Evidence for a direct functional antagonism of the selector genes *proboscipedia* and *eyeless* in *Drosophila* head development

Corinne Benassayag¹, Serge Plaza², Patrick Callaerts³, Jason Clements³, Yves Romeo⁴, Walter J. Gehring² and David L. Cribbs¹,*

¹Centre de Biologie du Développement-CNRS and Institut d’Exploration Fonctionnelle du Génome, 118 route de Narbonne, Bâtiment 4R3, F-31062 Toulouse Cedex 04, France
²Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland
³Department of Biology and Biochemistry, University of Houston, 369 Science and Research Bldg. 2, Houston TX 77204-5001, USA
⁴IBCG-CNRS, Université Paul Sabatier, 118 route de Narbonne, F-31062 Toulouse Cedex, France
*Author for correspondence (e-mail: cribbs@cict.fr)

Accepted 15 October 2002

SUMMARY

Diversification of *Drosophila* segmental and cellular identities both require the combinatorial function of homeodomain-containing transcription factors. Ectopic expression of the mouthparts selector *proboscipedia* (*pb*) directs a homeotic antenna-to-maxillary palp transformation. It also induces a dosage-sensitive eye loss that we used to screen for dominant Enhancer mutations. Four such Enhancer mutations were alleles of the *eyeless* (*ey*) gene that encode truncated EY proteins. Apart from eye loss, these new *eyeless* alleles lead to defects in the adult olfactory appendages: the maxillary palps and antennae. In support of these observations, both *ey* and *pb* are expressed in cell subsets of the prepupal maxillary primordium of the antennal imaginal disc, beginning early in pupal development. Transient co-expression is detected early after this onset, but is apparently resolved to yield exclusive groups of cells expressing either PB or EY proteins. A combination of in vivo and in vitro approaches indicates that PB suppresses EY transactivation activity via protein-protein contacts of the PB homeodomain and EY Paired domain. The direct functional antagonism between PB and EY proteins suggests a novel crosstalk mechanism integrating known selector functions in *Drosophila* head morphogenesis.

Key words: Hox, Pax6, *proboscipedia*, Maxillary palps, Protein-protein, Differentiation

INTRODUCTION

Homeobox-containing genes encode transcriptional regulators involved in many developmental processes including specification of unpatterned tissues. These include the highly conserved Hox gene class (Gehring et al., 1994; Manak and Scott, 1994). The Hox genes that specify the segments reiterated along the antero-posterior axis of diverse animals (Lawrence and Morata, 1994; McGinnis and Krumlauf, 1992) are viewed as selectors that control morphogenetic processes through the coordinate regulation of batteries of downstream target genes (Garcia-Bellido, 1975; Garcia-Bellido, 1977). Indeed, the *Drosophila* Hox genes encode homeodomain transcription factors whose patterned and overlapping expression leads to differential activation of target genes in individual segments (Botas, 1993; Graba et al., 1997; Morata, 1993).

Despite the identification of several target genes, important gaps remain in our understanding of the pathway from patterned Hox gene expression to the development of diverse segmental morphologies (Mann, 1995). For example, the poor DNA sequence specificity exhibited by Hox transcription factors in vitro contrasts with their highly specific effects in vivo. One source of enhanced Hox specificity derives from their joint action with cofactors such as *extradenticle* and its mammalian PBX family counterparts that enhance sequence selectivity and alter in vivo specificity (Chan et al., 1994; Passner et al., 1999; Pinsonneault et al., 1997; Rauskolb et al., 1993; van Dijk and Murre, 1994). An important role for stoichiometric combinations of homeotic proteins with functional partners is supported by observations suggesting that Hox protein levels can be crucial for their developmental effects (Cribbs et al., 1995; Greer et al., 2000; Roch and Akam, 2000; Smolik-Utlaut, 1990). A combinatorial coding model predicts that different Hox genes and their partners acting together can specify novel cell or segment identities, deploying developmental pathways that are different to those specified by one gene product acting alone.

The *Drosophila* *proboscipedia* (*pb*) gene codes for a conserved homeodomain protein required for the correct development of the adult fly mouthparts (Cribbs et al., 1992) where two distinct appendages, the labial and maxillary palps, show a dose-sensitive requirement for *pb* gene function (Kaufman, 1978; Pultz et al., 1988). These appendages derive
from the labial and antennal imaginal discs, respectively. Labial palp differentiation requires the combinatorial action of two Hox genes, pb and Scr (Percival-Smith et al., 1997). In contrast, pb appears to be necessary and sufficient for the differentiation of maxillary palps, and ectopic PB expression induces a dose-sensitive transformation of antennae to maxillary palps (Cribbs et al., 1995). Another consequence of ectopic PB expression is a striking dose-sensitive eye loss. The observation that PB protein incapable of DNA binding can induce this eye loss suggested that context-specific protein-protein interactions are central to this defect (Benassayag et al., 1997). We attempted to identify specific protein partners of PB by isolating dominant Enhancer mutations for the PB-protein interactions are central to this defect (Benassayag et al., 1997). We attempted to identify specific protein partners of PB by isolating dominant Enhancer mutations for the PB-dependent eye loss. Four such mutations are new alleles of the eyeless gene encoding a D. melanogaster Pax6 homolog.

The Pax gene class encodes proteins with a DNA-binding Paired domain that may be coupled with a class-specific homeodomain (Bopp et al., 1986; Frigerio et al., 1986; Gehring et al., 1994; Manak and Scott, 1994; Strachan and Read, 1994; Stuart et al., 1994). Reduced Pax6 function in mammals leads to the defective eye structures typical of the dominant congenital disorder Aniridia in humans (Ton et al., 1991), or Small eye (Sey) in mice (Hill et al., 1991). Further roles for Pax6 in mouse development are revealed in homozygous mutants lacking nasal structures or showing brain defects (Glaser et al., 1994; Hogan et al., 1988; Schmahl et al., 1993). The spectrum of abnormalities seen in Sey mice thus indicates that Pax6 is required in eye, nose and brain development. Loss-of-function eyeless mutations in Drosophila lead to reduced or defective eye structures, whereas targeted expression of EY protein induces ectopic eyes (Halder et al., 1995; Quiring et al., 1994). Recent work has also revealed that apart from this central role in forming the Drosophila eye, eyeless is also required for brain development (Callaerts et al., 2001; Kurusu et al., 2000; Noveen et al., 2000).

To better understand the roles of the eye selector gene eyeless (ey) and the basis of its functional relationship with proboscipedia (pb) in normal development, we analyzed the molecular structures and the mutant phenotypes of the four newly identified eyeless mutations. These alleles code for truncated EY proteins and are expected to affect all aspects of ey function. The mutant phenotypes indicate that eyeless is required for adult maxillary palp differentiation. Furthermore, both EY and PB accumulate in the maxillary primordium beginning early in pupal development. We show that PB-induced eye loss occurs via post-translational repression of EY-mediated target gene transcription. Furthermore, we detect direct protein-protein binding in vitro involving the PB homeodomain and the EY Paired domain. Direct protein-protein binding in a modified yeast two-hybrid analysis is likewise sufficient to diminish or abolish EY transcriptional activation from a target promoter, sine oculis. These genetic and molecular results support a direct functional antagonism between the Hox gene pb and the Pax gene ey in maxillary palp development and provide evidence for a functional link integrating eyeless with the position-specific Hox gene network.

MATERIALS AND METHODS

Genetic screen
The X-linked HSPB:2.5 element (and HSPBγ derived from it) is expressed normally in the labium and maxillary palps, and ectopically in tissues including the antennae, legs, wings and eyes. PBγ expression in the eyes provokes a dosage-sensitive eye loss without an antenna-to-maxillary palp transformation (Benassayag et al., 1997). Phenotypically normal males carrying one HSPBγ element were mutagenized with 35 mM EMS or 7 mM DEB as described (Lewis and Bacher, 1968), then crossed en masse with homozygous HSPBγ females (eyes slightly reduced). Progeny were screened for dominant enhancers by selecting females (2×HSPBγ) with strongly reduced eyes. Candidate females (presumed genotype: 2×HSPBγ; Ey+/+) were re-crossed with HSPBγ males. Candidates yielding a significant fraction of female progeny with strong eye reduction were retained.

Fly strains
All stocks and crosses were maintained at 25°C on standard yeast-agar-cornmeal medium. Specific fly lines or alleles used were: so32AcZ (Cheyette et al., 1994), dpp-Gal4 (Staehling-Hampton et al., 1994), UAS-ey (Halder et al., 1995), ey-GAL4 (Halder et al., 1998), UAS-pb 49.1 (Apbin and Kaufman, 1997), HSPBγ and wt (Benassayag et al., 1997; Cribbs et al., 1995), pbGal4 UASlacZ/CyO (constructed by L. Joulia). The eya and so alleles eyaA1, eya2, so1 and so31 were from L. Pignoni and L. Zipursky; eyaM12 and eya1 were from Henry Sun; eya2 and eya4 were as described (Quiring et al., 1994); and Df(4)Ba was provided by E. Frei and M. Noll (Kronhann et al., 2002). Specific genotypes generated for this publication were: UAS-pb; UAS-ey and so32Ac/CyO; dpp-GAL4/TM6B, Hu Tb.

Phenotypic analysis
Detailed phenotypic analysis was by light microscopy (Zeiss Axiophot) after mounting dissected samples in Hoyer’s medium, or by scanning electron microscopy (SEM). For SEM analysis, adult flies were stored in 95% ethanol until dissection of heads. Preparation and mounting were as described (Benassayag et al., 1997).

Molecular characterization of eyeless mutations
The molecular characterization of eyeless mutations involved PCR amplification of all the exons from intronic primers, and sequencing of the cloned products. For a detailed description, see Callaerts et al. (Callaerts et al., 2001).

Histology
In situ hybridizations on larvae or on prepupae were performed using digoxigenin-labeled probes according to Sturtevant and Bier (as described at http://www-bier.ucsd.edu/imagdisc.html). Antibody stainings were performed according to Halder et al. (Halder et al., 1998) at the following dilutions: rabbit α ey (1/500), rabbit α pbE9 (1/175), mouse α eya (1/50), mouse α dac (1/200) and mouse α β-gal (1/500).

Pull down experiments
GST fusion proteins were produced and purified according to the manufacturer’s specifications (Pharmacia). Quantification of the GST fusion protein was monitored by Coomassie staining to ensure equivalent amounts of GST fusion proteins in the assay. 35S-labeled proteins were synthesized using a coupled in vitro transcription translation kit (TNT Promega). The binding conditions were modified from Chen et al. (Chen et al., 1997): Glutathione resin-bound GST proteins were pelleted and washed three times with 500 µl of binding buffer (20 mM Hepes, 1 mM DTT, 0.1 mM EDTA, 2.5 mM MgCl2, 150 mM NaCl, 0.05% (v/v) NP40, 10% (v/v) glycerol, 100 µg/ml BSA). Labeled proteins were incubated in 0.4 ml binding buffer with approximately 10 µg of bound GST fusion proteins for 2 hours at 4°C. The resin was washed five times with 1 ml of binding buffer, then bound 35S proteins were eluted by boiling for 3 minutes in 25 µl of loading buffer, fractionated by SDS-PAGE and visualized by autoradiography (from 15 to 36 hours). We measured bound labeled
proteins eluted compared with the input by quantitation on a Phospho-imagery.

‘One and a half hybrid’ experiments

One-hybrid experiments were performed using the MATCHMAKER one hybrid system (Clontech) according to the manufacturer’s specifications. The Ncol-Smal fragment of ey cDNA was subcloned in frame with the Gal4DB in the pAS vector. The PB fragment (amino acids 128-305) was fused to the Gal4 transactivation domain in the pACT vector, pAS and pACT are from Durfee et al. (Durfee et al., 1993).

Plasmid constructions

Plasmids used for GST fusion protein production: pEn24-4 (Bourbon et al., 1995) leads to GST-EN fusion protein (EN-374/552) containing GST-EN protein, ends generated by a partial HincII digestion and the plasmid circularized. This construct expresses a truncated protein containing the first 245 amino acids, including the paired Domain (27 kDa). To obtain the EYAPD protein, ends generated by a partial HindIII digestion (position 773) and a complete XbaI digestion of pBSN-eyesless were blunt ended by filling in and the plasmid circularized. This construct expresses a truncated protein containing the 245 first amino acids, including the Paired Domain (27 kDa). To obtain the EYAPD protein, pBSN eyesless was digested with NcoI (made blunt by filling in) and BamH1 (made blunt by exonuclease treatment) and recircularized. This construct generates a protein of 681 amino acids (75 kDa) deleted for 157 amino acids (between 18-174). To generate the EYAPBAM4D protein, pBSN eyesless was digested with NcoI (making blunt by filling in) and NruI and recircularized. It leads to a protein of 387 amino acids (43 kDa) deleted for 451 amino acids (18-468).

Construction of a pb-GAL4 driver: GAL4 coding sequences were placed with pb control elements within 6.2 kilobase pair (kb) Sall fragment harboring pb exons 1 and 2, intron 1, and regulatory sequences in intron 2 (Cribbs et al., 1995; Kapoun and Kaufman, 1995). The circular form of the 6.2 kb fragment subcloned in the plasmid pGEM2 was used for amplification by PCR. One primer, situated in exon 1, was oriented upstream and contained restriction sites for BamH1 and NotI; the second primer, situated at the 3’ end of exon 2, was oriented downstream and contained restriction sites for NotI and Xhol. The amplified product of 6 kb was digested with NotI, ligated and transformed into E. coli. This plasmid is deleted for the 3’ end of exon 1, intron 1 and exon 2. GAL4 coding sequences removed from pGATB (Brand and Perrimon, 1993) by digestion with BamH1 and NotI were inserted into the pb plasmid cut with the same enzymes. Finally, an EcoRI/Sall fragment containing pb exon 1 as transcriptional fusion with GAL4 coding sequences, plus known intron 2 regulatory elements, was inserted into CaSpeR3 cut with EcoRI and Xhol. This pb-GAL4 plasmid was injected into flies together with a plasmid supplying transposase, and transgenic lines established by standard methods. The properties of these lines will be described in detail elsewhere (L. Jouia, H. M. Bourbon and D. L. C., unpublished).

RESULTS

Isolation of new eyeless mutations by functional interaction with proboscipedia

The pb homeotic function is required for correct development of the labial and maxillary palps composing the adult mouthparts. Its selector function in head development is revealed by ectopic expression of PB protein from a pb mini-gene, HSPB, that induces a reliable dose-dependent transformation of antennae to maxillary palps (Ant→Mx) (Cribbs et al., 1995). Ectopic PB also induces a dose-sensitive eye loss. We previously screened for PB mutations of sites essential for homeotic transformation. A mutant transgene, HSPB (Arg5→His5 in the homeodomain N terminus) was identified that induces dose-sensitive eye loss even though it no longer directs the Ant→Mx transformation (Fig. 1A-C). This eye-loss does not require PB DNA-binding ability, suggesting that specific proteins required for eye development are antagonized by direct contact with PB (Benassayag et al., 1997).

In a search for such functional protein partners of pb also involved in head morphogenesis, we screened for dominant mutations enhancing the observed eye defect. We employed the mutant transgene HSPB, because PB protein retains the capacity of wild-type PB to induce eye loss but is less toxic for flies. Males hemizygous for the X-linked HSPB element are normal (Fig. 1A), and homozygous females (two transgene copies) are nearly so (Fig. 1B). In contrast, complete eye loss occurs in females carrying four copies (Fig. 1C). HSPB males were treated with the chemical mutagens EMS or DEB and crossed with homozygous females (two copies of HSPB). We then selected candidate female progeny (two copies) with a strong eye loss resembling four copies (Fig. 1C). Among approximately 80,000 F1 HSPB/HSPB females screened, approximately 20 new Enhancer mutations were retained. These mutations represent at least five loci, distributed on all four chromosomes. Three of the Enhancer mutations were new alleles of Notch (Boube et al., 1998). Four En(sy) alleles mapped to the fourth chromosome and were identified as alleles of the eye development gene eyeless based on complementation analyses with three previously characterized eyeless alleles, ey2, ey4 and Df(4)BA (Kronhamm et al., 2002; Quiring et al., 1994).

Specific genetic antagonism between pb and ey in PB-induced eye loss

The strength of the new ey alleles (eyJD, eyEH, ey11 and eyDI4a) was compared with ey2 and Df(4)BA for its interaction with HSPB (Fig. 1D). On crossing HSPB/Y, ey*/+ males with HSPB/HSPB females, half the female progeny should be HSPB/HSPB; ey*/+ and eye loss could attain at most 50%. In this test, sensitized females heterozygous for the eye-null allele ey2 showed 12% eye loss; similarly, heterozygotes for the recently described deletion Df(4)BA (Kronhamm et al., 2002) showed 13% eye loss. In contrast, most of the new alleles showed a more marked interaction, eyJD, eyDI4a and eyEH interacted similarly with HSPB (Fig. 1D; eye loss between 25 and 29%), although ey11 was markedly weaker (6%). These results suggest that the eyJD, eyEH and eyDI4a alleles are stronger than ey11 in general agreement with the results of complementation tests employing ey2, ey4 and Df(4)BA (Callaerts et al., 2001) (data not shown). Thus three of the new ey alleles interact more strongly with HSPB than the described null alleles. To examine the specificity of the observed eye loss, we tested for genetic interactions with sine oculis (so), eyes absent (eya) and eye gone (eyg) (Bonini et al.,
prominent players in the eye differentiation cascade (Fig. 1D) (Halder et al., 1998; Niimi et al., 1999; Zimmerman et al., 2000). None showed a marked interaction with PB<sup>Y</sup> (Fig. 1D). The remaining unidentified Enhancer loci from our screen complement mutants of all three genes, and thus do not appear to correspond to characterized members of the eye hierarchy. In conclusion, these results support a specific genetic antagonism between pb and eyeless/Pax6 in programmed eye loss.

### The new eyeless alleles encode truncated proteins

The eyeless locus encodes a transcription factor with two highly conserved DNA-binding motifs, a paired domain (PD) and a homeodomain (HD) (Callaerts et al., 1997). Two of the best characterized eyeless alleles, ey<sup>2</sup> and ey<sup>R</sup>, result from insertion of mobile elements within eye-specific regulatory sequences (Quiring et al., 1994). In contrast, the four new eyeless alleles isolated by their interaction with PB all affect the EY protein itself, based on the molecular lesions identified by PCR amplification and sequencing of genomic exons (Callaerts et al., 2001) (Fig. 1E). Sequence analysis revealed that ey<sup>JD</sup> (EMS-induced) harbors a point mutation and encodes a protein truncated in the homeodomain after the first helix. The DEB-induced ey<sup>D1Da</sup> removes the C-terminal 156 amino acids (Fig. 1E), whereas for a second DEB-induced allele, ey<sup>EH</sup>, a 6 bp sequence in exon 4 (positions 423-428 in exon 4) is replaced by a single T nucleotide (Fig. 1F).

The resulting frameshift should yield a truncated protein of 115 amino acids deleted at the C-terminal end of the paired domain (Fig. 1E). ey<sup>11</sup> allele harbors an EMS-induced point mutation in the splice acceptor site of intron 8 (Fig. 1F). Translation of unspliced mRNA retaining intron 8 is expected to terminate at a stop codon immediately after the exon 8, resulting in a protein truncated by 199 amino acid (Fig. 1E). This molecular characterization thus confirmed the identity of these Enhancer mutations as ey alleles that should affect the EY protein itself. In the case of ey<sup>JD</sup> and ey<sup>D1Da</sup> this point has been validated (Callaerts et al., 2001).

### eyeless function is required for adult maxillary and antennal development

To better understand the relationship between PB and EY in normal development, we examined the phenotypic effects of ey mutations in the sensitized HSPB<sup>Y</sup> genetic context. Two copies of the HSPB<sup>Y</sup> transgene (the sensitizing condition) showed no marked effect (Fig. 1B). In contrast, pharate adult females with 2X HSPB<sup>Y</sup> and homozygous for ey<sup>JD</sup> showed strong maxillary palp and antennal defects (Fig. 2B,C). In some cases, the maxillary palp, whose identity is indicated by the distinctive distal bristles, remains adjoined to the antennal appendage (Fig. 2C, arrowheads). However, differentiation of the proboscis (which likewise depends on pb function) is not affected (Fig. 2B, 'lb'). Thus in the sensitized context, ey mutations can provoke strong defects of the maxillary and antennal development.
antennal appendages. The phenotype of Fig. 2B in which the appendages are poorly resolved, suggests that eye\textsuperscript{+} may participate in partitioning imaginal disc cells into antenna and maxillary palp during morphogenesis.

To confirm a role of eyeless in this process, we removed HSPB\textsuperscript{sy} from the genetic background to examine the effects of the new eyeless mutations alone. All four eye alleles appear recessive in a non-sensitized background as shown for eye\textsuperscript{JD} (Fig. 2A,D), and can be interpreted as loss-of-function mutations in accord with their molecular lesions. All give homozygous escapers with visible defects, allowing us to compose an allelic series, from weakest to strongest: eye\textsuperscript{1+} > eye\textsuperscript{D1Da} > eye\textsuperscript{EH} > eye\textsuperscript{JD}. Analysis of the phenotypes of hemizygotes with Df(4)BA led to the same conclusion.

eye\textsuperscript{JD} homozygotes display eye reduction or loss (Fig. 2G), low viability and strong brain defects associated with abnormal behavior (Callaerts et al., 2001). Furthermore, a minority of surviving eye\textsuperscript{JD} homozygotes (10-20%, after outcrossing) show alterations in the size and/or shape of maxillary palps and antennae (Fig. 2G-I). The altered maxillary palps of eye\textsuperscript{JD} homozygotes still harbor the two characteristic sensilla trichodea (Fig. 2H, arrowheads), suggesting that maxillary identity per se is not affected. Reduced, malformed maxillary palps are often accompanied by enlarged, misshapen antennae (compare Fig. 2E and 2H; 2F and 2I). Similar although weaker defects were likewise detected for eye\textsuperscript{EH} homozygotes, as well as for certain trans-heterozygous combinations with other Enhancer alleles in the sensitized background (not shown). The reciprocal effect of eye\textsuperscript{JD} on appendages that derive from the same antennal imaginal disc constitutes evidence for a potential role for eye in apportioning the maxillary portion of this disc. The defects observed in eye\textsuperscript{JD} homozygotes appeared stronger than in the hemizygous combination, eye\textsuperscript{JD}/Df(4)BA. Thus, the truncated protein may have a limited antimorphic character not detected in the presence of wild-type protein.

**EY and PB proteins are both expressed in the pupal maxillary primordium**

Although mutant phenotypes implicated both pb and now eye in maxillary development, no gene expression had been detected in the maxillary portion of the antennal disc in the third instar larvae. We analyzed PB and EY expression later, during the prepupal stage when maxillary and antennal structures evaginate from the eye-antenna imaginal disc (Fig. 3A). Using a rabbit anti-PB serum directed against the C-terminal region (anti-E9), PB accumulation was detected in the central part of the maxillary primordium beginning approximately eight hours after puparium formation, during evagination of the antennal and maxillary appendages from the composite disc (Fig. 3B). EY protein as visualized by a rabbit anti-EY serum accumulates in the same primordium and, within discrimination, at the same time (Fig. 3C). This expression appeared to be limited to the borders of the primordium (Fig. 3C, higher magnification), rather than the center as for PB (Fig. 3B, higher magnification). Results of tests for co-expression of the two proteins were mitigated: in situ hybridization or immunostaining experiments were inconclusive, whereas available antibodies that gave acceptable signals in this tissue were both rabbit polyclonal antisera. To address whether endogenous pb and eye

![Fig. 2.](image_url)
patterns in the maxillary palps may overlap, we utilized a *pb-GAL4* mini-gene based on previous descriptions of the *pb*-promoter region (Kapoun and Kaufman, 1995). Using a *pb-GAL4* driver insertion to direct β-galactosidase expression (*pb-GAL4*→*UAS-lacZ*), we examined the patterns of *lacZ* expression in the antennal disc (see Kapoun and Kaufman, 1995). This expression is not visible in E.

To further characterize the molecular basis of the functional relationship between the two genes, the epistatic relations of *pb* to *ey* were tested. *Eyesless* mRNA accumulates normally in *pb*− embryos, third instar larvae or pre-pupae, whereas PB protein accumulated normally in *ey* mutants (data not shown). These observations argued against a transcriptional cross-regulation effect by the nuclear EY and PB transcription factors.

The mechanism of PB-induced eye loss was examined employing UAS/GAL4-mediated expression. The eye loss is temporally specific, because drivers expressed early during eye differentiation (*ey-GAL4, dpp-GAL4*) led to substantial or full eye loss (Fig. 4A; compare parts 1 and 6), whereas the later-acting GMR-GAL4 driver had little effect (not shown). We next examined the effects of PB on the expression of several identified genes acting early in the eye cascade (see Fig. 4A). Despite a drastic reduction of the L3 eye disc, early PB eye-expression (*dpp-GAL4*→*UAS-PB*) does not eliminate *ey* mRNA accumulation there (Fig. 4A, part 7). In contrast, expression of the *ey* target genes *so, eya* and *dac* was strongly reduced or abolished (Fig. 4A, parts 8,9,10, respectively). One possible explanation is that PB represses the same ensemble of targets activated by EY. If so, removing *pb* activity in labial discs could lead to expression of these eye genes there. Indeed, novel *dac* expression is detected in the labial discs of *pb*− larvae in accord with the appearance of ectopic legs (Abzhanov et al., 2001). In contrast, no corresponding expression was detected for *eya* or *so* in mutant labial discs (compared with endogenous expression in the eye). These results thus raise an alternative possibility, namely that PB simultaneously suppresses expression of all three EY targets by antagonizing EY activity. This could occur via a post-transcriptional mechanism, acting at the level of EY protein (model, Fig. 4B).

To test whether PB represses EY transcription factor activity, the GAL4 system was used to direct expression of these proteins, singly or together, in several imaginal discs under a common driver (*dpp-GAL4*). EY activity was monitored by formation of adult eyes, and by the expression of the known target gene *sine oculis* (*so*) (employing an enhancer trap line that fully recapitulates normal *so* expression in the eye imaginal disc (Cheyette et al., 1994). Targeted PB expression induces eye loss, abnormalities in legs, antennae and wings (see Fig. 4C, part 1) (Aplin and Kaufman, 1997), and diminished *so* expression in the eye imaginal disc (Fig. 4C, parts 1.2). Targeted expression of EY in the same tissues induces supernumerary eyes (Fig. 4C, part 4) and *so-lacZ* expression in several tissues including the antennal
pb'ey in head development

In vitro protein binding is mediated by the EY Paired domain and the PB homeodomain

To ask whether PB protein interacts directly with EY, we first tested for stable in vitro binding in ‘pull-down’ experiments. Glutathione S-transferase (GST) protein was produced intact, transcription and translation. No binding was detected between immobilized GST protein and any of the probe proteins tested (EXD, EY, SO and EYA) (Fig. 5B, lanes 5–8). In contrast, GST-EN fusion protein effectively bound the homeodomain-containing EXD protein (Fig. 5B, lane 13) (van Dijk and Murre, 1994), but not the homeodomain-containing EXD and SO nor the nuclear protein EYA (Fig. 5B, lanes 14,15,16). On testing with the same probes, efficient binding of GST-PB to EXD was observed (Fig. 5B, lane 9), as predicted based on the YPWM motif present in PB and implicated in binding to the homeotic co-factor EXD (Johnson et al., 1995). On testing the three proteins of the eye cascade, PB binding was detected with EY (Fig. 5B, lane 12), but not with EYA or SO (Fig. 5B, lanes 10,11). These data support direct, specific contacts between EY and the PB homeodomain-containing region.

The EY protein contains two DNA-binding domains, a paired domain (PD) and a homeodomain (HD) (see Fig. 1E).
Of these, the PD has been shown to interact with other transcription factors (Bendall et al., 1999; Fitzsimmons et al., 1996; Jun and Desplan, 1996; Plaza et al., 1997; Plaza et al., 2001). To define sequences within EY required for interaction with PB, we tested deleted forms of EY lacking the PD, the HD, or the region containing both, for their ability to bind the GST-PB fusion protein. EY deleted of its HD retains PB-binding capacity comparable to the intact protein (Fig. 5C, lane 6). In contrast, EY protein deleted for the PD (ΔPD), with or without the HD and linker, showed little or no interaction with PB (Fig. 5C lanes 7,8). This finding supports a crucial role for the PD in binding with PB.

A PB protein entirely deficient for in vitro DNA binding but harboring the Arg5→His mutation can still induce eye reduction, suggesting that this point mutation might favor specific protein contacts with some context-specific eye protein compared to wild-type PB HD (Benassayag et al., 1997). The GST-PB and GST-PB<sup>Δ</sup> HD proteins were therefore compared for their relative binding affinities to full-length or deleted forms of 35<sup>S</sup>-labeled EY protein (lanes 5,9) and truncated forms of EY lacking the HD (lanes 6,10), PD (lanes 7,11), or HD and PD (lanes 8,12), incubated with GST-PB (lanes 5,8) or GST-PB<sup>Δ</sup> HD (lanes 9,12), carrying the homeodomain R5H mutation indicated in A. Aliquots (5 µl) of in vitro translation products are shown in lanes 1-4. In this experiment, GST-EN bound 13% of the input 35<sup>S</sup>-labeled EXD protein, whereas GST-PB bound 10% of input 35<sup>S</sup>-labeled EY. (C) The interaction requires the EY PD and PB HD. GST ‘pull-down’ assays were performed in the same conditions as in B, with 35<sup>S</sup>-labeled full-length EY proteins (lanes 5,9) and truncated forms of EY lacking the HD (lanes 6,10), PD (lanes 7,11), or HD and PD (lanes 8,12), incubated with GST-PB (lanes 5,8) or GST-PB<sup>Δ</sup> HD (lanes 9,12), carrying the homeodomain R5H mutation indicated in A. Aliquots (5 µl) of in vitro translation products are shown in lanes 1-4. (D) Yeast one-and-a-half hybrid experiment. A strain carrying the integrated reporter vector pLucZ:so containing one copy of the so10 enhancer upstream of the minimal promoter of the yeast iso-1-cytochromeC gene. Vectors were pAS and pACT. pAS-EY, EY encoding vector. pACT-PB (126-306, see in A) is fused to the GAL4 activation domain. Lane 1, control with empty vector; lane 2, transactivation of the enhancer so10 with EY; lane 3, co-expression of EY and PB inhibits EY-dependent so10 transactivation, whereas PB does not activate so10 enhancer.

**PB inhibits EY-dependent transcriptional activation in yeast**

To address whether this protein-protein interaction can contribute to the inhibition of EY activity in a cell, a ‘one-and-a-half’ hybrid assay in yeast was performed to test the effects of PB on EY-mediated activation of a *sine oculis* (*so*) transcription enhancer. The reporter gene harbors the so10 fragment of 400 bp, an eye-specific element directly regulated by EY (Niimi et al., 1999), upstream of the *lacZ* reporter. EY expressed from the pAS plasmid activates so10-*lacZ* (Fig. 5D lane 2). PB sequences were fused to the GAL4 trans-activation domain in pACT-PB, using the same PB HD-containing peptide as for the pull-down experiments (amino acids 126-
DISCUSSION

We previously observed that ectopically expressed homeotic PB protein, even a form bereft of DNA-binding capacity, can suppress eye development in a dose-sensitive manner (Benassayag et al., 1997). Genetic and molecular results presented here indicate a central role for direct contacts between conserved domains of the Hox selector protein PB and the eye selector EY. One physiological situation where the interaction between pb and ey is likely to be relevant was identified, based on their genetic interaction, mutant phenotypes and expression patterns in forming the adult antennae and maxillary palps.

**ey function in maxillary palp development**

The present work has identified a previously unrecognized role for ey in the development of the maxillary palps and antennae. The mutation employed for most of our experiments here, eyJD, behaves as a strong allele affecting viability, formation of the adult eyes and brain mushroom bodies (Callaerts et al., 2001), but also of antennal and maxillary differentiation. Consistent with a late requirement for ey in the antennal disc, ey expression in the maxillary primordium appears in early stages of metamorphosis when both eye-antennal discs have fused, and the antennal and maxillary appendages start to evaginate. After evagination, the maxillary primordium migrates to join the labial disc in forming the adult mouthparts, whereas the antennal primordium remains near the eye. When a contiguous epidermal cell layer has been completed the head sac is abruptly evaginated under the internal pressure (Jürgens and Hartenstein, 1993). Maxillary expression in early stages of pre-pupal metamorphosis was limited to the boundary between the maxillary primordium and the antenna, and ey mutant phenotypes often involved simultaneously reduced palps and enlarged antennae. These reciprocal effects are consistent with communicating cell populations, suggesting that ey may contribute to a partitioning of the antennal disc permitting the establishment of two separate appendages. Further analysis of this process will require new maxillary-specific markers permitting the fates of these cells to be followed.

**pb/ey antagonism in maxillary versus antennal development**

In the present work, starting from a dose-sensitive eye loss provoked by ectopic PB, we identified new ey alleles isolated as eye loss Enhancers. These mutations reveal a role for ey, and a potential biological relevance for this Hox-PAX6 interaction, in the development of the antenna and maxillary sensory palps. ey loss-of-function defects in the sensory palps are exacerbated by PB<sup>583</sup>. The most direct interpretation of the enhanced ey loss-of-function phenotype with HSPB<sup>583</sup> is that the newly isolated alleles retain a partial function that can be negated by adequate PB levels. The molecular characterization of these alleles is consistent with this hypothesis, because all four alleles should encode truncated proteins that contain most or all of the interacting PD.

To better understand the in vivo relationship between these two selector genes, we sought to define the effects of double mutants for pb and ey. Although homozygotes for pb<sup>583</sup> or for the new ey mutants showed viabilities of up to 50% compared with heterozygotes, we have never obtained a double mutant adult for any of the four ey alleles. This result, although suggesting that the double mutant is synthetic lethal, does not offer insight into the tissue(s) implicated in this lethality.

One tissue in which an interaction is clearly indicated from our analysis is the maxillary palp primordium, where we detected a dynamic expression of EY and of PB (directly or via the pbGAL4 driver) during pre-pupal development. Transient early co-expression of PB and EY in pre-pupae was limited to a small number of cells, whereas later expression appears exclusive. This result can be rationalized in two ways: first, co-expressing cells might be rapidly eliminated by apoptosis, through a coordinate gene-activation process triggered by a Hox-Pax dimer; second, co-expression of the EY and PB transcription factors could induce a developmental pathway interference resulting in a G1 cell-cycle arrest, as recently suggested by Jiao et al. (Jiao et al., 2001). These possibilities are not fully exclusive. Indeed, one or both mechanisms could serve to refine the boundaries between antennal and maxillary cell populations within the antennal disc.

**Multiple roles of eyeless in head development**

In vertebrates, Pax6 has multiple known or inferred roles in eye, brain and nasal development (Grindley et al., 1995; Quinn et al., 1996). Apart from the fly eye, several groups have identified an eyeless function required for development of the mushroom bodies, neural structures important for olfactory perception and learning (Callaerts et al., 2001; Kursus et al., 2000; Noveen et al., 2000). Our study describes a specific role for EY in concert with PB in the maxillary and antennal appendages that are derived from the antennal disc and constitute the adult olfactory system. A recent analysis of mutations producing headless flies revealed a new role for Drosophila Pax6 in head morphogenesis and thereby suggests a requirement of ey for the development of all structures derived from eye-antennal discs (Kronhamn et al., 2002). A parallel can be drawn with the work presented here because both cases involve mutations truncating the EY protein which induces head defects. Interestingly, because these truncated EY proteins still contain the PD, we cannot exclude the fact that the phenotypes obtained reflect an allele-specific antimorphic effect of the PD. Taken together, these results strongly suggest that eyeless, apart from its known role in eye morphogenesis, may also play multiple other roles in head formation (notably for brain and olfactory sensory systems).
The development of olfactory and visual systems has several common features in Drosophila. Both systems are derived from the composite eye-antennal imaginal disc. Moreover, both have similar signal transduction pathways and appear to share regulatory networks (Carlson, 1996; Gaines and Carlson, 1995; Smith, 1996). However, when we examined the expression of ey, so, eya and dac in the pre-pupal maxillary primordium, only ey expression was detected. This observation suggests that ey acts there via a distinct combinatorial code of regulatory genes compared with eye development. One possibility proposed in this study is that ey activity is modulated by other co-factors or transcription factors whose activity is likely sensitive to PB. In this light, it is worth noting that other Enhancer mutations isolated in our screen also similarly affect maxillary palps, singly or in combination with ey. It will be of fundamental interest to better understand the molecular basis for how a single protein might function in multiple, distinct networks.

**HOX-PAX6 interaction as a general regulating mechanism**

The ey mutants studied here were identified as dominant enhancers of pb-induced eye reduction. Consistent with the antagonism observed in vivo, EY and PB proteins interact directly in vitro, via the EY Paired domain and the PB homeodomain. This interaction with PB that renders EY unable to activate its downstream target genes can be extended to other homeotic genes because Antp, Scr, Ubx, abdA and AbdB repress eye development while increasing apoptosis in the eye disc, and their protein products likewise interact in vitro with EY protein (Plaza et al., 2001). This suggests a combinatorial interaction of homeodomain-containing proteins (Hox and Pax) to specify a given body segment (Plaza et al., 2001). An inhibition through physical association has been proposed between Pax6 and En-I during eye development in quail (Plaza et al., 1997), and between Pax3 and Msx1 for muscle development in chicken (Bendall et al., 1999). Moreover, a similar inhibitory mechanism involving a Hox protein HD has recently been reported in vertebrates (Abramovich et al., 2000; Kataoka et al., 2001); in contrast, physical interaction with Hox-B1 protein led to increased Pax6 activity in Hela cells (Mikkola et al., 2000), raising the possibility that additional context-dependent partners modulate the action of Hox-Pax combinations to generate functional diversity. Based on our genetic and molecular data, we propose that variations on a PD-HD interface can serve to mediate combinatorial or hierarchical functional relationships among Hox and Pax genes in normal development.

The recent paper of Jiao et al. proposed that ectopic expression of Hox and Pax (and various other) transcription factors in the eye induces a generic developmental pathway interference (Jiao et al., 2001). This cellular process was proposed as a general surveillance mechanism that eliminates most aberrations in the genetic program during development and evolution, but is not a consequence of specific protein collaboration. However, the existence of such a mechanism does not preclude the possibility of more specific interactions such as we describe. The results presented here appear to favor a specific role for discrete protein-protein interactions rather than an indirect interference mechanism. Indeed, (i) by analysing the residues of PB protein involved in its homeotic function, we identified a PB5 protein with diminished DNA binding but still able to inhibit eye development; (ii) using this mutant in a genetic screen to isolate PB functional partners, we isolated four independent eyeless mutations, all of them leading to a shortened EY protein; (iii) genetic interaction tests showed that PB5-induced eye loss is highly sensitive to levels of ey function but independent of several other eye-determining genes including eyg, eya or so; and (iv) ectopically expressed PB interferes with ey activity in the eye imaginal disc by inhibiting so and eya activation without affecting ey transcription or EY accumulation.

In conclusion, our results suggest that a specific Hox/Pax interaction between PB and EY is involved in a normal developmental process defining the boundary between the antenna and maxillary palp. More generally, the formation of such protein couples could afford a sensitive and delicate measure of the balance of Pax6 level, permitting a finely tuned integration to generate distinct transcriptional outputs during development.

We thank Fabienne Pituello for her critical reading of the manuscript, Laurent Joulia for providing a pbGAL4 line and Henry Sun, Larry Zipursky, Laura Pignoni, Erich Frei, Markus Noll, Uwe Walldorf and the Indiana University Drosophila Stock Center for various stocks and reagents used in the course of this study. The monoclonal antibodies directed against Eya protein (developed by S. Benzer and N. Bonini) and Dac protein (developed by G. M. Rubin) used in this work were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. We thank Agnès Lepage for her expert technical assistance; Bruno Savelli for his generous gifts of time and competence in our ongoing war against the computer; and Thom Kaufman for sharing helpful discussions and unpublished data. Two eyeless alleles were identified by undergraduate students during a fourth-year genetics course at the Université Paul Sabatier, Toulouse, France (ey10D: Jenny Durrieu de Madron; eyEH, Emma Hill). This work benefitted from the ongoing support of the Centre National de Recherche Scientifique (CNRS) and grants from the Association pour la Recherche sur le Cancer (ARC) and from Retina France. C. B. benefitted from the crucial and timely support of the Fondation pour la Recherche Médicale (FRM) in the form of a short-term fellowship. S.P. was supported by an EMBO long-term fellowship.

REFERENCES


