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Direct regulatory interaction of the *eyeless* protein with an eye-specific enhancer in the *sine oculis* gene during eye induction in *Drosophila*

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SUMMARY

The *Pax-6* gene encodes a transcription factor with two DNA-binding domains, a paired and a homeodomain, and is expressed during eye morphogenesis and development of the nervous system. *Pax-6* homologs have been isolated from a wide variety of organisms ranging from flatworms to humans. Since loss-of-function mutants in insects and mammals lead to an eyeless phenotype and *Pax-6* orthologs from distantly related species are capable of inducing ectopic eyes in *Drosophila*, we have proposed that *Pax-6* is a universal master control gene for eye morphogenesis. To determine the extent of evolutionary conservation of the eye morphogenetic pathway, we have begun to identify subordinate target genes of *Pax-6*. Previously we have shown that expression of two genes, *sine oculis* (*so*) and *eyes absent* (*eya*), is induced by *eyeless* (*ey*), the *Pax-6* homolog of *Drosophila*. Here we present evidence from ectopic expression studies in transgenic flies, from transcription activation studies in yeast, and from gel shift assays in vitro that the EY protein activates transcription of *sine oculis* by direct interaction with an eye-specific enhancer in the long intron of the *so* gene.

Key words: Drosophila, Eye development, *eyeless*, *sine oculis*

INTRODUCTION

Pax-6 genes have been cloned from representatives of eight animal phyla, which to date includes homologs from genomes of human (Ton et al., 1991), mouse (Walther and Gruss, 1991), rat (Matsuo et at., 1993), chicken (Goulding et al., 1993), quail (Martin et al., 1992), *Xenopus* (Hirsch and Harris, 1997), zebrafish (Krauss et al., 1991; Puschel et al., 1992), ascidian (Glardon et al., 1997), sea urchin (Czerny and Busslinger, 1995), squid (Tomarev et al., 1997), *Drosophila* (Quiring et al., 1994; Czerny et al., 1999), *Caenorhabditis elegans* (Chisholm and Horvitz, 1995; Zhang and Emmons, 1995), ribbonworm (Loosli et al., 1996) and planaria (Callaerts et al., 1999). *Pax-6* genes encode a transcription factor with two DNA binding domains, a homeodomain and a paired domain, both of which have been highly conserved during evolution. Although the consensus target site for each DNA binding domain has been determined separately by in vitro binding assays (Epstein et al., 1994; Czerny and Busslinger, 1995), only a limited number of target genes of *Pax-6* have been identified (Sheng et al., 1997; for review, see Cvekl and Piatigorsky, 1996).

In mammals, congenital diseases known as *Aniridia* (humans) and *Small eye* (mice and rats) are caused by loss-of-function mutations of *Pax-6* in heterozygotes, whereas homozygous embryos lack eyes and nostrils completely, have brain and spinal cord malformations, and die prior to birth. In *Drosophila*, loss-of-function mutations in the *eyeless* (*ey*) locus, found to encode a *Pax-6* homolog (Quiring et al., 1994), also show eye defects (Hoge, 1915). In gain-of-function *Drosophila* mutants, ectopic eyes are formed on the antennae, legs, wings and halteres of the fly (Halder et al., 1995). Because ectopic expression of *Pax-6* homologs from human, mouse, squid and sea squirts (ascidians) are capable of inducing ectopic eyes in *Drosophila* (Halder et al., 1995; Tomarev et al., 1997; Glardon et al., 1997), we have proposed that *Pax-6* may serve as a universal master control gene for eye morphogenesis in metazoa. This implies a monophyletic origin of the eye as a sensory organ and contradicts the previously held view that the various eye types have evolved independent of one another (Salvini-Plawen and Mayr, 1977). Besides *Pax-6*, which resides at or near the top of the hierarchy or cascade of genes controlling eye morphogenesis, a large number of subordinate genes encoding transcription factors, signaling molecules and structural proteins are hypothesized to be required in this process. To what extent this genetic cascade is evolutionarily conserved in the various eye types is currently unknown. In order to approach this problem, we have set out to identify subordinate target genes of *Pax-6*. We have found that the *Pax-6* genes of *Drosophila* and the silkworm underwent duplication (Czerny et al., 1999) and that the two paralogs, *eyeless* (*ey*) and *twin-of-eyeless* (*toy*), are differentially expressed. During embryonic development, *toy* appears to be expressed prior to *ey* and is required at least for activation, if not also maintenance, of *ey*. Downstream of *ey*, we have identified the

sine oculis (*so*) and *eyes absent* (*eya*) genes (Halder et al., 1998), but it is not known whether *ey* interacts directly with these downstream targets. In this paper, we show by the results of experiments performed both in vivo and in vitro that the EY protein activates transcription of *so* by binding specifically to an eye-specific enhancer in the *so* gene. The requirement for induction of *so* by *ey* is demonstrated with the so^1 mutation, a deletion of 1.3 kb in the eye-specific enhancer region that leaves the coding region intact. In so^1/so^1 mutant flies, *ey* fails to induce *so* expression and the formation of ectopic eyes. In contrast, *toy* induces both ectopic *so* expression and ectopic eyes in a so^1/so^1 homozygous mutants. We discuss these findings on the genetic control of eye morphogenesis in *Drosophila* in the context of those obtained in vertebrates.

MATERIALS AND METHODS

Fly strains

Flies were reared on standard medium at 25°C. Embryos of the *yellow white* strain (*y ac* w^{1118}) were used as recipients for DNA injection for generation of transgenic lines. The stock carrying *so-lacZ* line (Cheyette et al., 1994) was kindly provided by Larry Zipursky. Flies carrying the dpp^{blink}*-Gal4* transgene (line C40.6, Staehling-Hampton et al., 1994) were gift from Michael Hoffmann. UAS-*ey* and UAS-*toy*, ey^2 and so^1 have been described in Quiring et al. (1994), Cheyette et al. (1994) and Halder et al. (1995). The molecular lesions of the ey^2 and so^1 mutants were described previously by Quiring et al. (1994) and Cheyette et al. (1994), respectively.

Construction of *so5-lacZ* and *so10-lacZ* and generation of transgenic flies

All *so* genomic fragments were cloned in the same orientation upstream of the truncated hsp70 promoter in a P-element *lacZ* reporter plasmid, pcβ (kindly provided by Markus Affolter). The *so*5 fragment was isolated as a 398 bp *Eco*RI-*Bam*HI restriction fragment from a *so* genomic DNA (Cheyette et al., 1994). The 398 bp fragment was subcloned into pcβ with the *Eco*RI-*Bam*HI linker. The *so*10 fragment was isolated as a 428 bp *Eco*RI-*Asp*718 fragment from a *so* genomic DNA. The fragment was subcloned into the *Asp*718 site of pcβ with the *Eco*RI-*Asp*718 linker. At least ten transformant lines of each construct were generated and analyzed as described by Rubin and Spradling (1982).

Ectopic *eyeless* expression and β-galactosidase detection

Virgin females homozygous for HS-*ey* (Halder et al., 1995) were crossed with males homozygous for the *lacZ* reporter transgenes. Eggs laid during 1 hour were collected and incubated at 25°C. Heat-shock cycles were applied starting at 81 hours after egg laying. One cycle consisted of a heat shock at 38°C for 45 minutes followed by recovery at 25°C for 3 hours 15 minutes. The heat-shocked larvae were allowed to recover for 3 hours prior to dissection. Detection of β-galactosidase was carried out in 0.5 ml of 10 mM $NaH_2PO_4{\cdot}H_2O/Na_2HPO_4{\cdot}2H_2O$ (pH 7.2), 150 mM NaCl, 1 mM $MgCl_2{\cdot}6H_2O$, 3.1 mM $K_4[Fe^{II}(CN)_6]$, 3.1 mM $K_3[Fe^{III}(CN)_6]$, 0.3% Triton X-100 and 12.5 μl of 8% X-gal in DMSO; subsequently, the dissected larvae were washed several times in PBS and the discs were mounted in glycergel (Dakopatts) for microscopic examination. All lines derived from the same DNA construct gave similar patterns and intensities of staining.

In situ hybridization

Digoxigenin-labeled DNA probes were prepared from full length *so* cDNA (Cheyette et al., 1994) and hybridized to whole-mount imaginal discs. Hybridization was carried out in 50% formamide, 5× SSC, 100 μg/ml of sonicated salmon sperm DNA, 100 μg/ml of tRNA, 50 μg/ml of heparin and 0.1% Tween 20 overnight at 48°C. After washing several times with PBS-0.1% Tween 20, discs were incubated with an anti-digoxigenin antibody coupled with alkaline phosphatase and the staining reaction allowed to proceed for 2 hours.

Yeast one-hybrid system

DNA-binding analysis by the yeast one-hybrid system was carried out essentially as described by Mastick et al. (1995). *ey* embryonic cDNA (Quiring et al., 1994) was inserted into an activator plasmid (pBM258T; Mastick et al., 1995) under the control of the *pGAL1* promoter, which is activated by galactose and repressed by glucose. Fragments of the *so* genomic gene were inserted into a reporter plasmid (pHR307a; Mastick et al., 1995). EY induced by galactose, bound to its target site, in turn induces *HIS3* expression, which allows for growth on medium lacking histidine. *URA3* and *TRP3* were used for the selection of the activator and reporter constructs, respectively. As a control, two or four copies of a consensus PRD binding site (5′-TTCACGCATGAGTTCCT-3′) were inserted into the reporter plasmid pHR307a.

Gel shift assay

EY protein was synthesized using a coupled in vitro transcription translation kit (Promega) with the T7 primer and *ey* embryonic cDNA as template (Quiring et al., 1994). Crude protein extracts were prepared from heat-induced and control (non-induced) HS-*ey* embryos. Genomic fragments depicted in Fig. 3A were divided into subfragments (approximately 120 bp each) by PCR and tested for EY binding activity by gel shift assay. For *so*5, four subfragments 1-120, 101-220, 201-320 and 301-398 (compare Fig. 4) were generated with oligonucleotide primers (20-mers) at both the 5′ and 3′ ends of each PCR product. Subfragments 1-120, 101-220, 201-320 and 301-428 fragments were similarly generated for *so*10 probes. Purified PCR products were radioactively labeled with polynucleotide kinase and [γ-^{32}P]ATP. For super shift experiments, 1 μl of rabbit anti-EY antiserum (kindly provided by Uwe Walldorf) was added. Binding reactions were carried out in 20 μl of 10 mM Tris-HCl pH 8.0, 50 mM KCl, 4 mM dithiothreitol, 10% glycerol and incubated at room temperature for 15 minutes and analyzed by electrophoresis through 6% native polyacrylamide gels followed by autoradiography. Both binding and electrophoresis were performed at room temperature.

RESULTS

Heat-shock *eyeless* induces ectopic expression of *sine oculis*

Although *sine oculis* (*so*) has been shown to be required during normal and ectopic eye development and to act downstream of *ey* (Halder et al., 1998), whether *ey* controls *so* directly or indirectly remains to be determined. If *so* is under the direct control of *ey*, ectopic expression of *so* should be induced as an immediate-early response to the ectopic expression of *ey*. To determine whether such an immediate-early response is induced, a *so-lacZ* enhancer trap line that reflects the expression pattern of the endogenous *so* gene (Fig. 1; Cheyette et al., 1994) and a heat-shock-*eyeless* (HS-*ey*) line were crossed and the transheterozygous larvae tested for ectopic *lacZ* expression after application of several cycles of heat shock. After 2 cycles of heat shock, ectopic expression of β-galactosidase in the *so-lacZ* line was barely detectable in wing and leg imaginal discs (Fig. 2A). However, ectopic expression was strongly induced after 3 cycles of heat shock (Fig. 2B, arrow). The site of ectopic expression of β-galactosidase in the *so-lacZ* discs corresponds to the location of the ectopic eyes in

Fig. 1. β-galactosidase expression in imaginal discs of late third instar larvae bearing a *sine oculis*(*so*)-*lacZ* transgene (Cheyette et al., 1994). The pattern of expression of the transgene in the eye disc (left) and the leg disc (right) reflects the expression pattern of the endogenous *so* gene, except that expression in the developing chordotonal organs is not detected by in situ hybridization with *so* cDNA (see Fig. 7A). Posterior is to the left and dorsal is up for all discs. β-galactosidase was detected only at background levels in wing imaginal discs (center).

the adult flies (data not shown). In contrast, when the *so-lacZ* line was crossed to *y ac* w^{1118} flies as a control, no ectopic expression of β-galactosidase was detected upon heat shock (Fig. 2D-F). This result indicates that the expression of *so* is induced immediately upon induction of *ey* expression. Interestingly, the capacity to induce ectopic eyes was much reduced in HS-*ey* lines as compared to Gal4 driver lines. Under the conditions used, no ectopic eyes were formed after 2 or 3 cycles of heat shock. However, after 6 cycles of heat shock, small ectopic eyes were detected on wings and legs (data not shown).

eyeless protein binds to an eye-specific enhancer and the promoter region of *sine oculis* and activates transcription in a yeast one-hybrid system

The time course of induction of *so* by *ey* in the heat-shock experiments suggests, but does not prove, a direct interaction. Therefore, we examined whether EY binds directly to regulatory regions of *so* in a yeast one-hybrid system and thereby activates transcription. For expression of the one-hybrid fusion protein, *ey* cDNA was inserted downstream of the *pGAL1* promoter of the yeast activator plasmid (see Materials and Methods). We first tested two promoter regions and a putative eye-specific enhancer region of *so*. Four fragments from these regions (Fig. 3A, upper row) were inserted upstream of *HIS3* in the yeast reporter plasmid. The binding activity of EY to the *so* genomic fragments was assayed by growth of yeast on medium lacking histidine and containing galactose. Two out of four fragments (*so* fragments 2 and 4) were positive in the first test (Fig. 3B). These two positive fragments were subdivided (Fig. 3A, middle and lower rows) and tested again by the same method. Ultimately, two EY binding regions, fragments 5 and 10, were identified (Fig. 3B). Each fragment is approximately 400 bp in length and contains a putative PRD binding site (Fig. 4A,B, shaded boxes). The

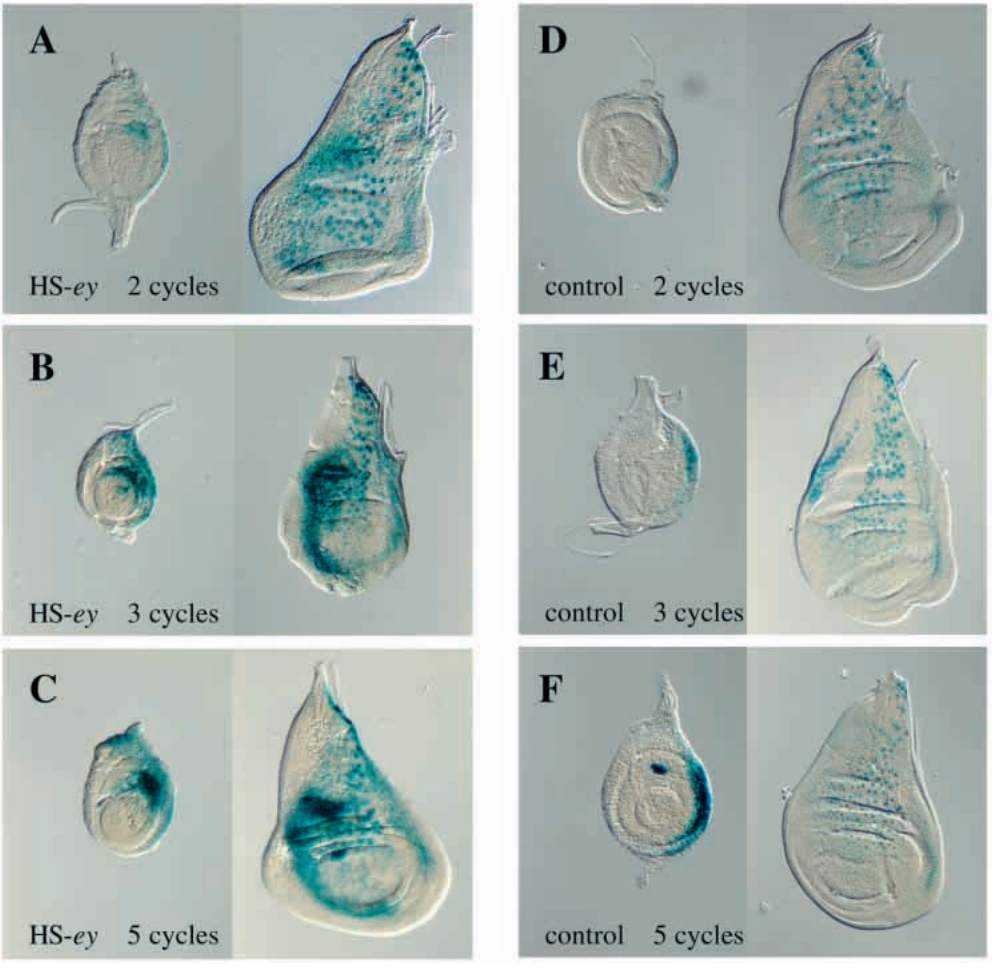

Fig. 2. β-galactosidase expression in *sine oculis*(*so*)-*lacZ* line transheterozygous for HS-*ey* (A-C) and *y ac* w^{1118} (D-F) as control. Foreleg discs are shown on the left, wing discs on the right of each panel. After 2 cycles of heat shock, little difference is observed between HS-*ey* and the control discs (A,D). With an additional cycle of heat shock, ectopic β-galactosidase expression was clearly induced in both leg and wing discs by HS-*ey* (B) in comparison with the control discs (E). Following 5 cycles of heat shock, strong ectopic β-galactosidase expression was observed in HS-*ey* (C), whereas no ectopic expression was observed in control wing discs (F). Note, that the increased staining in the leg disc is confined to the periphery of the disc and a spot marking the presumptive chordotonal organs (regions that express the transgene in the absence of ectopic *ey*; see Fig. 1 right) and there is no ectopic expression in the interior of the disc as in C.

sequences of the putative sites are at maximum 88% homologous (fragment 5 site) and 70% (fragment 10 site) with the consensus PRD binding site sequence reported by Czerny and Busslinger (1995) (Fig. 4C). As a further control, we tested the PRD consensus binding site in the yeast one-hybrid system and found that EY does indeed recognize this sequence (Fig. 3B). These results demonstrate that EY binds directly to the *so* locus (fragments 5 and 10), putatively at PRD consensus sequences and activates reporter gene transcription in a yeast one-hybrid system.

eyeless protein selectively binds a subfragment of the eye-specific enhancer in *sine oculis* in vitro

To narrow down further the EY binding region, fragments 5 and 10 were subdivided each into four subfragments approximately 120 bp in length and analyzed for EY binding activity by gel shift assay with crude protein extracts from heat-induced HS-*ey* embryo. In these assays, only one subfragment (Fig. 4 underlined) of genomic fragment 10, which was derived from the *so* eye-specific enhancer, revealed a strong reduction in migration rate consistent with the formation of a specific protein-DNA complex (Fig. 5, lane 6), but the four subfragments of genomic fragment 5, derived from the *so* promoter region, failed to bind protein in the extract even though the *so* promoter region was positive in the yeast one-hybrid system (data not shown). The apparent complex was observed with heat-shocked extracts from HS-*ey* embryos (compare lanes 6 and 7) but not with extracts from heat-shocked *y ac* w^{1118} control embryos (data not shown). Competition with unlabeled oligonucleotide (lane 8) revealed that the complex was specific. To confirm that the complex observed with crude HS-*ey* extracts was due to binding of the EY protein, band shifts were performed with EY protein produced by in vitro transcription and translation. A shifted complex was observed to co-migrate with the heat-shock-induced species (compare lanes 2 and 6), demonstrating conclusively that with heat-shock-induced extracts the specific complex was formed by EY. Furthermore, addition of antiserum against EY to binding reactions with both crude and in vitro synthesized protein prevented formation of the complex (lanes 5 and 9). These results indicate that EY binds directly to the *so* eye-specific enhancer region in vitro. Because the fragment that is bound by EY contains a putative PRD consensus binding sequence, we tested a PRD consensus binding site fragment (Czerny and Busslinger, 1995) by gel shift assay and again observed specific complex formation with heat-shock-induced extracts.

eyeless induces ectopic expression of *sine oculis* by interaction with the eye-specific enhancer region in vivo

To determine whether EY binds to *so* genomic fragments 5 and 10 in vivo, transgenic reporter lines *so*5-*lacZ* and *so*10-*lacZ* were generated with a P-element vector. Although β-galactosidase expression of the *so-lacZ* control

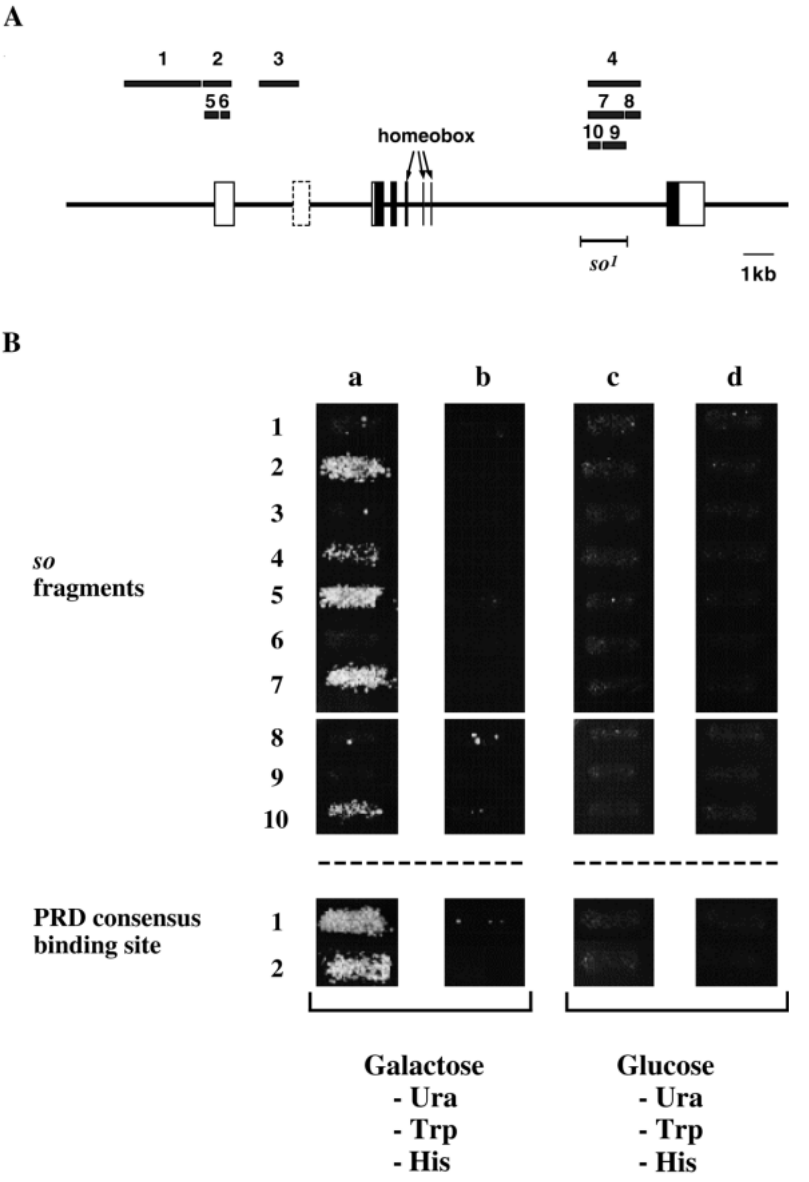

Fig. 3. EY protein binds to the *so* enhancer and promoter regions and activates reporter gene transcription in a yeast one-hybrid system. (A) Genomic map of *so*. Exons are depicted as boxes with the protein coding region shown in black. The dotted box represents an exon transcribed from a second internal promoter. The extent of the so^1 deletion is indicated below the map. The bars above represent genomic DNA fragments tested in the one-hybrid assay (see below). The 5′ fragments 1, 2, 3, 5 and 6 encompass two promoter regions and the more 3′ fragments 4, 7, 9 and 10 are clustered in a *so*-enhancer region, which is deleted in the so^1 mutation. (B) Yeast one-hybrid analysis. a and b were carried out on galactose medium, which induces EY protein expression, c and d on glucose medium in which EY is not induced. Both media lack uracil, tryptophan and histidine. (a) Reporter gene expression allowing growth is observed with two promoter fragments (2 and 5) and with three enhancer fragments (4, 7 and 10). No growth is observed without an *ey* producer plasmid (b) or without EY expression in glucose medium (c,d). The consensus PRD binding sequence also allows EY protein to bind and to activate transcription. PRD binding sequence 1 contains four repeats of the consensus sequence and 2, two repeats.

A

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GAATTCTGAG TTATCATCAG TTCATATATG TGTAATCATG AACTGTGTGT   50
GCCCAGTGAC TTTATAAAAG TCAGTGAATG TAAGTCTCGC TTCACTTGAT  100
TCCATTATTT TCCTATAACG TTTTTGGAAA GTGACGCACA AAGTGTCGGT  150
GCATCCCACT GTGCAGTTCA CCCCCTTGGT GTTCGCTCAC TCACACACTC  200
GCACGACCAG TCGGCCATCT CGCTCTGGCG CACGCACACC GCCAGCGGCA  250
CCAACCATTT TCTGCCTTTT GTCTCGGCCG TTTTCGGTTC GATTAGGTTT  300
GAGAACCGGA GAGTTCTCAG TTGTTTTCGA GATTCCGTCG CGTGCGGTTC  350
GCTGCCCGTT CTACCCACGT CGCTCGCTTA GTAATCCAGG CCGGATCC
       398
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B

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GAATTCCTTG GAAGCCACAG TCCAGTCCCA GTTCTCCACT CATTCGTGTG   50
AAAGTTAGTT GGGGTTATGT CATGGGCCAT TCTCAATTCT TCTATTTTCC  100
CAATCAATTG ACATCAGATC GATGCGAAAG ATGTCTTGCC AAAATGTTAA  150
ATACCTTTAT AGACCAACTC ACTCACGTGG CAAACTCGTT TGTGGCCCAA  200
CCCACCGATT CACCCCTCAC CGATTCCGTT TCCAGTTAAA CATAATCCGA  250
TCATGCATTA TTACCAAGCT AATGAATCTG CTTAAGACAG AGCCCGGCTA  300
CAAAAAACCA ATTGGGCAAA CAAGTAAAAA TTAATTCCCC CTCACTGGGC  350
ACAACTCTGG ACCCAAAAAG AAATATGAAA AGAAATGTGC GGGAAGAAAA  400
GCCCGAGGAA CCTTCAGCCA AAGGTACC
                        428
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C

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A  C   G T   GACC
 NG ANT A GCG
G  A   C A   TGAA
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Fig. 4. Nucleotide sequence of *so* promoter region and eye-specific enhancer region. The sequence of *so*5 (A) and *so*10 (B) fragments which were found to be positive in the yeast one-hybrid system. The two 17 bp sequences (shown shaded), 84-100 in *so*5 fragment and 379-395 in *so*10, correspond to putative PRD binding sites. Nucleotides indicated in bold in A and B match the consensus PRD binding site sequence shown in C (Czerny and Busslinger 1995). The putative PRD binding site in *so*10 is contained in a subfragment (underlined) formed to bind EY by gel shift assay (see below).

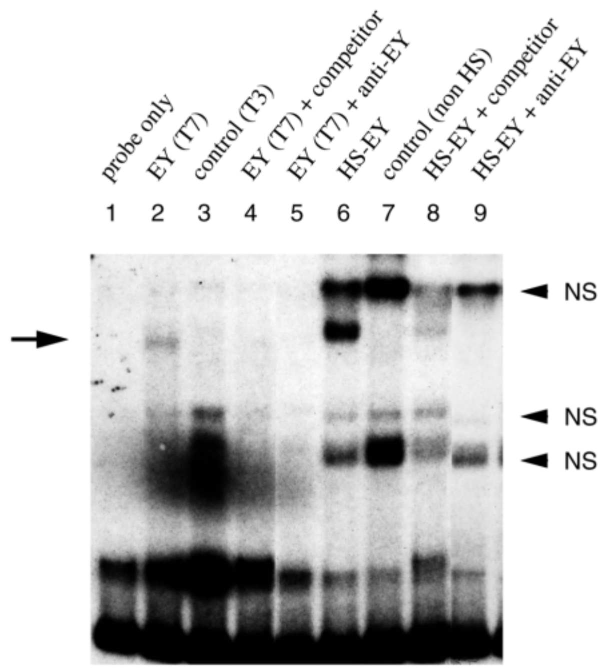

Fig. 5. Gel shift assays. Genomic fragments *so*5 and *so*10 were each subcloned as four approximately 120 bp fragments which were tested for EY binding activity by gel shift assays. Only *so*10-4 (underlined in Fig. 4B) was found to be positive in this assay. The in vitro transcription-translation products with T7 primer (lanes 2, 4 and 5) and with T3 primer (negative control, lane 3) are shown. Crude protein extracts were prepared from heat-shocked HS-*ey* embryos (lanes 6, 8 and 9) and control (no heat shock) HS-*ey* embryos (lane 7). Addition of unlabeled oligonucleotide (lanes 4 and 8) competed for the formation of the specific complexes observed in lanes 2 and 6 (arrow). Addition of anti-EY antiserum appeared to inhibit the specific DNA binding activity of EY (lanes 5 and 9). NS, non-specific complexes.

line was detected only in eye discs and leg discs (Fig. 1), expression in the *so*5-*lacZ* line was detected in eye-antennal, leg and wing discs (Fig. 6A). Expression of *so*10-*lacZ* was confined to the eye imaginal disc (Fig. 6D) and its pattern very similar to the endogenous *so* expression pattern in the eye disc, i.e. both anterior and posterior to the morphogenetic furrow of the eye disc, indicating that this 428 bp region harbors an eye-specific enhancer of *so* responsible for the selective expression of *so* in the eye disc. This result is in agreement with the eye-specific phenotype (no eyes or eyeless) of the so^1 mutant in which this region is deleted (Cheyette et al., 1994). We also determined whether HS-*ey* can induce ectopic expression of β-galactosidase in the *so*5-*lacZ* and the *so*10-*lacZ* lines. The staining pattern of *so*5-*lacZ* line was unaltered after 2 cycles of heat shock (Fig. 6B) and additional cycles of heat shock failed to induce ectopic expression of β-galactosidase (Fig. 6C). In contrast, ectopic expression of β-galactosidase was detected after 2 cycles and 3 cycles of heat shock in both wing and leg discs from the *so*10-*lacZ* line (Fig. 6E,F), demonstrating clearly that ectopic expression of EY by heat shock induces ectopic expression of the *so*10 reporter gene. These results show that EY controls *so* expression through the eye-specific enhancer in the *so* gene in vivo.

eyeless protein is unable to induce *so* expression in homozygous so^1 enhancer deletion mutants

If EY protein interacts exclusively with the eye-specific enhancer in *so*, induction of *so* expression by EY should be abolished in homozygous so^1/so^1 deletion mutants that lack the enhancer (cf. Fig. 3). Indeed, as shown by in situ hybridization, EY is not capable of inducing *so* expression (Fig. 7B,D). This finding is consistent with the observation that EY cannot induce ectopic eyes in so^1/so^1 mutants (Halder et al., 1998 and this work), but in marked contrast to results with *twin of eyeless* (*toy*), a second *Pax-6* homolog found in *Drosophila* and other higher insects (Czerny et al., 1999). Even though EY and TOY share 90% and 95% amino acid sequence identity in their paired and homeodomains, respectively, the two proteins differ significantly in their DNA binding properties. TOY can ectopically activate *so* transcription in so^1/so^1 mutants (Fig. 7C) and also induces ectopic eyes (Fig. 7E). This indicates that EY and TOY do not interact with the same regulatory regions in *so*.

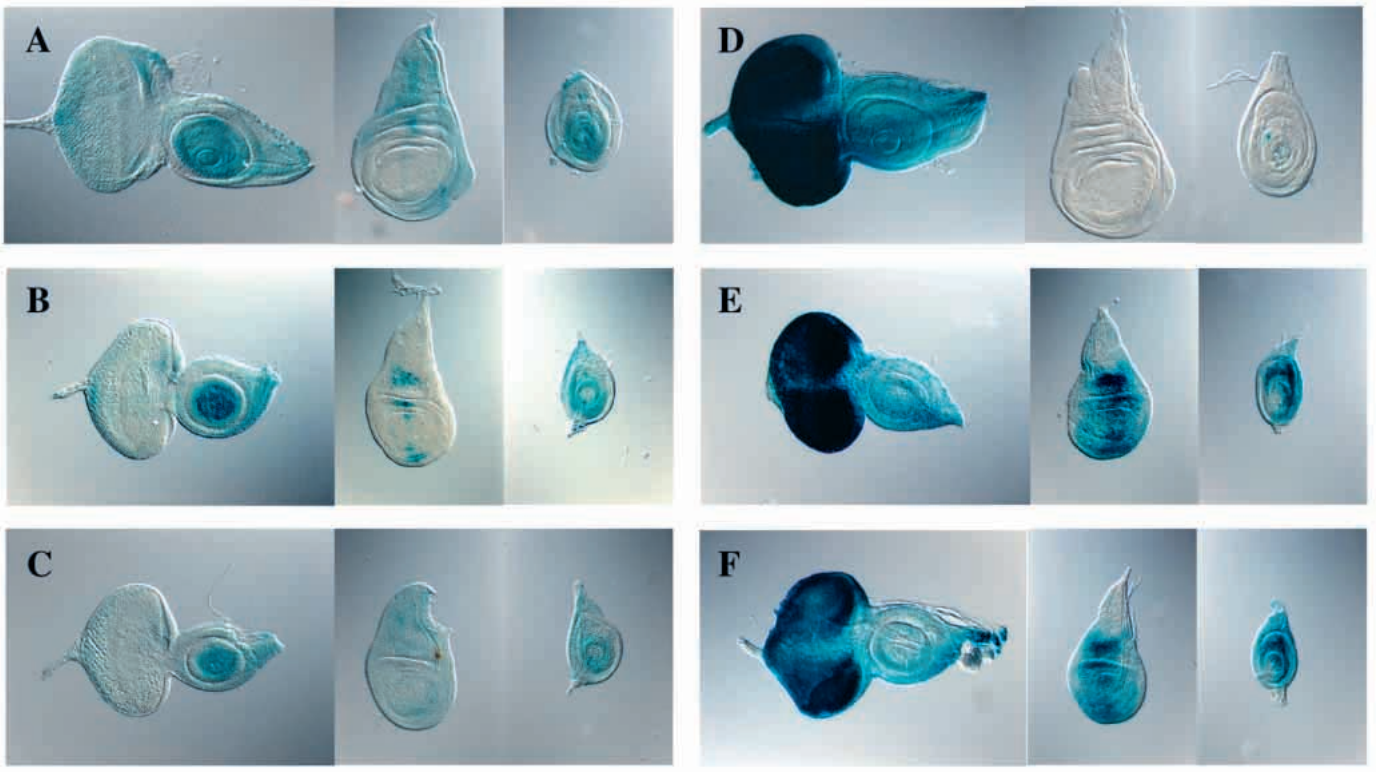

Fig. 6. β-galactosidase expression in eye-antennal (left), wing (center) and leg (right) discs of late third instar larvae carrying *so*5-*lacZ* (A-C) and *so*10-*lacZ* (D-F) transgenes. Detection of β-galactosidase activity in control (no heat shock) *so*5-*lacZ* (A) and *so*10-*lacZ* (D) discs. Two cycles (B,E) and three cycles (C,F) of heat shock. The staining pattern in the eye disc of *so*10-*lacZ* (D) is similar to that of the enhancer trap line *so-lacZ* (cf. Fig. 1). The staining pattern of *so*5-*lacZ* (A) is unrelated to that of *so-lacZ* and does not reflect *so* gene expression (B,C). The *so*5-*lacZ* construct was not inducible by HS-*ey*, whereas ectopic expression of β-galactosidase was clearly detected in wing and leg discs of *so*10-*lacZ* after 2 cycles of heat shock (E) and became more intense with three cycles.

DISCUSSION

Several target genes of Pax-6 in vertebrates have already been identified: mouse and chicken *αA-crystallin*, chicken *δ1-crystallin*, guinea pig *ζ-crystallin* and mouse *N-CAML1* (Cvekl et al., 1994, 1995a,b; Richardson et al., 1995; Chalepakis et al., 1994). In *Drosophila*, *rhodopsin* is a candidate target gene (Sheng et al., 1997), although the evidence for direct interaction is inconclusive. Interestingly, all of these target genes encode protein products for terminal differentiation in eye morphogenesis. However, *Pax-6* acts on the developing eye primordia from the earliest stages of morphogenesis, it may not only regulate genes involved in terminal differentiation, but also transcription factors functioning earlier to regulate further downstream genes. This includes *Pax-6* itself which was shown to autoregulate its own promoter (Plaza et al., 1995). We have shown here that *ey* directly regulates *so*, which therefore functions downstream in the eye morphogenetic pathway.

The *so* gene encodes a homeodomain protein required for the development of the entire visual system, including the compound eye, the ocelli, the optic lobe of the brain and the

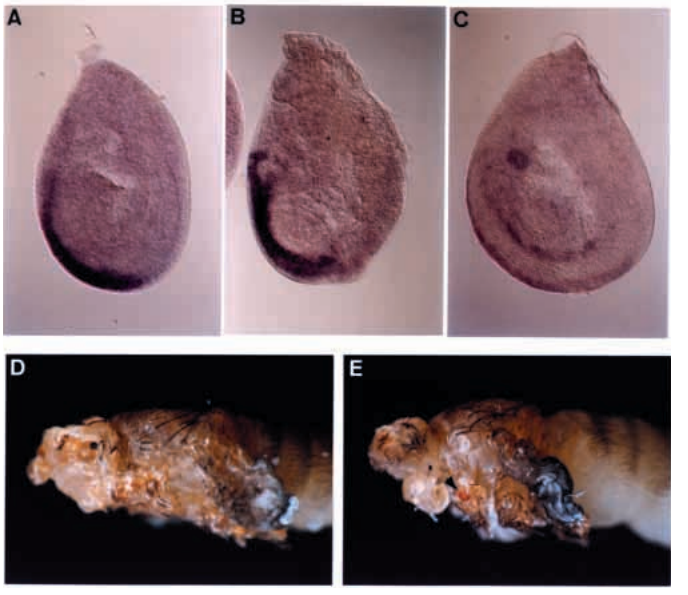

Fig. 7. Analysis of *so* transcription in homozygous so^1/so^1 mutants. (A-C) In situ hybridizations with a *so* cDNA probe in leg imaginal discs. (A) Wild type. Ectopic expression of *ey* (B) did not lead to any detectable ectopic expression of *so*. Ectopic expression of *toy* (C) produces a single spot of ectopic *so* expression. Ectopic eyes are induced by ectopic expression of *toy* (E, arrowhead), but not of *ey* (D) in a so^1/so^1 homozygous mutants. *ey* and *toy* were expressed ectopically with dpp^{blink}-Gal4 in the Gal4/UAS system (Brand and Perrimon, 1993).

larval photoreceptor designated as Bolwig's organ (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Pignoni et al., 1997). *so* acts downstream of *ey* (Halder et al., 1998) and physically interacts with another transcription factor encoded by *eyes absent* (*eya*) to form a protein complex that feeds back on *ey* expression and is also capable of inducing ectopic eyes (Bonini et al., 1993, 1997; Pignoni et al., 1997). We have identified *so* and *eya* as mediators of the eye-inducing activity of *ey* (Halder et al., 1998). Both *so* and *eya* are induced by *ey* and the expression of both genes is required independently during ectopic eye induction, but not vice versa. We have proposed a model in which *ey* induces the initial expression of both *so* and *eya* in the eye disc, after which *so* and *eya* participate in a positive feedback loop that regulates the expression of all three and possibly more genes.

In this paper, we present evidence for a direct interaction between EY and the eye-specific enhancer of *so*, which lends support to the above model. The evidence is based upon the yeast one-hybrid system in which EY protein is expressed by a driver plasmid and tested for its ability to bind a putative DNA binding site and activate reporter gene transcription. DNA fragments from both the eye-specific enhancer and the promoter of *so* were recognized as target sites. These results were confirmed for the eye-specific enhancer by gel shift assays in vitro, which in combination with the yeast one-hybrid system, allowed us to narrow down the binding site to a fragment of 120 bp containing a putative *Pax-6* paired domain binding sequence. The functional importance of the eye-specific enhancer of *so* is demonstrated in vivo by means of the so^1 mutant, which deletes a 1.3 kb region including the enhancer, but leaves the coding sequences intact. In so^1 homozygous flies, *ey* is neither capable of inducing *so* transcription nor can it induce ectopic eyes. In contrast to *ey*, its paralog *toy* induces both ectopic *so* transcription and ectopic eyes in a so^1 mutant background. This indicates that *ey* and *toy* regulate *so* by different mechanisms.

Sine oculis homologs have been identified in humans (Boucher et al., 1996), chicken (Bovolenta et al., 1996) mouse (Oliver et al., 1995), zebrafish (Seo et al., 1998; Kobayashi et al., 1998) and medaka fish (Loosli et al., 1998) as well as in planarians (P. Callaerts and E. Salo, personal communication). However, because several paralogs have been identified both in mammals and *Drosophila* that form a small gene family, which genes are true orthologs and which are paralogs is not clear in all cases. Nevertheless, several members of the vertebrate *so/Six* family are expressed during eye morphogenesis and their functional importance in eye development is clearly illustrated by the experiments of Oliver et al. (1996) who showed that ectopic expression of the mouse *Six3* gene in medaka fish embryos can induce ectopic lens formation. Therefore, at least some of the *sine oculis* homologs play an important role in eye morphogenesis, but the exact evolutionary relationships between the genes and their functions remains to be elucidated. Since recent studies of Bonini et al. (1997) indicate that the role of *eya* in eye development has also been conserved between flies and vertebrates, it seems that several important circuits of gene regulation have been conserved during evolution of the eye developmental pathway even though different types of eyes are formed in insects and vertebrates.

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