The *eyeless* homeodomain is dispensable for eye development in *Drosophila*

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***Pax-6* genes, known to be essential for eye development, encode an evolutionarily conserved transcription factor with two DNA-binding domains. To corroborate the contribution of each DNA-binding domain to eye formation, we generated truncated forms of the *Drosophila Pax-6* gene *eyeless* and tested their capacity to rescue the ey^2 mutant. Surprisingly, EY deleted of the homeodomain rescued the ey^2 mutant and triggered ectopic eyes morphogenesis. In contrast, EY lacking the paired domain failed to rescue the ey^2 mutant, led to truncation of appendages, and repressed *Distal-less* when misexpressed. This result suggests distinct functions mediated differentially by the two DNA-binding domains of *eyeless*.**

[*Key Words*: *eyeless*; *Pax-6*; eye development; paired domain; homeodomain]

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The animal body plan is set up during embryogenesis by a combinatorial genetic interaction between selector genes. The *Hox* gene family is responsible for the anterior-posterior segmentation pattern of the embryo. First, the segmentation genes establish a repetitive pattern of body segments. Then, the *Hox* genes specify the identity of each segment and induce another class of selector genes that determine the different appendages or organs within a given segment. The exclusive expression of those genes gives each organ its specific identity. Recently, several *Drosophila* genes have been identified as being capable of inducing organogenesis when ectopically expressed. *Vestigial* is essential for wing and haltere identity (Kim et al. 1996), *Distal-less* (*Dll*) for leg identity, and in combination with *extradenticale* and *homothorax* for antenna determination (Casares and Mann 1998; Gonzalez-Crespo et al. 1998). The selector gene for eye morphogenesis is the *Pax-6* gene (Halder et al. 1995).

Pax genes encode nuclear transcription factors that play a key role in organogenesis (Dahl et al. 1997). They are characterized by a structurally conserved DNA-binding domain known as the paired domain (PD). The Pax family is subdivided into different subgroups, according to the presence or absence of additional conserved domains, namely, a paired-like homeodomain (HD) or a truncated paired-like homeodomain and an octapeptide (Strachan and Read 1994). The paired domain is a bipartite DNA-binding domain, subdivided into a N- and C-terminal part referred to as the PAI and RED domain, respectively (Jun and Desplan 1996). PAI, RED, and HD consist of three α-helices each, with the third helix contacting the bases in the major groove of the DNA (Xu et al. 1999).

Pax-6 contains a paired domain, and a paired-like homeodomain, but lacks the octapeptide (Ton et al. 1991). The role of *Pax-6* appears to be evolutionarily conserved during eye development in both mammals and flies. An important function for *Pax-6* in mammalian eye development was deduced from the disruption of eye development in homozygous *Small eye* (*Sey*) mice carrying a *Pax-6* mutation (Hill et al. 1991). In humans, heterozygous mutations of Pax-6 are known to cause various forms of congenital eye abnormalities, such as Aniridia or Peter's anomaly, and a complete absence of eyes in homozygous mutants. Further analysis in mice revealed an early expression in most structures of the developing eye (Walther and Gruss 1991; Grindley et al. 1995). Beside its role in eye morphogenesis, Pax-6 has important functions in the development of the brain and the spinal cord.

In contrast to vertebrates, in which multiple protein isoforms derived from a single *Pax-6* gene are found, in *Drosophila* two *Pax-6* genes, *ey* (Quiring et al. 1994) and *twin of eyeless* (*toy*) (Czerny et al. 1999), and a related gene called *eye gone* (*eyg*) have been identified. Like *toy* and *ey*, *eyg* is a Pax class transcription factor and has a RED domain and a paired-class HD, but lacks the N-terminal arm of the PAI domain (Jun et al. 1998). High-affinity binding assays with the RED domain revealed a binding specificity similar to the one described for the *Pax-6* 5a isoform (Epstein et al. 1994; Jun et al. 1998). This splice form contains a 14-amino-acid insertion in

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the PD found in all vertebrate *Pax-6* genes analyzed so far. No *Pax-6* splice forms have been reported in *Drosophila*, suggesting that this organism solves the complex regulation of development by gene duplication and modification rather than by differential splicing.

Two hypomorphic mutants of *eyeless*, ey^2 and ey^R, both inactivating the eye-specific enhancer, result in partial to complete loss of compound eyes (Quiring et al. 1994). The early expression pattern of *toy* and *ey* and the mutant phenotypes of *ey* suggest that *Pax-6* is also a crucial regulator of the development of the insect eye. Gain-of-function experiments in which *toy*, *ey*, or *Sey* are ectopically expressed, lead to the formation of ectopic eyes on *Drosophila* appendages (Halder et al. 1995; Czerny et al. 1999). Misexpression of *Pax-6* in *Xenopus* leads to the formation of ectopic eye structures (Altmann et al. 1997; Chow et al. 1999). The conservation of *Pax-6* genes in the animal kingdom, their ability to induce ectopic eyes, and their mutant phenotypes puts them high up in the genetic hierarchy of eye development.

The three genes *sine oculis* (*so*), *eyes absent* (*eya*), and *dachshund* (*dac*) encode evolutionarily conserved nuclear proteins that are essential for *Drosophila* eye development (Bonini et al. 1993; Cheyette et al. 1994; Mardon et al. 1994). All three genes are activated by EY and are required for EY-induced formation of ectopic eyes, which suggests that these genes act downstream of *ey* in the eye development pathway (Bonini et al. 1997; Shen and Mardon 1997; Halder et al. 1998). This hypothesis was confirmed for *so*, which is a direct target gene of *ey* (Niimi et al. 1999). In contrast, *toy* acts upstream of *ey*, because ectopic expression of *toy* induces *ey*, but not vice versa (Czerny et al. 1999). Whereas the crosstalk between *toy*, *ey*, and their downstream targets has been shown, the relationship between *eyg*, *ey*, and *toy* remains unclear.

The PD and the HD are the most conserved regions within the Pax-6 protein, pointing out evolutionary constraints imposed to maintain specific binding to target genes. It has been suggested that the protein might activate target genes either through the PD, the HD, or both. Alternatively, both domains could work in a cooperative manner to regulate their target genes (Jun and Desplan 1996). Furthermore, recent evidence indicates that these two DNA-binding domains are also involved in protein–protein interactions (Plaza et al. 2001). Despite the importance of *Pax-6* for eye development, the respective functions of the two DNA-binding domains are unknown. Thus, understanding their contribution in vivo is crucial for the correlation of the different mutations to their respective phenotypes. To unravel the functional role of the PD and the HD in EY, we generated different truncated forms and tested their capacity to rescue an *ey* mutant eye phenotype. We show that the PD within the EY protein is essential and sufficient for the induction of eye development and that the HD within the EY protein is sufficient to repress the selector gene *Dll*. Thus, *Pax-6* can exert a dual function as an activator and a repressor via its two different DNA-binding domains.

Results

The eyeless *homeodomain is not required to rescue* ey^2 *mutants and to induce ectopic eyes*

It has been shown previously that full-length *ey* cDNA was able to efficiently rescue the eye phenotype of ey^2 mutants when expressed in the eye disc under the control of the eye-specific *ey* enhancer (Halder et al. 1998). To assess the contribution of the EY DNA-binding domains to eye development, we expressed mutant *ey* cDNAs with ey-enhancer Gal4 in an *ey* mutant background. We generated *ey* cDNA under the control of the UAS promoter (Brand et al. 1994), which lacked either the paired box (*ey*Δ*PD*) or the homeobox (*ey*Δ*HD*) (Fig. 1G). When expressed in the eye disc, *ey*Δ*PD* was not able to rescue the eye phenotype of ey^2, but enhanced the eye reduction, leading to a complete loss of compound eye in 64% of the flies (Fig. 1B). Unexpectedly, *ey*Δ*HD* rescued the eye phenotype at an even higher efficiency than the full-length *ey*-cDNA (Fig. 1A,C), suggesting that the HD of *ey* was dispensable for eye formation in the ey^2 mutant background. Similar results were obtained in the *ey* null mutant $ey^{J5.71}$ (data not shown) isolated recently in our laboratory by an EMS mutagenesis screen. This mutant is RNA and protein null (see also Figs. 2F and 3C, below) due to a 9-kb deletion in the 5′ region of the gene ($ey^{J5.71}$; S. Flister, U. Kloter, and W.J. Gehring, unpubl.). To corroborate these findings, we performed ectopic expression experiments in a wild-type background. We found that misexpression of *ey*Δ*PD* by dpp^{blink}–Gal4 in various imaginal discs did not lead to the formation of any eye structures, but generated severely truncated appendages (Fig. 1D). In contrast, misexpression of *ey*Δ*HD* resulted in ectopic eye formation at the same efficiency as full-length *ey* (Fig. 1E). These results confirmed our finding that the PD was sufficient to induce the eye developmental pathway and suggested that the HD might act as a repressor for a gene involved in leg development.

To further characterize the function of the HD, we generated the two following constructs: one lacking PD and HD (*ey*Δ*PD*+Δ*HD*) and one lacking the PD and carrying two point mutations of amino acids directly involved in DNA binding of the HD (point mutations, S50A and N51A) (*ey*Δ*PDPMHD*). Misexpression of both *ey*Δ*PD*+Δ*HD* and *ey*Δ*PDPMHD*, by dpp^{blink}–Gal4 did not induce any appendage truncation (data not shown). Previously published results had shown that the nuclear localization signals are contained within the DNA-binding domains of *Pax-6* (Carriere et al. 1995). To ensure that our point-mutated construct was still localized in the nucleus, we performed immunofluorecent analysis. We found that *ey*Δ*PD*+Δ*HD* was no longer transported into the nucleus, whereas *ey*Δ*PDPMHD* was nuclear (data not shown). Therefore, we concluded that the truncated appendages we observed by misexpression of *ey*Δ*PD* are due to DNA binding of the HD.

To ensure that the Gal4 system used during our rescue assay does not over produce the *ey* constructs and that the ectopically induced proteins were correctly ex-

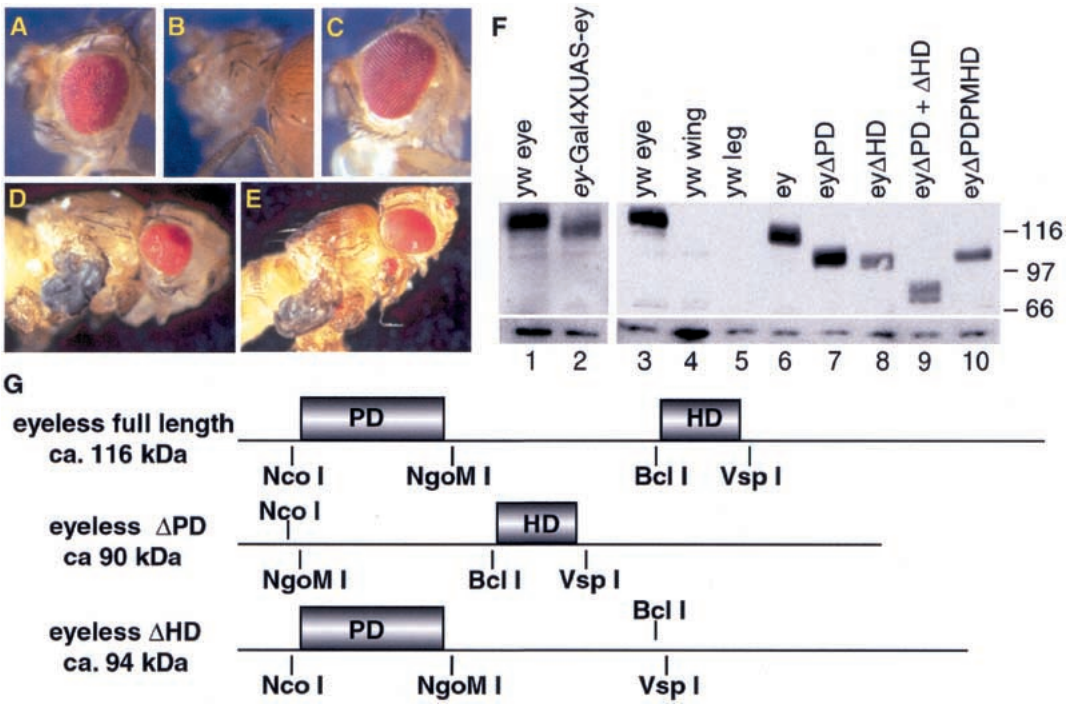

Figure 1. Rescue and ectopic expression of the different *ey* constructs. (*A–C*) Different UAS constructs were crossed to *ey* enhancer Gal4 in an ey^2 mutant background. Three independent crosses were done with two independent *ey*–Gal4 driver lines. Percentages given relate to an eye size that is >80% of the wild-type eye size. They represent the average of six measurements, in which at least 70 flies were analyzed. (*A*) Rescue with full-length *ey* (50%). (*B*) No rescue with *eyΔPD*. (*C*) Rescue with *eyΔHD* (79%). (*D,E*) Ectopic expression of UAS constructs with dpp^{blink}–Gal4 in wild-type background. (*D*) Misexpression of *eyΔPD* leads to truncation of appendages. (*E*) Misexpression of *eyΔHD* leads to formation of ectopic eyes. (*F*) Western blot analysis on third instar discs of EY proteins expressed during the rescue (lanes *1,2*) and the ectopic expression (lanes *3–10*) experiments. (Lanes *1,3*) *yw* control eye discs; (lane *2*) eye discs expressing *ey* with *ey* enhancer Gal4 in an ey^2 mutant background; (lanes *4,5*) *yw* control wing and leg discs, respectively; (lanes *6–10*) leg discs expressing the various *ey* constructs by dpp^{blink} Gal4; (lane *6*) misexpression of full-length *ey*; (lane *7*) misexpression of *eyΔPD*; (lane *8*) misexpression of *eyΔHD*; (lane *9*) misexpression of *eyΔPD+ΔHD*; (lane *10*) misexpression of *eyΔPDPMHD*. Molecular weight marker is indicated at *right*. The Western blot below indicates the loading control with an anti-β-Tubulin antibody. (*G*) Schematic representation of the *ey* constructs. The *ey*-cDNA was used to generate the different deletions by use of the indicated restriction sites. The size of the new proteins is indicated in kilodaltons next to the schematically drawn cDNA.

pressed, we performed Western analysis using an α-EY antibody. We analyzed eye discs of ey^2 mutants that expressed full-length *ey* by *ey*-enhancer Gal4, and leg discs of third instar larvae that misexpressed the various EY proteins in the dpp^{blink} domain and compared them with endogenous EY levels. We found that the Gal4 system does not over express *ey* in our rescue experiment (Fig. 1F, lanes 1 and 2) and that our ectopically induced proteins were expressed with the expected molecular weight and at comparable levels (Fig. 1F, lanes 3–10). Therefore, we concluded that the phenotypes obtained were due to the misexpression of the different mutated *ey* constructs.

eyΔHD *is sufficient to trigger eye development in the absence of endogenous* ey

Regulatory feedback loops between *ey* and its downstream genes *so*, *dac*, and *eya* have been demonstrated in an ectopic situation (Chen et al. 1997; Pignoni et al. 1997). Therefore, we tested whether during *eyΔHD*-induced ectopic eye development, the endogenous intact *ey* gene might get activated and in turn be responsible for the ectopic eye formation. We tested for the presence of endogenous *ey* by RT–PCR analysis in leg imaginal discs in which *ey* and *eyΔHD* were misexpressed in a wild-type background. First, we used a set of primers able to detect both intact *ey* and *eyΔHD* (Fig. 2A) (primer 1 + 2; Fig. 2D). Full-length *ey* was detected only in *yw* eye discs (Fig. 2A, lane 1), and in leg discs in which it had been misexpressed (Fig. 2A, lane 4). It was absent in *yw* control wing (Fig. 2A, lane 2) and leg discs (Fig. 2A, lane 3), and in leg discs in which *eyΔHD* had been misexpressed (Fig. 2A, lane 6). To exclude template competition, we repeated the RT–PCR experiment using an additional set of primers able to prime only the intact *ey* and not the ΔHD transcript (Fig. 2B) (primer 1 + 3; Fig. 2D). Again, we failed to detect any full-length *ey* (Fig. 2B, lane 6) upon misexpression of *eyΔHD*. This experiment shows that *eyΔHD* is not able to induce full-length *ey*, suggesting that in an ectopic situation, the homeodomain is dispensable during the larval stages of eye development.

To test whether the HD would also be dispensable during the pupal stages of development, we ectopically

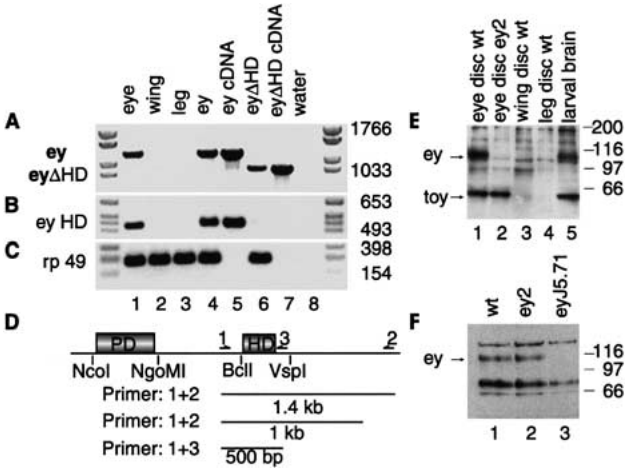

Figure 2. RT–PCR analysis to test for the presence of *ey HD* and molecular analysis of the *ey* mutants. (*A–C*) RT–PCR on leg discs of third instar larval stage in which *ey* and *eyΔHD* were misexpressed. (Lanes *1–3*) *yw* controls. (Lane *1*) Eye; (lane *2*) wing; (lane *3*) leg discs; (lane *4*) misexpression of full-length *ey*; (lane *5*) *ey* cDNA control; (lane *6*) misexpression of *eyΔHD*; (lane *7*) *eyΔHD* cDNA control; (lane *8*) water control. (*A*) Detection of full-length *ey*, primer 1 + 2 of *D*. (*B*) Detection of *ey* HD fragment, primer 1 + 3 of *D*. (*C*) rp49 control. In lane *6*, in which *eyΔHD* was misexpressed, we failed to detect full-length *ey* (*A*) and the HD fragment (*B*), whereas rp49 was expressed normally. (*D*) Schematic drawing of *ey* cDNA and the respectively used primers for the RT–PCR in *A* and *B*. (*E,F*) Molecular analysis of *ey* mutants. (*E*, lanes *1–5*) Western blot analysis of ey^2 mutants of third instar larval stage with an anti PD antibody; (lanes *1,2*) eye discs of wild-type and ey^2, respectively. EY is only detectable in wild-type in contrast to TOY. (Lanes *3,4*) Wing and leg discs control of wild type; (lane *5*) larval brain of wild type, both proteins are expressed. (*F*, lanes *1–3*): Western blot analysis of adult head with an anti EY antibody. (Lane 1) Wild type; (lane *2*) ey^2; (lane *3*) $ey^{J5.71}$. EY could be detected in wild type and ey^2 , but not in $ey^{J5.71}$.

misexpressed *eyΔHD* in ey^2 mutants. There, we expect no endogenous EY to be present in the tissue that gives rise to the eye, because the eye-specific enhancer of the gene is disrupted (Quiring et al. 1994). All *ey* mutants characterized so far show a partial to complete loss of the compound eye, although to a highly variable degree. As an additional control, we used the *eyeless* null mutant $ey^{J5.71}$. As revealed by in situ hybridization, *ey* is not expressed in the embryonic eye-anlagen and in the larval eye discs of ey^2 mutants, whereas *toy* expression remains unaffected (Quiring et al. 1994; Czerny et al. 1999). Western blot analysis on ey^2 eye discs confirmed that EY is not detectable, in contrast to TOY (Fig. 2E, lanes 1–5). Western blot analysis of adult heads revealed that EY is absent in $ey^{J5.71}$ mutants, but present in ey^2 mutants (Fig. 2F, lanes 1–3). To elucidate whether *ey* is expressed at all in the adult eye, we performed in situ hybridization on cryosections of adult heads. We found that *ey* expression in the adult head is restricted to the brain area (Fig. 3A–C). Therefore, the residual expression of EY in ey^2 adult heads is due to the expression of the gene in the brain, ey^2 being a mutation affecting only the eye-specific enhancer of the gene (Quiring et al. 1994). This result allowed us to analyze the role of the *ey* homeodomain during the late stages of eye development. Misexpression of *eyΔHD* in ey^2 induced ectopic eyes at the same efficiency as full-length *ey* (Fig. 3D,E). The same result was obtained in $ey^{J5.71}$ mutants (data not shown), showing that the HD of *ey* is not required during ectopic eye development.

Because *Drosophila* has two *Pax-6* genes, we wanted to exclude the possibility that *eyΔHD* would activate *toy* and that the TOY–HD would be responsible for the ectopic eye development. Although it has been demonstrated that in an ectopic situation *ey* does not activate *toy*, we performed in situ hybridization on leg discs of ey^2 mutants in which *eyΔHD* was misexpressed. However, we could not detect ectopically induced *toy* transcripts (Fig. 3F,G), ruling out the possibility that the TOY–HD may functionally replace EY–HD during ectopic eye development. Thus, these results show that *eyΔHD* induces ectopic eyes independently of endogenous *toy* and *ey*.

ey *target genes are induced via the paired domain*

Our data predict that *ey* downstream target genes required for eye development are activated by the paired domain. Therefore, we tested the expression of *so* as a

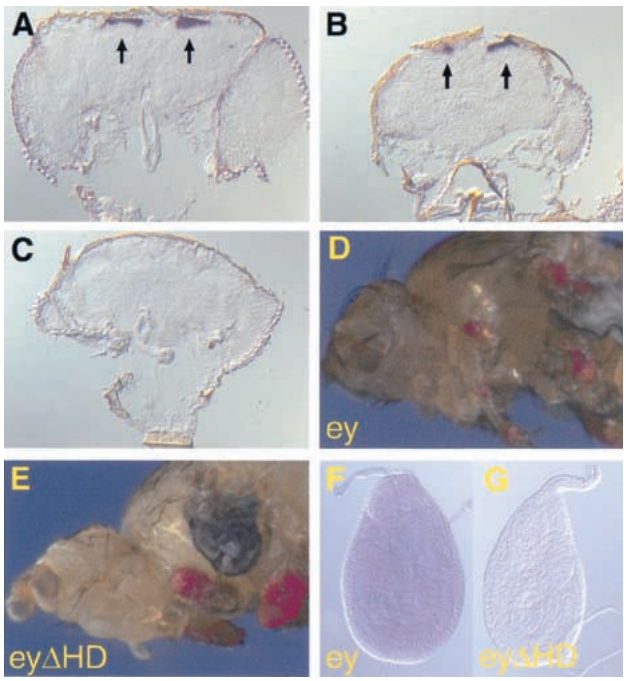

Figure 3. Expression analysis of *ey* and ectopic eyes in ey^2 mutants. (*A–C*) In situ hybridization on cryosections of adult heads with an anti-sense Dig *ey* probe. Expression was only detected in the brain (arrows), but not in the retina. (*A*) Wild type; (*B*) ey^2; (*C*) $ey^{J5.71}$. (*D,E*) Ectopic eyes induced by *ey* and *eyΔHD*, respectively with dpp^{blink}–Gal4 in an ey^2 mutant background. (*F,G*) In situ hybridization with an anti-sense Dig *toy* probe on third instar leg discs of ey^2 mutants in which *ey* and *eyΔHD* were misexpressed with dpp^{blink}–Gal4. In both cases, *toy* could not be detected.

direct target gene of *ey* (Niimi et al. 1999). The various *ey* constructs were misexpressed by *dpp*^{blink}–Gal4. Induction of *so* expression was detected by *lac-Z* staining of an enhancer trap line (Cheyette et al. 1994). *so* was ectopically activated by the full-length (Fig. 4B) as well as the EYΔHD (Fig. 4D) protein. It was not ectopically activated when the paired domain was missing (Fig. 4C), showing that the PD within the EY protein is sufficient to induce its direct target *so*.

dac is an indirect target gene of *ey* and misexpression of *dac* has been shown to be in part responsible for appendage truncation and to be able to induce ectopic eye development (Shen and Mardon 1997). We repeated the misexpression experiments done for *so* and used antibodies to detect both EY and DAC. Like *so*, DAC is only ectopically activated if the PD in EY is present (Fig. 4F–H), as expected for an essential gene in eye development. This suggests that in our case, the truncated appendages are not due to DAC misexpression, because it does not get ectopically activated by *ey*Δ*PD*.

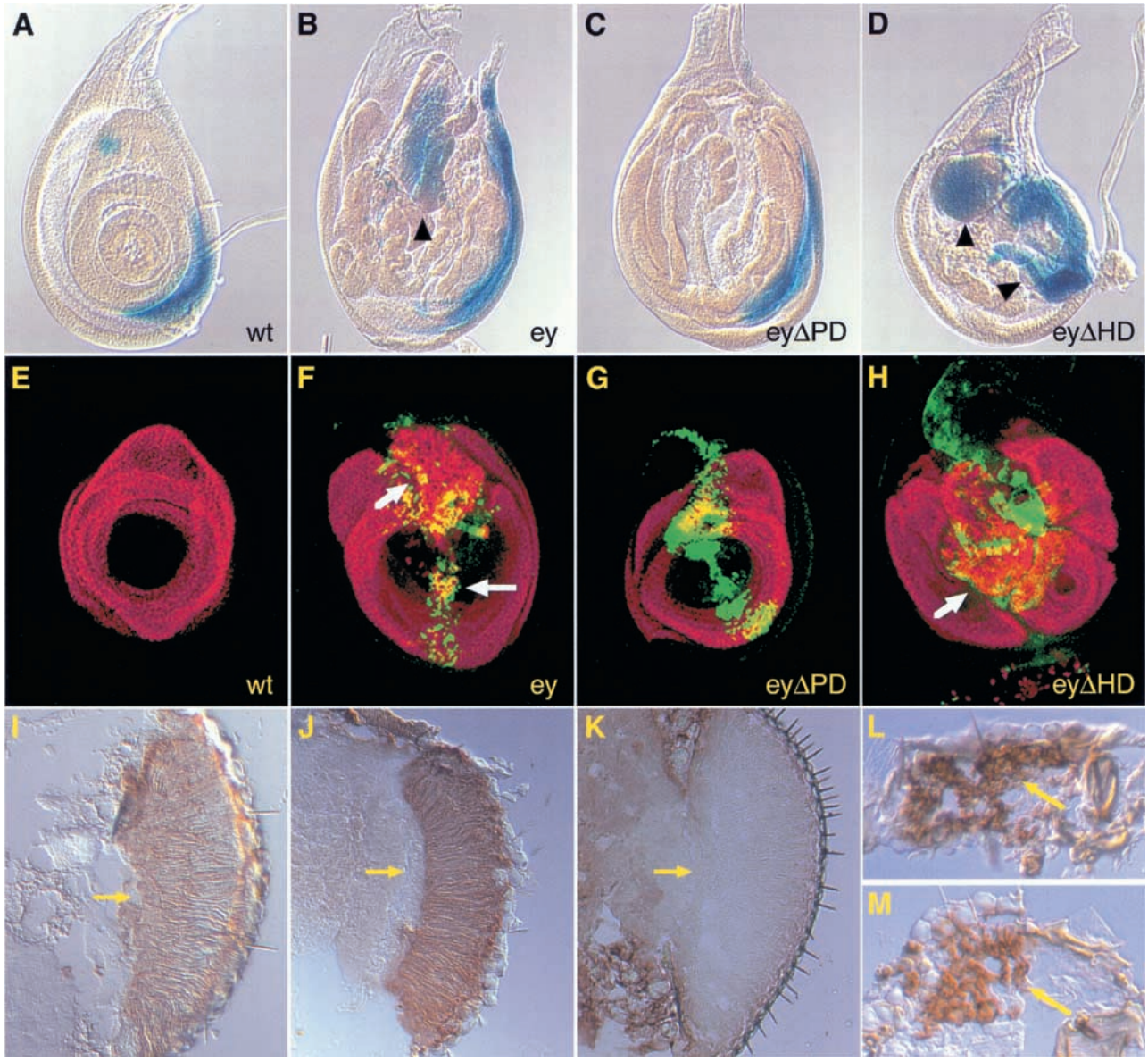

Figure 4. Activation of downstream genes of *ey* required during eye development. (*A–D*) Induction of *so* enhancer trap line by misexpression of the various *ey* constructs. (*E–H*) Confocal sections showing the induction of DAC by misexpression of the various *ey* constructs (DAC, red; EY, green). All panels show late third instar larval leg discs in which the various constructs were misexpressed by *dpp*^{blink}–Gal4. (*A*,*E*) Wild-type expression pattern; (*B*,*F*) misexpression of *ey* (arrowhead and arrow highlight regions of induction); (*C*,*G*) misexpression of *ey*Δ*PD* (*so* and DAC are both not induced); (*D*,*H*) misexpression of *ey*Δ*HD* leads, in both cases, to a stronger induction than with full-length *ey*; (*I–M*) Rhodopsin-1 expression in various mutants and in ectopic eyes. The expression of Rhodopsin was monitored with an α-Rhodopsin-1 antibody on cryosections; (*I*,*J*) Rhodopsin-1 expression in the retina in *ey*^{2} (*I*) and *ey*$^{J5.71}$ (*J*) mutants; (*K*) no staining in the *rhodopsin-1* mutant *ninaE*; (*L*,*M*) Rhodopsin-1 expression in ectopic eyes induced *ey* (*L*) and *ey*Δ*HD* (*M*) in an *ey*^{2} mutant. Arrows indicate the retina.

Next, we asked whether the ectopic eyes generated by *ey*Δ*HD* also express late marker genes of eye development. *rhodopsin-1* has been proposed to be directly regulated by the homeodomain of *ey* (Sheng et al. 1997). We therefore analyzed the presence of *rhodopsin-1* in ectopic eyes generated by *ey*Δ*HD* in ey^2 mutants, in which we showed that neither EY–HD nor TOY–HD are required for ectopic eye development. Immunostainings on cryosections by use of an α-Rhodopsin-1 antibody revealed that Rhodopsin-1 is expressed in the retina of ectopic eyes generated by *ey* and *ey*Δ*HD* in ey^2 mutants (Fig. 4L,M). Rhodopsin-1 expression was also detected in the eyes of both ey^2 and $ey^{J5.71}$ mutants, but not in the eyes of the *rhodopsin-1* mutant *ninaE* (Fig. 4I–K). This indicates that the expression of Rhodopsin-1 is independent of the homeodomain of *ey* and does not require the presence of EY in the adult eye. It strengthens the hypothesis that *rhodopsin-1* is likely to be activated by another paired type HD containing a gene other than *ey*.

The ey *homeodomain is able to repress* distal-less

Because the homeodomain of the Pax-6 proteins is highly conserved, we wondered about its function during development. It has been shown previously that *ey* is able to repress *Dll* in an ectopic situation (Kurata et al. 2000). So far, our experiments have suggested that the HD may confer gene repression. Therefore, we tested whether *Dll* repression by *ey* is mediated by the homeodomain. We ectopically expressed *ey*, *ey*Δ*PD*, *ey*Δ*HD*, and *ey*Δ*PDPMHD* on all appendages with dpp^{blink}–Gal4 and monitored EY and DLL expression by antibody detection. Ectopic expression of *ey* (data not shown) and *ey*Δ*PD* (Fig. 5A) were able to repress *Dll* expression in the respective areas of overlap, in contrast to ectopic expression of *ey*Δ*HD* (Fig. 5B) and *ey*Δ*PDPMHD* (data not shown). This result shows that the HD of *ey* mediates *Dll* repression by DNA binding. Therefore, we conclude that the truncated appendages are due in part to the repression of *Dll*.

Discussion

Pax-6 is one of the most important transcription factors controlling eye development. It has two evolutionarily conserved DNA-binding domains and, therefore, we considered it a priori unlikely that one of the two domains would be sufficient to induce all genes required for eye development. However, the rescue of the ey^2 mutant by *ey*Δ*HD* led us to the conclusion that the HD of *ey* could be dispensable for eye formation. To corroborate this finding, we switched to ectopic expression experiments. We showed that *ey*Δ*HD* induces ectopic eyes in wild-type and in an *ey* mutant background. All target genes tested are only activated in the presence of the PD. In addition, misexpression of *ey*Δ*HD* was not able to induce endogenous *ey* in wild-type or endogenous *toy* in an *ey* mutant background. To corroborate our hypothesis that the HD of *ey* was dispensable, we further characterized the ey^2 mutant, showing that endogenous full-length *ey* is not expressed in cells that give rise to the eye. Therefore, together with the rescue experiment, we conclude that the expression of a PD containing EY protein is sufficient to induce eye development and that the HD of *ey* is dispensable for target gene activation and EY-mediated eye morphogenesis. Sequence analysis of *Pax-6* mutations in patients with Aniridia or Peter's anomaly showed that point mutations of *Pax-6* affecting eye development are mostly located in the PD (Glaser et al. 1992; Hanson et al. 1994; Martha et al. 1994). This raises the question of the function of the homeodomain being so highly conserved among the *Pax-6* genes. Obviously, the HD might have an essential function in the development of the brain and the ventral nerve cord. We were able to show in an ectopic situation that the repression of *Dll* (at the RNA level) is mediated by EY–HD. This may explain why we observed truncated appendages when *ey*Δ*PD* was misexpressed, and it suggests that the EY–HD can confer repression during development. The repression might be achieved either directly or by activation of a repressor. We do not rule out the possibility that the HD might be able to activate gene expression in organs other than the eye. In vertebrates, different splice forms of *Pax-6* have been characterized, some of them lacking the paired domain (Carriere et al. 1993). In *Caenorhabditis elegans*, one splice form without the paired domain was found to be important for the development of the peripheral nervous system (Zhang and Emmons 1995). Therefore, the homeodomain of *Pax-6* might play a major role during nervous system develop-

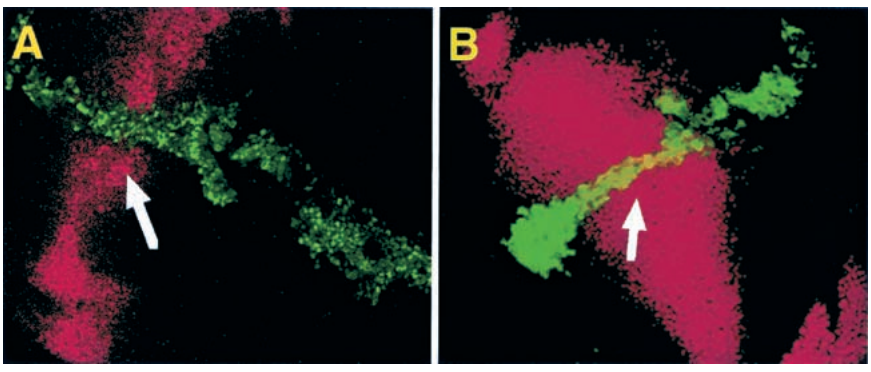

Figure 5. DLL repression by ectopic expression of *ey*Δ*PD*. (*A*,*B*) Confocal sections of antibody staining on wing discs in which the different *ey* constructs were misexpressed with dpp^{blink}–Gal4 (DLL, red; EY, green). (*A*) Misexpression of *ey*Δ*PD* leads to a repression of DLL in the respective areas of overlap (arrow: yellow staining), whereas misexpression of *ey*Δ*HD* does not (*B*).

ment in *Drosophila* also. Thus, eye development could provide the selective pressure directed toward the conservation of the PD, whereas the nervous system put constrains on the HD. This would connect one protein to the development of two different organ systems, both of which are required for vision.

Our results show that each domain of the *Drosophila Pax-6* gene *ey* can function separately, and that simultaneous binding of both domains, on the same regulatory element, is not required. Similar deletion analysis have also been done for other homeodomain containing proteins (Fitzpatrick et al. 1992; Ananthan et al. 1993; Bertuccioli et al. 1996). In contrast to this analysis, in which the lack of the HD was not able to fully rescue endogenous protein function, in the case of *ey*, the HD is dispensable to rescue an *ey* mutant eye phenotype. Thus, these results might reflect different ways of actions for the different homeodomain-containing proteins. Therefore, we would like to propose a model for *ey* in which the same transcription factor can act as a repressor and an activator via its two different DNA-binding domains in the context of different organs. The question of how the protein exerts its different functions might be explained by recruiting different cofactors as has been shown recently for *Pax-5* (Eberhard et al. 2000). We are currently searching for such interacting factors.

Materials and methods

Molecular methods

Western blot and cloning procedure were done according to the standard protocol described in Sambrook et al. (1989). The embryonic *ey* cDNA (E10) was deleted between S_{19}-GT-A_{181} for *ey*Δ*PD* by use of the *Nco*I–*Ngo*MI sites. The amino acids GT were inserted in the linker to connect the two restriction sites. The *ey*Δ*HD* was deleted between L_{405}–T_{523} using the sites *Bcl*I–*Vsp*I and also by connecting them with a linker. The double deletion contains the same deletion regions. The point mutations S_{50} to A_{50} (TCA to GCA); N_{51} to A_{51} (ACC to GCC) were done by standard PCR experiments. Each construct generated was confirmed by sequencing. Western blot experiments were done with a rabbit α-EY antibody at a dilution of 1:200, in which the antibody was preabsorbed with larval tissue or with a rabbit α-PD of squid Pax-6 antibody at a dilution of 1:500. Each lane was loaded with extracts from 10 discs, 5 adult heads, or 5 larval brains, respectively. Extracts of all Western were boiled for 6 min. Correct transfer was tested by ponceau red staining. Additionally, equal loading was tested with an α-β-Tubulin antibody at a dilution of 1:10. The secondary antibody for detection of the signal was used at a dilution of 1:2000 (HRP-coupled swine α-rabbit antibody from DAKO A/S), and the signal was revealed by use of a chemoluminescence kit (Amersham). The RT–PCR was performed as follows: total RNA was extracted from discs (with Trizol; Life Technologies) and then reverse transcribed (80 leg discs per 800 μL of Trizol). The single-stranded cDNA was amplified by SMART PCR cDNA Synthesis Kit (Clontech). The PCR was further performed as follows: 28 cycles of, 20 sec at 95°C, 20 sec at 57°C, 13 sec at 72°C for rp49; 20 sec at 95°C, 30 sec at 55°C, 1 min 40 sec at 72°C for primer 1 + 2; 20 sec at 95°C, 20 sec at 58°C, 13 sec at 72°C for primer 1 + 3 by use of the Taq polymerase from Pharmacia. Primer sequences. Primer 1, 5′-AGTCCGATGAAACGGG-3′; Primer 2, 5′-CCTAGACCCACGGTGAG-3′; Primer 3, 5′-GGGACCCCCAGCTGATCCGG-3′; rp49 sens, 5′-CGAACAAGCGCACCCGC-3′; rp49 antisens, 5′-CGCAGGCGACCGTTGGGG-3′.

Histology

In situ hybridization on sections was performed as described in Janssens and Gehring (1999). In situ hybridization on discs, β-Galactosidase staining and antibody stainings on cryosections were performed as described in Ashburner (1989). Antibody staining on discs were performed as described in Halder et al. (1998). The concentration of the antibodies was as follows: rat α-EY 1:500 (Haldes et al. 1998); mAb α-DLL 1:20 (Wu and Cohen 1999); mAb α-DAC 1:100 (Mardón et al. 1994).

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