Eyg and Ey Pax proteins act by distinct transcriptional mechanisms in *Drosophila* development

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***Drosophila* has two pairs of Pax genes, *ey/toy* and *eyg/toe*, that play different functions during eye development. *ey* specifies eye fate, while *eyg* promotes cell proliferation. We have determined the molecular basis for the functional diversity of Eyg and Ey. Eyg and Ey act by distinct transcriptional mechanisms. They use different DNA-binding domains for target recognition. Most interestingly, Eyg acts exclusively as a repressor, whereas Ey is an activator. Several vertebrate Pax proteins are known to switch between activator and repressor activities, but none as repressors only. Eyg may be the first Pax protein as a dedicated repressor. Vertebrates produce a Pax6 isoform, Pax6-5a, differing from Pax6 in DNA-binding properties and functions and structurally similar to Eyg/Toe. We found that Pax6-5a acts as an activator like Ey, but has DNA-binding specificity like Eyg.**

The EMBO Journal (2005) 24, 2602–2612. doi:10.1038/sj.emboj.7600725; Published online 23 June 2005

Subject Categories: chromatin & transcription; development

Keywords: *Drosophila*; eye; Pax; transcriptional repressor

Introduction

Pax6 genes are highly conserved in evolution and are important for eye development (Gehring, 2002; Pichaud and Desplan, 2002). Pax6 belongs to the Pax family of transcriptional factors. Pax6 contains two DNA-binding domains: a PAIRED domain (PD) and a homeodomain (HD). The PD can be further divided into two DNA-binding subdomains, PAI and RED, plus a linker (L) region linking the two. In vertebrates, Pax6 produces two protein isoforms by alternative splicing of an alternative exon 5a (Walther and Gruss, 1991), which is conserved from amphibian to mammals (Jaworski *et al*, 1997). The use of the exon 5a in Pax6-5a causes a 14 aa insertion effectively truncating the PAI subdomain of the PD and causing a change in DNA-binding specificity. The Pax6 uses the PAI to bind to the target site (P6CON), while the Pax6-5a uses the RED to bind to a different target site (5aCON) (Epstein *et al*, 1994a,b; Kozmik *et al*, 1997; Duncan *et al*, 2000). The 5a isoform is important for eye development, since both 5a-specific mutations and elevated

5a expression cause eye phenotypes (Epstein *et al*, 1994b; Singh *et al*, 2002). The Pax6 and Pax6-5a mutants have different eye and brain defects (Singh *et al*, 2002; Haubst *et al*, 2004). Overexpression of Pax6 and Pax6-5a also causes different developmental effects and gene expression (Duncan *et al*, 2000; Chauhan *et al*, 2002; Haubst *et al*, 2004). These suggest that they play different developmental functions.

Drosophila has a pair of Pax6 genes, *eyeless* (*ey*) and *twin of eyeless* (*toy*) (Halder *et al*, 1995; Czerny *et al*, 1999), and another pair of Pax genes, *eye gone* (*eyg*) and *twin of eyg* (*toe*) (Jun *et al*, 1998; Jang *et al*, 2003), that are similar to *ey/toy*. The two pairs of Pax genes play different functions in eye development. The major difference is that *ey/toy* specifies eye fate, while *eyg* is primarily responsible for eye growth (Chao *et al*, 2004; Dominguez *et al*, 2004). Loss-of-function mutations in *toy*, *ey*, and *eyg* all cause eye loss (Quiring *et al*, 1994; Kronhamn *et al*, 2002; Jang *et al*, 2003). Although no *toe* mutation has been reported, RNAi on *toe* also caused eye reduction (Chuen-Chuen Jang and Y Henry Sun, unpublished results). *eyg* is a major mediator of the Notch (N) signaling to promote cell proliferation in the larval eye disc (Chao *et al*, 2004; Dominguez *et al*, 2004). *eyg* induces the expression of *unpaired* (*upd*), which encodes a secreted signal that acts over long range through the Dome/Jak/STAT signaling pathway to induce global cell proliferation (Tsai and Sun, 2004). When N signaling in the eye disc is blocked, the eye cannot develop. The phenotype can be partially rescued by expressing *eyg* or *toe*, but not by *ey* or *toy* (Chao *et al*, 2004; Dominguez *et al*, 2004), suggesting that *eyg/toe*, but not *ey/toy*, are primarily responsible for the control of cell proliferation in the eye disc.

Ectopic expression of *ey* or *toy* can induce the formation of ectopic eye, through the retinal determination gene network (Halder *et al*, 1995, 1998; Czerny *et al*, 1999), which includes *sine oculis* (*so*), *eyes absent* (*eya*), *dachshund* (*dac*), *decapentaplegic* (*dpp*), and *hedgehog* (*hh*) (Silver and Rebay, 2005). In the wild-type third instar eye disc, differentiation begins at the posterior margin and progresses toward the anterior end. The front of the differentiation wave is marked by the morphogenetic furrow (MF). When *ey* or *toy* is ectopically expressed in other imaginal discs, ectopic MF and retinal differentiation can be induced, leading to ectopic eyes (Halder *et al*, 1995; Czerny *et al*, 1999). Thus, *ey* and *toy* can confer eye fate to the normally non-eye tissues. In contrast, ectopic expression of *eyg* can only induce extra eye field from the lateral margins of the eye disc, but not in other imaginal discs (Jang *et al*, 2003). The ectopic eye field is due to ectopic MF initiation when the *wingless* (*wg*) expression in the lateral margins is suppressed by *eyg* (Hazelett *et al*, 1998). So *ey/toy*, but not *eyg*, has the ability to specify eye fate.

A fundamental question is how these Pax proteins with similar structures are capable of performing different

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Received: 18 March 2005; accepted: 2 June 2005; published online: 23 June 2005

functions. An obvious possibility is that they recognize different target genes. Ey and Toy have complete PDs (Halder *et al*, 1995; Czerny *et al*, 1999), but Eyg and Toe have a truncated PAI (Jun *et al*, 1998; Jang *et al*, 2003). This difference confers different DNA-binding preferences (Jun *et al*, 1998). The HDs also recognize slightly different target sites. The HDs of Ey and Pax6 prefer the P3 site (Czerny and Busslinger, 1995; Sheng *et al*, 1997), while the HD of Eyg prefers the P2 site (Jun *et al*, 1998). Since Toy and Ey have been shown to bind directly to the *ey* and *so* enhancers, respectively, and activate their expression, it is expected that they act as transcriptional activators (Czerny *et al*, 1999; Niimi *et al*, 1999; Punzo *et al*, 2002). The Eyg C-terminus is Pro/Ser/Thr-rich, a characteristic of the Pax activation domain (Kozmik *et al*, 1993; Chalepakos *et al*, 1994; Dorfler and Busslinger, 1996; Tang *et al*, 1998), so Eyg is expected to also be an activator. Their functional differences may simply be that they activate different target genes.

When the vertebrate *Pax6* genes are misexpressed in *Drosophila*, Pax6 induces ectopic eye development, while Pax6-5a induces massive overgrowth, suggesting that the two isoforms have different *in vivo* functions (Dominguez *et al*, 2004). Since Pax6 and Pax6-5a are structurally similar to Ey/Toy and Eyg/Toe, respectively, the interesting possibility is that the two vertebrate Pax6 isoforms produced from a single gene are functionally equivalent to the two pairs of *Drosophila* Pax proteins, respectively (Dominguez *et al*, 2004; Silver and Rebay, 2005).

In this study, we tried to determine the molecular basis for the functional difference between Ey and Eyg. Since the structural requirement for Ey has been reported (Punzo *et al*, 2001, 2004), we started with analyzing the structural requirement of Eyg, and compared it with Ey. A series of truncation and mutant constructs were expressed in trans-

genic flies and tested for their *in vivo* functions, based on a number of criteria. Surprisingly, we found that Eyg acts as a transcriptional repressor, whereas Ey acts as a transcriptional activator. Moreover, based on the domain requirements and the analysis of a series of Ey-Eyg domain swap constructs, we showed that the DNA-binding specificity of Ey is contributed by the PAI and L regions of the PD, whereas Eyg requires its L, RED, and HD. Thus the functional diversities of Ey and Eyg are determined by the differences of their DNA target specificities, and by opposite transcriptional activities. We also found that the human PAX6-5a is not functionally equivalent to Eyg, but acts as an activator in flies. Whereas the vertebrate PAX6 and PAX6-5a can function as either transcriptional activator or repressor, our results strongly suggest that in flies the two types of transcriptional activities are delegated to Ey and Eyg, respectively.

Results

Functional analysis of Eyg

We first analyzed the structural requirement of Eyg. We made a series of *eyg* constructs and expressed these transgenes in flies using the *GAL4/UAS* system (Brand and Perrimon, 1993) to test their functions *in vivo*. Three criteria for Eyg functions were used: (1) the ability to rescue *eyg* mutant. We used an *eyg*-*GAL4*, *eyg*²²⁻² (Jang *et al*, 2003), which drove expression in a pattern that closely mimics the *eyg* expression (Figure 1A and Supplementary Figure 1A). *eyg*²²⁻² is also an *eyg* hypomorphic mutant (Jang *et al*, 2003). *eyg*²²⁻² in combination with the null allele *eyg*^{M3-12} caused a range of phenotypes in the pharate adults: 20% are headless, 35% are eyeless (Figure 1B), and 45% have eyes smaller than 400 ommatidia. Furthermore, 100% have a grooved thorax phenotype (Supplementary Figure 1B; Aldaz *et al*, 2003). In *UAS-eyg/+*;

Figure 1 *In vivo* assays for Eyg functions. (A) *eyg*-*GAL4* driving *UAS-gfp* expression (abbreviated as *eyg*>*gfp*; green) reflected the *eyg* expression pattern in late third instar eye disc. Relative position with *Elav* (red) and *wg-lacZ* (blue) was shown. (B) A total of 35% of *eyg*²²⁻²/*eyg*^{M3-12} mutant were eyeless, and none had eyes larger than 400 ommatidia. (C) *eyg*>*eyg554* could fully rescue the *eyg*²²⁻²/*eyg*^{M3-12} mutant eye. The shift in the distribution of the phenotypic classes was summarized in Supplementary Table I. (D) *dpp*>*gfp* showed that *dpp*-*GAL4* drives expression (green) in the lateral margins of the eye disc. (E) *dpp*>*eyg554* could induce extra ventral eye (arrow), at a frequency similar to that of the full-length *eyg* cDNA (Jang *et al*, 2003). (F) *dpp*>*eyg554* suppressed *wg-lacZ* expression (purple) in the dorsal and ventral margins of the eye disc (arrow).

*eyg*²²⁻²/*eyg*^{M3-12} flies, the *eyg*-GAL4 drove the expression of UAS-*eyg* (abbreviated as *eyg*>*eyg*) in its endogenous expression domain and could rescue the eye and thorax phenotypes of *eyg*²²⁻²/*eyg*^{M3-12} (not shown). (2) The ability to induce extra ventral eye, when driven by *dpp*-GAL4 (abbreviated as *dpp*>*eyg*; Jang *et al*, 2003). (3) The ability of *dpp*>*eyg* to suppress *wingless* (*wg*) expression at the dorsal and ventral margins of the eye disc (Jang *et al*, 2003). For all analysis of Eyg constructs, the results were summarized in Figure 2A.

For the *eyg* mutant rescue experiments, the distribution of the eye phenotypes was summarized in Supplementary Table I.

The *eyg* cDNA has three in-frame start codons, with the potential of encoding proteins of 670, 643, and 554 amino acids, respectively (Figure 2A). All three ORFs (Eyg670, Eyg643, and Eyg554) showed activity comparable to the full-length *eyg* cDNA (Figures 1C–F, Supplementary Figure 1C, and summarized in Figure 2A). The shortest form, *eyg*554, was used as the starting point for all subsequent

Figure 2 Structural-functional analysis of Eyg. (A) Summary of the various constructs and the results of their *in vivo* functional assays. The full-length *eyg* cDNA was shown on top. The three potential start codons and the domain structure of the encoded protein were indicated. If the third ATG is used as the start codon, then the truncated PAI is residues 1–35, the L region is 36–50, the RED is 51–102, and the HD is 232–291. The various Eyg deletion and mutant constructs were shown. F: Flag tag; N: NLS from the SV40 large T antigen. *Within the RED, indicated mutations in four DNA-contacting residues (S92A, S94A, S95A, and N97A). *Within the HD, indicated mutations in two DNA-contacting residues (S281A and N282A). Results from the *in vivo* functional assays were summarized to the right. In eye and thorax rescue of *eyg*²²⁻²/*eyg*^{M3-12}, F: complete or nearly complete rescue; P: partial rescue; DN: dominant-negative effect; ND: not determined. The distribution of eye phenotypes for each construct was summarized in Supplementary Table I. For the induction of extra ventral eye, ++: about 20% of flies have extra ventral eye; +: about 10%; -: 0%; #: only small red spots were induced at low frequency. For the repression of *wg-lacZ* expression, ++: strong repression; ±: weak repression; -: no repression; ND: not determined. Consequences of EygΔPD (B, C), EygΔHD (D, E), EygMHD (F, G), and EygMPD plus EygMHD (H, I) expression in the rescue of the *eyg* mutant eye (B, D, F, H), and suppression of *wg-lacZ* (C, E, G; *wg-lacZ*: blue; *Elav*: red; white arrow indicated suppression; yellow arrowhead indicated no suppression) were shown. (I) Coexpression of EygMPD and EygMHD could induce extra ventral eye. (J) GST-Eyg[PD] could bind to 5aCON, and this binding could be competed out with 200× excess cold 5aCON. In contrast, GST-EygMPD failed to interact with the 5aCON. (K) GST-Eyg[HD] could bind to P2CG, and this binding can be competed out with 200× excess cold P2CG. In contrast, GST-EygMHD failed to interact with P2CG. The concentrations of proteins were increased in two-fold steps (GST-Eyg[PD]: from 6.7×10^{-8} to 1.1×10^{-6} M; GST-Eyg[HD]: from 1.5×10^{-8} to 2.4×10^{-7} M). The concentrations of GST and mutant proteins were the same as the highest concentrations of the wild-type proteins.

constructs in this study. Eyg554 was located in the nucleus (Supplementary Figure 2). In all subsequent constructs, a Flag epitope and a nuclear localization signal (NLS) were added to the N-terminus to make sure that the transgenic proteins are nuclear and can be easily detected by anti-Flag antibody (Supplementary Figure 2).

Both the PD and HD are important for Eyg functions

We first tested the functional requirement for the two DNA-binding domains: PD and HD. When the PD was deleted (Eyg Δ PD), the *eyg* mutant was not rescued but enhanced. None had eyes larger than 100 ommatidia (Figures 2A and B). The grooved thorax phenotype was also enhanced (Supplementary Figure 1D). These effects were also seen in wild-type background, suggesting that Eyg Δ PD can antagonize endogenous Eyg functions. Eyg Δ PD could still suppress *wg* (Figure 2C) but failed to induce extra ventral eye (not shown). Thus, the PD is important for most of the Eyg functions except the *wg* repression. We further dissected the PD. Deletion of the PAI (Eyg Δ PAI) did not affect Eyg functions, whereas deletion of the PAI and L (Eyg Δ PAI Δ L) caused a failure to rescue *eyg* mutant (Figure 2A). Deletion of the RED (Eyg Δ RED), or deletion of both the L and RED (Eyg Δ L Δ RED), caused effects similar to those of Eyg Δ PD (Figure 2A). These results suggest that both the L and RED, but not the PAI, are critical for functions of the Eyg PD.

When the HD was deleted (Eyg Δ HD), the mutant eyes were partially rescued, but no extra ventral eye was induced (Figures 2A and D). Mutant thorax was partially rescued (Supplementary Figure 2E). Interestingly, *wg* was suppressed only at the ventral margin (Figure 2E, white arrow), but not at the dorsal margin (Figure 2E, yellow arrowhead). These results suggest that the HD is important for part of the Eyg functions.

To understand the importance of DNA-binding abilities of both the PD and HD, several of their DNA contacting residues, based on the X-ray crystal structure of the human PAX6 PD (Xu *et al.*, 1999) and the study of the Ey HD (Punzo *et al.*, 2001), were mutated. Individual PD and HD could bind to their respective consensus binding sites (Figures 2J and K). Mutations in their DNA contacting residues resulted in the loss of binding activity (Figures 2J and K). When DNA-binding residues of the PD were disrupted (Eyg Δ PD), the effects were similar to those of Eyg PD (Figure 2A), suggesting that DNA binding of the PD is important for its functions.

When DNA-binding residues of the HD were mutated (Eyg Δ HD), 21% of mutant eyes were fully rescued, but no extra ventral eye was induced (Figures 2A and F). Furthermore, the mutant thorax was fully rescued, although there were still many extra macrochaetes at lateral regions (Supplementary Figure 2F). *wg* expression in both margins of the eye disc was suppressed (Figure 2G). Since Eyg Δ HD showed more Eyg activities than Eyg Δ HD, these results suggest that DNA binding contributes to HD functions, but the HD has additional function independent of its DNA binding, perhaps through interaction with other proteins. When both the PD and HD DNA-binding sites were mutated (Eyg Δ PD Δ HD), it exhibited a dominant-negative effect. It did not rescue mutant eyes, nor induced extra ventral eyes, but rather caused a headless phenotype with 100% penetrance (Supplementary Table I), similar to the phenotype of the *eyg*^{M3-12} homozygote (Jang *et al.*, 2003). The dominant-

negative effect suggests that although the Eyg Δ PD Δ HD lost DNA-binding ability, it retained the ability for protein interaction and interfered with the functions of the endogenous Eyg. These results suggest the importance of protein interaction, in addition to DNA binding, in Eyg functions.

Interestingly, the PD and HD could function in *trans*. Coexpression of the Eyg Δ PD and Eyg Δ HD could fully rescue the mutant eye (Figure 2H) and thorax (Supplementary Figure 2G). When coexpressed by *dpp*-GAL4, *wg* was suppressed (not shown) and extra ventral eye could be induced in the ventral head region (Figure 2I), similar to the phenotype caused by *dpp*>*eyg*. These results suggest that the PD and HD may be used independently on different target genes.

Eyg acts as a transcriptional repressor

Eyg is predicted to be a transcriptional factor. We tested directly whether it acts as a transcriptional activator or repressor. Constructs were made that fuse either the VP16 activation domain or the Engrailed repression domain to Eyg554 (called Eyg-VP16 and Eyg-En, respectively; Figure 2A). Fusion with the VP16 activation domain is expected to force Eyg-VP16 to activate Eyg target genes, while Eyg-En represses Eyg target genes. When expressed in transgenic flies, Eyg-En functioned like Eyg554 (Figures 2A, 3A, and B and Supplementary Figures 1H and 3A). In contrast, *eyg*>*eyg*-VP16 in the *eyg* mutant caused a headless phenotype with 100% penetrance (Figure 3C) and enhanced the thorax phenotype (Supplementary Figure 1I), similar to that of the *eyg*^{M3-12} homozygote. These effects were also seen in wild-type background (not shown), suggesting that Eyg-VP16 acts to antagonize endogenous Eyg functions. Consistent with this, *dpp*>*eyg*-VP16 failed to suppress *wg* (Figure 3D), did not induce extra ventral eye, but caused a reduction of the endogenous eye (Supplementary Figure 3B). Coexpression of Eyg-VP16 and Eyg (using *dpp*-GAL4) gave an intermediate phenotype (Supplementary Figure 3C). Taken together, these results strongly suggest that Eyg acts as a transcriptional repressor. In contrast, similar experiments showed that Ey acts as a transcriptional activator in ectopic eye induction (Supplementary Figure 5).

We further tested whether the endogenous Eyg protein exhibits repressive activity. Two copies of the *ey* eye-specific enhancer (Hauck *et al.*, 1999) were used to drive GFP expression uniformly behind the MF in the eye disc (Supplementary Figure 3D). When three or six copies of the Eyg PD-binding site (Jun *et al.*, 1998) were added, GFP expression was noticeably reduced in the *eyg*-expressing domain in the eye disc (Figure 3E and Supplementary Figure 3E). The repression domain was broader than the *eyg* expression domain based on *in situ* hybridization and the reporter gene (Jang *et al.*, 2003), suggesting that the GFP reporter is now responding to a low level of Eyg, which escaped detection by other methods. These results demonstrate the repressor activity of the endogenous Eyg protein.

Eyg has two independent regions with repressor activity

We developed an *in vivo* assay for the transcriptional repressor activity. The assay was composed of a driver and a reporter (Figure 4A). The driver consisted of the Gal4 DNA-binding domain (Gal4DBD) fused to the different domains to

Figure 3 Eyg acts as a transcriptional repressor. (A) *eyg > eyg-en* fully rescued the *eyg²²⁻²/eyg^{M3-12}* mutant eye. (B) *dpp > eyg-en* repressed *wg-lacZ* expression in lateral margins of the eye disc (arrows; *wg-lacZ*: blue; Elav: red). (C) *eyg > eyg-VP16* did not rescue the *eyg²²⁻²/eyg^{M3-12}* mutant eye, but caused a headless phenotype. (D) *dpp > eyg-VP16* did not repress *wg-lacZ* (yellow arrowheads; *wg-lacZ*: purple; Elav: red). (E) When six copies of the Eyg PD consensus binding site were added to the *ey-gfp* reporter, GFP expression (green) was suppressed in the *eyg* expression domain (*eyg-lacZ*: blue). An arrow indicated MF.

be tested (domain X), and was placed under the control of a *wg* eye enhancer that drove expression in the lateral margins of the eye disc (W11; J-G Yao, Jaeseob Kim, and Y Henry Sun, unpublished results). The reporter *W9-UAS-gfp* was under the control of a similar *wg* eye enhancer (W9; Supplementary Figure 4A) with the addition of five tandem Gal4-binding sites (UAS). Transgenic flies harboring the driver and reporter were examined. If domain X has repressor activity, then Gal4DBD-X will bind to UAS sites of the reporter construct and repress its expression. As a negative control, the *W11-GAL4DBD*, with no X domain, did not repress the reporter expression (Figure 4B and Supplementary Figure 4A). As a positive control, addition of the En repressor domain (Gal4DBD-En) repressed the GFP expression in both the eye disc (Figure 4C) and salivary gland (not shown). We then tested four regions of the Eyg protein: PD, B, HD, and C (Figure 4 and Supplementary Figure 4). The C-terminus strongly repressed the reporter expression in both tissues (Figure 4D and not shown). When the C-terminus was

deleted and DNA binding of both the PD and HD was mutated, the *EygΔC[mPDmHD]* still revealed weak repression activity (Figure 4E). Thus, the N-terminal part of Eyg also had repressor activity. Since the individual PD, B, and HD had no repressor activity (Supplementary Figures 4B–D), the repressor activity of the N-terminal part may require the collaboration of more than one domain. These results showed that Eyg has two independent regions with repressor activity.

Although the C-terminus of Eyg has strong repressor activity, its activity may be redundant for Eyg functions. Three constructs that partially or completely deleted the C-terminus (*Eyg438*, *Eyg325*, and *EygΔC*) still retained the Eyg functions (Figure 2A). When the Eyg C region was expressed alone (*Eyg[C]*), it caused a dominant-negative effect (Figure 2A), suggesting that the C-terminus does play a role, perhaps through protein interaction. These results suggest that the two halves of Eyg have redundant repressor activity.

We also developed an *in vivo* assay for transcriptional activation activity of the different domains (Supplementary Figures 5G–O). None of the Eyg domains had activation activity (not shown), suggesting that Eyg can only act as a repressor.

Functional specificity of Ey is determined by PAI, L, and C-terminus

Ey and Eyg are functionally distinct based on several over-expression phenotypes. *dpp > ey* can induce ectopic eyes in adult appendages and *so¹⁰-lacZ* expression in imaginal discs and suppress *Dll-lacZ* expression (Halder *et al.*, 1995; Niimi *et al.*, 1999; Kurata *et al.*, 2000), whereas *dpp > eyg* cannot (Jang *et al.*, 2003). Instead, *dpp > eyg* induces extra ventral eye (Jang *et al.*, 2003). *dpp > ey* does not have this effect. To understand which region(s) of Ey contribute to the functional specificity distinct from that of Eyg, we made a series of domain swaps between Ey and Eyg. Ey and Eyg were each divided into six regions, namely PAI, L, RED, B, HD, and C (Figure 5A). In the chimera constructs, the parts from Ey and Eyg were labeled E and G, respectively. The results were summarized in Figure 5A.

We first tested which of the DNA-binding domains of Ey is required for its functional difference from Eyg. Replacing the RED, HD, or both, of Ey by the corresponding domain from Eyg (EEGEEE, EEEEGE, and EEGEGE, respectively) had no effect on Ey functions (Figures 5A and E–G). These findings are consistent with previous reports that the Ey HD is dispensable for eye development (Punzo *et al.*, 2001, 2004) and that DNA binding of the PAX6 PD is primarily through the PAI, and DNA binding by the RED is masked in the presence of the PAI (Epstein *et al.*, 1994a). However, *EyΔRED* lost the ability to induce ectopic eyes (not shown), suggesting that the RED may still play a role in DNA recognition, or in maintaining the protein structure of the PD.

In contrast, whenever the swaps involved the PAI (in GEEEEE, GGEEEEE, GGGE, and GGGE; Figure 5A), the chimeras failed to induce ectopic eye. These results suggest that the Ey PAI is required for Ey functions. In support of this, deletion of the Ey PAI (*EyΔPAI*) also abolished Ey functions (Figures 6F and G). However, when the PAI, the PAI plus L, or the entire PD of Eyg was substituted by that of Ey (EGGGGG, EEGGGG, and EEGGGG, respectively), they did not cause

Figure 4 Eyg has two redundant repression domains. (A) The *in vivo* repression assay used a driver and a *gfp* reporter. Both were regulated by similar *wg* enhancers to express in the lateral margins of the eye disc and in the salivary gland (not shown). The driver contained the GAL4 DNA-binding domain (GAL4DBD) fused to the different domains to be tested. *Indicated mutations in the DNA-binding residues in the PD and HD. (B, C) Expression of the *gfp* reporter was not repressed by the Gal4DBD alone (B), but was repressed when the Gal4DBD was fused to the En repressor domain (C). (D) Eyg[C] showed strong repressor activity. (E) EygΔC[mPDmHD] had weaker repressor activity.

ectopic eyes (Figure 5A) as Ey did, indicating that the Ey PD is not sufficient to determine the functional specificity. Further addition of the HD (EEEGEG) still did not induce ectopic eye (Figure 5A). These results suggest that the non-DNA-binding domains may be critical for Ey functions.

We next tested the roles of the non-DNA-binding domains B and C. When the Ey B was replaced (EEEGEE), it could still induce ectopic eyes, but of smaller size (Figure 5A). Therefore, the B region is not critical for the functional specificity of Ey, but is required for the full activity of Ey. Whenever the C-terminus of Ey was replaced by the Eyg C (in EGGGGG, EEGGGG, EEEGGG, EEEGEG, EEEEEG, GEEEGG, GGEEGG, GGEGGG, GGGGEG, GGELEG and GGEGGG), these failed to induce ectopic eye (Figure 5A), confirming that the Ey C-terminus is essential for Ey functions. In an *in vivo* activation assay, the C-terminus of Ey had strong activation activity, while the Toy C-terminus had weak activity (Supplementary Figures 5M–O).

Both PAI and C are required for Ey functions, but individually or together (in EGGGGE) they were not sufficient to induce ectopic eye (Figure 5A). Since the L between the PAI and RED is known to contact DNA (Xu *et al*, 1999) and is divergent between Ey and Eyg (Jun *et al*, 1998), it may play a critical role. When the PAI, L, and C from Ey were all swapped into Eyg, EEGGGE was capable of inducing ectopic eyes in appendages (Figure 5B) as well as activation of *so* (Figure 5C), *Eya*, and *Dac* (not shown) and suppression of *Dll* (Figure 5D). However, sizes of these ectopic eyes and the level and area of gene activation were significantly smaller than those induced by Ey. These results suggest that the combination of the PAI, L, and C is sufficient to determine the functional specificity of Ey, but the level of activity requires additional domains. This requirement could be satisfied with the addition of the B domain. The EEGEGE showed full Ey

activity (Figures 5E–G). In a yeast one-hybrid assay that directly tests the transcriptional activation of Ey (Niimi *et al*, 1999), when the PAI, L, and C were from Ey (EEGGGE, EEGEEE, EEEEGE, and EEGEGE), these chimeras could activate reporter gene expression by directly binding to Ey target sites (Figure 5H). Thus, the PAI, L, and C together are required and sufficient for Ey functional specificity.

Functional specificity of Eyg is determined by L, RED, B, and HD

We then tested which domain(s) in Eyg is critical in determining its functional difference from Ey. When the Eyg PAI was replaced with the Ey PAI (EGGGGG), it induced extra ventral eye, similar to Eyg (Figure 5A). When both the PAI and L of Eyg were replaced, the EEGGGG chimera could not induce extra ventral eye (Figure 5A). Thus, the masking of the RED DNA recognition (Epstein *et al*, 1994b) may require both the PAI and L regions. The Eyg RED could be replaced by the Ey RED (GGEGGG) without affecting its function in rescuing *eyg* mutant (Figure 5A). This suggests that the DNA-binding specificities of the RED in Ey and Eyg are identical, but this domain is used in Eyg and masked in Ey. These results are consistent with the results from the deletion experiments (Figure 2A). The Eyg HD could not be functionally replaced by the Ey HD, because GGGGEG and GGEGEG chimeras lost their abilities to rescue *eyg* mutant (Figure 5A). The Eyg B region is also important, since the ability to rescue *eyg* mutant was abolished when the B was replaced (in GGEGGG; Figure 5A). The B region is not involved in DNA binding, so is probably required for the repressor activity of Eyg. Although the C of Eyg could be deleted, replacing it with the C from Ey (GGGGGE) abolished Eyg functions (Figure 5A). This is consistent with our finding that the C of Ey is an activation domain and would reverse the Eyg

Figure 5 Domain swaps between Ey and Eyg. (A) The various Ey-Eyg domain swap constructs were shown to the left. The two proteins were each divided into six domains: PAI, L, RED, B, HD, and C. Ey was drawn in black and was indicated by E. Eyg was drawn in white and was indicated by G. Results of functional assays were shown to the right. For the induction of ectopic eyes (when driven by *dpp*-GAL4), E indicated the Ey-type ectopic eyes on appendages, e indicated weaker Ey-type activity, and G indicated the Eyg-type extra ventral eye on the head. Activation of *so*¹⁰-lacZ and repression of *Dll*-lacZ (when driven by *dpp*-GAL4) reflected Ey activities. Rescue of *eyg*²²⁻²/*eyg*^{M3-12} mutants reflected *eyg* functions. (B–D) *dpp* > EEGGGE induced very small ectopic eyes in adults (B, arrows), weakly induced *so*¹⁰-lacZ expression in the wing disc (C), induced small ectopic eye (white arrow; red: Elav⁺ cells), and weakly suppressed *Dll*-lacZ (yellow arrowhead; blue: β-gal⁺ cells) in the leg disc (D). (E–G) *dpp* > EEGEGE induced ectopic eyes (E, arrows), induced *so*¹⁰-lacZ in the wing disc (F), induced ectopic eye development (white arrow; red: Elav⁺ cells), and repressed *Dll*-lacZ (yellow arrowhead; blue: β-gal⁺ cells) in the leg disc (G). Scale bar: 100 μm (C, F) and 50 μm (D, G). *dpp* > gfp (green) indicated *dpp*-expressing cells. (H) Yeast one-hybrid assay. A synthetic Ey PD-binding site (4xEy[PD]) and the natural Ey PD-binding site (*so*¹⁰) were linked to the *HIS3* reporter gene. When grown in glucose, the chimeras were not expressed, and cells grew poorly. When the Ey-Eyg chimeras were induced by galactose, if they could bind to the Ey binding sites, they could induce *HIS3* expression and allow cell growth in His⁻ medium. Ey and the chimeras that contain the PAI, L, and C from Ey were able to direct binding to the Ey binding sites and activate reporter expression.

repressor activity. In summary, the L, RED, and HD of Eyg are important for the functional specificity of Eyg.

PAX6-5a target specificity is like Eyg but acts as an activator like Ey

The vertebrate Pax6-5a has a disrupted PAI subdomain, similar to that of Eyg (Figure 6A). We tested whether Pax6-5a is functionally equivalent to Eyg. Expression of human PAX6-5a (hPAX6-5a) driven by *dpp*-GAL4 caused eye reduction (Figure 6B), but did not induce extra ventral eye as *dpp* > *eyg* would. *dpp* > hPAX6-5a (Dominguez *et al*, 2004) and *dpp* > hPAX6-5a-VP16 (Figure 6C) induced massive overgrowth in the wing disc, whereas *dpp* > *eyg* induced only slight overgrowth in the wing discs (Figure 6E). The finding

suggests that hPAX6-5a acts as a transcriptional activator in flies. In addition, *eyg* > hPAX6-5a did not rescue *eyg* mutant, but enhanced the phenotype (Figure 6D). These results indicate that hPAX6-5a is not functionally equivalent to the fly Eyg.

hPAX6 induces retinal differentiation whereas hPAX6-5a induces massive overgrowth in flies (Dominguez *et al*, 2004). Their functional difference is likely due to the difference in the PAI. We tested this on Ey. Deletion of the Ey PAI (EyΔPAI) failed to induce ectopic eye, but caused strong eye reduction (Figure 6F) and massive overgrowth in the wing discs (Figure 6G), similar to that caused by hPAX6-5a. When the entire Ey PD was replaced by the Eyg PD, it also caused similar phenotypes as EyΔPAI did (Figures 6H and I). These

Figure 6 PAX6-5a is functionally equivalent to EyΔPAI. (A) The hPAX6-5a has a 14 aa insertion disrupting the PAI. Similarly, Eyg has a truncated PAI. EyΔPAI: Ey with the PAI deletion; GGGEEE: the Ey PD replaced by the Eyg PD. (B) *dpp > hPAX6-5a* caused a reduction of the eye, without induction of extra ventral eye. (C) Both *dpp > hPAX6-5a* (not shown) and *dpp > hPAX6-5a-VP16* induced massive overgrowth. (D) *eyg > hPAX6-5a* enhanced the *eyg²²⁻²/eyg^{M3-12}* mutant eye and thorax phenotypes. (E) *dpp > eyg554* caused only weak overgrowth effect. (F, G) *dpp > eyΔPAI* caused an eyeless phenotype (F) and induced massive overgrowth (G). (H, I) *dpp > GGGEEE* caused phenotypes similar to those caused by *dpp > hPAX6-5a*, *dpp > hPAX6-5a-VP16*, and *dpp > eyΔPAI*. (C, E, G, I) In all wing discs, *dpp* expression domain was visualized by *dpp > gfp* (green). Blue: *wg-lacZ*; scale bar: 100 μm.

results suggest that hPAX6-5a is equivalent to EyΔPAI. The lack of the PAI probably allowed the RED to bind to DNA. Thus, the difference between hPAX6 and hPAX6-5a is in their target gene specificities, as determined by the usage of different DNA-binding domains.

Discussion

The results presented in this study demonstrate that Ey and Eyg use distinct mechanisms for transcriptional regulation. There are two major differences between Ey and Eyg. First, Ey and Eyg bind to different target sites using different combinations of DNA-binding domains. Second, Ey acts as a transcriptional activator, whereas Eyg acts as a transcriptional repressor.

Ey and Eyg use different combinations of DNA-binding domains for target recognition

Ey specificity, compared with Eyg, is determined by its PAI and L domains. *In vitro* studies show that when the intact PAI is present, the DNA binding of the RED is masked (Epstein *et al*, 1994b). However, we found that *in vivo* the RED of Ey may still play a role in target recognition or in maintaining the PD structure. The HD is not required for most of Ey functions, although it is required to suppress *Dll* expression (Punzo

et al, 2001, 2004). In contrast, Eyg uses the L, RED, and HD domains for target recognition. The L region is important for the functional specificity of both Ey and Eyg. Although the L region has been shown to contact DNA by X-ray crystallography (Xu *et al*, 1999), its functional role has not been demonstrated until now. The non-DNA-binding B region is not involved in target specificity, but in both Ey and Eyg it is required for the full activity. The different DNA-binding domains may be used independently or in a combinatorial fashion. For example, the Ey PD, but not the HD, is used to induce ectopic eye (Punzo *et al*, 2001, 2002). The Ey HD is required for the suppression of *Dll* in order to block the antennal fate during eye development (Punzo *et al*, 2001, 2004). For Eyg, the PD and HD are redundantly involved in *wg* suppression. The finding that the Eyg PD and HD could function in *trans* suggests that the two domains may bind independently to different target genes.

Eyg acts as a repressor, while Ey acts as an activator

Our results strongly suggest that Eyg acts exclusively as a transcriptional repressor. This is based on three sets of results: (1) Expression of the obligatory repressor Eyg-En in *eyg* expression domains completely rescued the *eyg* mutant defects in eye, head, and thorax development, while

Eyg-VP16 caused dominant-negative effects. Moreover, *ey>eyg⁵⁵⁴* and *ey>eyg-en* could rescue headless homozygous *eyg^{M3-12}* to approximately 500–600 ommatidia in size (not shown). (2) Ectopic expression of Eyg-En caused phenotypes similar to those of Eyg, while Eyg-VP16 caused opposite effects. Thus, in all functional assays that we tested, Eyg-En behaved just like Eyg, while Eyg-VP16 had the opposite effects. (3) Eyg has two sets of repression domains but has no activation domain.

Ey is known to activate directly *so* and the *rhodopsin* genes (Sheng *et al*, 1997; Niimi *et al*, 1999; Papatsenko *et al*, 2001). Our Ey-VP16 and Ey-En results suggest that, at least for inducing ectopic eye development, Ey functions as an activator. The suppression of *Dll* transcription by Ey is suggested to be indirect (Punzo *et al*, 2001, 2004). We found that Ey-VP16 could repress *Dll*, while Ey-En could not (Figures 5B and D), strongly suggesting that Ey probably activates a repressor of *Dll*. These results suggest that Ey in general acts as an activator. Ey uses its C-terminus for the transactivation activity. We also found that the Toy C-terminus has only weak transactivation activity (Supplementary Figure 5O), providing an explanation on why the Toy C-terminus cannot functionally substitute for the Ey C-terminus (Punzo *et al*, 2004).

Comparison with PAX6 and PAX6-5a

Both Pax6-5a and Eyg have a disrupted PAI and similar DNA-binding preference. It was proposed that PAX6-5a is functionally equivalent to Eyg (Dominguez *et al*, 2004). Our results ruled out this interesting possibility. We showed that expression of Eyg and hPAX6-5a caused different effects, and that hPAX6-5a failed to rescue *eyg* mutant. In addition, hPAX6-5a acted as a transcriptional activator in flies. Thus, hPAX6-5a is not functionally equivalent to Eyg. In contrast, hPAX6-5a functioned like the Ey construct without an intact PAI, suggesting that hPax6-5a uses the RED for DNA binding. Thus, both hPAX6 and hPAX6-5a acted as activators, but induced different target genes through use of different DNA-binding domains.

Eyg is the first dedicated repressor Pax protein

Pax6 genes from a variety of organisms can induce ectopic eyes when expressed in *Drosophila*, suggesting that the functional mechanism is conserved and they likely act also as activators in flies. In vertebrate development, Pax6 and Pax6-5a can also act as transcriptional repressors (Duncan *et al*, 1998; Cui *et al*, 2004). Pax2, Pax3, Pax4, Pax5, and Pax8 can also function as repressors, in addition to being activators (reviewed in Underhill, 2000). The switch between functioning as activators and repressors may depend on interaction with cofactors. For example, Pax3 interacts with Daxx and HIRA (Magnaghi *et al*, 1998; Hollenbach *et al*, 1999). Eyg is not only the first repressor Pax in *Drosophila*, but also the first example of a dedicated repressor Pax protein. Our results also suggest that Ey may function exclusively as an activator, at least for inducing ectopic eye development. Thus, the activator and repressor roles are delegated to proteins encoded by two different genes, *ey* and *eyg*, respectively.

Materials and methods

Fly stocks

The following flies were used: *dpp-GAL4* (Staehling-Hampton *et al*, 1994), *ey-GAL4* (Quiring *et al*, 1994), *Dll-lacZ* (a gift by Grace Panganiban), *so¹⁰-lacZ* (Niimi *et al*, 1999), *UAS-ey* (Halder *et al*, 1995), *UAS-eyg* (Jang *et al*, 2003) and *eyg* mutants: *eyg²²⁻²* (*eyg-GAL4*) and *eyg^{M3-12}* (Jang *et al*, 2003). Other fly stocks were obtained from the Bloomington *Drosophila* Sock Center.

DNA construction and generation of transgenic flies

pUAST-flag was derived by inserting the Flag epitope coding sequence from *pflag-CMV-2* (Stratagene) into the *EcoRI* and *XhoI* sites of *pUAST* (Brand and Perrimon, 1993). *pUAST-flag-nls* contains the coding sequence of the NLS of the SV40 T-antigen in the *HindIII* site of *pUAST-flag*. DNA fragments of all *ey*, *eyg*, and *hPAX6-5a* variants were PCR-amplified and cloned into *pUAST-flag* or *pUAST-flag-nls*. Detailed construction process is available upon request. For *in vivo* activation and repression assays, the coding sequence of 1–93 amino acids of the yeast Gal4 DNA-binding domain, which was derived from the KREG vector (Nibu *et al*, 1998), replaced *gfp* from the pH-Stinger vector (Barolo *et al*, 2000). For *W11-GAL4DBD*, the *wg* W11 fragment was cloned into *EcoRI* and *XhoI* sites of this pH-Stinger derivative. Different regions of *toy*, *ey*, *eyg*, *en*, and *VP16* were placed behind *GAL4DBD*. For *W9-5UAS-gfp*, five copies of *UAS* derived from *pUAST* were inserted into the *XhoI* site of *W9-gfp*. Germline transformants of these constructs were as described previously (Jang *et al*, 2003). Three to six independent transgenic lines for each construct were generated and tested. Since Gal4 induction is temperature-dependent, the induction was carried out at 18, 25, and 29°C to sample the effect of induction at different levels.

Protein purification and electrophoretic mobility gel shift assay

The coding sequences of the PD, mPD, HD, and mHD of Eyg were cloned into pGEX-4T-3. These GST fusion proteins were purified from *Escherichia coli* BL21(DE3) according to the manufacturer's recommendations (Amersham-Pharmacia). Protein concentration was confirmed by CDB, Coomassie blue, and western blotting assays. In gel shift assays, the 5aCON duplex (Jun *et al*, 1998) was used as probe for the Eyg PD, and the P2CG duplex (Jun *et al*, 1998) was used as probe for the Eyg HD. 5' ends of these probes were labeled with [γ -³²P]ATP. GST-Eyg fusion proteins and probes were mixed in the binding buffer (50 mM KCl, 1 mM DTT, 10 mM Tris pH 7.5, 10% glycerol, 50 ng/μl poly(dI.dC), 2 μg/μl BSA, 1 mM EDTA, and 2 mM EGTA) and incubated at room temperature for 20 min. The mixtures were run in 6% native polyacrylamide gel, and the dried gel was exposed onto X-ray film for observation of band shift.

Yeast one hybrid

The yeast one-hybrid system (Niimi *et al*, 1999) has a *HIS3* reporter fused to either a *so¹⁰* enhancer or four copies of the Ey target site and a driver (pBM258T vector, in which expression of gene of interest is controlled by galactose-inducible *GAL1* promoter). These are kind gifts from Makiko Seimiya. *ey-eyg* chimeras were inserted into the *BamHI* and *XbaI* sites of the pBM258T vector. The mixtures of reporters and drivers were cotransformed into yeast SEY6211 strain and selected with –Ura/–Trp/–His SD medium. Expression of Ey-Eyg chimeras was repressed by glucose and induced by galactose.

Immunohistochemistry

Immunostaining of larval tissues was as described (Chao *et al*, 2004). Primary antibodies were rabbit anti-β-gal (1:1000; Cappel), mouse anti-Flag-M2 (1:1000; Kodak), mouse anti-Elav (1:500), rat anti-Elav (1:500), mouse anti-Eya (1:100), and mouse anti-Dac (1:100) from the Developmental Studies Hybridoma Bank. Secondary antibodies were Cy3- or Cy5-conjugated IgG (1:200; Jackson Lab.). Some samples were also stained with FITC-phalloidin (1:1000; Invitrogen) or Hoechst (1:1000). Samples were scanned on a Zeiss LSM510 confocal microscope.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank James Shen, Cheng-Ting Chien, Der-Hwa Huang, Meng-Chao Yao, Steve Cohen, Bill McGinnis, Kwang-Wook Choi, Hugo Bellen, Walter Gehring, and Lydia Michaut for valuable suggestions on the manuscript, and the Bloomington Stock Center, Jaeseob Kim, Alan Laughon, and Grace Panganiban for providing the fly stocks, Makiko Seimiya and Walter Gerhing for providing the yeast

one-hybrid constructs, Xian-Jie Yang for the *hPAX6-5a* cDNA, Michael Levine for the *GAL4DBD* cDNA, and MY Cheng for the yeast strain. We thank Chun-Lan Hsu and Yu-Chi Yang for preparing fly food and maintaining fly stocks and Chiou-Yang Tang and Hui-Yin Su for microinjection. This study was supported by grants to YHS from the National Science Council (NSC 91-2312-B-001-006, 92-2312-B-001-006, and 93-2312-B-001-016) of the Republic of China.

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