Development 126, 383-395 (1999)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV9634

Regulation of *Pax6* expression is conserved between mice and flies

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Accepted 23 October; published on WWW 14 December 1998

SUMMARY

***Pax6* plays a key role in visual system development throughout the metazoa and the function of *Pax6* is evolutionarily conserved. However, the regulation of *Pax6* expression during eye development is largely unknown. We have identified two physically distinct promoters in mouse *Pax6*, P0 and P1, that direct differential *Pax6* expression in the developing eye. P0-initiated transcripts predominate in lens placode and corneal and conjunctival epithelia, whereas P1-initiated transcripts are expressed in lens placode, optic vesicle and CNS, and only weakly in corneal and conjunctival epithelia. To further investigate their tissue-specific expression, a series of constructs for each promoter were examined in transgenic mice. We identified three different regulatory regions which direct distinct domains of *Pax6* expression in the eye. A regulatory element upstream of the *Pax6* P0 promoter is required for expression in a subpopulation of retinal progenitors and in the developing pancreas, while a second regulatory element upstream of the *Pax6* P1 promoter is sufficient to direct expression in a subset of post-mitotic, non-terminally differentiated photoreceptors. A third element in *Pax6* intron 4, when combined with either the P0 or P1 promoter, accurately directs expression in amacrine cells, ciliary body and iris. These results indicate that the complex expression pattern of *Pax6* is differentially regulated by two promoters acting in combination with multiple *cis*-acting elements.**

We have also tested whether the regulatory mechanisms that direct *Pax6* ocular expression are conserved between mice and flies. Remarkably, when inserted upstream of either the mouse *Pax6* P1 or P0 promoter, an eye-enhancer region of the *Drosophila eyeless* gene, a *Pax6* homolog, directs eye- and CNS-specific expression in transgenic mice that accurately reproduces features of endogenous *Pax6* expression. These results suggest that in addition to conservation of *Pax6* function, the upstream regulation of *Pax6* has also been conserved during evolution.

Key words: *Pax6*, Developmental expression, Eye development, Eye imaginal disc, *eyeless*, Evolutionary conservation, Pancreas, Retina, Spinal cord, Transgenesis

INTRODUCTION

The eye forms during vertebrate embryogenesis through a series of inductive interactions involving neuroectoderm, surface ectoderm, neural crest and mesoderm cell populations. This process, which has long served as a paradigm for embryonic induction, is poorly understood at the molecular level. Pax6, a member of the paired domain family of transcription factors, has been identified as a key regulator of eye development in both vertebrates and invertebrates, and the regulatory pathways of eye development controlled by *Pax6* genes appear to be conserved throughout the metazoa (reviewed by Glaser et al., 1995; Hanson et al., 1995; Halder et al., 1995a,b; Zuker, 1995). The *Pax6* gene is expressed in essentially all vertebrate ocular structures, beginning in the early embryo with the acquisition of lens-forming bias in the anterior neural plate, and proceeding in sequence through the optic vesicle, lens, cornea, iris, and neural retina as these elements form (Walther and Gruss, 1991; Martin et al., 1992; Grindley et al., 1995; Davis and Reed, 1996; Koroma et al., 1997). In addition to the eye, *Pax6* is transcribed in mitotic cells of the spinal cord and the developing cortex of the central nervous system (CNS) and in the endocrine pancreas (Walther and Gruss, 1991; Turque et al., 1994; St-Onge, 1997; Sander et al., 1997). The tissue-specificity of *Pax6* expression suggests the existence of a highly coordinated system of transcriptional regulatory control elements.

It has been suggested that Shh or a closely related signaling molecule emanating from midline tissue in ventral forebrain either directly or indirectly inhibits the expression of *Pax6* in the eye (Macdonald et al., 1995). In ventral spinal cord, *Pax6* is one of several genes that mediate the ability of Shh to specify progenitor cell fate (Ericson et al., 1997). *Pax6* has also been shown to be subject to repression by Activin A in the spinal cord (Pituello et al., 1995). In *Drosophila* there are two *Pax6* homologs, *eyeless* (*ey*) and *twin of eyeless* (*toy*), with *toy* acting upstream of *ey* in *Drosophila* eye development since targeted expression of *toy* in imaginal discs induces *ey* expression (Czerny et al., 1997). In quail *Pax6*, two promoters have been identified as well as a phylogenetically conserved 216 bp region in intron 4 that can function as an enhancer in cultured

neuroretina cells (Plaza et al., 1995a,b). In the mouse *Pax6* gene, a lens placode control element has been recently identified and the element is conserved in human *PAX6* (Williams et al., 1998). To date, however, little else is established about the differential regulation of *Pax6* expression in the multiple tissues of the developing vertebrate eye.

To understand the regulation of *Pax6*, we have isolated the mouse *Pax6* gene, defined the structures and major transcriptional start sites of two promoters that reside within the gene and generated transgenic mice expressing a *lacZ* reporter gene under the control of three different sets of regulatory elements. Furthermore, we have tested whether the regulatory mechanisms controlling *Pax6* expression are conserved between mammals and insects. Our results show that the complex pattern of mouse *Pax6* expression is differentially regulated by two promoters, P0 and P1, acting in combination with distinct regulatory elements, and that some of the regulatory mechanisms that control *Pax6* expression are indeed conserved between mice and flies.

MATERIALS AND METHODS

Cloning and characterization of the murine *Pax6* gene

Hybridization screening of a mouse genomic library with *Pax6* cDNA probes yielded 9 overlapping clones which included exons 0-9, and a restriction map of the isolated genomic fragments was established (Fig. 1A). Three *Pax6* cDNA splice forms containing different 5′-UTRs were isolated from mouse α-TN4 lens cells by RT-PCR. These splice forms correspond to splicing of exons 0 and 2, exons 1 and 2 and a third form utilizing an exon designated exon 1′ that contains exon 1 and 2 and the intervening intron (Fig. 1A).

Mapping of transcription initiation sites

For RNase protection assays, a 605 bp *Bam*HI-*Hin*dIII genomic fragment for P0 and a 760 bp *Bam*HI-*Pst*I genomic fragment for P1 were subcloned into pBluescript II KS+ and used to synthesize antisense RNA probes (Fig. 1B). Hybridization was carried out at 50˚C and the protected fragments were separated on a 6% sequencing gel. For primer extension assays, an antisense oligonucleotide complementary to the region +154 to +121 of exon 0 or the region +201 to +168 of exon 1 cDNA was end-labeled with T4 polynucleotide kinase. Total RNA (50-100 μg) isolated from α-TN4 lens cells using RNAzol B (Biotecx Laboratories) was hybridized at 55˚C and the reaction was performed as described by Xu et al. (1994).

In situ hybridization

The P0- and P1-specific in situ probes were made from exon 0 or exon 1, respectively. PCR fragments containing 154 bp of exon 0 (from nucleotide position +1 to +154, as shown in Fig. 1C) or 201 bp of exon 1 (from nucleotide position +1 to +201, as shown in Fig. 1D) were subcloned into pBS KS+ and used to generate antisense riboprobes with T7 RNA polymerase. A *Pax6* 3′-UTR probe detecting all *Pax6* splice forms was also employed. Tissue section in situ hybridizations, high-stringency washing and RNase treatment were performed as described by Xu et al. (1997), except that hybridization was at 63°C for 16 hours and RNase treatment was at 37°C for 1 hour.

Reporter constructs

Constructs used for transgenic animals were produced in a multistep process. As indicated in Table 1, for P0, a 4 kb *Eco*RI fragment containing 3.3 kb of upstream region and exon 0 and approx. 600 bp of intron 1 (p3.3P0-lz), a series of 5′ deletion mutants containing various upstream regions (p2.7P0-lz, p2.35P0-lz and p2.3P0-lz), or a 2.2 kb *Hin*dIII fragment (p1.9P0-lz) were subcloned into the promoterless β-gal vector pNASSβ (Clontech) and used to generate transgenic mice. For P1, a 4 kb *Xba*I-*Pst*I fragment containing 3.8 kb of upstream region and exon 1 and approx. 15 bp downstream of exon 1 (p3.8P1-lz), and a series of 5′ deletion mutants containing various upstream regions (p3.3P1-lz, p3.1P1-lz, p2.9P1-lz and p2.0P1-lz) were subcloned into pNASSβ (and used to generate transgenic mice. Construct p0.53P1-lz contains 530 bp upstream, exons 1 and 2, and 2.5 kb of intron 2. To analyze the intron 4 region, a 500 bp region located in *Pax6* intron 4 was subcloned, sequenced and inserted into a *Xho*I site of construct p3.3P0-lz or p3.8P1-lz, and the resulting constructs pα3.3P0-lz or pα3.8P1-lz used to generate transgenic mice.

Generation of transgenic mice

Pax6 promoter constructs were linearized and gel purified by electroelution and phenol/chloroform extraction and resuspended in 10 mM Tris-HCl (pH 7.5), 0.25 mM EDTA. Transgenic animals were produced by injecting DNA into male pronuclei of fertilized oocytes of inbred FVB/N mice. Mice carrying the transgene were genotyped by PCR using tail DNA and primers to *lacZ* (forward primer 5′-GTTGCGCAGCCTGAATGGCG-3′, reverse primer 5′-GCCGTCACTCCAACGCAGCA-3′; the PCR product is 433 bp). The number of independent lines for each construct is indicated in Table 1. Transgene expression was analyzed in the F_1 embryos obtained by timed matings with wild-type FVB/N females taking the day of plug discovery as E0.5. For transient transgenic assays, embryos were isolated 13-14 days after injection using embryonic membranes for genotyping.

X-gal staining, BrdU pulse-labeling and immunohistochemistry

Embryos from E9.5-15.5 or eyes from E16.5 to adult were isolated in 1×PBS and fixed for 30 minutes in 2% paraformaldehyde, 0.01% sodium deoxycholate, 0.02% NP-40, 1× PBS buffer (pH 7.3). After rinsing with 1× PBS, specimens were stained overnight at room temperature in 1 mg/ml X-gal (Sigma), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 2 mM $MgCl_2$ in 1× PBS (pH 7.3). Stained samples were rinsed in 1× PBS, dehydrated through ethanol, cleared in xylene and embedded in paraffin. Histological sections were cut at 10 μm and counterstained with Ehrlich's hematoxylin.

To label retinal progenitors, timed pregnant mice at E12.5 and 13.5 were injected i.p. twice at 1-hour intervals with 5-bromodeoxyuridine (BrdU; Sigma) in PBS at 100 mg/kg and processed as described by Xiang (1998) except that sections were denatured with 1 N HCl.

For immunostaining, sections were cut at 5-8 μm and treated with 10% normal goat serum in PBS at room temperature for 2 hours prior to addition of antisera, then incubated at 4°C with primary antibody. Antibodies were obtained as follows: mouse monoclonal anti-BrdU (Sigma), guinea pig anti-insulin antiserum (Fitzgerald), rabbit anti-glucagon (Biodesign), biotinylated secondary antisera and Avidin reagents (Vector Laboratories). Anti-BrdU antibody was detected using biotinylated anti-mouse antiserum followed by HRP-coupled Avidin D and staining with diaminobenzidine (DAB) (Fig. 3G,N). Anti-glucagon antibody was detected using biotinylated anti-rabbit antiserum followed by HRP-coupled Avidin D and staining with DAB. Anti-insulin antibody was detected using biotinylated anti-guinea pig antiserum, followed by AP-coupled Avidin D and staining with Vector Red. Immunostained sections were cleared, mounted in Permount and viewed using DIC.

Cloning of the *Drosophila ey* intron 1 region

A genomic fragment containing *ey* intron 1 was cloned by PCR and sequenced (forward primer 2E, 5′-ggaaTTCATACTTCG-CCACAACTTACTACCATTTAACC-3′ located in the exon 2; reverse primer 3E, 5′-TACTTGCAGAATTCGAGAAATATCACATGGCCG-3′ located in the exon 3; Quiring et al., 1994). The positions of two transposon insertions that cause *ey* mutations (ey^R and ey^2; Quiring et al., 1994) were determined by cloning and sequencing the

genomic fragments from DNAs of ey^R (forward primer 5′-ATTTAGCCTTTTAGCTTT-3′ located in the *blastopia* element (Frommer et al., 1994) and the 3E reverse primer) and ey^2 (forward primer 5′-TTGCATTTCGTAGCTTGAAAGAAACACGTC-3′ located in the *doc* element (O'Hare et al., 1991; Driver et al., 1989), and the 3E reverse primer). ey^R and ey^2 flies were obtained from the *Drosophila* Stock Center, Bloomington, IL. The sequence of entire *ey* intron 1 from *Drosophila hydei* was determined after isolation of *D. hydei ey* cDNA by RT-PCR using primers to *D. melanogaster ey* exons 1 and 2, then PCR amplification of *D. hydei* genomic DNA using primers to sequences in *D. hydei ey* exons 1 and 2. GenBank Accession numbers for intron 1 sequences of *ey* are AF089733 for 1.2 kb region of *D. melanogaster* and AF098329 for 5.5 kb region of *D. hydei*.

Generation of transgenic flies and X-gal and antibody staining

The 1.2 kb *Sal*I intron 1 fragment of *Drosophila ey*, or 0.5 or 0.28 kb deletion fragments, were cloned into the *Bam*HI site in the polylinker 5′ to an *hsp*27 basal promoter in pETWnuclacZ (Table 2). The pETWnuclacZ vector contains *Drosophila hsp27* basal promoter sequences fused to a nuclear-tagged *lacZ* reporter in the vector pCaSpeR. *lacZ* constructs were injected into the pole cell region of *yw* embryos along with helper plasmid, and transgenic flies selected by eye color (Spradling, 1986).

For analysis of mouse *Pax6* fragments in transgenic flies, a 3 kb *Xba*I-*Xho*I fragment containing the P1 upstream regulatory region or the 500 bp intron 4 enhancer was inserted into a *Bam*HI site upstream

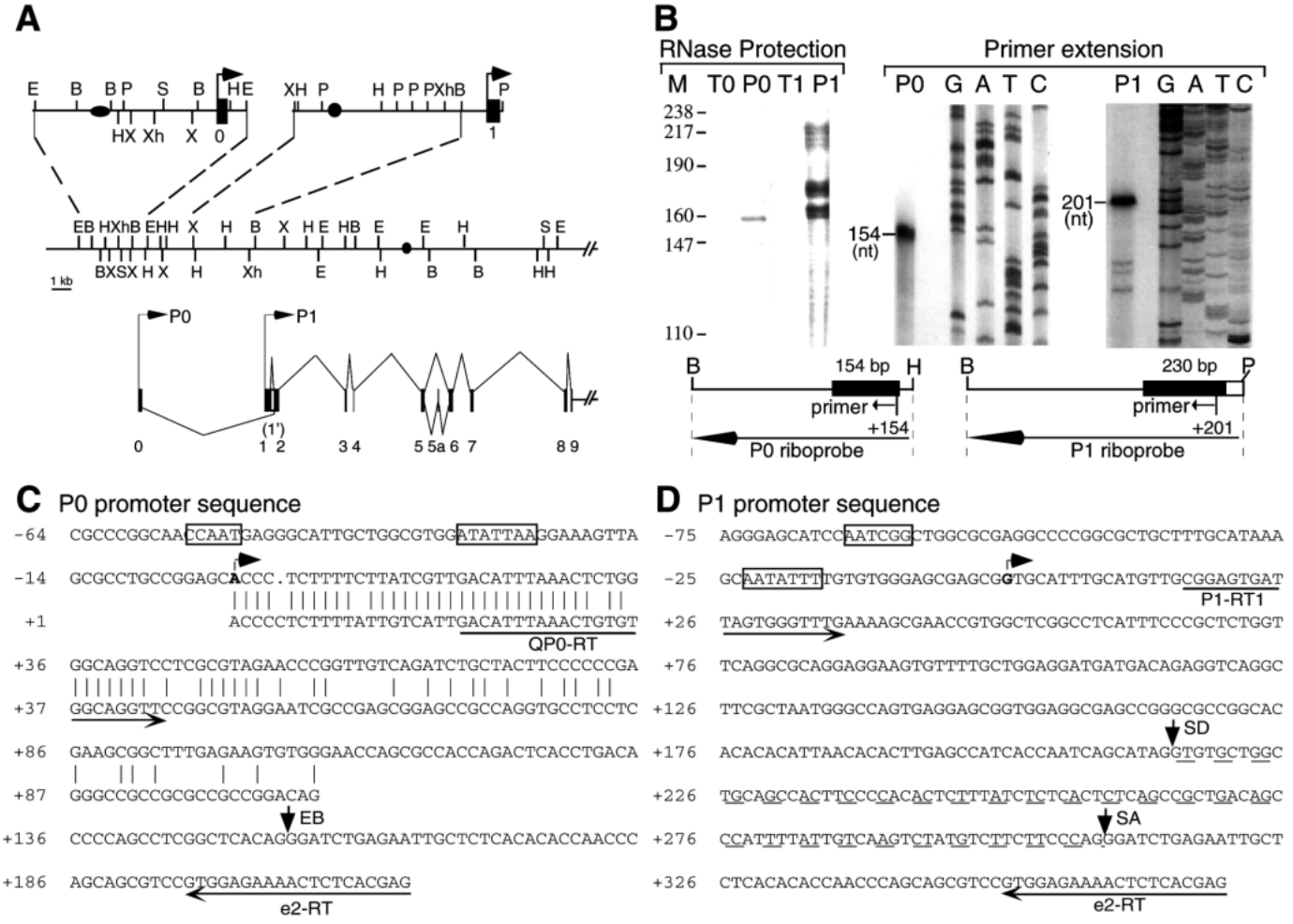


Fig. 1. (A) Partial genomic structure of the mouse *Pax6* gene. Transcriptional start sites and exons are shown. P0-initiated transcripts contain exons 0 and 2, while P1-initiated transcripts contain exons 1 and 2, with (exon 1′) or without the intervening intron. 'α' is an enhancer region within intron 4 (see text and Plaza et al., 1995b). B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xba*I; Xh, *Xho*I. (B) Transcriptional start sites of mouse *Pax6* transcripts. RNase protection: lane T0, P0 riboprobe hybridized to 50 μg yeast tRNA; lane P0, P0 riboprobe hybridized to 50 μg total RNA from mouse α-TN4 lens cells; lane T1, P1 riboprobe hybridized to 50 μg yeast tRNA; lane P1, P1 riboprobe hybridized to 50 μg total RNA from mouse α-TN4 lens cells. Size markers are indicated. Primer extension: 100 μg of total RNA from mouse α-TN lens cells was hybridized to the primers. Extension products and sequencing ladder are shown. The riboprobes and primers are schematically indicated. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I. (C) Sequence of the mouse P0 promoter region (upper sequence) and comparison to quail P0 (Plaza et al., 1995a). The transcriptional start site is indicated by an arrow and TATA-like and CAAT sequences are boxed. EB, exon boundary between exon 0 and exon 2. Exon 0 is not highly conserved except near the start site. (D) Sequence of the P1 promoter region. The transcriptional start site is indicated by an arrow. SD/SA, splice donor/acceptor between exon 1 and exon 2. Exon 1′ contains exon 1, the region between exon 1 and exon 2 (dashed-underline), and exon 2. The P1 promoter upstream sequence is highly conserved over a 1 kb region compared with human (Xu and Saunders, 1997). The GenBank accession numbers for the genomic fragments are: P0 promoter, AF008212 for 0.9 kb region from +0.3 to –0.6 kb and AF098639 for 2.1 kb region from –1.8 to –3.9 kb; P1 promoter, AF008211 for 1.2 kb region from +0.4 to –0.8 kb and AF098640 for 4.3 kb region from +0.4 to –3.9 kb; intron 4 region, AF008213.

of the *hsp27* basal promoter in pETWnuclacZ and used to generate transgenic flies (Table 2). X-gal staining in imaginal discs was performed as described (Hiromi and Gehring, 1987). For antibody staining of imaginal discs, larvae were dissected in cold PBS and fixed in PEM (100 mM Pipes, pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA, 4% formamide) for 30 minutes on ice. Discs were then washed 4 times for 15 minutes in PBT (PBS with 0.3% Triton X-100) on ice and blocked in PBTN (2% normal goat serum (Vector Laboratories) in PBT) for at least 30 minutes. Discs were incubated with primary antibody (mAb α-β-gal (Promega) 1:1000, or in some experiments, mouse anti-Dachshund 1:20, in PBTN at 4°C overnight, then washed six times at room temperature. Discs were stained according to directions in the Vectastain ABC kit (Vector Laboratories), using biotinylated or Cy2- or Cy3-conjugated secondary antibodies.

RESULTS

The mouse *Pax6* gene contains two 5′ promoters

Several distinct *Pax6* cDNAs containing either exon 0 or exon 1 at their 5′-ends were isolated (Fig. 1A). To determine whether the corresponding transcripts initiated from different promoters, their transcription start sites were determined by RNase protection and primer extension. RNase protection with exon 0- or exon 1-specific riboprobes yielded a 154 bp protected fragment for exon 0 and several protected fragments for exon 1, the longest being 230 bp (Fig. 1B). The assignment of initiation sites was confirmed by primer extension. An antisense oligonucleotide complementary to either exon 0 or exon 1 resulted in 154 nt or 201 nt extension products respectively (Fig. 1B). These results indicate that exon 0 and exon 1 transcripts are initiated from two distinct promoters, P0 and P1. The initiation of transcripts containing exon 0 is at an A, 30 bp downstream of the putative P0 TATA box, while that of transcripts containing exon 1 is at a G, 23 bp downstream of the putative P1 TATA box (Fig. 1C,D). Moreover, the P0 and P1 promoter regions function as promoters in cultured cells (data not shown).

P0- and P1-initiated transcripts are differentially expressed during ocular development

To study whether the mouse P0 and P1 *Pax6* promoters direct differential *Pax6* expression during eye development, we performed in situ hybridization with probes specific to either P0- or P1-initiated transcripts, as well as with a third probe located in the 3′-UTR region which detects all *Pax6* transcripts. P0 transcripts were observed abundantly in the lens placode at E9.5 (Fig. 2A). In contrast, P1 transcripts are abundant in both the lens placode and optic vesicle (Fig. 2B). Consistent with these results, a *Pax6* 3′-UTR probe detected expression in both the lens placode and optic vesicle with stronger expression in the lens placode (Fig. 2C). After E9.5, P0 transcripts increased in the retina, and by E13.5 became comparable in abundance and spatial distribution to P1 transcripts in lens and retina (data not shown). At E17.5, both P0 and P1 transcripts are strongly expressed in lens epithelial cells and developing neuroretina, but P0 transcripts are more abundant in the inner layer, while P1 transcripts are distributed uniformly (Fig. 2D,E). Lastly, in the developing corneal and conjunctival epithelia, P0 transcripts are more abundant than P1 transcripts, and both transcripts are also differentially distributed in CNS development (Fig. 2G,H and data not shown). The differential and overlapping expression of the two *Pax6* promoters suggests that their expression is controlled by both promoter specific regulatory elements, and by elements capable of interacting with both promoters with different efficiencies.

A regulatory element upstream of the *Pax6* P0 promoter directs expression in a subset of retinal progenitors and in the developing pancreas

To further investigate the tissue-specific expression of these two promoters, a series of *Pax6*-lacZ constructs were examined in transgenic mice (Table 1). For the P0 promoter, we first analyzed

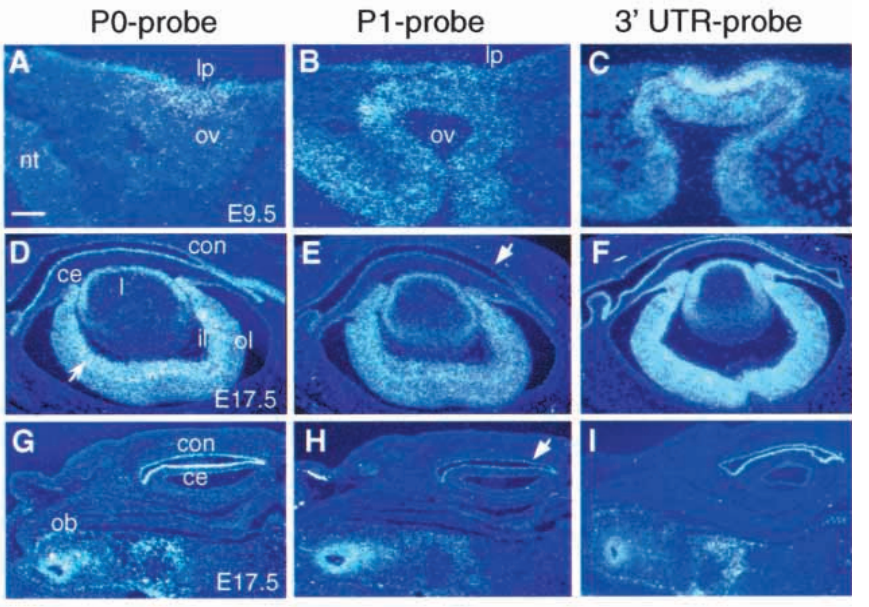


Fig. 2. P0- and P1-initiated *Pax6* transcripts are differentially expressed during eye development. (A-I) Radioactive in situ hybridizations of transverse sections showing specific expression of P0- and P1-initiated transcripts, and also total *Pax6* expression detected by a general 3′-UTR probe. (A) At E9.5, P0 transcripts are expressed abundantly in lens placode (lp), but at low or background levels in optic vesicle (ov). (B) P1 transcripts are detected in both structures. (C) Using the 3′ UTR probe, *Pax6* is expressed in both lens placode and optic vesicle at E9.5, but appears stronger in the lens placode, reflecting summation of the patterns shown in A and B. (D) At E17.5, P0 transcripts are observed in lens (l), in inner (il) and outer (ol) retinal layers, and in corneal and conjunctival epithelia (ce, con). In retina, P0 transcripts are more abundant in the inner layer (arrow). (E) P1 transcripts are observed in lens, uniformly in neural retina and weakly in corneal and conjunctival epithelium (arrow). (F) Total *Pax6* expression in lens, corneal and conjunctival epithelium and neuroretina; retinal expression is stronger in the inner than in the outer layer, reflecting summation of the patterns shown in D and E. (G) The expression of P0 transcripts in the corneal and conjunctival epithelium is higher than that of P1 transcripts, indicated by an arrow in H. For comparison, P0 and P1 transcripts are detected at equal levels in olfactory bulb (ob). (I) Total *Pax6* expression in corneal and conjunctival epithelium. Scale bar, (A-C) 40 μm; (D-I) 100 μm.

the *lacZ* expression during eye development from construct p3.3P0-lz containing 3.3 kb of upstream sequence, exon 0 and part of intron 1 (Table 1). *lacZ* expression was weakly detected in a few cells of the central retina at E11.5 (data not shown), but by E12.5-13.5, expression became stronger and was restricted to a subpopulation of retinal cells (Fig. 3A-D). This expression pattern, present in 6 of 7 permanent independent transgenic lines, reproduces a subdomain of endogenous *Pax6* expression in the retina, since *Pax6* is expressed in all retinal cells at these stages (data not shown; Walther and Gruss, 1991). By E15.5, the expression remained only in a few retinal cells (Fig. 3E,F) and became undetectable thereafter. Based on the timing of expression in relation to known birthdating data, the *lacZ*-positive cells represent a subpopulation of retinal progenitors. To test this hypothesis, S-phase cells in E12.5 and 13.5 retinas were pulse-labeled with BrdU for 2 hours and double-labeled with X-gal and BrdU antibody. In retinas from both stages, approximately 40% of *lacZ*-positive cells (33/84 cells at E12.5) incorporated BrdU (Fig. 3G), indicating that they are retinal progenitors. The identification of a specific element that directs expression in a subset of retinal progenitors suggests that not all retinal progenitors are equivalent.

To further define the regulatory element in the P0 promoter upstream region, a series of 5′ deletion constructs were analyzed in transgenic mice. Deletion of 0.95 kb of 5′ flanking sequence from construct p3.3P0-lz (construct p2.35P0-lz) had no effect on *lacZ* expression in the eye (Table 1). However, deletion of an additional 50 bp of 5′ flanking region (construct p2.3P0-lz) resulted in a significant reduction of *lacZ* expression in the eye, with only very weak staining (data not shown). Further deletion of 0.4 kb of 5′ flanking region abolished the expression in the eye, indicating that the region between 2.35 and 1.9 kb upstream of the P0 promoter is critical for directing *Pax6* expression in the retina.

In addition to the eye, the same P0 regulatory element also directs strong *lacZ* expression in the developing pancreas (Fig. 4, Table 1). Initial *lacZ* expression was detected at E9.5, coincident with the appearance of the dorsal pancreatic bud and pancreatic progenitor cells (Fig. 4A). At E10.5, *lacZ* is expressed in the dorsal and ventral pancreatic buds (Fig. 4B and data not shown). As the pancreatic epithelium grows and develops into a branched structure, *lacZ* expression persists (Fig. 4C,E,F). By E15.5 when distinct exocrine and endocrine compartments can be identified, expression of *lacZ* became restricted to the endocrine cells (Fig. 4D). In adult pancreas, *lacZ* expression was detected in peripheral cells in the islets of Langerhans (data not shown).

The mouse islet comprises four distinct α, β, δ and γ endocrine cell populations, which produce glucagon, insulin, somatostatin and pancreatic polypeptide (PP), respectively. *Pax6* is expressed in pancreatic epithelial cells and later on in all mature endocrine cells and is required for differentiation of all four cell types (St-Onge et al., 1997, Sander et al., 1997). To determine which endocrine cell types express *lacZ*, we performed double labeling with glucagon or insulin. Cells expressing both *lacZ* and glucagon could be detected as early as E10.5 (data not shown). At E13.5, *lacZ*-positive cells expressed glucagon strongly, while only a few *lacZ*-positive cells expressed insulin (Fig. 4E,F). Some *lacZ*-positive cells coexpressed neither glucagon nor insulin, suggesting they could be either δ or γ cells. Thus, the same P0 upstream regulatory region required for *Pax6* expression in retinal progenitors is also necessary for *Pax6* expression in pancreatic progenitors at E9.5 and, thereafter, in differentiating α and β cells.

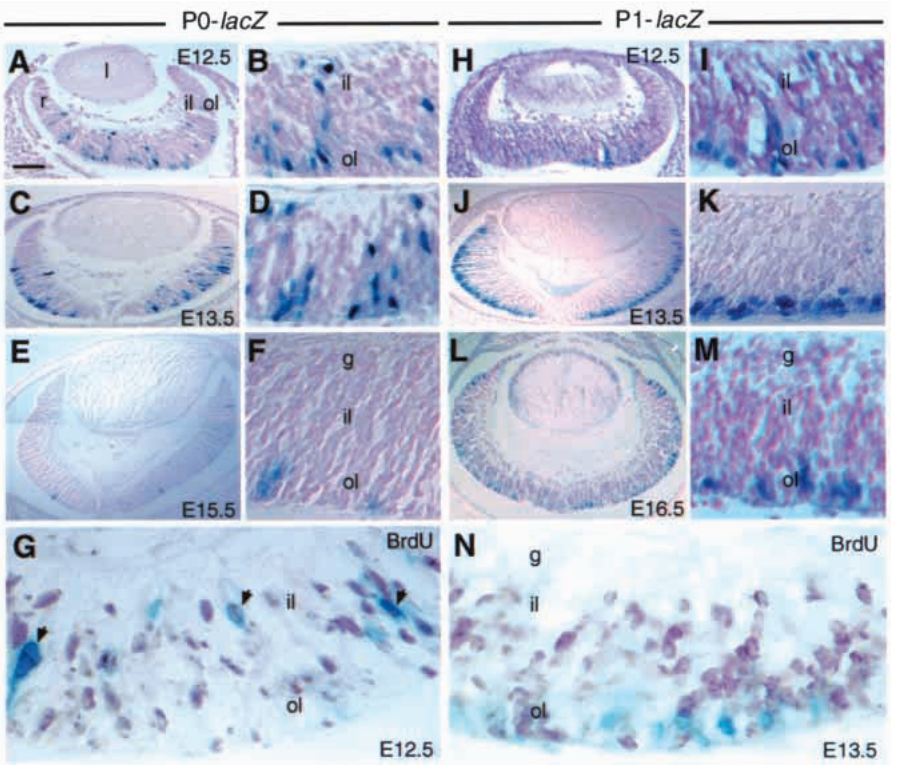
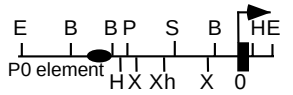


Fig. 3. P0 and P1 regulatory elements direct *lacZ* expression in distinct retinal cell populations. (A-N) Transverse sections showing *lacZ* expression during eye development. At E11.5, *lacZ* expression directed by the P0 element is weakly detected in a few central retinal cells (data not shown). At E12.5 (A,B) and E13.5 (C,D), *lacZ* expression is stronger in a subpopulation of retinal cells in the ventricular zone (E12.5) and in the inner (il) and outer (ol) retinal layers (E13.5). (E,F) Only a few retinal cells still express *lacZ* by E15.5. (G) Retinal section from BrdU-labeled E12.5 embryo double labeled with X-gal (blue) and anti-BrdU antibody (purple). Approximately 40% of the *lacZ*-positive cells (33/84) are labeled by BrdU antibody. Arrows indicate retinal cells that are clearly co-stained. (H,I) *lacZ* expression directed by the P1 element is first detected at E12.5 in a subset of retinal cells. (J,K) Most *lacZ*-positive cells are in the outer aspect of the retina at E13.5, corresponding to the prospective photoreceptor layer. (L,M) *lacZ*-positive cells are reduced in number in the retinal outer layer at E16.5 and expression is lost at E18.5. (N) Retinal section from BrdU-labeled E13.5 embryo double labeled with X-gal (blue) and anti-BrdU antibody (purple). Most of the *lacZ*-positive cells are not labeled with BrdU and are therefore postmitotic. Abb: l, lens; g, ganglion cell layer. Scale bar, (A,H,C,J) 64 μm; (B,I,D,K,F) 8 μm; (L) 96 μm; (M) 12 μm; (G,N) 5 μm.

Table 1. Ocular expression of *Pax6*-lacZ transgenes in mice

Construct		Ocular expression	Ocular expression/ total no. transgenic lines
A. P0			
p3.3P0-lz	-3.3	+; retinal progenitors (and pancreas)	6/7
p2.7P0-lz	-2.7	+; retinal progenitors (and pancreas)	6/10
p2.35P0-lz	-2.35	+; retinal progenitors (and pancreas)	10/18*
p2.3P0-lz	-2.3	+/-; retinal progenitors (and pancreas)	3/5*
p1.9P0-lz	-1.9	---	0/5*
pα3.3P0-lz	-3.3	+; amacrine cells, ciliary body and iris	5/9
B. P1			
p3.8P1-lz	-3.8	+;developing photoreceptors	5/6
p3.3P1-lz	-3.3	+; developing photoreceptors	4/7*
p3.1P1-lz	-3.1	+; developing photoreceptors	5/8*
p2.9P1-lz	-2.9	-; ectopic expression only	0/9
p1.3idP1-lz	-3.3	+; developing photoreceptors	1/9*
p0.9idP1-lz	-3.1	+; developing photoreceptors	2/9*
p2.0P1-lz	-2.0	-; ectopic expression only	0/10*
pP0.53P1-lz	-0.53	---	0/7*
pα3.8P1-lz	-3.8	+; amacrine cells, ciliary body and iris	2/2

Constructs containing various promoter regions were used for making transgenic mice (see Methods). In construct p1.3idP1-lz, 1.3 kb fragment between -0.7 and -2.0 was deleted. In construct p0.9idP1-lz, 0.9 kb fragment between -2.0 and -2.9 was deleted. +, strong expression; +/-, very weak expression; -, no ocular expression. *Transient transgenic assays were performed for these constructs at E13.5-14.5; embryos were dissected out from the recipients 13 or 14 days after injection. The total number of independent transgenic lines for each construct is indicated. GenBank accession numbers for P0 and P1 promoter regions are indicated in Methods.

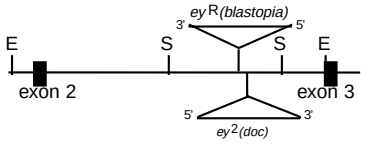
A regulatory element upstream of the *Pax6* P1 promoter directs expression in developing photoreceptors

For the P1 promoter, we first analyzed *lacZ* expression during eye development from construct p3.8P1-lz containing 3.8 kb upstream of P1 and exon 1 (Table 1). *lacZ* expression was first detected in the eye at E12.5 in a subpopulation of retinal cells (Fig. 3H,I). At E13.5, *lacZ* expression localized to the outer neuroretina corresponding to the prospective photoreceptor layer and by E16.5, the *lacZ*-positive cells within the outer layer were reduced (Fig. 3J-M). By E17.5, *lacZ* was only detected in cells in the outermost portion of the retina, with more positive cells peripherally than centrally, and was undetectable after birth (data not shown). In addition, the *lacZ* expression was also observed in a subset of nasal epithelial cells during nasal development (data not shown).

To further identify the regulatory element in the P1 promoter upstream region, a series of 5′ and internal deletion constructs were analyzed in transgenic mice. As indicated in Table 1, 5′ deletion of up to 0.7 kb from the –3.8 kb construct had no effect on *lacZ* expression. Further deletion of 0.2 kb of 5′ flanking region, however, resulted in loss of *lacZ* expression in the eye, indicating that the P1 regulatory element resides within 3.1 to 2.9 kb upstream of the promoter (see Table 1). Constructs that contained internal deletions of either a 1.3 kb *Hind*III-*Xho*I fragment or a non-overlapping 0.9 kb region of 5′ flanking region retained *lacZ* expression in the eye. This spatiotemporal pattern of *lacZ* expression correlates with the period of cone cell genesis (between E12 to E18) and the early expression of

Table 2. Expression of *ey*-lacZ and *Pax6*-lacZ transgenes in mice and flies

ey

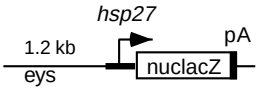
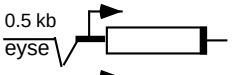
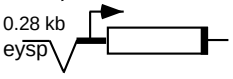
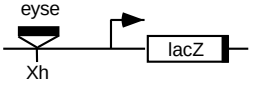
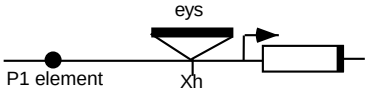
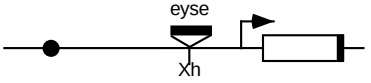
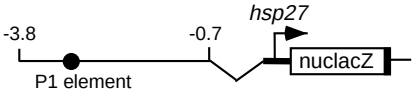
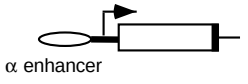



```
Dm 5'- TTCATACGGACGGACGGACATCGCTAACTTGACTCGGTTATATTTTATTTTTTGCTGTTA 60

       CATACTGTTAATACGAATCTAGTATACCCTTTTATTCTATGAGTAACGGGTATAAGTATA 120
                                  blastopia
       TGAACGTATAAGTATGTATGTACATGTATCTATGTTTTTCTGCTGCTCCGATGATTTTCT 180
                                                              doc
       TTCCAATAAAGCGGTAAATTGATACGGGGACTTTAGGATTTTACTTTCTATAAGTCCAAA 240
                 ||||||||||  || |||||||| | |       |     ||
Dh               AAATTGATACGCAGATTTGGGGATTATCC.......CACAATCAA

Dm TACTCGGAAATTTGTATAAAACCCTTCCATTTTTATGCTCGACCTGAGCGACGAGTGCTG 300
   |||| |||||||||| | ||| ||          ||| ||||| |    ||||| |  ||
Dh TACTAGGAAATTTGTTTCAAATCC.........ATGGTCGACTT....GACGATTTGTG

Dm CCCATTT....AGCCTTTTAGCTTTTCCCTTT 328
   |    ||    |  ||||| | ||  ||  ||
Dh CGACCTTTCAAAATCTTTTTGGTTGACCGCTT -3'
```

Construct	Eye and CNS expression	Eye and CNS expression/ total no. transgenic lines
A. *ey* enhancer in flies		
peys-nlz 	+; eye primordia in embryos, in eye disc at third instar larval stage	9/11
peyse-nlz 	same as above	12/15
peysp-nlz 	same as above	7/8
B. *ey* enhancer in mice		
peyse1.7P0-lz -1.7 	+; retina and CNS	5/8*
peys3.8P1-lz -3.8 	+; retina, tip of the optic cup, CNS +/-; lens	2/3* 2/3*
peyse3.8P1-lz -3.8 	+; retina and CNS +; lens	3/5 1/5
C. *Pax6* cis-elements in flies		
p3.1mp1-nlz 	*Drosophila* eye imaginal disc	2/9
p0.5α-nlz 	*Drosophila* optic lobe	9/14

The 1.2 kb *Sal*I intronic fragment, a 500 bp or a 280 bp (from nucleotide position 1 to 280, shown as *Dm* sequence) deletion fragment of the *Drosophila ey* gene, which contains the *blastopia* and *doc* transposon insertions and flanking sequence, was inserted upstream of *hsp27* promoter, respectively, and tested in transgenic flies. The 1.2 kb *Sal*I intronic fragment or a 500 bp deletion fragment was also inserted into a *Xho*I site of constructs p3.8P1-lz or p1.7P0-lz, respectively, and tested in transgenic mice.
+, strong expression; +/-, weak expression; -, no expression. *Transient transgenic assays were performed for the constructs at E13.5; embryos were dissected out from the recipients 13 days after injection. The total number of independent transgenic lines for each construct is indicated. *Dm, Drosophila melanogaster,* GenBank accession number AF 089733 for 1.2 kb fragment; *Dh, Drosophila hydei,* GenBank accession number AF 098329 for 5.5 kb fragment.

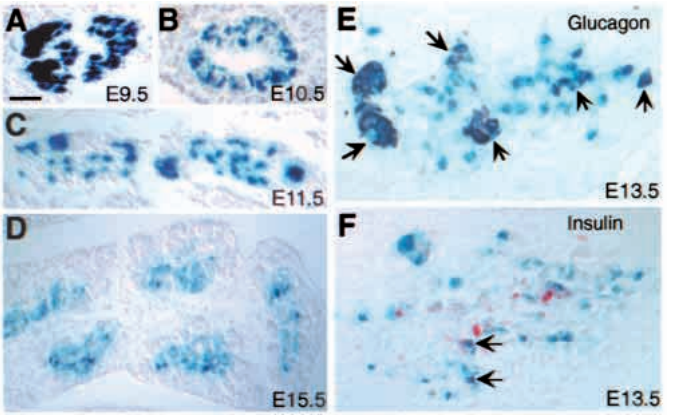


Fig. 4. The P0 regulatory element directs *lacZ* expression in the developing pancreas. **(A)** A transverse section shows strong β-gal activity in the dorsal pancreatic bud at E9.5. (B) Sagittal section showing *lacZ* expression in pancreatic epithelium at E10.5. (C) Transverse section showing *lacZ* expression in pancreatic epithelium as it develops into a branched structure at E11.5. (D) *lacZ* expression in developing pancreas is restricted to endocrine cells at E15.5 when distinct exocrine and endocrine compartments can be identified. (E) *lacZ*-positive cells (blue) express glucagon (purple, arrows) at E13.5. (F) Only a few *lacZ*-positive cells express insulin (red, arrows) at E13.5. Scale bar, (A) 40 μm; (B) 25 μm; (C) 80 μm; (D) 96 μm; (E,F) 60 μm.

a photoreceptor-specific homeobox gene *Crx* (Furukawa et al., 1998; Freund et al., 1998), suggesting that the *lacZ*-positive cells could be differentiating cone cells. To determine whether the *lacZ*-expressing cells are pre- or post-mitotic, E12.5 and E13.5 embryos were pulse-labeled with BrdU and the retinas double-stained with X-gal and anti-BrdU antibody. No retinal cells were found to be positive for both β-gal and BrdU (Fig. 3N). Although we cannot exclude the possibility that a small number of *lacZ*-positive cells are mitotic, we conclude that a *Pax6* P1 upstream element is sufficient to direct expression in a specific subset of differentiating postmitotic photoreceptors.

An intron 4 enhancer confers expression in amacrine cells, ciliary body and iris

A phylogenetically conserved 216 bp intronic retinal enhancer of quail *Pax6* (*Pax-QNR*) has been previously identified in cultured quail neuroretina (QNR) cells (Plaza et al., 1995a,b). To test whether this region directs retina-specific expression in vivo, a 500 bp mouse fragment within intron 4 (α enhancer; see Fig. 1A) containing the conserved 216 bp region was subcloned and inserted upstream of either the P0 or P1 promoter in different orientations and assayed for expression in transgenic mice (Table 1). Both P0- and P1-*lacZ* constructs containing the intron 4 region showed identical expression patterns in the eye distinct from those conferred by the P0 and P1 upstream elements.

The intron 4 containing transgenes were first expressed in the developing eye at E12.5 in a subpopulation of retinal cells (Fig. 5A,B). At E15.5, *lacZ* was strongly expressed in the peripheral retina, but in the central retina, only a few *lacZ*-positive cells were found in the outer layer (Fig. 5C and data not shown). In the peripheral retina, *lacZ* is expressed in both outer and inner layers and particularly strongly at the anterior tip of the optic cup which gives rise to the ciliary body and iris (Fig. 5C, arrows). The observed pattern of ciliary body and iris expression accurately defines elements of endogenous *Pax6* expression (see Fig. 2). At E17.5, the *lacZ* expression remains strong in the anterior tip of the optic cup, in some cells in the outer layer and also in amacrine cells as well as their processes in the inner plexiform layer (Fig. 5D,E). At P2-P4 and in the adult eye, expression was observed in the ciliary body and iris and in the amacrine cells as well as their processes in the inner plexiform layer of the retina (Fig. 5F and data not shown). Since the *lacZ* vector does not have a nuclear localization signal, it is likely that the X-gal staining in amacrine cells is cytoplasmic and can diffuse into the inner plexiform layer. Thus, our results show that the 500 bp intron 4 region can function with either the P0 or P1 promoter as an orientation-independent, in vivo regulatory enhancer element capable of directing *Pax6* expression in amacrine cells, ciliary body and iris.

A *Drosophila ey* eye regulatory region accurately reproduces *Pax6* eye and CNS expression in transgenic mice

Although the *Pax6*-controlled eye-regulatory hierarchy is partly conserved between vertebrates and invertebrates (Halder et al., 1995a,b; Zuker, 1995), it is unknown whether the regulation of *Pax6* is also evolutionary conserved. To examine this idea, we isolated and assayed a potential *Drosophila ey* regulatory region, first in transgenic flies to verify that it could

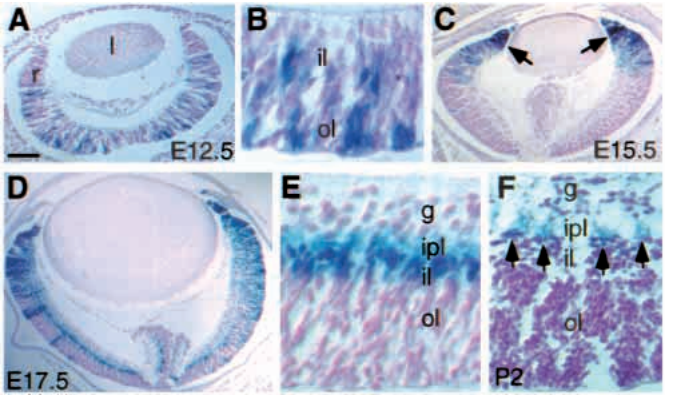


Fig. 5. A 500 bp intron 4 enhancer region directs *lacZ* expression in amacrine cells, ciliary body, iris and lens germinative zone during eye development. Transverse sections are shown. (A,B) lacZ is expressed in a subpopulation of retinal cells at E12.5. l, lens; r, retina. (C) At E15.5, there is strong *lacZ* expression in peripheral retina (arrows) while only a few cells are stained in the outer portion of the central retina (data not shown). (D,E) At E17.5, *lacZ*-positive cells are in peripheral retina in both inner and outer layers. In the central retina, some *lacZ*-positive cells are present in the outer layer (ol), but fewer than in peripheral retina. The *lacZ* expression in the inner layer (il) is in amacrine cells and their processes in the inner plexiform layer (ipl). Some *lacZ*-positive cells in the ganglion cell layer (g) are probably displaced amacrine cells (data not shwon), since *lacZ* is not expressed in ganglion cells. (F) *lacZ* is expressed in amacrine cells and their processes in the inner plexiform layer at P2. The staining remains in amacrine cells and their processes in adult eyes, and is also present in the ciliary body and iris at P2 and in the adult eye (data not shown). No expression is observed in ganglion cells (g), the outer nuclear layer (ol) or in the corneal epithelium at any time. Scale bar, (A) 60 μm; (B) 10 μm; (C) 80 μm; (D) 96 μm; (E) 16 μm; (F) 20 μm.

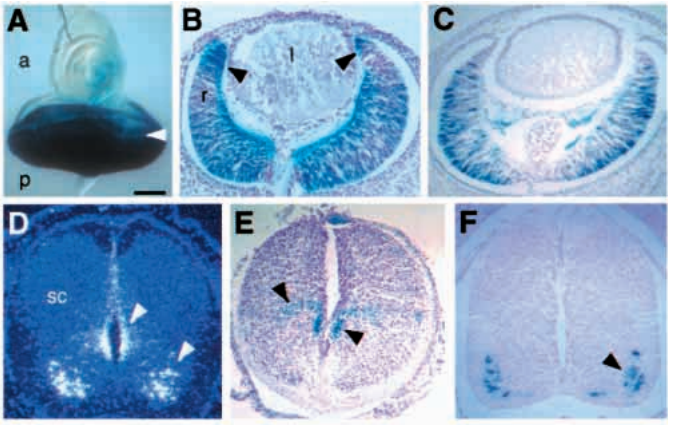


Fig. 6. The eye regulatory element of *Drosophila ey* gene directs eye and CNS expression in transgenic mice. (A) The 500 bp eye enhancer located in *ey* intron 1 drives *lacZ* expression in the eye disc at the third larval instar. The position of the morphogenetic furrow is indicated (arrowhead). Sectioning of anti-β-galatactosidase stained eye discs shows that the *ey* transgene is expressed appropriately anterior to the furrow in epithelial progenitors, but is expressed ectopically posterior to the furrow in the peripodial membrane and apically located photoreceptor precursors (data not shown). a, anterior; p, posterior. (B) Transverse section showing *lacZ* expression of a 1.2 kb intronic *ey* fragment and mouse P1 promoter in developing mouse eye at E13.5 (Table 2). *lacZ* is expressed throughout the neuroretina and strongly in the peripheral optic cup region fated to become ciliary body and iris (arrows). Some lens epithelial cells are also weakly stained (data not shown). (C) Transverse section showing *lacZ* expression driven by a 500 bp intronic *ey* fragment inserted upstream of minimal P0 promoter in a subset of retinal cells at E13.5 (Table 2). Similar expression was also detected with the same *ey* fragment inserted upstream of the P1 promoter. (D) In situ hybridization showing endogenous mouse *Pax6* expression in a transverse section of E13.5 spinal cord (sc). *Pax6* is expressed in the ventral ventricular zone and in migratory cells (arrowheads). (E,F) E13.5 transverse sections showing *lacZ* expression driven by the 1.2 kb *Sal*I intronic *ey* fragment at two different levels of the spinal cord. Expression is detected in the ventricular zone (E), in migratory cells (F), or both, depending on section level, reproducing endogenous *Pax6* expression. Scale bar, (A) 60 μm; (B,C) 80 μm; (D-F) 40 μm.

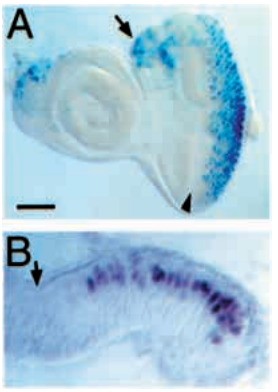


Fig. 7. Enhancer activity of the mouse *Pax6* P1 upstream region in developing *Drosophila* photoreceptors. (A) X-gal stained 3rd instar larval eye-antennae disc from a transgenic fly line expressing the mouse P1 upstream region-*hsp27* transgene. The position of the morphogenetic furrow is indicated by an arrowhead. Positive staining is present in prospective ocelli (arrow) and in individual ommatidia commencing several rows posterior to the furrow. (B) Transverse section of an anti-β-galactosidase-stained 3rd instar eye disc showing expression in developing photoreceptors. The arrow indicates the position of morphogenetic furrow. Anterior is to the left. Scale bar, (A) 70 μm; (B) 12 μm.

indeed function as an enhancer, and then in transgenic mice to test for functional conservation.

In *Drosophila*, two transposon insertions within the first intron of the *ey* gene result in mutant alleles, ey^2 and ey^R, which are characterized by a loss of expression in optic primordia and eye disc but not in brain (Quiring et al., 1994). We determined that the transposon integration sites in ey^2 and ey^R genomic DNAs reside 84 bp apart (Table 2). Sequence comparison of equivalent regions in *D. melanogaster* and *D. hydei* revealed two blocks of high sequence conservation flanking the *doc* transposon integration site (Table 2). Neither conserved sequence, however, conforms to Toy binding sites recently identified in this region (M. Busslinger, personal communication). 1.2 kb *Sal*I, 0.5 or 0.28 kb fragments containing this region were then inserted upstream of an *hsp27* basal promoter and analyzed for expression in the third instar eye-antenna imaginal disc of transgenic flies (Table 2). With respect to staining in the eye disc, the behavior of all three fragments was identical. *lacZ* expression was detected in cells anterior to the morphogenetic furrow and more strongly in cells posterior to furrow (Fig. 6A; also see Table 2), in agreement with previous observations for a larger 3.6 kb intron 1 region of *ey* (Quiring et al., 1994; Halder et al., 1998). These results delimit the *ey* eye enhancer region in transgenic flies to a 280 bp region encompassing the *doc* and *blastopia* insertions (Table 2).

Next, to test whether this regulatory region of *Drosophila ey* can function in mammals as part of a conserved mechanism for regulating *Pax6* expression, the 1.2 kb *Sal*I and 500 bp *ey* deletion fragments were inserted upstream of either the mouse *Pax6* P0 or P1 promoters and analyzed in transgenic mice (Table 2). Remarkably, both P0 and P1 transgenes containing either *ey* fragment directed *lacZ* expression in the eye (Fig. 6B,C). When inserted upstream of a P1 promoter also containing the P1 element, the 1.2 kb *Sal*I fragment directed *lacZ* expression throughout most of the neuroretina with particularly strong expression in the peripheral optic cup (Fig. 6B, arrowheads). The peripheral optic cup expression is similar to that observed for transgenic lines containing the intron 4 enhancer element (compare Figs 5C and 6B). Some lens epithelial cells were also weakly stained and in addition to the eye, β-gal activity was also detected in the optic recess (data not shown). The smaller 500 bp *ey* fragment also directed *lacZ* expression in the eye, but expression was restricted to a subset of retinal cells. Similar results were obtained regardless of whether the 1.2 or 0.5 kb fragments were inserted upstream of the P1 promoter, also containing the P1 upstream element, or upstream of a minimal P0 promoter that alone cannot direct reporter gene expression (Table 2; Fig. 6C).

In addition to the eye, *lacZ* expression was also observed during CNS development (Fig. 6D-F and data not shown) where it also faithfully reproduced aspects of endogenous mouse *Pax6* expression. In the spinal cord, *Pax6* is normally

expressed in the ventral neural tube and in mitotic migratory neuroblasts (Fig. 6D). The 1.2 kb *ey*-directed *lacZ* transgene is also expressed in the ventral neural tube and in mitotic migratory neuroblasts (Fig. 6E,F). These results indicate that the *Drosophila ey* eye-enhancer region contains both retinal and CNS enhancer elements. Moreover, the regulatory mechanisms acting through the *cis*-acting sequences present within this regulatory region appear to have been sufficiently conserved in evolution to permit accurate function in mice.

Lastly, we also analyzed whether the regulatory mechanisms responsible for mouse *Pax6* eye expression can operate in *Drosophila*. A 3.1 kb *Xba*I-*Xho*I fragment upstream of the P1 promoter containing the mouse photoreceptor element was cloned upstream of an *hsp27* basal promoter and analyzed in the *Drosophila* eye imaginal disc at the third larval instar. The P1-lacZ transgene is expressed in prospective ocelli and in differentiating photoreceptor clusters posterior to the morphogenetic furrow (Fig. 7A,B). Thus, although the mouse *Pax6* P1 element does not reproduce endogenous fly *ey* expression which is normally restricted to anterior of the morphogenetic furrow, it does direct expression in developing photoreceptors in both species. In addition, when assayed in transgenic flies, the mouse *Pax6* intronic enhancer directs *lacZ* expression in two discrete glial cell populations in the medullary cortex of the optic lobe (data not shown). These results indicate that the mouse *Pax6* P1 and intronic regulatory regions can function as enhancers in the *Drosophila* visual system.

DISCUSSION

Mammalian *Pax6* is controlled by multiple distinct promoters and regulatory elements

Our results describe the expression capabilities of two promoters and three regulatory regions in the mouse *Pax6* gene; of the three regulatory regions, two are novel and none have been previously analyzed in vivo. In the quail *Pax6* gene, no differences were observed in the spatial distribution of P0 and P1 initiated transcripts, and only P1 transcripts were found in the pancreas (Plaza et al., 1995a). In contrast, our results clearly show that in mouse embryos P1 and P0 transcripts are differentially expressed in the developing eye and CNS and that P0-initiated transcripts are very likely to be expressed in the pancreas, since a pancreatic regulatory element resides upstream of the P0 promoter. In quail, a neuroretina-specific enhancer element, denoted the α region, has been suggested to act in QNR cells specifically on the P0 promoter (Plaza et al., 1995b). In contrast, in vivo, the homologous mouse *Pax6* intron 4 enhancer acts equally well with either the P0 or P1 promoter. However, it should be noted that the transgenic approach employed here may not be capable of discerning subtle effects related to the position or interdependence of individual regulatory elements. Considered with the recent identification of a *Pax6* lens and corneal enhancer (Williams et al., 1998), and a forebrain regulatory element upstream of the human *PAX6* P1 promoter (T. Glaser and R. M., unpublished data), our identification of additional elements controlling *Pax6* retinal, ocular and pancreatic expression means that many of the elements controlling the tissue-specific expression of *Pax6* are accounted for.

An interesting feature of the P0 and P1 promoters is that their differential expression is not absolute. For example, although P0-initiated transcripts predominate in corneal and conjunctival epithelia, P1-initiated transcripts are still detectable. One possibility is that the two *Pax6* promoters arose by intragenic duplication and thus might contain similar tissue-specific regulatory elements. However, sequence comparison reveals little similarity between the P0 and P1 proximal promoter regions. Therefore, a more attractive explanation is that expression of both promoters is influenced by common regulatory elements which act on both promoters but with different efficiencies. The intron 4 and the P0 and P1 upstream elements are strong candidates for such roles.

The intronic and P0 and P1 regulatory elements direct *lacZ* expression in distinct retinal and ocular cell populations. The P0 regulatory element is currently delimited to a small region 2.3 kb upstream of the P0 promoter. Consistent with the results from BrdU labeling experiments, the onset and location *lacZ* expression directed by the P0 regulatory element suggests that the *lacZ*-positive cells are retinal progenitors. Mouse *Pax6* is expressed in all retinal progenitor cells at early stages; therefore, expression of the P0-lacZ transgene is faithful to a subset of endogenous *Pax6* expression (Walther and Gruss, 1991; Davis and Reed, 1996). Retinal progenitors are multipotent, with the onset of differentiation commencing with terminal mitosis (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Belecky-Adams et al., 1996). The identified P0 element could therefore indicate either that there are different types of multipotent progenitors or that, alternatively, some restriction in cell fate occurs even in progenitors. Fate mapping will be required to definitively address this issue. Significantly, a 50 bp deletion from –2.35 to –2.3 kb resulted in markedly reduced but still detectable transgene expression in both retina and pancreas, suggesting that sequences immediately flanking –2.3 kb are critical for directing *lacZ* expression. Thus far, we have not resolved the elements directing retinal and pancreatic expression. This raises the interesting possibility that the same regulatory factors may control *Pax6* expression in both retinal and pancreatic progenitors.

Recently, an evolutionarily conserved 341 bp mouse *Pax6* regulatory element was identified that controls lens placode and corneal ectoderm expression (Williams et al., 1998). This enhancer resides between 3.9 and 3.5 kb upstream of the P0 promoter, just upstream of our longest 3.3 kb P0 construct. The identification of a *Pax6* lens placode enhancer upstream of the P0 promoter is consistent with our results demonstrating that P0-initiated transcripts are preferentially expressed in lens placode and corneal epithelium. However, a discrepancy between our results and those of Williams et al. is that retinal and pancreatic expression from their P0 transgenes was not detected. Although technical differences relating to staining time or the embryonic stages examined may explain this, another explanation is that our P0 transgenic constructs also contain exon 0 and part of the adjacent intron (see Fig. 1C; also Methods). These sequences are not present on the transgenes assayed by Williams et al. (1998). Thus, even though the requisite element identified by our 5′ deletion analysis maps to a specific region upstream of P0, exon 0 may also contain *cis*-regulatory elements essential to *Pax6* retinal and pancreatic expression. In this case, two distinct regulatory elements

separated by over 2 kb would act cooperatively to reproduce endogenous pancreatic and a subset of retinal *Pax6* expression.

The P1 regulatory element resides between 3.1 and 2.9 kb upstream of the P1 promoter. This element directs *lacZ* expression in postmitotic retinal cells that migrate to the outermost aspect of the retinal outer layer, coinciding temporally and spatially with cone cell genesis between E11 and E18. Although cone cells constitute only about 3% of the total photoreceptor cell population and the P1 transgene is expressed in a majority of outer layer cells at E13.5, this could reflect a maximal level of cone differentiation prior to the onset of rod differentiation, which mainly occurs postnatally (Sidman, 1961; Carter-Dawson and LaVail, 1979). Consistent with the fact that *Pax6* is not expressed in differentiated photoreceptors (Koroma et al., 1997; Belecky-Adams et al., 1997), *lacZ* expression was not observed after birth when most rod photoreceptors are born; nor was transgene expression detected in other retinal cell types. Furthermore, the P1-lacZ transgene expression correlates with the early expression pattern of a cone and rod specific homeobox gene *Crx* (Furukawa et al., 1998). Thus, while conclusive proof requires cell lineage analyses, our data suggest that the P1-lacZ transgene expressing cells are likely to be differentiating cone cells. Interestingly, a potentially analogous, post-mitotic pre-rod stage of rod photoreceptor differentiation has been identified in vitro (Ezzeddine et al., 1997; Neophytou et al., 1997). Previously it was found that *PAX6* overexpression in transgenic mice resulted in an absence of photoreceptors (Schedl et al., 1996). *Pax6* may thus participate in photoreceptor differentiation, and the P1 element might regulate this function.

The third regulatory region analyzed in these experiments is located within *Pax6* intron 4 and directs *lacZ* expression in the anterior tip of the optic cup which gives rise to ciliary body, iris and in amacrine cells. The expression in these ocular structures remains through adult stages and accurately reproduces the expression of endogenous *Pax6*. Interestingly, introduction of the intron 4 region between the P0 or P1 upstream elements and their respective promoters suppressed the expression patterns directed by those elements; a similar interference phenomenon has been described in the β-globin locus (Hanscombe et al., 1991). Nonetheless, these results clearly show that the intron 4 region can function in vivo as an orientation-independent, eye-specific enhancer, and further highlight the modular organization of *Pax6* regulatory control elements.

The upstream mechanisms regulating *Pax6* expression are conserved between insects and mammals

Pax6 genes have been shown to be key regulators of eye development and the eye-regulatory pathways controlled by *Pax6* genes appear to be conserved across metazoa (Zuker, 1994; Halder, 1995b). However, the mechanisms regulating *Pax6* expression in different species may be dissimilar. To test whether the regulation of *Pax6* is evolutionarily conserved, we analyzed an eye-regulatory region of the *Drosophila ey* gene in transgenic mice, and of the P1 regulatory and intron enhancer elements of mouse *Pax6* in transgenic flies. Our results indicate that the intronic regulatory region of *Drosophila ey* functions in a conserved manner both in flies and mice to direct key aspects of early ocular and CNS expression. Recent results indicate that in *Drosophila* a second *Pax6* gene called *twin of eyeless* (*toy*) exists which encodes a protein even more similar to vertebrate Pax6 than Ey (M. Busslinger, personal communication). Although *toy* expression slightly precedes that of *ey* during embryonic development, expression of both genes is virtually identical during eye imaginal disc development. Thus, it seems likely that similar results would be obtained even if an equivalent analysis was performed employing eye-specific regulatory elements from *toy*.

In the case of the 1.2 kb *ey* transgene, *lacZ* was expressed not only throughout much of the retina, but also in the peripheral optic cup which will give rise to the ciliary body and iris. The peripheral optic cup expression recapitulates the *lacZ* transgene expression directed by the mouse *Pax6* intron 4 enhancer region. A smaller 500 bp *ey* fragment directed retinal expression in mice, but failed to confer peripheral optic cup expression. Thus, any functional homologies involved in regulating expression in peripheral optic cup must reside in sequences present on the 1.2 kb but not the 500 bp *ey* fragment. Sequence comparison between this region of the *ey* and mouse intronic enhancers does reveal a region of potentially significant sequence identity. An 80 bp sequence in the 3′ portion of the 1.2 kb fragment exhibits 66% identity with a sequence present within intron 4 enhancer which includes two binding sites identified in quail, designated DF-3 and -4 (Plaza et al., 1995b). It is possible that these sequences are conserved because they bind evolutionarily conserved transcription factors involved in the spatial regulation of *Pax6* gene expression during peripheral optic cup development. Thus, the molecular pathway acting through the intron 4 enhancer may be potentially conserved not only between mouse and quail, but also between mouse and fly.

Although the *ey* regulatory elements appear to function in mice with considerable fidelity, the results from reciprocal experiments analyzing mouse *Pax6* elements in *Drosophila* were not as clear. A mouse fragment containing the *Pax6* P1 element was, however, able to direct *lacZ* transgene expression in the *Drosophila* eye imaginal disc. This expression was restricted specifically to differentiating photoreceptors posterior to the morphogenetic furrow, which is not a site of endogenous Ey expression (Halder et al., 1998). Curiously, our analysis and that of others (Halder et al., 1998) reveals that the *ey* transgenes also exhibit anomalous expression posterior to the furrow. The basis for the *ey* transgenic results is unclear, but could reflect β-galactosidase perdurance or the absence of a repressive function from the *ey* transgene. In the case of the mouse P1 element, it is interesting that the cells that activate the P1 element in both the *Drosophila* eye imaginal disc and the mouse retina are developing photoreceptors. In fact, in the *Drosophila* adult eye and in Bolwig's organ, a component of the larval visual system, *ey* is expressed in photoreceptors (Sheng et al., 1997). Thus, although the expression of the mouse *Pax6* P1 upstream fragment in *Drosophila* does not reproduce endogenous *ey* expression, the mechanisms regulating photoreceptor differentiation in mice and flies may still be conserved.

Why do the *Drosophila* elements reproduce *Pax6* expression in mouse but not vice versa? One possibility is that non-homologous regions of the two genes are being compared. While the *ey* enhancer reproduces significant aspects of

endogenous *Pax6* expression in eye and spinal cord, it cannot be entirely assumed that, with the possible exception of peripheral optic cup, the homologous murine elements have been examined in flies. In addition, it seems possible that even in the event of conserved pathways, specific differences in individual recognition sequences may preclude reciprocal regulatory interactions. Finally, the regulatory cascades themselves are not likely to be entirely conserved. It seems plausible that the archetypal set of *Pax6* regulatory elements evolved in such a way as to preserve their original functions and to acquire newer, more highly specialized functions unique to individual species. The P1 upstream element in mouse *Pax6* may provide an example of such a more recently acquired element, and could represent an example of convergent evolution whereby a regulatory element homologous to the mouse P1 element also evolved in *Drosophila*, but in a gene different from *ey*.

In sum, our results define the behavior of specific *Pax6*-regulatory elements and strongly suggest that parts of the regulatory cascades for *Pax6* and *ey* expression are conserved between mice and flies. In addition, these results provide an important basis for studies aimed at identifying transcription factors that regulate the individual elements that generate the complex expression pattern of *Pax6* during oculogenesis.

We thank Drs G. Rubin, U. Walldorf and G. Mardon for kindly providing the Elav, Eyeless and Dachshund antibodies, Stephen Gisselbrecht and Kwang-Hyun Baek for assistance with the fly injections and Dr Louis Pasquale for help in analyzing the intron 4 transgenic mouse lines. We also thank Drs Connie Cepko and Takahisa Furukawa (Harvard Medical School) for reading the manuscript, Drs Sam Kunes and Georg Halder for helpful discussions, and Drs Meinrad Busslinger and Patrick Callaerts for communicating results in advance of publication. A. M. M. is an Assistant Investigator of the Howard Hughes Medical Institute. This work was supported by NIH grant 1RO1 EY10123.

REFERENCES

Belecky-Adams, T., Cook, B. and Adler, R. (1996). Correlations between terminal mitosis and differentiated fate of retinal precursor cells in vivo and in vitro: analysis with the 'window labeling' technique. *Dev. Biol*. **178**, 304-315.

Belecky-Adams, T., Tomarev, S., Li, H.-S., Ploder, L., McInnes, R. R., Sundin, O. and Adler, R. (1997). *Pax6, Prox1* and *Chx10* homeobox gene expression correlates with phenotypic fate of retinal precursor cells. *Invest. Ophthalmol. Vis. Sci.* **38**, 1293-1303.

Carter-Dawson, L. D. and LaVail, M. (1979). Rods and cones in the mouse retina. *Comp. Neur*. **188**, 263-272.

Czerny, T., Halder, G., Callaerts, P., Kloter, U., Gehring, W. J. and Busslinger, M. (1997). *twin of eyeless*, a second *Pax6* gene of *Drosophila*, acts upstream of *eyeless* in the control of eye development. Cold Spring Harbor Symposium, p51.

Davis, J. A. and Reed, R. R. (1996). Role of Olf-1 and Pax6 transcription factors in neurodevelopment. *J. Neurosci*. **16**, 5082-5094.

Driver, A., Lacey, S. F., Cullingford, T. E., Mitchelson, A. and O'Hare, K. (1989). Structural analysis of Doc transposable elements associated with mutations at the white and suppressor of forked loci of Drosophila melanogaster. *Mol. Gen. Genet.* **220**, 49-52.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell **90***, 169-180.

Ezzedine, Z. D., Yang, X., DeChiara, T., Yancopulos, G. and Cepko, C. (1997). Postmitotic cell fated to become rod photoreceptors can be respecified by CNTF treatment of the retina. *Development* **124**, 1055-1067.

Freund, C. L., Gregory-Evans, C. Y., Furukawa, T., Papaioannou, M., Looser, J., Ploder, L., Bellingham, J., Ng, D., Herbrick, J. S., Duncan, A., et al. (1998). Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (*CRX*) essential for maintenance of the photoreceptor. *Cell* **91**, 543-553.

Frommer, G., Schuh, R. and Jackle, H. (1994). Localized expression of a novel micropia-like element in the blastoderm of *Drosophila melanogaster* is dependent on the anterior morphogen bicoid. *Chromosoma* **103**, 82-89.

Furukawa, T., Morrow, E. M. and Cepko, C. L. (1998). *Crx*, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. *Cell* **91**, 531-541.

Glaser, T., Walton, D. S., Cai, J., Epstein, J., Jepeal, L. and Maas, R. L. (1995). *PAX6* gene mutations in aniridia. In *Molecular Genetics of Ocular Disease*. (ed. J. Wiggs), pp. 51-82. New York: Wiley Liss, Inc.

Grindley, J. C., Davidson, D. R. and Hill, R. E. (1995). The role of Pax-6 in eye and nasal development. *Development* **121**, 1433-1442.

Halder, G., Callaerts, P. and Gehring, W. J. (1995a). Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. *Science* **267**, 1788-1792.

Halder, G., Callaerts, P. and Gehring, W. J. (1995b). New perspectives on eye evolution. *Curr. Opin. Genet. Dev.* **5**, 602-609.

Halder, G., Callaerts, P., Flister, S., Walldorf, U., Kloter, U. and Gehring, W. J. (1998). Eyeless initiates the expression of both *sine oculis* and *eyes absent* during *Drosophila* compound eye development**.** *Development* **125**, 2181-2191.

Hanscombe, O., Whyatt, D., Fraser, P., Yannoutsos, N., Greaves, D., Dillon, N. and Grosveld, F. (1991). Importance of globin gene order for correct developmental expression. *Genes Dev.* **5**, 1387-1394.

Hanson, I., Brown, A. and van Heyningen, V. (1995). Pax6: more than meets the eye. *Trends Genet.* **11**, 268-272.

Holt, C. E., Bertsch, T. W., Ellis, H. M. and Harris, W. A. (1988). Cellular determination in the *Xenopus* retina is independent of lineage and birth date. *Neuron* **1**, 15-26.

Hiromi, Y. and Gehring, W. (1987). Regulation and function of the Drosophila segmentation gene fushi tarazu. *Cell* **50**, 963-974.

Koroma, B. M., Yang, J. -M. and Sundin, O. H. (1997). The *Pax6* homeobox gene is expressed throughout the corneal and conjunctival epithelia. *Invest. Ophthalmol. Vis. Sci.* **38**, 108-120.

Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I. and Wilson, S. W. (1995). Midline signalling is required for *Pax* gene regulation and patterning of the eyes. *Development* **121**, 3267-3278.

Martin, P., Carriere, C., Dozier, C., Quatannens, B., Mirabel, M., Vandenbunder, B., Stehelin, D. and Saule, S. (1992). Characterization of a pairedbox- and homeobox-containing quail gene (*pax-QNR*) expressed in the neuroretina. *Oncogene* **7**, 1721-1728.

Neophytou, C., Vernallis, A. B., Smith, A. and Raff, M. C. (1997). Müller-cell-derived leukaemia inhibitory factor arrests rod photoreceptor differentiation at a postmitotic pre-rod stage of development. *Development* **124**, 2345-2354.

O'Hare, K., Alley, M. R. K., Cullingford, T. E., Driver, A. and Sanderson, M. J. (1991). DNA sequence of the *Doc* retroposon in the white-one mutant of *Drosophila* melanogaster and of secondary insertions in the phenotypically altered derivatives white-honey and white-eosin. *Mol. Gen. Genet.* **225,** 17-24.

Pituello, F., Yamada, G. and Gruss, P. (1995). Activin A inhibits *Pax6* expression and perturbs cell differentiation in the developing spinal cord in vitro. *Proc. Natl. Sci. USA* **92**, 6952-6956.

Plaza, S., Dozier, C., Turque, N. and Saule, S. (1995a). Quail *Pax6* (*Pax-QNR*) mRNAs are expressed from two promoters used differentially during retina development and neuronal differentiation. *Mol. Cell. Biol*. **15**, 3344-3353.

Plaza, S., Dozier, C., Langlois, M. and Saule, S. (1995b). Identification and characterization of a neuroretina-specific enhancer element in the quail Pax6 (Pax-QNR) gene. *Mol. Cell. Biol*. **15**, 892-903.

Quiring, R., Walldorf, U., Kloter, U. and Gehring, W. (1994). Homology of the *eyeless* gene of *Drosophila* to the *Small eye* gene in mice and *Aniridia* in humans. *Science* **265**, 785-789.

Sander, M., Neubüser, A., Kalamaras, J., Ee, H. C., Martin, G. R. and German, M. S. (1997). Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. *Genes Dev.* **11**, 1662-1673.

Schedl, A., Ross, A., Lee, M., Engelkamp, D., Rashbass, P., van Heyningen, V. and Hastie, N. D. (1996). Influence of *PAX6* gene dosage on development: overexpression causes severe eye abnormalities. *Cell* **86**, 71-82.

Sheng, G., Thouvenot, E., Schmucker, D., Wilson, D. S. and Desplan, C. (1997). Direct regulation of *rhodopsin 1* by Pax-6/eyeless in *Drosophila*: evidence for a conserved function in photoreceptors. *Genes Dev.* **11**, 1122-1131.

Sidman, R. L. (1961). Histogenesis of the mouse retina studied with thymidine-3H. In *The Structure of the Eye* (ed. G. Smelser), pp. 487-506. New York: Academic Press.

Spradling, A. C. (1986). P element-Mediated Transformation. In *Drosophila: A Practical Approach* (ed. D. B. Roberts), pp. 175-197. Oxford, Washington DC: IRL Press.

St-Onge, L., Sosa-Pineda, B., Chowdhury, K., Mansouri, A. and Gruss, P. (1997). *Pax6* is required for differentiation of glucagon-producing α-cells in mouse pancreas. *Nature* **387**, 406-413.

Turque, N., Plaza, S., Radvanyi, F., Carriere, C. and Saule, S. (1994). *Pax-QUR/Pax6*, a paired box- and homeobox-containing gene expressed in neurons, is also expressed in pancreatic endocrine cells. *Mol. Endocrinol.* **8**, 929-938.

Turner, D. L. and Cepko, C. (1987). A common progenitor for neurons and glia persists in rat retinas late in development. *Nature* **328**, 131-136.

Walther, C. and Gruss, P. (1991). *Pax6*, a murine paired box gene, is expressed in the developing CNS. *Development* **113**, 1435-1449.

Wetts, R. and Fraser, S. E. (1988). Multipotent precursors can give rise to all major cell types of the frog retina. *Science* **239**, 1142-1145.

Williams, S. C., Altmann, C. R., Chow, R. L., Hemmati-Brivanlou, A. and Lang, R. A. (1998). A highly conserved lens transcriptional control element from the *Pax6* gene. *Mech. Dev.* **73**, 225-229.

Xiang, M. (1998). Requirement for Brn-3b in early differentiation of postmitotic retinal ganglion cell precursors. *Dev. Biol.* **197**, 155-169.

Xu, P.-X., Fukuta, K., Takiya, S., Matsuno, K., Xu, X. and Suzuki, Y. (1994). Promoter of the *POU-M1/SGF-3* gene involved in the expression of Bombyx silk genes. *J. Biol. Chem.* **269**, 2733-2742.

Xu, P.-X., Woo, I., Her, H., Beier, D. R. and Maas, R. L. (1997). Mouse *Eya* homologues of the *Drosophila eyes absent* gene require *Pax6* expression in lens and nasal placode. *Development* **124**, 219-231.

Xu, Z.-P. and Saunders, G. F. (1997). Transcriptional regulation of the human *PAX6* gene promoter. *J. Biol. Chem*. **272**, 3430-3436.

Zuker, C. S. (1994). On the evolution of eyes: would you like it simple or compound? *Science* **265**, 742-743.