD-Gal4 drives expression mimicking *eyg* expression (LHW and YHS, unpublished results). The following antibodies were used in this study: rat anti-ELAV, mouse anti-Eyg (gift of Ntalia Azpiazu), IgG conjugated Cy3. Adult eyes were prepared for scanning and light microscopy as essentially described in Kumar et al., 1998. Developing imaginal discs, salivary glands and embryos were prepared for light and confocal microscopy as essentially described in Yao and Sun, 2005 and Jang et al., 2003.

Generation of eyg and toe protein variants

Schematic drawings of Eyg deletion, Toe deletion and Eyg/Toe chimeric proteins are diagramed in Fig. 9. An alignment of the Eyg and Toe proteins, along with a demarcation of the individual domains, is provided within the Supplemental Data Section Fig. S1). Our full-length Eyg protein is 525 amino acids in length and represents the shortest functional isoform. Each protein domain was originally defined by Jun and Desplan, 1996. The N-terminal (NT) region consists of residues 1–13, the PD domain consists of residues 14–104, the B region contains residues 105–231, the HD domain contains residues 232–291 and the C-terminal (CT) region contains residues 292–525. The N-terminal deletion (Eyg ΔNT) contains amino acids 14–525, the paired domain deletion construct (Eyg ΔNT+PD) contains amino acids 105–525, the B domain deletion construct (Eyg ΔB) contains amino acids 1–104 fused to residues 232–525, the homeodomain deletion construct (Eyg ΔHD) contains amino acids 1–231 fused to 292–525 and the C-terminal deletion construct (Eyg ΔCT) contains amino acids 1–291. In addition we made two multiple domain deletion constructs. The combined N and C terminal deletion constructs (Eyg ΔNT+CT) contains amino acids 14–291 and the triple N terminal, B domain and C-terminal deletion construct (Eyg ΔNT+B+CT) contains amino acids 14–104 fused to residues 232–291.

Our full-length TOE protein is 640 amino acids in length. The N-terminal region consists of residues 1–142, the PD domain consists of residues 143–233, the B region consists of 234–383, the HD domain consists of residues 384–443 and the C-terminal region consists of residues 444–640. The N-terminal deletion (Toe ΔNT) contains amino acids 143–640, the paired domain deletion construct (Toe ΔNT+PD) contains amino acids 234–640, the B domain deletion construct (Toe ΔB) contains amino acids 1–233 fused to residues 384–640, the homeodomain deletion construct (Toe ΔHD) contains amino acids 1–383 fused to 444–640 and the C-terminal deletion construct (Toe ΔCT) contains amino acids 1–443. In addition we made two multiple domain deletion constructs. The combined N and C terminal deletion constructs (Toe ΔNT+CT) contains amino acids 143–443 and the triple N terminal, B domain and C-terminal deletion construct (Toe ΔNT+B+CT) contains amino acids 143–233 fused to residues 384–443.

We made a series of chimeric proteins in which single or multiple domains of Eyg were replaced with the corresponding domains of Toe. The Eyg/Toe NT chimera contains amino acids 1–142 of TOE fused to residues 14–525 of Eyg,

the Eyg/Toe PD chimera was generated by replacing the PD of Eyg with amino acids 143–233 from Toe, the Eyg/Toe B chimera was generated by replacing the B domain of Eyg with amino acids 234–383 of Toe, the Eyg/Toe HD chimera was generated by replacing the HD of Eyg with amino acids 384–443 of Toe and the Eyg/Toe CT chimera contains amino acids 1–291 of Eyg fused to residues 444–640 of Toe. The Eyg/Toe NT+CT chimera was generated by replacing the NT and CT regions of Eyg with amino acids 1–142 and 444–640 of Toe while the Eyg/Toe PD+HD chimera was generated by replacing the PD and HD of Eyg with amino acids 143–233 and 384–443 of Toe.

With the exception of three constructs (Eyg ΔNT+PD, ΔHD and Eyg ΔCT) each of the remaining deletion and chimeric constructs are new and novel lines. The Eyg ΔNT+PD, Eyg ΔHD and Eyg ΔCT constructs, while independently generated, are similar to those described in Yao and Sun, 2005. The results reported here, using these lines, are in agreement with those in the earlier report.

Real-time PCR

Total RNA was isolated from 50 third instar larval eye-antennal discs. 1 μg of total RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Roche). 1 μl of the RT reaction was amplified using the SYBR Green PCR Master Mix (Roche Diagnostics, Germany) according to its manufacturer's instructions. mRNA was quantified using a LightCycler (Roche Diagnostics, Germany). Thermocycling conditions were 95 °C for 10 min and 45 cycles of [95 °C/10 s; 60 °C/5 s; 72 °C/9 s]. Fluorescence was detected at the end of the extension phase. Melting curve analyses were performed at the end of the amplification to confirm the specificity of the amplified products and lack of primer dimers. The expected lengths of amplified products were verified in gel electrophoresis and sequenced. Quantified PCR was performed using the *eyg*-specific primer set (5′-AGGCAAGAGTTCAGGTGTGG-3′ and 5′-CAACGGCTGCTGAGGTG-3′) and *toe*-specific primer set (5′-GGCCAGGGTGCAGGTT-3′ and 5′ TTGCTGGTGCTGTACGGATA-3′), respectively.

Absolute quantification of transcripts

Two stand curves for *eyg* and *toe*, respectively, were generated using serial dilutions of a known amount of the corresponding cDNA. Plasmids containing *eyg* cDNA and *toe* cDNA were linearized. The copy number was estimated by optical density according to the molar mass derived from the plasmid size. Different dilutions were made to obtain 10^2, 10^3, 10^4, 10^5 and 10^6 copies in 1 μl. All standards were amplified in duplicates. Target mRNA copy numbers were calculated based on the standard curves generated by LightCycler software Version 4.05. The same cDNA samples were examined in five independent PCR. When comparing different cDNA samples, the fluorescence intensity was normalized to ribosomal gene *rp49*. The primers for *rp49* were 5′-TACAGGCCCAAGATCGTGAA-3′ and 5′-ACGTTGTGC ACCAG-GAACTT-3′.

toe miRNA generation

The 22 nucleotides (nt. 1464 to 1485) of the *toe* coding sequence (underlined in oligo 1 and 2) were selected as a target sequence. To generate the *toemiRNAi* construct, the below four long primers were synthesized. The PCR product of oligo 1 and 2 was amplified using oligos 3 and 4. The final PCR product was digested with *Eco*RI and *Not*I and then cloned into pUAST.

[GGCAGCTTACTTAAACTTAATCACAGCCTTTAATGTGAAGCAGC-CATATCCGTACCGCTAAGTTAATATACCATATC] — oligonucleotide 1
[AATAATGATGTTAGGCACTTTAGGTACGAAGCAGCCATATCCG-TACAGCTAGATATGGTATATTAACTTAGCGG] — oligonucleotide 2
[GGCGAATTCATGTTTAAAGTCCACAACTCATCAAGGAAAAT-GAAAGTCAAAGTTGGCAGCTTACTTAAACTTAATCA] — oligonu-cleotide 3
[GGCGCGGCCGCATCCAAAACGGCATGGTTATTCGTGTGC-CAAAAAAAAAAAAAATTAAATAA TGATGTTAGGCACTT] — oligo-nucleotide 4

In situ hybridization

Staged embryos and third instar larvae were prepared for *in situ* hybridization (detailed protocols are available upon request). Non-radioactive labeled antisense RNA probes were used for *in situ* hybridization (Tautz and Pfeifle, 1989). The pBluescript-SKII-lune plasmid was linearized and transcribed to generate *eyg* antisense RNA probes (Jun et al., 1998). The EST clone, pOT2a-toe was similarly transcribed to synthesize the *toe* antisense RNA probe. Both probes were denatured and hybridized to either embryos or imaginal discs. All hybridizations and washes were done at 65 °C. Specific details of the RNA probes and hybridization conditions are available upon request.

Results

Expression of eyg and toe during development

eyg transcripts are distributed within several embryonic tissues as well as the leg, wing and eye-antennal imaginal discs (Jones et al., 1998; Jun et al., 1998; Aldaz et al., 2003). Here we have characterized the expression pattern of *toe* and compare it to that of *eyg* (Fig. 1). *eyg* and *toe* transcripts are first detected in stage 9 embryos within the salivary gland precursor (SGP) and a small cluster of cells within the dorsal head (Figs. 1A–C; Jones et al., 1998; Jun et al., 1998). The expression of *toe* transcripts in the SGP will persist through the rest of embryonic and larval development while *eyg* expression is terminated in late stage embryos and reinitiated later (Figs. 1D–I, Figs. 2J, K, Jones et al., 1998). By late stage 10 both transcripts are also found in identical patterns within the posterior spiracle (PS) and within a cluster of cells at the anterior edge of each thoracic and abdominal segment (Figs. 1D–F, Jones et al., 1998; Jun et al., 1998). Expression of *eyg* and *toe* expands to the larval antennal organ (AO) as well as the leg disc primordia by stage 12 (Figs. 1G–I, Jones et al., 1998; Jun et al., 1998). During the latter stages of embryogenesis both *eyg* and *toe* transcripts accumulate in the presumptive eye-antennal imaginal disc (Figs. 1J–L; Jones et al., 1998; Jun et al., 1998). Only two other members of the eye specification cascade, *ey* and *toy*, share this expression pattern (Quiring et al., 1994; Czerny et al., 1999). The remaining members are added sequentially during the larval development (Kumar and Moses, 2001). The only discernable difference between the expression patterns of either Pax6(5a) gene during embryogenesis is found within the SG: *eyg* expression is eliminated while *toe* transcriptional levels are maintained (Figs. 1J–L; Jones et al., 1998).

Within the developing larval eye-antennal discs both *eyg* and *toe* transcripts accumulate in identical patterns. Within the antennal segment both transcripts localize to the medial and distal segments while in the eye disc expression of both genes is found anterior to the morphogenetic furrow (Figs. 2A–C, Dominguez et al., 2004). Unlike the similarities found in the embryo, *eyg* and *toe* expression is somewhat different from that of *ey* and *toy*. The Pax6 transcripts are expressed broadly ahead of the advancing furrow (Quiring et al., 1994; Czerny et al., 1999). However, *eyg* and *toe* expression is restricted to a narrow domain of cells that straddle the dorsal–ventral compartment boundary and does not extend laterally (Figs. 2A–C, Dominguez et al., 2004). This difference in expression is

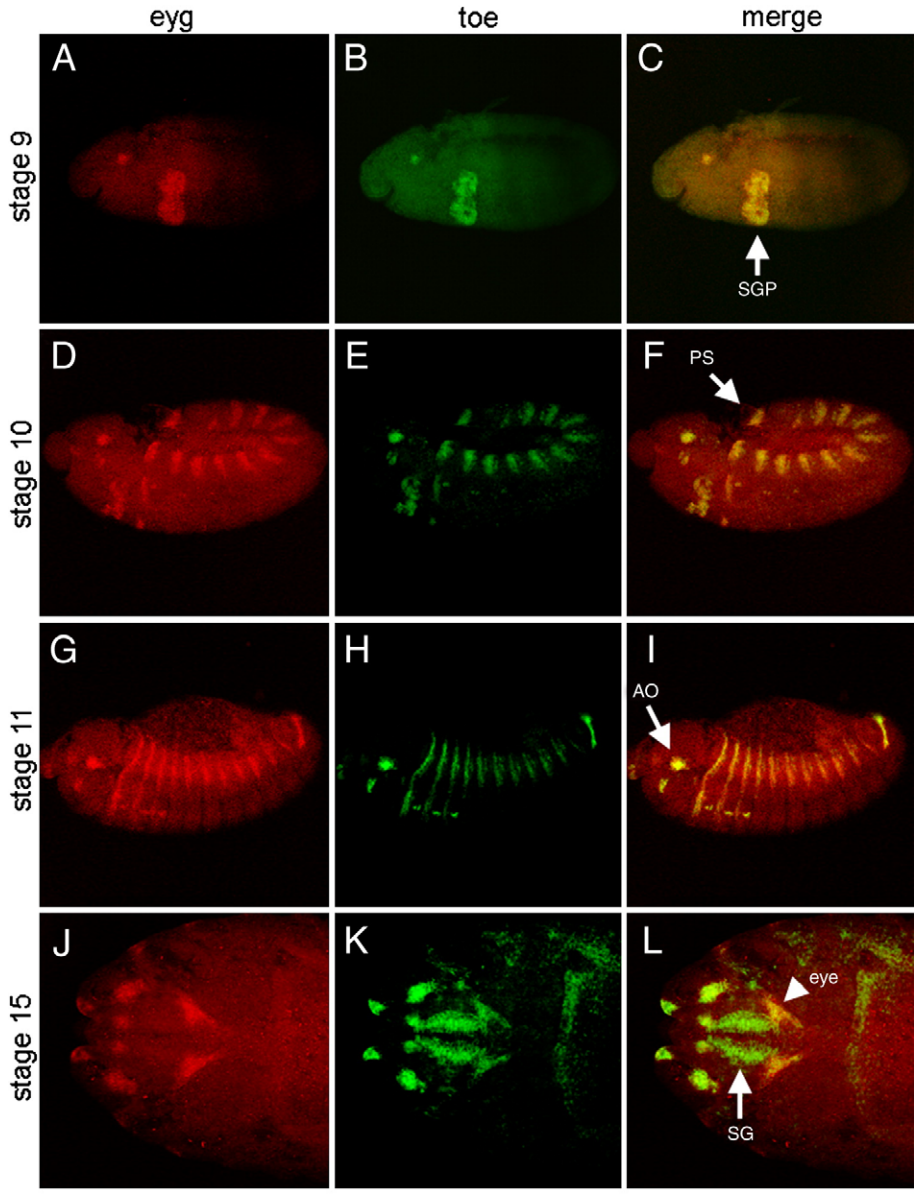


Fig. 1. *eyg* and *toe* are expressed in nearly identical patterns during embryogenesis. Transcriptional profile of *eyg* and *toe* during several stages of embryogenesis. Probes are listed at the top of each column. Embryonic stage is denoted at the right of each row. Note that the expression patterns are nearly identical except for the salivary glands in stage 15 embryos. SGP=salivary gland precursor, PS=posterior spiracle, AO=antennal organ, SG=salivary gland. Anterior is to the left.

likely due to the requirements of *eyg* (and possibly *toe*) in Notch mediated control of cell proliferation at the organizing center versus the role of *ey* and *toy* in tissue specification. Within the developing wing primordium both transcripts are expressed broadly within the notum and in two discrete regions within the presumptive wing (Figs. 2D–F). It is interesting that one of those areas is particularly susceptible to being transformed into retinal tissue in response to forced expression of *ey* (Fig. 2F, arrow). Both *eyg* and *toe* transcripts are also found within identical patterns of the leg primordium (Figs. 2G–I) and the anterior duct cells of the salivary gland (Figs. 2J, K; Jones et al., 1998). The results from this and other studies of *eyg* and *toe* expression suggest at first glance that these genes may play redundant roles within several developing tissues including the compound eye. It is unlikely, however, that these genes play completely surplus roles (at least in the eye) as *eyg* loss-of-function mutants show near complete loss of retinal tissue and forced expression of *toe* is insufficient to restore eye development to these flies (see below).

Quantitative contribution of eyg and toe mRNA transcripts

In order to further examine the contributions of *eyg* and *toe* to the development of the eye we used real-time PCR to measure the levels of each Pax6(5a) mRNA transcript in the eye-antennal disc (Fig. 3). We measured the combined levels of *eyg* and *toe* in normal eye-antennal imaginal discs and compared it to discs in which we forcibly expressed a microRNA that is predicted to target and reduce the levels of *toe* mRNA transcripts. This method was employed because a

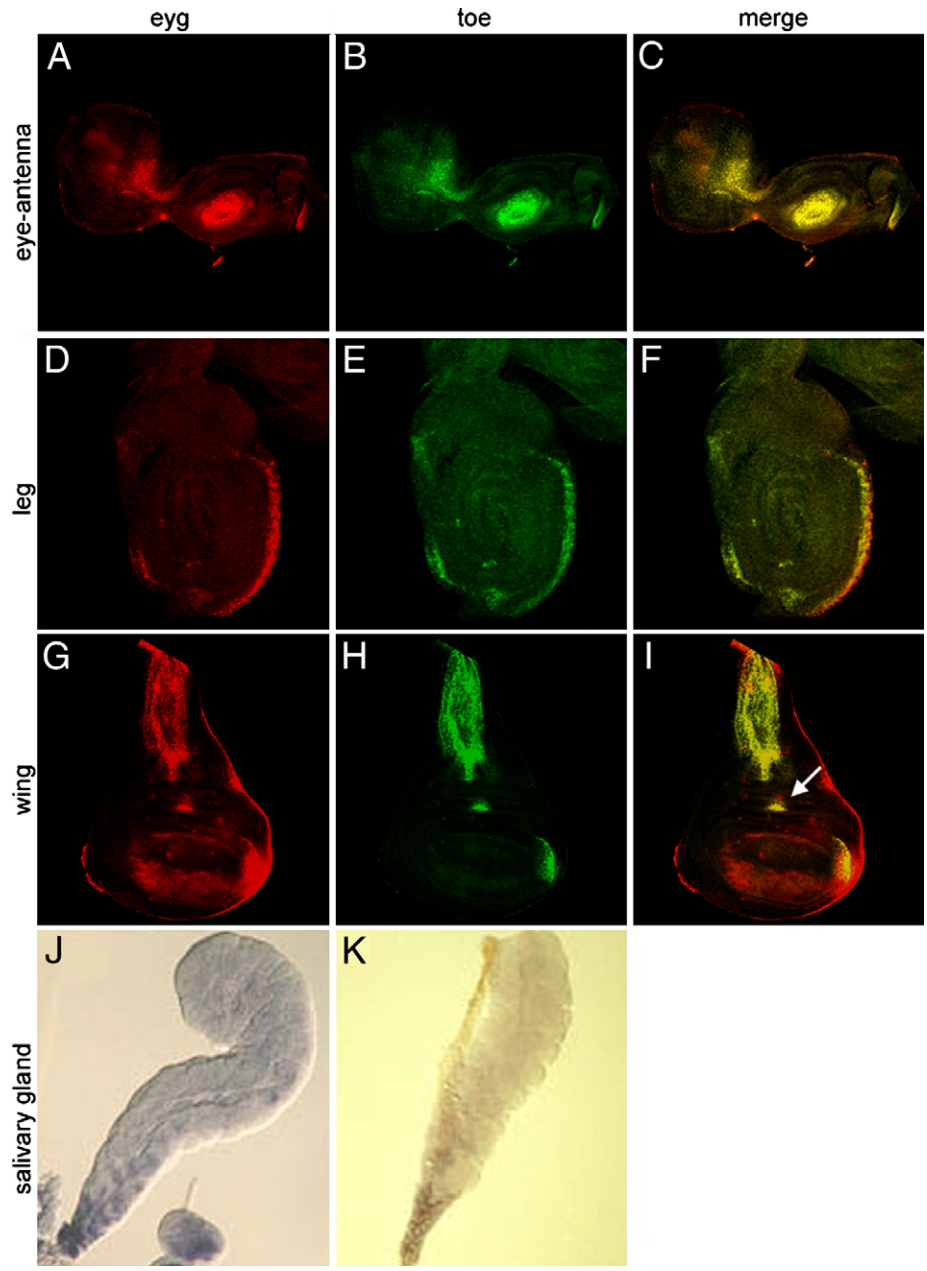


Fig. 2. *eyg* and *toe* are expressed in nearly identical patterns in salivary glands and imaginal discs. Transcriptional profile of *eyg* and *toe* during the third larval instar. Probes are listed at the top of each column. Tissue type is denoted at the right of each row. Note that the expression patterns are nearly identical. Arrow in panel I marks area of the wing disc in which ectopic eye development is supported by the expression of several retinal determination genes including *ey*.

toe specific loss-of-function mutation does not yet exist. Our results indicate that the vast majority (approximately 87%) of Pax6(5a) mRNAs are transcribed from the *eyg* locus (Fig. 3A). A direct comparison of *eyg* and *toe* levels in wild type eye-antennal discs confirms the unequal levels of Pax6(5a) expression (Fig. 3B). This relationship is maintained autonomously in individual eye and antennal discs (Fig. 3C). Finally, we set out to determine if the relative levels of *toe* are dependent upon *eyg* expression. In both *eyg* loss-of-function mutants and forced expression experiments the level of *toe* remained constant suggesting that *toe* levels are regulated independently of *eyg* (Fig. 3D). It appears that *toe* transcriptional regulation is also independent of *eyg* in several other tissues including the developing embryo and wing imaginal discs (Aldaz et al., 2003; Jang et al., 2003).

An anti-Pax6(5a) antibody recognizes the PD of Eyg and Toe

An *in vivo* comparison of the roles played by the two Pax6 (5a) genes has been hampered by the lack of available molecular markers for the distribution of Eyg and Toe proteins. To overcome this obstacle we generated a polyclonal antibody that recognizes full-length Eyg (data not shown). Since the antibody recognized a region of the eye disc in which both *eyg* and *toe* are expressed, and since both proteins share considerable sequence similarity within the DNA binding

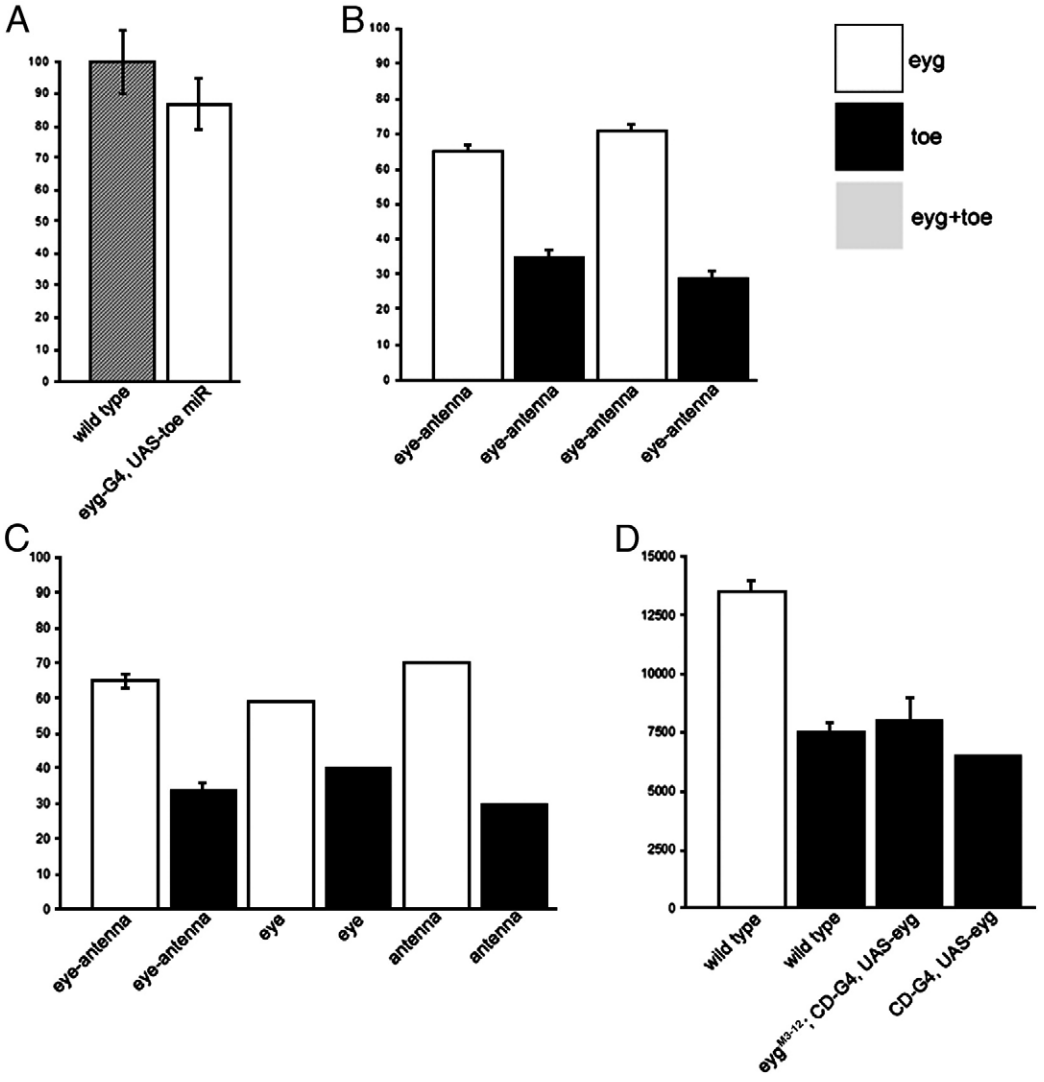


Fig. 3. *eyg* is expressed at a higher level than *toe* in eye-antennal disc. (A) Relative level of anti-Eyg/Toe immunostaining on third instar eye discs. The signal intensity in wild type ($n=12$) is taken as 100%. The signal intensity when Toe is efficiently knockdown (eyg-Gal4 driving UAS-toe miR; $n=7$) is reduced to about 87%. (B–D) Graphs depict the levels of *eyg* and *toe* in the developing eye and antenna as assayed by quantitative RT-PCR. White bars are *eyg* transcript level. Black bars are *toe* transcript level. (B) RT-PCR from eye-antennal disc from late third instar (left two bars) and second instar (right two bars). (C) When the eye and antenna discs were surgically separated, the *eyg* transcript is higher than *toe* in both eye and antennal discs. (D) Absolute *eyg* and *toe* transcript numbers were estimated from eye-antennal discs. *toe* transcript level was not significantly affected when *eyg* is overexpressed (CD-Gal4 driving UAS-eyg), indicate that *toe* is not transcriptionally regulated by *eyg*. $eyg^{M3\text{-}12}$ is a null mutation with a deletion beginning 23 bp upstream of *eyg* and extending 13 kb downstream of *eyg* transcription unit (Jang et al., 2003). Although the *toe* transcriptional unit is not affected, it is not clear whether *toe* expression is affected by the deletion. There is no eye-antennal disc in $eyg^{M3\text{-}12}$ to examine whether *toe* is affected. So we drove *eyg* expression by the CD-Gal4 in $eyg^{M3\text{-}12}$ to rescue the eye-antennal disc. In these rescued eye disc, *toe* transcript level is not significantly different from that in the wild type disc. This result clearly demonstrate that the $eyg^{M3\text{-}12}$ mutation affects only *eyg*.

domains we wanted to determine the specificity of the anti-Eyg antibody. We expressed *toe* along the A/P axis of the wing disc using a dpp-GAL4 driver. Our antibody not only recognized endogenous Eyg, which is found within the hinge region, but it also recognized the exogenously added Toe protein along the A/P axis (Figs. 4A, B). Thus the antibody we recovered recognizes both Eyg and Toe and will be referred to as anti-Pax6(5a).

The DNA binding domains of Eyg and Toe (PD and HD) are likely epitopes for the anti-Pax6(5a) antibody as they share a high degree of sequence similarity between the two proteins. We set out to determine which one of these two domains the antibody recognizes. We expressed individual domains of the Pax6(5a) proteins along the margins of the eye disc using an enhancer of the *wingless (wg)* gene. The anti-Pax6(5a) antibody was then used to detect the expressed protein segments. In addition to the endogenous Pax6(5a) proteins, the antibody only detected the exogenously added Eyg PD (Figs. 4C–F, arrows). The antibody failed to recognize a mutant Eyg PD thus confirming the specificity of anti-Pax6(5a) to the PD (Fig. 4G). The Pax6(5a) PDs share 96% sequence similarity and the antibody fails to recognize the remaining regions of Toe (Fig. 4H; data not shown). A mutated version of the Toe PD is also not recognized by the antibody (Fig. 4H). Together, our results

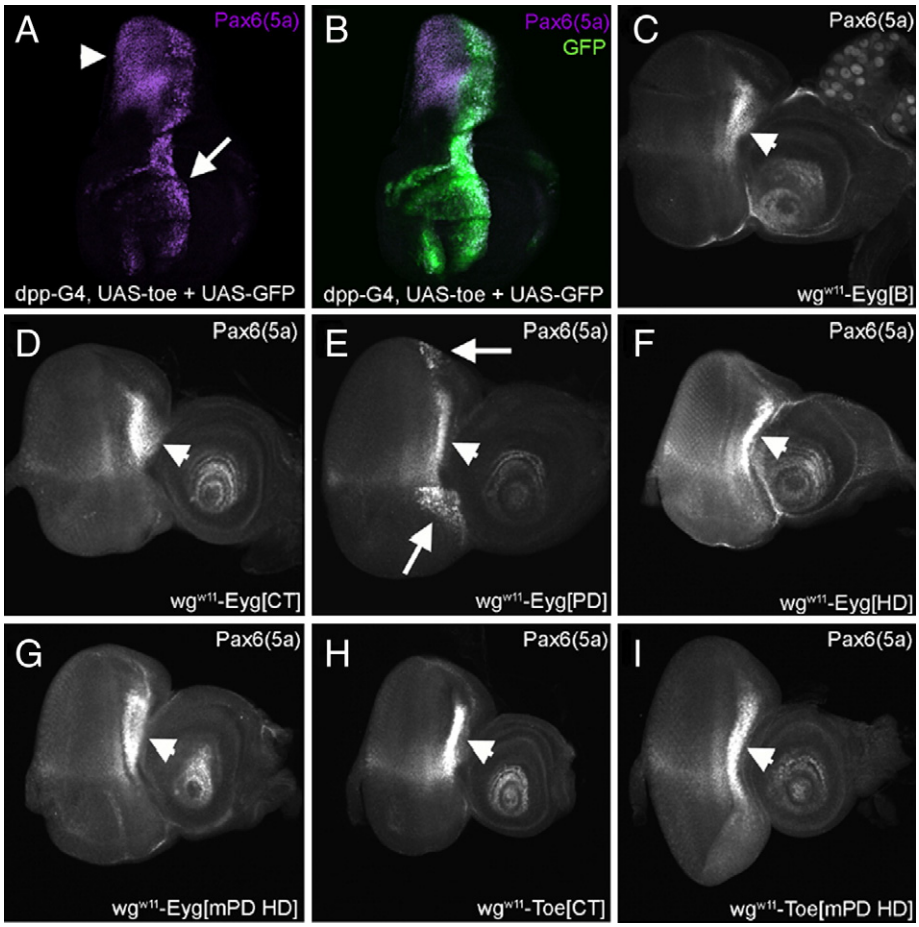


Fig. 4. The Eyg/Toe antibody recognizes the PD of Eyg and Toe. (A, B) Wing discs. (C–I) Eye-antennal discs. Arrowhead in all panels denotes endogenous Pax6(5a) expression in the wing and eye disc. Arrows in panels a and e indicate detection of exogenous Pax6(5a) protein. Genotype of each tissue is indicated in each panel. Note that wg^{w11} enhancer drives expression in the lateral regions of the eye disc ahead of the morphogenetic furrow. Anterior is to the right.

indicate that the anti-Pax6(5a) antibody recognizes the Eyg and Toe PDs.

As the anti-Pax6(5a) antibody recognizes both proteins we generated tagged proteins in which full-length Eyg and Toe are marked with the FLAG epitope tag in order to visualize each protein individually. When UAS-Eyg[flag] is combined with an *eyg-GAL4* driver the distribution of the marked protein can be followed in the imaginal discs and salivary glands (data not shown). Unfortunately, as a *toe-GAL4* line does not yet exist we are unable to specifically follow the distribution of Toe. However, combinatorial use of anti-Pax6(5a) with the Eyg [flag] is sufficient to differentiate between the distribution patterns of both proteins during normal and forced expression experiments.

Differential requirement for eyg and toe in the eye and thorax

The absence of *toe* loss-of-function mutants has been another obstacle to clearly defining the contributions that each gene makes to retinal development. To complement the study of existing *eyg* loss-of-function mutant phenotypes we made use of a microRNA (miRNA) that targets *toe* mRNA transcript thereby reducing Toe protein levels and potentially substituting for *toe* loss-of-function mutants. If the miRNA is co-expressed with FLAG tagged version of either Pax6(5a) protein, only the Toe levels are eliminated (Figs. 5A–F). Additionally, only salivary gland defects that results from the overexpression of Toe-Flag, are reversed by the miRNA (Figs. 5C, F). Eyg[flag] levels remain unaffected (Figs. 5G–I). Furthermore, expression of the miRNA in severe *eyg* mutants eliminates endogenous Toe protein from both salivary glands and several imaginal discs (Figs. 5J–O). Note that the images in M–O have been overexposed to indicate that Toe protein cannot be detected in the nucleus. Together, these results indicate that the miRNA selectively targets *toe* transcripts.

We then set out to determine what contribution, if any, to eye specification is made by *toe*. Using an *eyg-GAL4* driver we expressed 2 copies of the *toe* miRNA in regions of the developing eye that normally express both Pax6(5a) proteins. Interestingly, we did not observe any discernable defects suggesting that although Toe levels are being eliminated in the retina, the endogenous levels of Eyg is sufficient to fully support eye development. The thorax, which also requires *eyg*, is similarly unaffected by the expression of the miRNA under control of either the *eyg-GAL4* and/or tub-GAL4 (Figs. 6A, E). Since *eyg* and *toe* mRNA levels make up 87% and 13% of Pax6

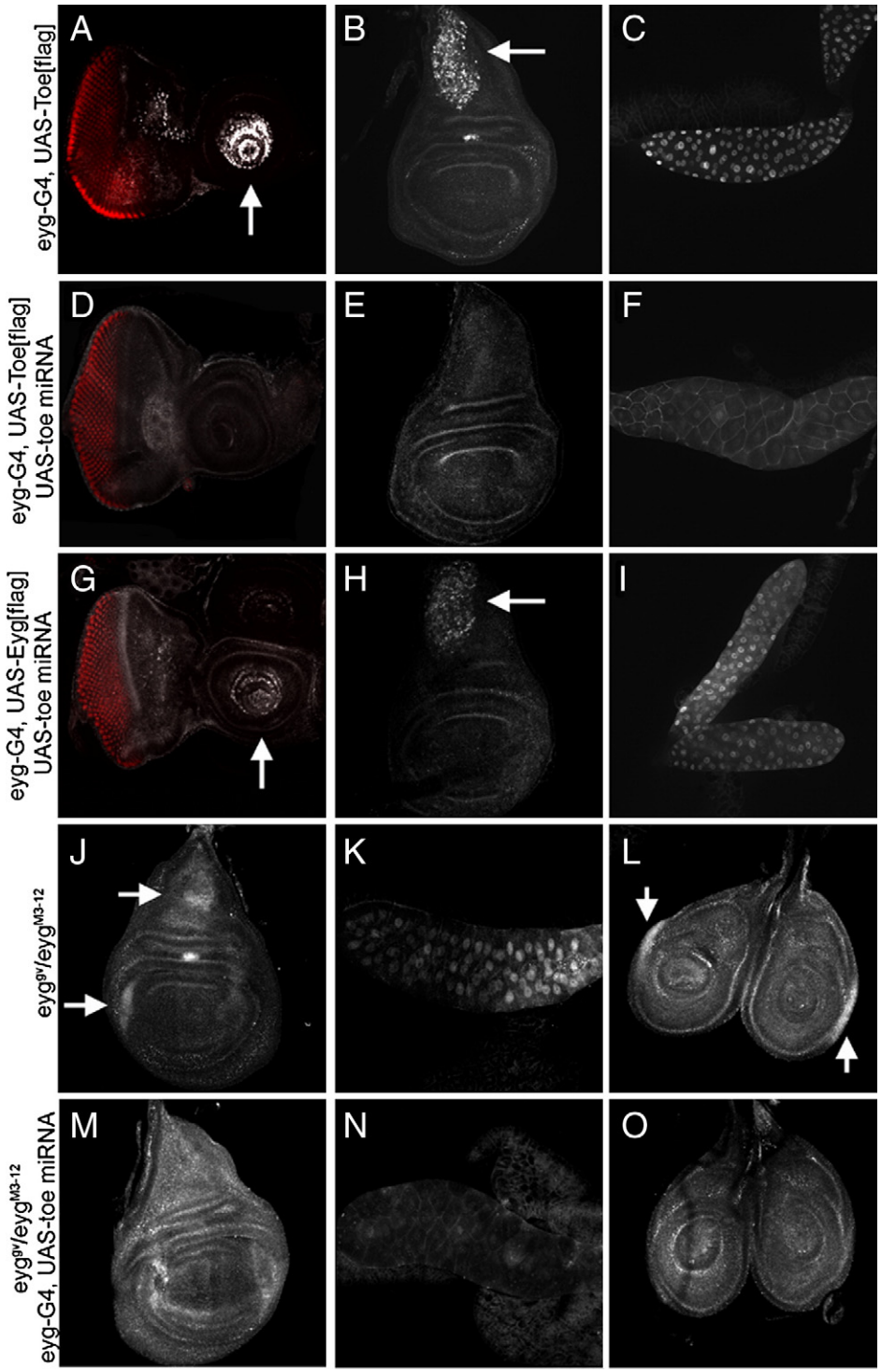


Fig. 5. An miRNA specifically knocked down Toe protein level. (A, D, G) eye-antennal discs. (B, E, H, J, M) wing discs. (C, F, I, K, N) salivary glands. (L, O) leg discs. Genotypes of each tissue are indicated at the left of each row. Arrows in each panel indicate exogenous Toe or Eyg. Note that panels M–O have been overexposed to show that there is Toe protein is down regulated (detected by anti-Flag in panels A–I, by anti-Eyg/Toe in panels J–O). Also note that the morphology of the salivary gland is rescued when the *toe* miRNA is coexpressed with a full-length *toe* construct (panel F). The RED stain in panels A, D, G is Elav.

(5a) levels respectively we expressed the miRNA in flies heterozygous for an *eyg* null mutant. In this situation the eye remains unaffected but the anterior–central region of the thorax does not develop (a phenotype that is not observed in *eyg* heterozygotes). This is visibly manifested as a severe groove within the thorax (Figs. 6B, F). The failure to develop the anterior–central portions of the thorax is reminiscent of the effect of severely diminished levels of *eyg* (Fig. 6G, Aldaz et al., 2003). Interestingly, 50% reductions in Eyg protein levels are not enough to severely alter the structure of the eye (Fig. 6C). However, if *eyg* and *toe* levels are simultaneously compromised, then development of both the head and

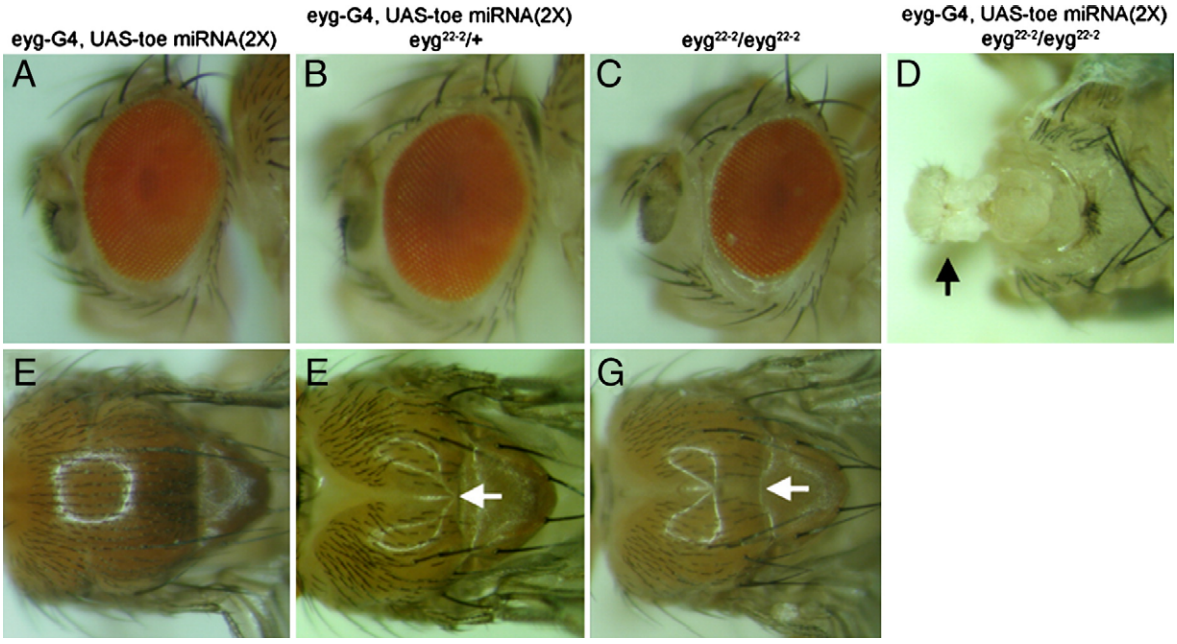


Fig. 6. *toe* and *eyg* are differentially required in the developing eye and thorax. (A–D) adult eye and head. (E–G) adult thorax. Genotype of each tissue is indicated at the top of each column. Arrow in panel D indicates the near complete inhibition of eye and head development. Arrows in panels F and G denote defects in thorax development. Anterior is to the left.

compound eyes is blocked (Fig. 6D). The thorax is much more sensitive to simultaneous reductions in Eyg and Toe levels than is the developing eye. Our results also suggest that removal of *toe*, on its own, has little to no effect on the development of either tissue. This is consistent with the minor contribution that the *toe* locus makes to the overall levels of Pax6(5a) mRNA (Fig. 3). These results suggest that Eyg and Toe proteins are differentially required in the eye and thorax.

Consistent with this hypothesis, we have noted 43 different situations in which expression of *eyg* and *toe* had differing phenotypic consequences (Supplemental Data — Table 1). For example, expression of *eyg* in the wing disc via a vg-GAL4 driver has no effect. However, expression of *toe* within the same domain leads to increased levels of cell death. Conversely, while expression of *toe* in the embryonic CNS and brain via the c768-GAL4 driver has no effect, expression of *eyg* leads to embryonic lethality. We have been able to exclude trivial explanations such as line strength and protein levels as reason for these disparities. Instead, our results further the contention that the Pax6(5a) proteins are differentially required during development. This contention is also supported by differential effects on the developing eye and wing in response to the expression of various Pax6(5a) deletion and chimeric proteins (Fig. 9; Supplemental Data — Table 2).

Toe is transcriptional repressor

Using Eyg-VP16 (transcriptional activator) and Eyg-En (transcriptional repressor) protein fusions, *eyg* has been shown previously to encode a dedicated transcriptional repressor (Yao and Sun, 2005). Based on the evolutionary relationship between both Pax6(5a) genes, *toe* is also predicted to encode a transcriptional repressor. To test this hypothesis we created transgenic flies that expressed full-length Toe fused to the VP16 activation domain (Toe-VP16) along the A/P axis of several imaginal discs using a dpp-GAL4 driver. The activity of this transcriptional-activating form of Toe failed to mimic the activity of wild type Toe protein in several assays. In fact, in certain instances Toe-VP16 appeared to induce dominant-negative phenotypes. For instance, in contrast to Toe, which can induce extra eye fields, the expression of the transcriptional-activating form of Toe failed to promote and support eye development in a forced expression assay (compare Figs. 7A to 10B). Second, Toe-VP16 induced the formation of abnormal antennal structures and extra machrochaetes on the thorax, which are likely dominant negative effects (Figs. 7B, C). Another dominant-negative effect is seen when the expression of Toe-VP16 in *eyg* hypomorphic mutants leads to the production of "headless" flies (Figs. 7D, E). These phenotypes are reminiscent of the effects observed when either the *toe* miRNA or Eyg-VP16 is individually expressed within the same *eyg* mutant background (Fig. 6D, Yao and Sun, 2005). It should be noted that expression of the *toe* miRNA induced dominant negative phenotypes, such as the production of headless flies, only when the genetic background was compromised for *eyg* function. As these animals are headless and die as pharate adults we were unable to assay the effects that the *toe* miRNA had on macrochaetes numbers and antennal structure. The induction of dominant negative phenotypes by Toe-VP16 in an otherwise wild type background is likely due to the strong activation of putative Toe target genes via the VP16 activation domain. We also expect that Toe-VP16 would also activate some Eyg targets while the *toe* microRNA would only affect levels of Toe mRNA and protein. This might also contribute to the stronger Toe-

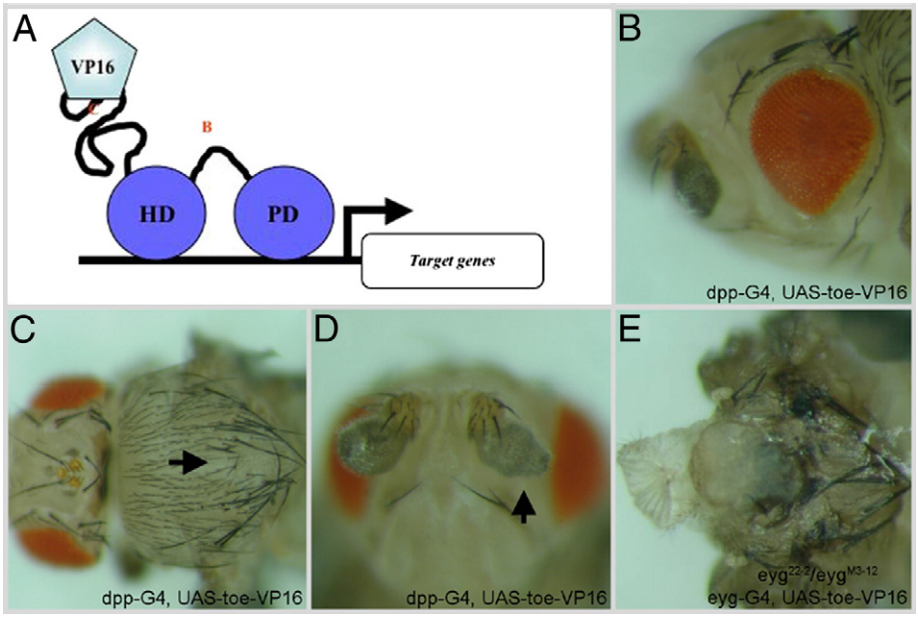


Fig. 7. Toe is a transcriptional repressor. (A) Schematic of VP16 fusion assay. (B–E) adult eye, thorax, antenna and head. Genotypes of each tissue are indicated at the top of each column. Arrows in panels C and D mark defects in thorax and antennal development. Anterior is to the left.

VP16 phenotype. As the activity of Toe-VP16 closely mimics that of Eyg-VP16 (Yao and Sun, 2005) our results are suggestive that Toe, like Eyg, functions as a transcriptional repressor.

Further evidence of Toe serving as a repressor comes from the partial rescue of the *eyg* loss-of-function retinal phenotype by expression of a chimeric protein in which Toe is fused to the Engrailed repressor (Toe-EN, data not shown). It should be noted that expression of Toe-EN failed to induce extra eye fields along the ventral surface of the head. There are two plausible explanations for these relatively mild effects. First, the Toe-EN construct may not be expressed at high enough levels to either fully rescue eyg mutants or induce extra eye fields. We think that this is unlikely as the EN domain that is fused to TOE is a strong transcriptional repressor and high levels of the fusion protein are not predicted to be required in this assay. Instead it may be that during normal development Toe functions as a repressor on some target genes and as an activator on others. Unlike Eyg, Toe may not function as a dedicated repressor. Instead it may have multiple functions with repression being one of its activities. This could account for several of the observed difference in the activities of the Pax6(5a) homologs (see below).

Mapping of the toe repressor domains

We set out to determine if the repressor domain(s) map to similar locations within Eyg and Toe. It has been previously demonstrated that Eyg contains two repressor domains: one maps to the CT region, the other is located in either the NT or the B regions of the protein (Yao and Sun, 2005; Figs. 8B–D). Expression of Eyg ΔB anterior to the furrow using an ey-GAL4 has a dominant negative effect on eye development (data not shown). Often times these dominant negative effects can be attributed to the deletion of either an activation or repressor domain. Here we show that the repressor domain within Toe is not located within the CT tail and may reside within the NT and B domains. The assay used by Yao and Sun and here is a bipartite system. In one half of the system a chimeric protein in which the GAL4 DNA binding domain is fused to either a full-length Pax6(5a) protein or an individual domain is expressed along the margins of the eye under the control of a *wingless (wg)* enhancer element. In the second half of the system the same *wg* enhancer directs the expression of a GFP reporter. A cluster of UAS sites separates the enhancer element from the reporter (Fig. 8A). In flies lacking the driver construct, GFP is expressed along the margins of the eye field (Fig. 8B). When a portion of Eyg containing a strong repressor (Eyg CT) is expressed in the same pattern, expression of the reporter is completely lost (Fig. 8C). If a weak or moderate repressor domain (Eyg ! CT mPDHD) is expressed then a reduction in reporter activity is observed (Fig. 8D). If now we express just the CT of Toe along the margins we see normal levels of the GFP reporter indicating that this domain does not contain any repressor activity (Fig. 8E). However, if the N-terminal portion of Toe containing mutated PD and HDs is expressed then strong repression of the reporter is seen. The most likely explanation is that a strong repressor domain resides within either the NT or the B domains (Fig. 8F). Thus it appears that a major functional difference between the Eyg and Toe proteins is the number and location of the repressor domains.

Molecular dissection of Eyg and Toe during normal development

As Eyg and Toe are derived from the same ancestral gene, are expressed in identical patterns and function as transcriptional

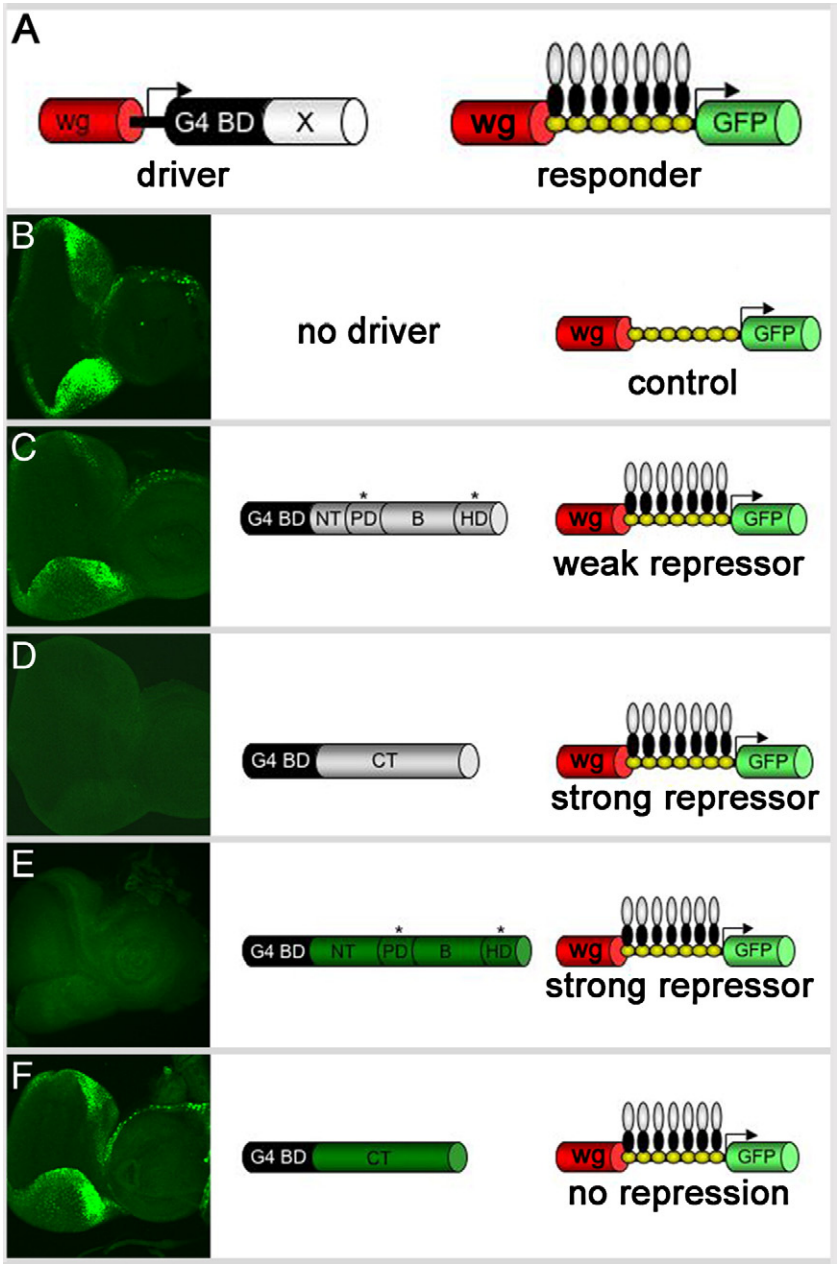
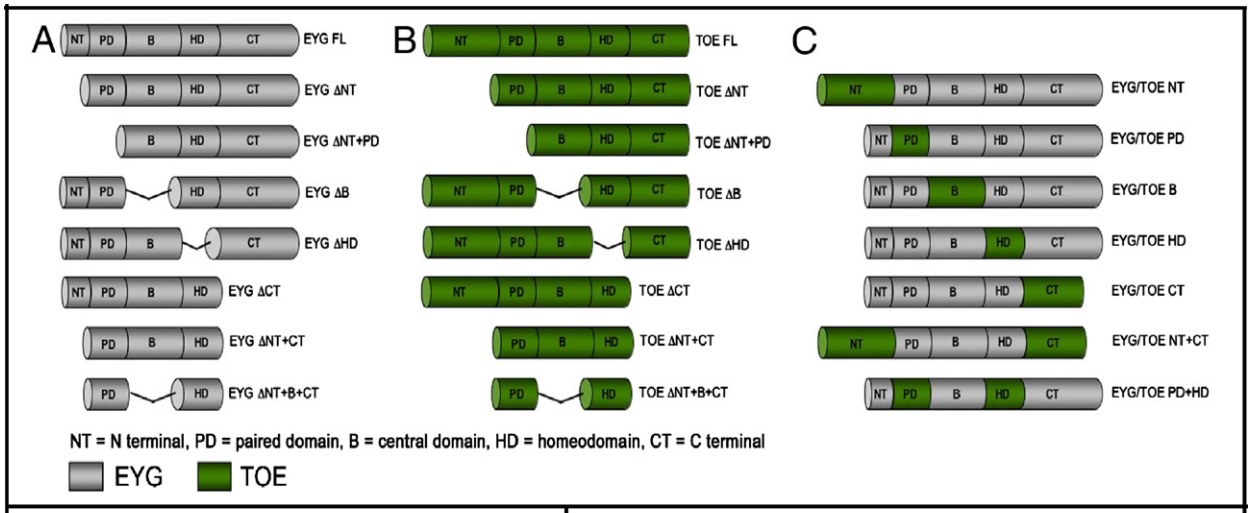


Fig. 8. Toe repressor activity resides in its N-terminal portion. (A) Schematic of repressor assay. (B–D) Third instar eye-antennal discs accompanied by schematic of driver/responder combinations. Note that wg-GAL4 drives expression in the lateral margins ahead of the morphogenetic furrow. Anterior is to the right.

repressors, yet have differential effects on eye and thorax development, we conducted a molecular dissection of both proteins in an effort to understand the biochemical basis that underlies these unique functions. These experiments which are aimed at elucidating the differences between two Pax6(5a) proteins (Eyg and Toe) extend those of Yao and Sun, 2005 which focused on the functional differences between the activities of Eyg and Pax6 (Ey). An alignment of the Eyg and Toe proteins, along with a demarcation of the individual domains, is provided within the Supplemental Data Section. The reagents that we generated for our studies include a series of protein deletions in which individual or multiple domains of either Eyg or Toe were removed. These Pax6(5a) variants were used to test the functional requirements for each domain. We also generated a series of chimeric Pax6(5a) proteins in which single or multiple domains of Eyg were deleted and replaced with the corresponding regions of Toe. These chimeric proteins were used to test the degree to which each domain has been functionally conserved. Diagrams of the Pax6(5a) deletions and chimeras are depicted in Figs. 9A–C. Each variant was assayed for the ability to rescue an *eyg*$^{[1]}$ loss-of-function mutant (Figs. 9 and 10) and to induce extra eye fields within ventral head segments (Figs. 9 and 11). It should be noted that for each deletion and chimera we tested multiple UAS insertion lines and conducted our experiments at several temperatures. We did this in an attempt to eliminate the



D

Domain requirements for normal eye development and the generation of extra eye fields

normal eye development
Eyg NT, PD, B
Toe PD, B, HD

extra eye fields
Eyg NT, PD, B, HD, CT
Toe NT, PD, B, CT

E

Rescue of eyg[1] mutants and induction of extra ventral eyes by Pax6(5a) deletion and chimeric proteins

Pax6(5a) Protein	rescue of eyg[1]/eyg[1]	induction of extra ventral eyes
Eyg FL	+	+
Eyg ΔNT	-	-
Eyg ΔNT+PD	-	-
Eyg ΔB	DN	-
Eyg ΔHD	+	-
Eyg ΔCT	+	-
Eyg ΔNT+CT	-	-
Eyg ΔNT+B+CT	-	-
Toe FL	-	+
Toe ΔNT	+	-
Toe ΔNT+PD	-	-
Toe ΔB	DN	-
Toe ΔHD	DN	+
Toe ΔCT	+	-
Toe ΔNT+CT	-	-
Toe ΔNT+B+CT	-	-
Eyg/Toe NT	+	+
Eyg/Toe PD	+	+
Eyg/Toe B	+	+
Eyg/Toe HD	+	-
Eyg/Toe CT	+	-
Eyg/Toe NT+CT	DN	+
Eyg/Toe PD+HD	+	+

Fig. 9. Functional assay of domains in Eyg and Toe. (A–C) Schematic of Eyg deletions, Toe deletions and Eyg/Toe chimeric proteins. (D) Summary of Eyg and Toe domain requirements in rescue and overexpression assays. (E) Summary of results from rescue of *eyg* loss-of-function mutants and extra eye field induction assays.

possibility that our results are affected by expression levels or insert integrity. We also crossed each construct to several GAL4 lines to ensure that each deletion or chimeric protein was functional.

Both wild type and all variants of Eyg and Toe were expressed in $eyg^{[1]}$ homozygous mutant retinas, which contain between 40 and 50 ommatidia (Fig. 10A). Flies that are homozygous for *eyg* null alleles die during embryogenesis and are therefore not appropriate for this particular assay. Expression of wild type Eyg but not Toe is sufficient to return $eyg^{[1]}$ mutant retinas to near wild type structure suggesting that these genes have functionally diverged since the duplication (Figs. 9D and 10B, C). These genes are thus unlikely to play redundant roles in eye development.

Requirements for the non-DNA-binding domains in normal eye development

We first proceeded to test the functional requirements of the non-DNA-binding domain. We started with deleting the sequences that lie upstream of the RED DNA binding domain in both proteins (Eyg ΔNT, Toe ΔNT). Toe ΔNT, but not Eyg ΔNT, restored eye development to $eyg^{[1]}$ mutant retinas (Figs. 9D and 10D, E) suggesting that not only is there a functional requirement for the NT region of both proteins but also that this region may functional distinguish the two Pax6(5a) proteins from each other. Surprisingly, expression of the Eyg/Toe NT chimera is also capable of rescuing $eyg^{[1]}$ (Figs. 9D and 10F). This result indicates that while the NT domain may functionally

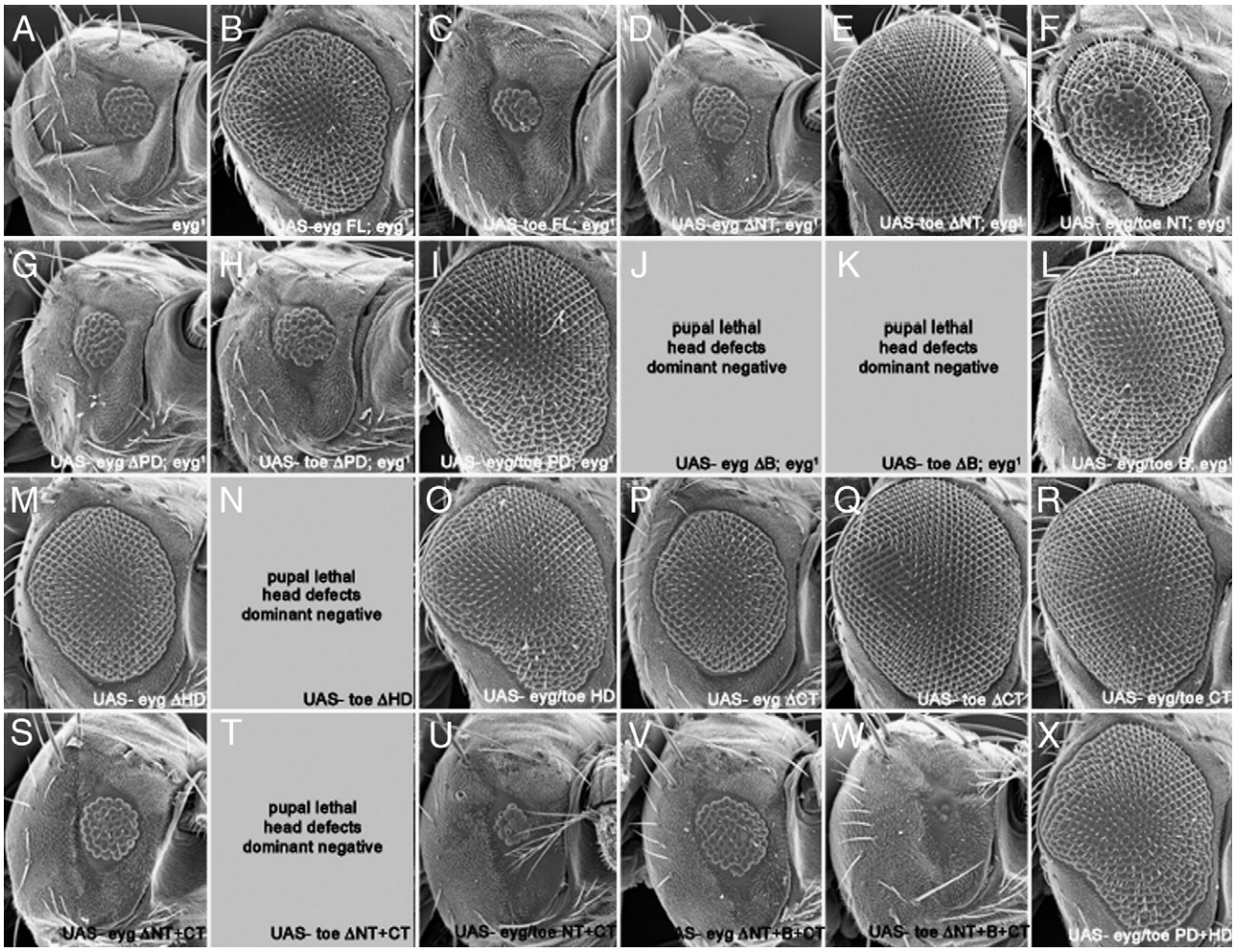


Fig. 10. Rescue of *eyg*^{1} loss-of-function mutants by expression of Eyg and Toe protein variants. Scanning electron micrographs of adult compound eyes. Genotypes of each animal are listed within each panel. All UAS lines were expressed using an ey-GAL4 driver. Anterior is to the right.

distinguish one Pax6(5a) from another, their function is still context dependent. Eyg contains two repressor domains, of which one lies within the first 443 amino acids of the protein (Yao and Sun, 2005). This region includes the NT, PD, B and HD as shown in Fig. 9A. Mutational analysis excluded the PD and HD regions thus leaving either the NT or B regions as possible sites for the repressor activity of Eyg. Our results raise the possibility that the repressor activity of Eyg resides within the NT region.

We then looked at the requirements for the B domain, a stretch of amino acids that lies between the PD and HD DNA binding motifs but is yet to be assigned a role in Pax protein function. Forced expression of constructs in which the B regions from Eyg and Toe were deleted individually or in combination with the NT and CT regions (Eyg ΔB, Toe ! B, Eyg ΔNT+B+CT, Toe ΔNT+B+CT) had a dominant negative effect on *eyg*$^{[1]}$ flies; the heads were severely deformed, the retinas were not restored to wild type and the flies died in their pupal cases (Figs. 9D and 10J–K, V, X). In contrast, *eyg*$^{[1]}$ mutant retinas were restored to near wild type levels when region B of Eyg was replaced with homologous region from Toe (Eyg/Toe B; Figs. 9D and 10L). These results suggest that the B domain is functionally essential for Pax6(5a) activity and has been functionally conserved between the two transcription factors. This conclusion is supported by the observation, from a related set of experiments in which the Pax6 proteins Ey and Toy do not function normally in the absence of the B domain. Proteins lacking this domain produce ectopic eyes that are less frequently observed and are significantly smaller in size than those produced by the full-length proteins (B.M. Weasner and J.P. Kumar, unpublished data). There is the possibility, however, that region B simply acts as a linker or spacer for the two DNA binding motifs and that deleting this region from any Pax protein may disrupt the normal structural configuration as the RED and HD motifs are brought together. One could interpret the rescue of *eyg*$^{[1]}$ by Eyg/Toe B as simply the result of restoring the spacing between the DNA binding motifs. We think that is rather unlikely as a similar chimera in which the B domains of EY and TOY have been interchanged appear to have acquired new activities and do not simply function as the parental Pax6 protein (B.M. Weasner and J.P. Kumar, unpublished data).

The C-terminal tail (residues 3′ of the HD) of Eyg but not Toe contains a transcriptional repressor domain (Yao and Sun, 2005; this report). We deleted the CT region in an attempt to

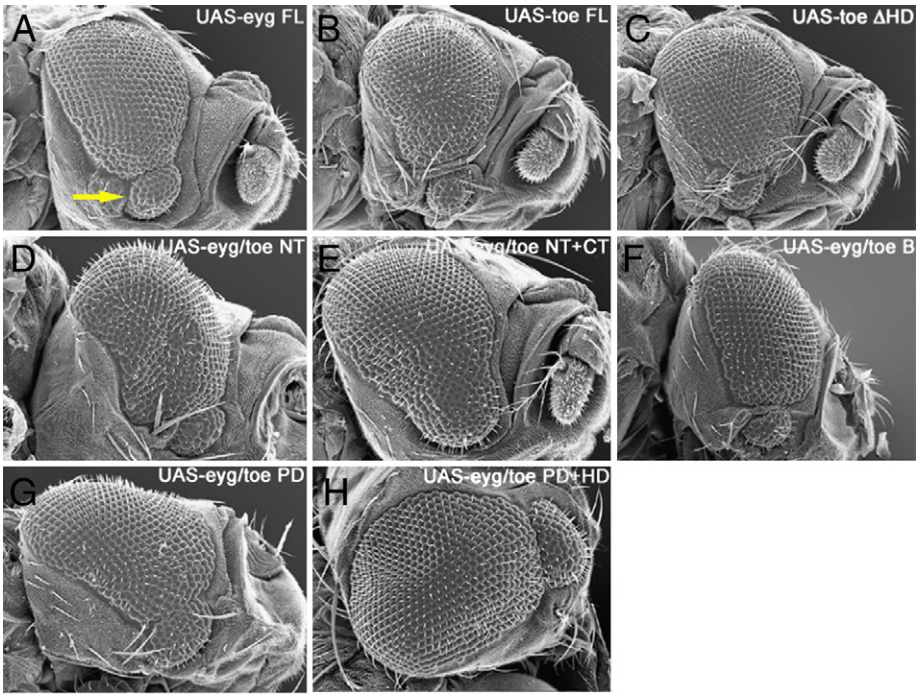


Fig. 11. Induction of extra eye fields by the expression of Eyg and Toe variants. Scanning electron micrographs of adult compound eyes and extra eye fields. Genotypes of each animal are listed within each panel. All UAS lines were expressed using a dpp-GAL4 driver. Yellow arrow in panel A indicates position of extra eye field. Anterior is to the right.

determine if this domain serves to functionally distinguish one Pax6(5a) protein from the other. Expression of Eyg ΔCT and Toe ΔCT fully restored eye development to *eyg*$^{[1]}$ mutant retinas (Yao and Sun, 2005; Figs. 9D and 10P, Q). While these results suggest that the CT is dispensable for Eyg function, it appears that the CT region is required for Toe activity. In fact, the results also suggest that there is a combinatorial interaction between the Toe NT and CT regions as the presence of both domains prevents Toe from rescuing the *eyg* mutants. Removal of either the NT or the CT is sufficient to then allow for rescue. These results are also intriguing as they suggest that the repressor domain within the CT of Eyg is not essential for its normal activity. Rather, it seems that the second repressor site, which is located in either the NT or B regions of the protein, is more essential to Eyg function. There appears, however, to be a genetic interaction between the NT and CT regions of both proteins. Expression of either Eyg ! NT+CT or Toe ΔNT+CT was insufficient to support eye development in *eyg[1]* mutants (Figs. 9D and 10S, T). This is in contrast to the near full rescue of *eyg*$^{[1]}$ retinas that is observed when either NT or CT regions of Toe are individually removed (Figs. 9D and 10E, Q). Such interactions are also evidenced by the inability of Eyg/Toe NT+CT to rescue *eyg*$^{[1]}$ flies when expression of chimeras involving individual domains (Eyg/Toe NT, Eyg/Toe CT) is sufficient to restore eye development (Figs. 9D and 10F, R, U).

Requirements of the RED and homeobox DNA binding domains in normal eye development

We were also interested in determining if functional distinctions between Eyg and Toe could be accounted for by differences in the use and requirements of the RED and HD motifs. Eye development could be restored to *eyg*$^{[1]}$ mutants through expression of Pax6(5a) variants that in which the RED domain was interchanged but not deleted (Yao and Sun, 2005; Figs. 9D and 10G–I). These results suggest that both Eyg and Toe exert their influence on transcription through the RED domain and that these domains have been functionally conserved. We similarly deleted and substituted the HDs and observed that expression of EYG ΔHD and Eyg/Toe HD rescued the small eye phenotype of *eyg*$^{[1]}$ mutants while Toe ΔHD failed in this respect (Yao and Sun, 2005; Figs. 9D and 10M–O). These results indicate that in contrast to absolute requirement for the RED domain it appears that the Eyg HD is completely dispensable for eye development. As a consequence Eyg primarily uses its RED domain to interact with DNA. There are several precedents for this observation. Several Pax genes including Drosophila pox meso and mammalian Pax1 and Pax9 completely lack the HD (Noll, 1993; Mansouri et al., 1999). Second, during eye development the HD of EY/Pax6 is also dispensable as an EY protein lacking the HD is sufficient to rescue loss-of-function ey mutants (Punzo et al., 2004). These results do not speak to the requirements of the Toe HD since the Toe full-length protein also failed to rescue. However, data presented below on the generation of extra eye fields indicates that Toe also does not make use of the HD (see below).

A large body of evidence indicates that a considerable degree of flexibility exists for the combinatorial use of DNA binding motifs by Pax proteins. We attempted to test the extreme limits of this feature by simultaneously replacing both the RED and HD regions of Eyg with the corresponding

domains of Toe. Surprisingly expression of the Eyg Toe PD+HD chimera rescued the structural defects of $eyg^{[1]}$ mutants (Figs. 9D and 10X). This result provides further evidence that the contextual framework motifs provided by the remaining non-DNA binding regions can influence how certain combinations of DNA binding domains are used during development.

Domain requirements for extra eye field induction

We set out to determine if Toe, like Eyg, is only capable of inducing extra eye fields adjacent to the developing endogenous retinal epithelium (as opposed to ectopic eye formation in other non-retinal tissues). We expressed each Pax6(5a) gene within 219 different developmental patterns and looked for the presence of retinal tissue. In the case of the full-length Eyg and Toe proteins, we were only able to induce extra eye fields adjacent to the normal compound eye (Figs. 11A, B; Jang et al., 2003). We were interested in determining if the domain requirements for the generation of extra eye fields are the same as those needed for the promotion of normal eye development. There is a precedent for the two processes requiring different protein domains. The activity of the CT regions of the SIX family proteins Sine Oculis and Optix (results of an ancient duplication) is an example. These regions are not interchangeable during normal eye development and in fact are thought to confer, in part, functional specificity upon SIX proteins. However, this is not the case for ectopic eye generation. The CT domains are in fact interchangeable under these conditions (Weasner et al., 2007). This result suggests that there are different molecular and biochemical requirements for normal and ectopic eye formation.

Of all the deletion constructs only the Toe ΔHD, in which the HD has been deleted, is capable of promoting the formation of an extra eye field (Fig. 11C). This implies that each domain of Eyg and all but the HD of Toe are required. This stands in contrast to our rescue assays in which the HD and CT regions of Eyg are dispensable for normal eye formation (Figs. 10M, P). Other differences were observed when the chimeric proteins were used to induce extra eye fields. In these cases the NT, B and PD domains can be individually substituted. Certain domain combinations (Eyg/Toe NT+CT and the Eyg/Toe PD+HD) could also be substituted successfully (Figs. 11D–H). Again, these requirements are dissimilar from those needed for Eyg and Toe to function properly during normal eye development. Our rescue assays concluded that all individual domains and only the PD+HD combination could be exchanged and still rescue $eyg^{[1]}$ mutants (Figs. 10F, I, L, O, R, X). As mentioned earlier, similar differences in domain requirements during normal eye development and ectopic eye formation (or extra eye field generation) are observed with other eye specification genes. These apparent disparities may reflect actual differences in the protein–protein interactions that occur between eye specification proteins and their cofactors. Such a model might imply that there is some flexibility in the path to producing an eye.

Discussion

In eye development the tasks of tissue specification and cell proliferation are regulated, in part, by the Pax6 and Pax6(5a) proteins respectively. In vertebrates, Pax6(5a) is generated as an alternately spliced isoform of Pax6. However, in *Drosophila* Pax6(5a) homologs are encoded by the *eyegone* and *twin of eyegone* genes. In this report we sought to determine the respective contributions that each gene makes to the specification of the fly. An initial analysis of transcriptional patterns indicates that both Pax6(5a) genes are expressed in identical patterns within the retina. However, *eyg* is expressed at a much higher level than *toe*. Not surprisingly, while mutations in *eyg* nearly delete the eye, a reduction in *toe* via miRNA treatments has no effects on its own. Simultaneous reductions in both genes, in contrast, result in a "headless" phenotype. Using a set of mini genetic screens and activator/repressor fusion assays we also demonstrated that both proteins function as transcriptional repressors. In total, these characteristics suggest that *eyg* and *toe* might play redundant roles in during development.

However, the high level of sequence divergence within the non-DNA binding domains hints that their functions may only be partially redundant. We set out to molecularly dissect both Pax6(5a) proteins and determine what, if any, differences exist between the activities of each protein. In two experimental contexts we were able to demonstrate that such differences between *eyg* and *toe* exist. First, a comparison of *eyg* and *toe* loss-of-function phenotypes indicated that *toe* played a greater role in the development of the thorax than the eye. Second, forced expression of both full-length proteins throughout the developing fly identified 43 different instances in which expression of one Pax6(5a) gene induced a different phenotype than the other. Taken together, these results hint that the roles of *eyg* and *toe* may be not be completely redundant.

We then set to determine which domain(s) might account for the differences seen in loss-of-function mutants and forced expression assays. We generated a set of deletion and chimeric proteins to dissect the requirement for each domain as well as the level of functional conservation. We attempted to rescue eyg^{1} mutants as well as generate extra eye fields with these protein variants. Our results indicate that Eyg and Toe make differential use of several domains. Many of these differences map to the non-DNA binding domains. We have also demonstrated that one possible mechanism for this is that Toe has only one repressor domain, while Eyg has two. Our prediction is that the differences in the non-DNA binding domains are the primary determinants of how each Pax6(5a) protein will influence development. It is less likely that the two DNA binding domains functionally distinguish one protein from another as there is an extremely high level of sequence conservation within these motifs. Thus our model for how Eyg and Toe Function is that both transcription factors bind to similar target genes but can differentially influence transcription through differing levels of repressor activity and/or interactions with disparate binding partners (Figs. 12A, B).

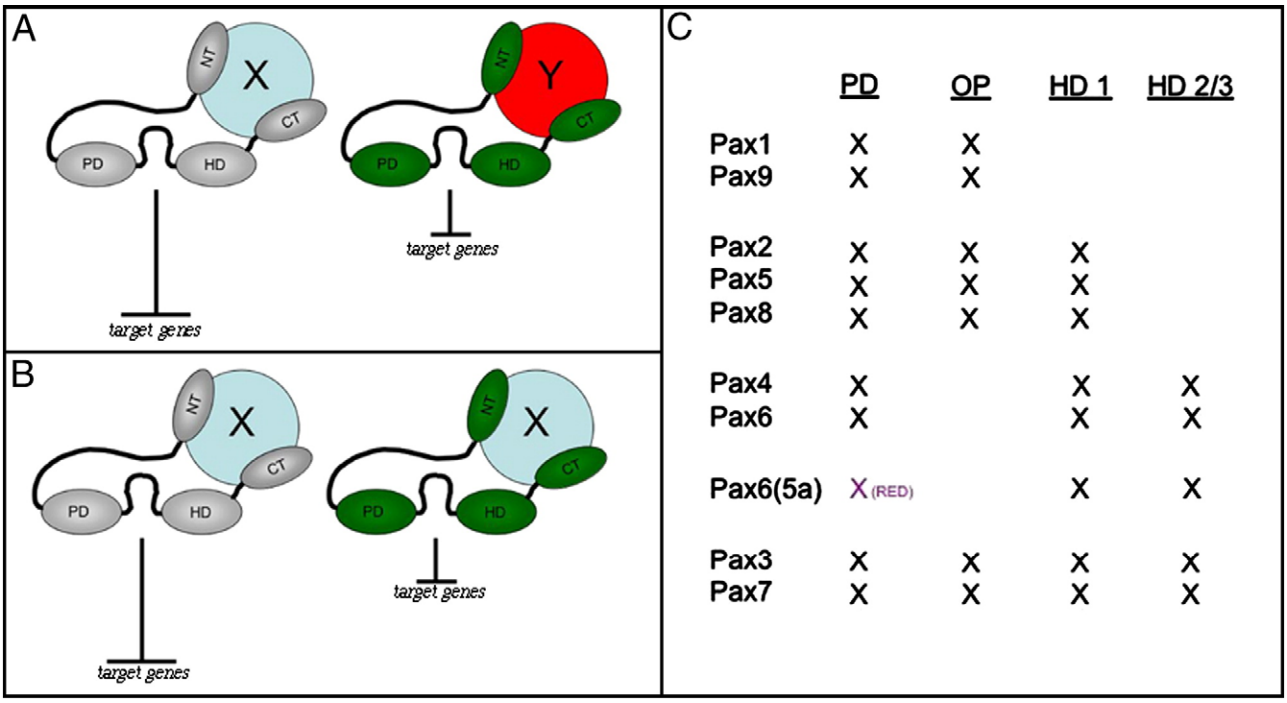


Fig. 12. Putative models for Pax6(5a) activity and structure of Pax subclasses. (A, B) Putative models. In model A the difference in the strength of repression is due to the interaction of Eyg and Toe with different binding partners. In model B these strength differences are simply due to different protein levels (binding partner is the same in this model). Note that the relative strength of repression can vary between the eye and thorax. (C) Table of mammalian Pax genes with a list of putative domains. PD=paired domain, HD=homeodomain, OP=octapeptide, HD1–3 refers to the three alpha helices.

These results may have broad implications for the activities of other Pax genes in both *Drosophila* and vertebrates. The fly genome contains two Pax6 genes, *eyeless (ey)* and *twin of eyeless (toy),* both of which also arose through a relatively recent duplication. Both share high degrees of homology within the DNA binding domains while having significantly lower levels of sequence conservation in the non-DNA binding regions (R. Datta and J.P. Kumar, unpublished data). Functionally, Ey and Toy have differing abilities to induce eye formation when expressed in non-retinal tissues (Halder et al., 1995a,b; Czerny et al., 1999). Some of these differences have been attributed to the C-terminal tail section of each protein (Punzo et al., 2004).

Mammalian Pax genes are grouped, in part, according to their structure (Fig. 12C). Individual classes are defined by the presence or absence of the octapeptide and the two DNA recognition (Paired and Homeobox) motifs. Like the fly genes, members within each Pax subclass share a very high degree of sequence conservation within the DNA binding domains thus they are likely to bind to very similar targets. Our results, if extended to these other Pax genes, would suggest that their activity could be distinguished by examining the localization of activation and repressor domains as well as the use of different binding partners.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.12.037.

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