

Spatial specification of mammalian eye territories by reciprocal transcriptional repression of Pax2 and Pax6

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SUMMARY

We have studied the molecular basis of the Pax2 and Pax6 function in the establishment of visual system territories. Loss-of-function mutants have revealed crucial roles for Pax2 in the generation of the optic stalk and for Pax6 in the development of the optic cup. Ectopic expression of Pax6 in the optic stalk under control of Pax2 promoter elements resulted in a shift of the optic cup/optic stalk boundary indicated by the presence of retinal pigmented cells on the optic stalk. By studying mouse embryos at early developmental stages we detected an expansion of Pax2 expression domain in the Pax6^{-/-} mutant and of Pax6 expression domain in the Pax2^{-/-} embryo. These results suggest that the position of the optic cup/optic stalk

boundary depends on Pax2 and Pax6 expression, hinting at a possible molecular interaction. Using gel shift experiments, we confirmed the presence of Pax2- and Pax6-binding sites on the retina enhancer of the Pax6 gene and on the Pax2 upstream control region, respectively. Co-transfection experiments revealed a reciprocal inhibition of Pax2 promoter/enhancer activity by Pax6 protein and vice versa. Based on our findings, we propose a model for Pax gene regulation that establishes the proper spatial regionalization of the mammalian visual system.

Key words: Knockout, Retina, Retinal pigmented epithelium, Pax, Mouse

INTRODUCTION

The primordia, which give rise to the complex architecture of the mammalian eye, are regionalized and specified by autonomous as well as inductive cues (Saha et al., 1992). After the early inductive events, distinct processes are involved in the formation of the eye. During a morphogenetic phase, invagination of the ventral part of the optic vesicle occurs. The ectodermally derived lens is encircled by the invaginated tissue at the most distal part of the optic cup. Subsequently, this process results in the formation of the optic globe, consisting of outer pigmented retina, neural retina and the lens (Saha et al., 1992). Ventrally, the eye and a part of the optic stalk remain transiently open, marked by the optic fissure. The closure of the optic fissure marks the end of the morphogenetic period (reviewed in Oliver and Gruss, 1997). The early and dynamic expression of Pax2 and Pax6 during eye formation suggests a potential role for these genes in eye regionalization (Nornes et al., 1990; Torres et al., 1996; Walther and Gruss, 1991). Pax6 is expressed in the optic primordium and later in all cells of the prospective retina, pigmented epithelium and lens epithelium (Walther and Gruss, 1991; Grindley et al., 1995). Mutations in Pax6 result in severe eye defects in mice (small eye) and humans (aniridia syndrome; Hill et al., 1991; Glaser et al., 1994; Hogan et al., 1988; Grindley et al., 1995). Pax6-

deficient mice completely lack eyes (Hill et al., 1991). In contrast to Pax6, Pax2 is expressed in the ventral half of the optic vesicle during early eye morphogenesis (Nornes et al., 1990; Torres et al., 1996). Shortly after the invagination of the optic cup it becomes confined to the optic stalk (Nornes et al., 1990; Torres et al., 1996). However, its expression persists throughout the whole morphogenetic phase at the lips of the optic fissure and extends into the optic stalk up to the brain (Nornes et al., 1990; Torres et al., 1996; Macdonald et al., 1995; Macdonald and Wilson, 1996). During this period, the developing optic cup/optic stalk border is marked by overlapping Pax2 and Pax6 expression domains (Nornes et al., 1990; Walther and Gruss, 1991). Mutations in the human PAX2 gene result in optic nerve colobomas (Sanyanusin et al., 1995). In mice deficient for Pax2, the optic nerves project only ipsilaterally to the superior colliculus (Torres et al., 1996). In addition, the Pax6-expressing pigmented epithelium of the retina has been shown to expand in the Pax2 mutant embryos, invading the optic cup/optic stalk boundary (Torres et al., 1996).

In the present study, we investigate the molecular mechanism by which the expression patterns of these transcription factors in the visual system become defined to form a sharp boundary between regions acquiring different fates. We tested a possible reciprocal transcriptional repression

between *Pax2* and *Pax6* in the regionalization and subsequent boundary formation between the optic cup and the optic stalk. We found that in the *Pax2*-deficient mice, the retinal pigmented epithelium (RPE) and all retinal compartments were expanded at the expense of the optic stalk, and they strongly expressed Pax6. Conversely, in the visual system of *Pax6*-deficient mice, all tissue that in wild-type animals expressed Pax6 had disappeared, leaving, as the only remnant, an optic stalk. In a dominant gain-of-function experiment in which *Pax6* cDNA was expressed in the ventral aspect of the optic stalk, we observed a proximal shift of retinal pigmented cells showing a disruption of the optic cup/optic stalk boundary and the potential of Pax6 to induce RPE development. We propose a molecular regulation between Pax2 and Pax6 by showing direct binding of Pax2 and Pax6 proteins on the *Pax2* and *Pax6* tissue-specific enhancers.

In order to elucidate the molecular mechanism(s) at the basis of these phenotypes, we used a cell culture system and demonstrate that Pax6 is sufficient to repress transcription of a reporter gene driven by *Pax2* enhancer sequences and vice versa.

MATERIALS AND METHODS

Generation and genotyping of transgenic mice

Pax2 enhancer/*lacZ* reporter transgenic animals were generated by microinjection of DNA into the paternal pronucleus of oocytes. The *Pax2* enhancer constructs contained 9.3 kb of upstream activating sequences of *Pax2* (the 3' end is at the *NotI* site 24 bp upstream of the ATG), followed by either the β -galactosidase or the Pax6 cDNA (Walther and Gruss, 1991), and the SV40 intron poly A sequence. The vectors used for microinjection were linearized using a unique *HindIII* site at the very 5' end of the *Pax2* promoter sequence and a *Sall* (*Pax6*) or *SfiI* (*lacZ*) at the 3' end of the construct. F₀ founder mice were genotyped using either β -galactosidase or the Pax6 cDNA (Walther and Gruss, 1991) a probe for Southern hybridization. DNA extraction from yolk sac or tail tip was performed as described (Kammandel et al., 1999). The analysis of *lacZ* expression in the mouse embryos (see Fig. 4D) was performed as described (Kammandel et al., 1999). *Pax6* enhancer/reporter transgenic embryos were generated and analysed as previously described (Kammandel et al., 1999).

Genetic and phenotypic analyses

Embryos were fixed in 4% paraformaldehyde and embedded in paraffin. The extra-embryonic membranes were genotyped by Southern analysis (Schwarz et al., 1997; St-Onge et al., 1997). The embryos were dissected, photographed, sectioned and finally stained with Cresyl Violet. X-Gal staining was carried out as described (Gossler et al., 1989). The staining reaction was left to proceed for 8 hours in order to obtain intense labeling of all structures expressing the transgene. For albumin-gelatine sectioning, the treated embryos were embedded in albumin-gelatine and vibratome-sectioned into slices 40 μ m thick. For paraffin sectioning, specimens were impregnated with paraffin wax, embedded and sectioned at 10 μ m.

In situ hybridization and immunohistochemistry

In situ hybridization experiments were performed following current protocols for [³⁵S]-labeled RNA probes (Stoykova and Gruss, 1994). The *Pax2*, *Pax6*, *Rx*, *Lhx2* and *Six3* probes have been described previously (Nornes et al., 1990; Walther and Gruss, 1991; Mathers et al., 1997; Furukawa et al., 1997; Porter et al., 1997; Oliver et al., 1995). In situ hybridization on whole embryos (whole mount in situ)

was performed following current protocols (Torres et al., 1995). Immunohistochemistry experiments were performed with a monoclonal anti-Pax6 antibody (DSHB) on 10 μ m-thick sagittal sections from paraffin-embedded e13.0 embryos using the alkaline phosphatase universal AK-5200 Vectastain kit (Vector Labs).

Gel mobility shift assays

Protein-DNA binding reactions for binding of Pax proteins to the *Pax6* retinal enhancer were performed as described (Chalepakis et al., 1991). Synthetic oligonucleotides of Ret and the mutant variant Ret/Mut were 3' end labeled with [³²P]dCTP using Klenow polymerase. The different Pax proteins were expressed under the control of hCMV promoter in transiently transfected cos-7 cells, as described (Maulbecker and Gruss, 1993), and whole-cell extracts were used. Saturation binding experiments were performed as described in current protocols.

Protein-DNA binding reactions for the *Pax2* optic stalk enhancer sequences were performed by mixing proteins and nucleic acids in binding buffer, in a final volume of 20 μ l. Proteins were produced using the PROMEGA TNT in vitro transcription/translation system and the supplied manufacturers protocol. The synthetic oligonucleotides (a-d) were 3' end labeled with [³²P]ATP using polynucleotide kinase. Binding experiments were performed using current protocols.

Cell culture

Cos-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and plated on 3.5 cm cell culture dishes. Twenty-four hours after the initial plating (approximately 70% confluence) the medium was changed and cells were plated in DMEM without fetal calf serum. Afterwards the cells were transfected with a total of 6 μ g DNA in each transfection using 10 μ l Lipofectamine reagent from Invitrogen BRL and the manufacturers transfection protocol. 12 hours after transfection the cells were supplemented with fetal calf serum to a final concentration of 10% and grown for further 12 hours. Twenty-four hours after transfection, the medium was exchanged with DMEM with 10% fetal calf serum and cells were grown for further 24 hours. Forty-eight hours after transfection the cells were washed with phosphate buffered saline (PBS) and subjected to X-gal staining procedure (Gossler and Zachgo, 1993). β -Galactosidase positive cells from each independent transfection were counted and cell numbers averaged following normalization of luciferase activity used as internal standard. The following amount of plasmid DNA was used in three independent experiments, carried out in doublets (1.5 μ g of P2e or P6e, 2 μ g of CMV-Pax2 or CMV-Pax6 expression plasmids, 2 μ g of Pax2 or Pax6 oligonucleotides and 0.5 μ g of a CMV-luciferase reporter plasmid). Bluescript SKII was used as internal standard. Finally bring the amount of transfected plasmid DNA to 6 μ g in each individual transfection. Co-transfections of unrelated oligonucleotides were used as controls in the competition experiments.

RESULTS

Retinal expansion at the expense of the optic stalk in *Pax2*-deficient embryos

In wild-type mouse embryos, Pax2 is expressed strongly in the ventral two thirds of the optic evagination. After day 9.5 of embryonic development (E9.5) the expression of Pax2 in the optic primordium is restricted to the cells of the optic stalk (Nornes et al., 1990). Subsequently, Pax2 expression labels the glial cells wrapped around the optic nerve with a sharp border of expression at the pigmented retina (RPE)/optic nerve boundary. The result is an exactly defined boundary in which

no cells expressing Pax2 show pigmentation (Torres et al., 1996). In *Pax2* mutant mice the differentiation of the optic stalk is altered (Torres et al., 1996). As a result, the sharp boundary between glial cells surrounding the optic nerve and retinal cells (including RPE and both layers of the neural retina) extend abnormally along the optic nerve towards the midline of the diencephalon. The phenotypic appearance is a huge optic cup lacking the glial cells around the optic nerve (see Fig. 1, right panels). The mutant optic nerve consists of a single bundle of axons (Torres et al., 1996).

The optic fissure, which is a transiently open structure, never closes in mutant embryos that show complete bilateral coloboma extending up to the diencephalon, owing to the

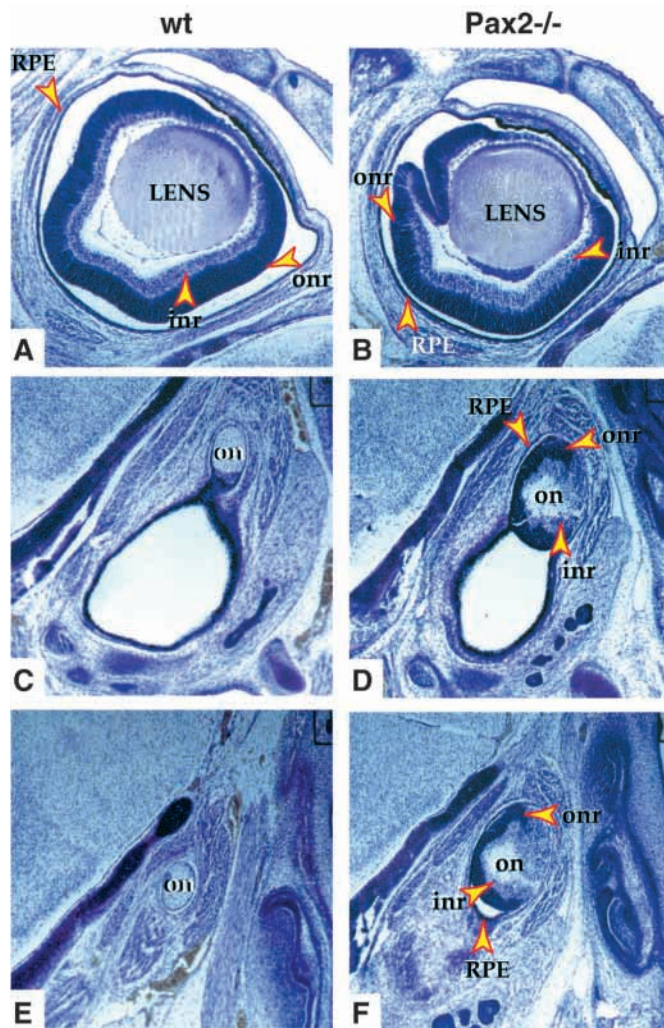


Fig. 1. Retinal expansion in *Pax2*-deficient mouse embryos. Cresyl Violet stained cross-sections through an E16.5 wild-type (A) and *Pax2* mutant (B) eye at the level of the eye cup are shown. Arrowheads indicate the three different retinal layers. (C,D) Sections of the optic nerve of the same eyes at a more medial level. The *Pax2* mutant optic nerve (D) is wrapped in all three retinal layers (arrowheads). The wild-type nerve (C) is wrapped in glial cells. (E,F) Sections through the same optic nerve close to the midline. The wild-type optic nerve (E) has its characteristic appearance, whereas the *Pax2* mutant optic nerve (F) is wrapped in the three retinal layers (arrowheads). inr, inner neuroretina; on, optic nerve; onr, outer neuroretina; RPE, retinal pigmented epithelium.

failure of contact-dependent dissolution of the basal lamina (Torres et al., 1996). Despite this fact, the morphological development of the neural retina appeared to be normal along the disto/proximal length of the optic nerve (see Fig. 1B,D,F).

Complementary phenotypes in *Pax2* and *Pax6* mutant mice

Once a part of the rostral neural tube wall has been determined to become the visual organ, a key decision involves the partitioning of this tissue into regions fated to form either eye cup or optic stalk. When comparing the mutant phenotypes of mice deficient either in *Pax2* or *Pax6*, we found striking complementary phenotypes with respect to the spatial organization of the visual organ, and associated with the complementary gene expression patterns of *Pax2* and *Pax6* (see Fig. 2A-T). In *Pax2*-deficient embryos, the optic primordium was able to form only retina and lens, giving the impression of an enormously elongated eye (Fig. 2C,D,G,H,K,L,O,P). In *Pax6*-deficient embryos, a reciprocal alteration was observed: the visual primordium forming the remnant of an optic stalk (Fig. 2S,T; Grindley et al., 1995). We ascertained the identity of the elongated retinal compartments after differentiation in the *Pax2* mutant mice by using retina-specific molecular markers (Fig. 2A-P). By contrast, the visual organ of *Pax6* mutant mice showed that all tissue that, in wild-type embryos at the same stage of development, would express *Pax6*, had disappeared (Fig. 2S,T). The remnant of the visual organ showed stalk morphology and expressed the molecular markers *Pax2* (Fig. 2S) and *Six3* (data not shown) at the same time as it did in control embryos during the primordial phase of eye morphogenesis. Our findings imply that in the absence of *Pax6*, the correct partitioning of the visual organ into stalk-specific tissue and cup (retinal tissue) cannot proceed.

Reciprocal expansion of *Pax2* and *Pax6* gene expression in the corresponding mutant embryos

The ectopic expression of *Pax6* in the expanded retina in *Pax2*-deficient embryos, together with a complementary phenotype in mice lacking *Pax6* function, suggest a possible reciprocal downregulation between *Pax2* and *Pax6* in the establishment of the optic cup/optic stalk boundary. In order to test for a reciprocal repression between *Pax2* and *Pax6* in vivo we performed whole-mount in situ hybridization studies on E9.5 embryos lacking either *Pax2* or *Pax6* function with *Pax6* and *Pax2* as probes, respectively (Fig. 3). As reported earlier a boundary between *Pax2* and *Pax6* expression is already established at E9.5 in wild-type embryos (Torres et al., 1996). At the same stage, the *Pax6* mutant embryos do not show an extensive regionalization phenotype in the optic anlage; the optic cup is formed and displays its characteristic shape (Grindley et al., 1995). However, if we compare the *Pax2* transcript distribution in wild-type and *Pax6* mutant mice, an expansion of *Pax2* expression along the margins of the optic cup could be detected in mice lacking *Pax6* function (Fig. 3, upper panel). Although gross morphological alterations were not present at that stage, ectopic expression of *Pax2* in the optic cup could be observed, suggesting the lack of *Pax2* gene repression by *Pax6* protein. In order to test if this possible repression was reciprocal, we performed the same experiment for *Pax6* transcripts in mice lacking *Pax2* function. Like *Pax6* mutant mice, mice lacking *Pax2* function do not show obvious

morphological alterations at E9.5 (Torres et al., 1996). However, if we compare the *Pax6* transcript distribution in

wild-type and *Pax2* mutant mice, a clear expansion of *Pax6* along the ventral part of the optic stalk could be seen (Fig. 3,

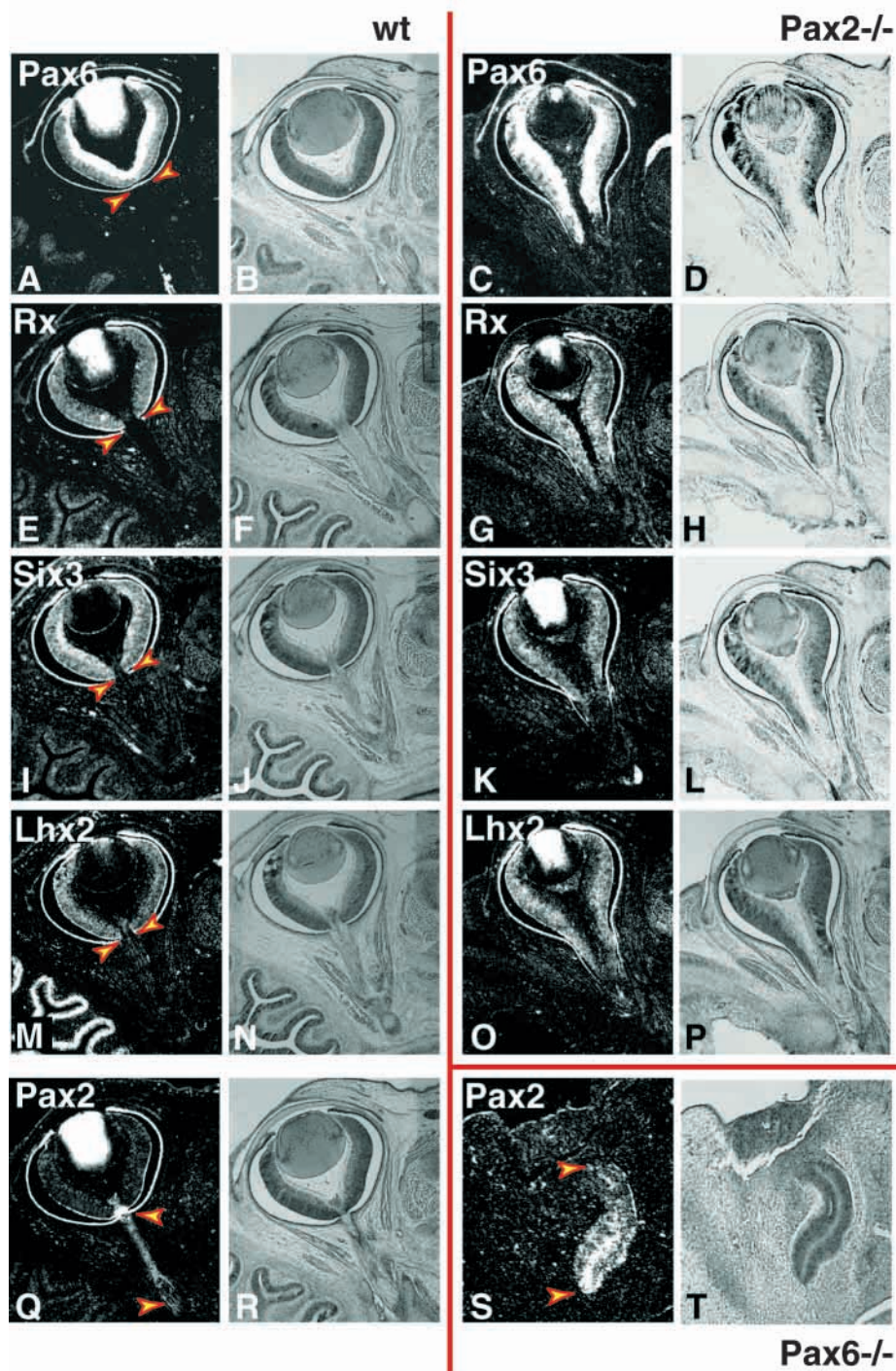


Fig. 2. Comparative in situ hybridization of cross-sectioned wild-type, *Pax2* and *Pax6* mutant eyes using retinal- and optic stalk-specific markers. *Pax6* expression in E13.0 wild-type (A,B) and in *Pax2*-deficient E13.0 eye (C,D). (E,F) *Rx* expression in the wild-type E13.0 eyes. (G,H) *Rx* expression in the *Pax2*-deficient E13.0 eye. (I, J) *Six3* expression in the wild-type E13.0 eye. (K,L) *Six3* expression in the *Pax2*-deficient E13.0 eye. (M,N) *Lhx2* expression in the wild-type E13.0 eye. (O,P) *Lhx2* expression in the *Pax2*-deficient E13.0 eye. Arrowheads show the eye/stalk boundary in the wild-type sections (A,E,I,M). (Q,R) *Pax2* expression in the wild-type eye. (S,T) *Pax2* expression in the *Pax6* mutant. Arrowheads show the extent of the optic nerve in the wild type (Q) and the remnant optic stalk of the *Pax6* mutant (S). The expression on the lens and RPE can often be due to an experimental artifact.

lower panel). These results suggest a possible reciprocal inhibition between *Pax2* and *Pax6* at the optic cup/optic stalk boundary.

Expansion of RPE in mice expressing *Pax6* under the control of the *Pax2* enhancer

We identified a *Pax2* enhancer element within the *Pax2* promoter region, capable of driving the expression of the β -galactosidase reporter gene in the optic stalk (see below). We used this enhancer sequence to express *Pax6* ectopically in the presumptive optic stalk cells, in order to test whether upregulation of *Pax6* expression in the adjacent *Pax2* territory could interfere with *Pax2* expression and to modulate the optic cup/optic stalk boundary formation in vivo. Two independent transgenic mice were generated and used to generate stable lines. On cross sections of eyes at E12.5 we observed, as shown in Fig. 4C-F,H, the presence of pigmented cells only in the dorsal part of the optic nerve. Radioactive in situ hybridization using either *Pax2* (Fig. 4C) or *Pax6* (Fig. 4E) riboprobes helped us to distinguish the optic nerve fibers, which strongly express *Pax2* mRNA but not *Pax6* mRNA (Fig. 4E). In cross sections of the optic nerve from transgenic animals, the refractant pigment cells were in direct contact with the fibers of the optic nerve (Fig. 4G,H). We therefore performed immunohistochemistry experiments with an antibody anti-*Pax6*, in order to show the ectopic expression of *Pax6* in distal and proximal cross-sections of the optic nerve (Fig. 4I,J). *Pax6* was ectopically expressed in the transgenic embryos in the dorsal aspect of the optic nerve in the region of the RPE and the surrounding presumptive glial cells. Our results suggest that the presumptive glial progenitor cells have the potential to differentiate along the retinal lineage upon ectopic *Pax6* expression. Interestingly, in the transgenic animals we detect only RPE but no cells of the neural retina. These phenotypic alterations are persistent up to adult stages, as shown in Fig. 4K,L. Similarly, injection of *Pax6* mRNA into *Xenopus* embryos has also been shown to induce RPE extension

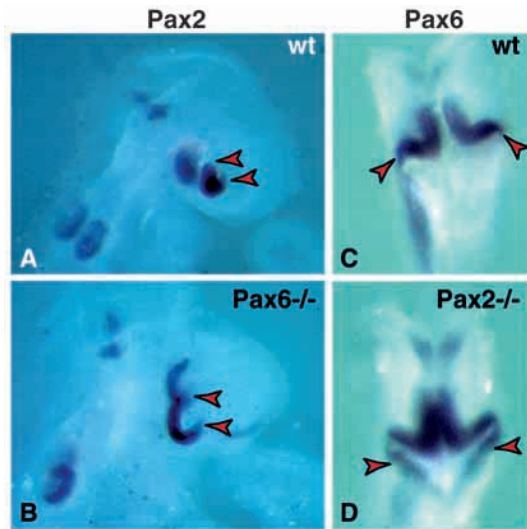
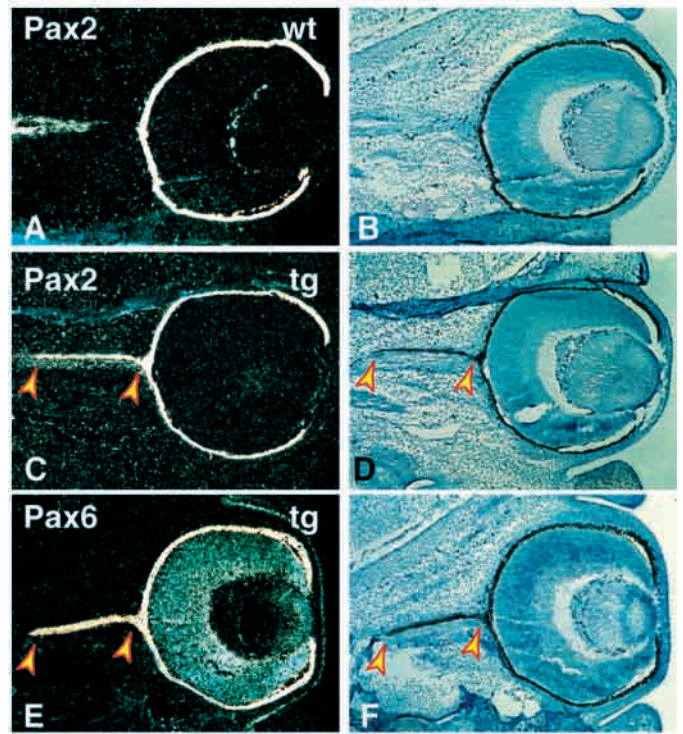


Fig. 3. Whole-mount in situ hybridization of *Pax2* and *Pax6* in E9.5 *Pax2* and *Pax6* mutant mice. **A** and **B** show the comparison between *Pax2* staining in the eye cup of wild-type (**A**) and *Pax6* mutant (**B**) mouse embryos (lateral view). Arrowheads point to the borders of *Pax2* gene expression at the level of the eye cup margins. **C** and **D** show the comparison between *Pax6* districution in the visual primordium of a wild-type (**C**) and a *Pax2* mutant (**D**) mouse embryos (ventral view). The arrowheads point to the ventral expansion of *Pax6* expression in the *Pax2* mutant (**D**) compared with the wild-type (**C**) embryo.



as a fine track of pigmented cells apparently associated with the optic nerve (Chow et al., 1999). However, only presumptive glial cells of the optic stalk were able to respond to the *Pax6* expression, as ectopic expression of *Pax6* in the otic placode did not induce retinal fates (data not shown).

Direct interaction between *Pax2*, *Pax6* and their corresponding transcriptional enhancers

In order to test a direct molecular interaction between *Pax2*, *Pax6* and their transcriptional elements, we sequenced the 9 kb upstream of the *Pax2* tsp (transcription start point) and the region corresponding to the retinal specific enhancer in the alpha region of *Pax6* intron 4 (Fig. 5A). The *Pax2* promoter

Fig. 4. Analysis of *Pax2* and *Pax6* transgenic E13.0 mouse embryos and adult optic nerves. **(A,B)** In situ *Pax2* expression in the wild-type optic nerve of a cross-sectioned E13.0 eye. **(C,D)** *Pax2* expression in the *Pax2-Pax6* transgenic optic nerve. **(E,F)** *Pax6* expression in the *Pax2-Pax6* transgenic optic nerve and the retina. **(A,C,E)** Dark field. **(B,D,F)** bright field. The arrowheads point to the ectopic retinal compartment in the *Pax2-Pax6* transgenic optic nerve **(C,D,E,F)**. The RNA expression on the RPE can often be due to an experimental artifact. **(G,H)** Cross-sections through a wild-type **(G)** and a *Pax2-Pax6* transgenic **(H)** optic nerve. The arrowheads point to the ectopic retinal pigmented epithelium **(H)**. **(I-J)** *Pax6* protein is ectopically expressed in the optic nerve of the transgenic embryos. Cross-sections through the optic nerve of an E13.0 *Pax2-Pax6* transgenic embryo were treated with anti-*Pax6* monoclonal antibody (staining in purple). The arrowheads define the limits of the expanded RPE in a distal **(I)** and more proximal **(J)** section. **(K,L)** Expansion of the RPE along the optic nerve of transgenic **(K)** and wild-type **(L)** adult mice. The arrowheads indicate the proximal limit of the RPE elongation. tg, transgenic; wt, wild type.

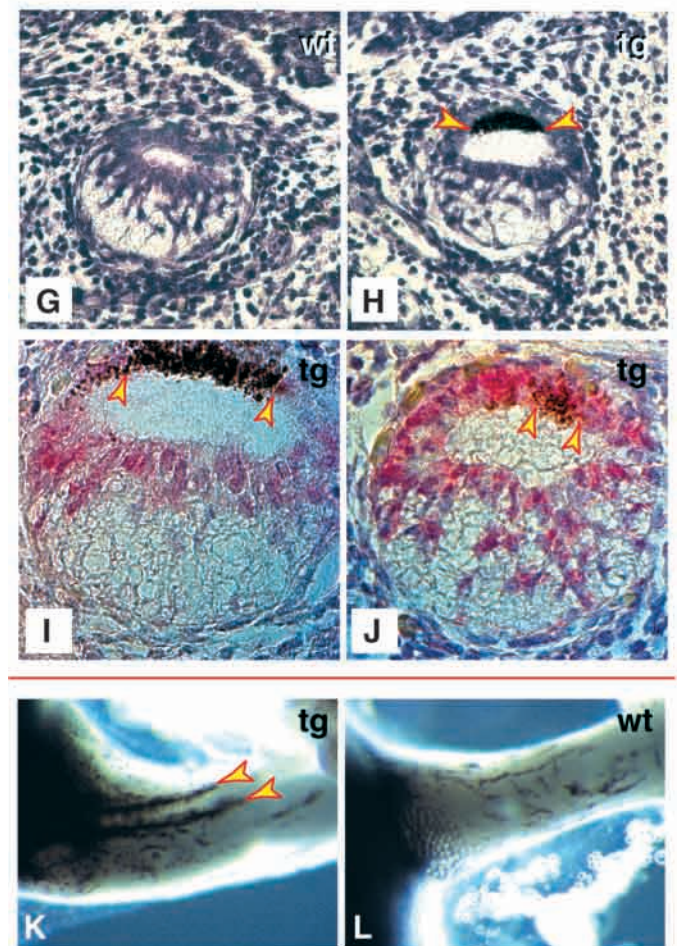
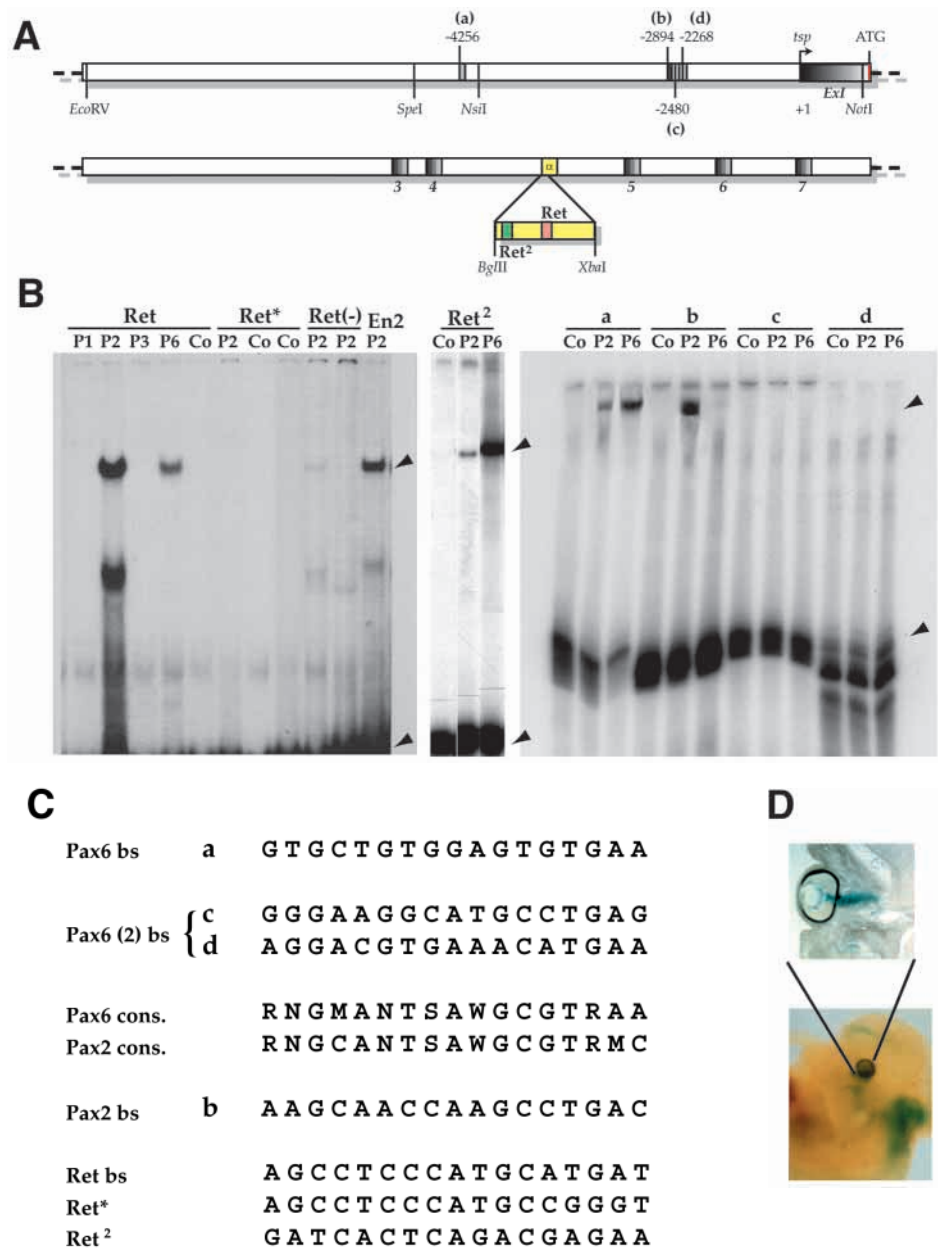


Fig. 5. Molecular analysis of Pax2 and Pax6 optic system-specific enhancer regions. (A) Pax2-(upper part) and Pax6-(lower part) tissue-specific enhancer regions. The Pax2 optic stalk-specific enhancer is located within 9 kb upstream to the transcription start point (*tsp*) of exon 1 (*Ex1*). The small dark-gray boxes (c,d) indicate the putative Pax6/2 binding sites found by sequence comparison. The small black box (b) indicates the single Pax2 binding site. The small gray box (a) indicates the Pax6-binding site. ATG is the start codon for the Pax2 coding sequence. The Pax6 retinal-specific enhancer (Ret) is located between the *Bgl*III and *Xba*I sites in the alpha region of intron 4 (α). The pink and the green boxes within the Ret element highlights the Pax2- and Pax6-binding sites found by sequence comparison (this work and Kammandel et al., 1999). (B) The left panel shows a gel shift assay showing binding of Pax2 and Pax6 proteins on the Pax6- and Pax2-binding sites located in the Pax6 retinal enhancer (Ret and Ret²). A mutated version of the binding site (Ret*) was used as a negative control. As controls, competition experiments were performed using different concentrations, from left to right, of the unlabeled oligonucleotides corresponding to the Ret element [Ret(-)]. Pax2 binding on the *en2* enhancer was also used as a positive control. The right panel shows binding of Pax2 and Pax6 proteins on the binding sites identified on the Pax2 enhancer by sequence comparison. The binding site (a) has the potential to bind both Pax2 and Pax6 protein, whereas the binding site (b) is able to bind Pax2 only. c and d are not able to bind Pax2 or Pax6 proteins. 'Co' indicates the basal control. The labeled oligonucleotides were incubated with protein pools in vitro generated without Pax2 and Pax6 expression vectors. (C) Sequence comparison between the identified binding sites, which were able to bind Pax2, Pax6 proteins or both, and the negative control site Ret*. (D) *lacZ* expression in the Pax2-*lacZ* transgenic embryo. The Pax2 promoter element shown in (A) is able to drive reporter gene expression along the optic nerve.



element was tested for its capability to drive reporter gene expression in optic nerve tissues in vivo (Fig. 5D). A putative Pax2-binding site and three putative Pax6-binding sites were found within the Pax2 enhancer region (at positions -2894 (c, Pax2) and -4256, -2480, -2268 (a,b,d, Pax6)). Two putative Pax2 and Pax6 binding sites were found within the Pax6 retinal enhancer region (Ret and Ret²) (Fig. 5A-C). Gel shift experiments showed direct interaction between in vitro translated Pax2 and Pax6 proteins, and end labeled DNA fragments corresponding to several putative binding sites (Fig. 5B; Czerny et al., 1993). For the Pax6 retinal enhancer, a mutated version of the Ret binding site was used as a control (Fig. 5B). We show that the Pax2 and Pax6 proteins could bind

both Ret and Ret² sites (Fig. 5B, left and middle panels). In addition Pax2 is able to bind the putative binding sites a and b located on its own enhancer, while Pax6 protein could only bind site a (Fig. 5B). The mutated Ret site (Ret*) as well as the other Pax6 putative binding sites (c,d) did not show any shifts when reacted with both proteins (see Fig. 5B,C; Czerny et al., 1993).

These results suggest that both Pax2 and Pax6 proteins can bind tissue-specific enhancer regions on both their own promoters and on the reciprocal ones, suggesting reciprocal regulation between both transcription factors and the potential for autoregulatory self-activation.

In order to confirm our findings in a cell culture system, we

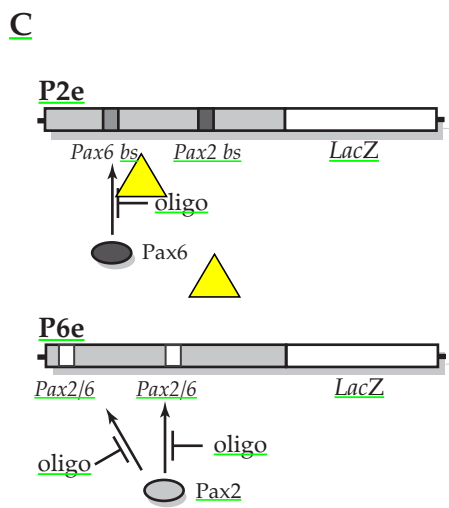
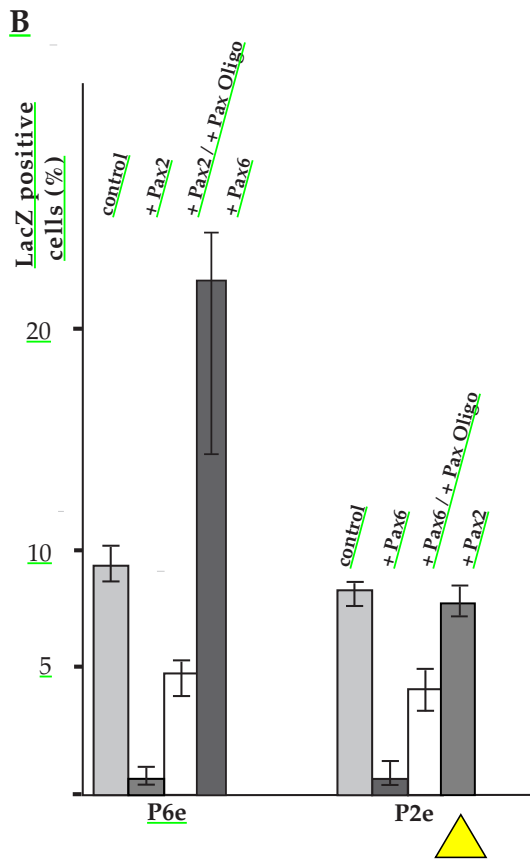
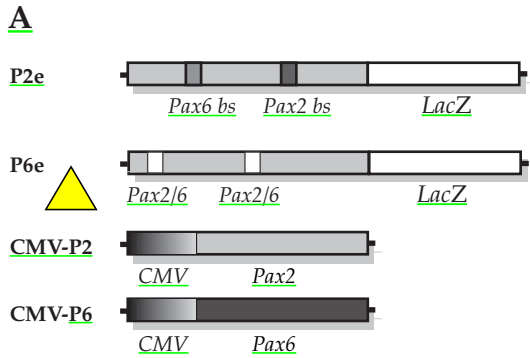


Fig. 6. The reciprocal repression between Pax2 and Pax6 in *cos-7* cells. (A) The constructs used in these experiments. CMV, human cytomegalovirus promoter; *lacZ*, reporter gene coding for β -galactosidase; Pax2, Pax2 cDNA, Pax6, Pax6 cDNA; Pax2, Pax2 binding site; Pax2, Pax6 binding site. (B) Histogram showing the relative percentage of X-gal stained cells, from different co-transfection experiments performed in doublets. Both P2e and P6e constructs are transcriptionally functional in *cos-7* cells. 100% indicates the highest number of stained cells obtained transiently transfecting them with construct P6e and P2e independently. All independent experiments were normalized to luciferase gene expression used as internal standard. (C) The molecular events that form the basis of the reporter gene expression shown in B. Pax6 binds to Pax6 bs on P2e and represses the expression of β -galactosidase; this effect can be compensated by the co-transfection of an oligonucleotide that specifically competes for Pax6 binding. Pax2 binds to Pax2 bs on the P6e and represses the expression of β -galactosidase; this effect is compensated by the co-transfection of oligonucleotide specifically competing for Pax2 binding.

tested the repressional activity of Pax2 and Pax6 proteins on their corresponding reciprocal tissue-specific enhancers by transient transfections of *cos-7* cells with the Pax2 and Pax6 enhancer constructs carrying the β -galactosidase reporter gene (P2e and P6e, respectively; Fig. 6A). Subsequently, we co-transfected the P2e construct with a CMV-Pax6 expression plasmid (Walther and Gruss, 1991) and found that *lacZ* activity, as revealed by X-gal staining, is reduced up to 90% when compared with P2e transfection alone (Fig. 6A,B). The same experiments were carried out with the P6e construct and a CMV-Pax2 expression vector (Fig. 6A,B) (Dressler et al., 1990). Comparable repression of the reporter gene expression was shown (Fig. 6B). In order to demonstrate that the repression effect we observed was specifically dependent on Pax2 and Pax6 proteins we co-transfected the oligonucleotides corresponding to Pax2- or Pax6-binding sites used for the band shift experiments. We could show a specific rescue of *lacZ* activity (Fig. 6B). After counting all the X-gal-stained cells out of three independent experiments carried out in parallel doublets, it was found that specific *lacZ* activity could be

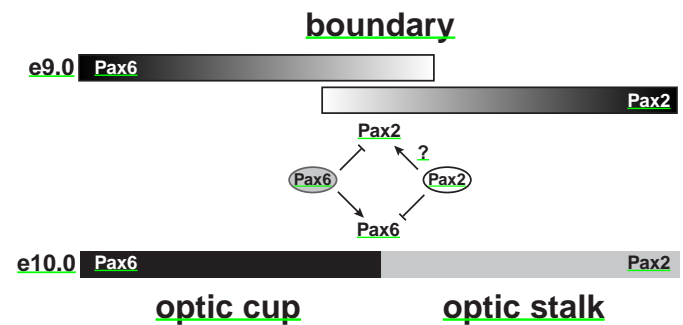


Fig. 7. A model for the reciprocal inhibition between Pax2 and Pax6 in the mammalian visual system. The shaded gray bars (upper part) schematically represent the Pax2 and Pax6 expression gradients in the optic vesicle at E9.0. The overlaps of the two boxes indicate the region of the prospective optic cup/optic stalk boundary. The black and the light gray bars (lower part) represent the finally differentiated optic cup and optic stalk at E10.0. In this model, Pax6 protein (gray circle) can repress Pax2 and enhances its own transcription. Conversely, Pax2 protein (white circle) can repress Pax6 and could enhance its own transcription.

rescued by up to 60% (Fig. 6B). In all the experiments, luciferase expression was used as an internal standard for transfection efficiency (data not shown). Co-transfection of P6e and CMV-Pax6 leads to a more than double *lacZ* expression. This evidence confirmed the assumption of an autoregulatory activation of Pax6e by the Pax6 protein. By contrast, co-transfection of P2e and CMV-Pax2 did not result in activation of the reporter gene. Fig. 6C schematically summarizes the molecular mechanisms implicated from the results of these co-transfection experiments.

DISCUSSION

The data presented provide evidence that the two paired box transcription factors Pax2 and Pax6 cooperate in a molecular network to establish the boundary between the optic stalk and the optic cup in mammals by reciprocally controlling their expression levels.

Pax2 and Pax6 expression domains in the mutant embryos

The *Pax2* and *Pax6* mutants do not exhibit gross morphological alterations at E9.5 regarding the spatial organization of optic cup and stalk (Torres et al., 1996; Grindley et al., 1995). We therefore tested for possible gene expression alterations in the respective mutants. We showed an expansion of Pax2 expression at the margins of the optic cup in *Pax6* mutants, suggesting that Pax2 expression fails to become restricted to the optic cup/optic stalk border. Later on in development the primordial cup disappears; nevertheless, strong staining for Pax2 remains. We attribute the expansion of Pax2 expression in the *Pax6* mutant at E9.5 as well as the remaining expression of Pax2 in later stages of the *Pax6* mutant to a failure of Pax6 to repress *Pax2* transcription. In *Pax2* mutants, Pax6 expression appears to be expanded towards the roof of the diencephalon, suggesting a failure of Pax2 protein to repress *Pax6* transcription. In comparison with the *Pax6*^{-/-} phenotype, the late phenotype of *Pax2* mutants is different, since expansion of Pax6 and other retinal-specific genes results in the formation of a correctly differentiated retina. Unlike the optic cup (which degenerates in the *Pax6* mutants), the primordial optic stalk acquires the fate of neural retina in the *Pax2* mutant. Our findings imply that the presumptive optic stalk has the potential to give rise to retina upon expression of retinal-specific genes. However, in the presence of Pax2 this can not happen, because *Pax6* is repressed.

In principle, the expression of *Pax6* into the putative Pax2-domain of the *Pax2*^{-/-} stalk could be due to a general elongation of cup-specific tissues into the stalk, depleted of Pax2-positive cells at early stages. Unfortunately, the *Pax2*-mutant allele does not contain any reporter activities, which would help us to better characterize the Pax6-positive cell population in the stalk. However, the RPE expansion we observed in the *Pax2*-*Pax6* transgenic optic nerve at E13.0 in presence of Pax2 endogenous expression seems to confirm our model (see below).

Ectopic expression of Pax6 in the optic stalk leads to retinal differentiation

Mice lacking Pax6 function do not develop retina (Hill et al.,

1991). In accordance with this finding it has been shown that in zebrafish with experimentally reduced number of Pax6-expressing cells in the optic vesicle, retinal differentiation is restricted to cells that retain Pax6 protein (Macdonald et al., 1995). It has therefore been suggested that Pax6 might be required by cells within the optic vesicle to initiate retinal development (Macdonald et al., 1995; Macdonald and Wilson, 1996).

Experiments carried out in *Drosophila* provided us with evidences that Pax6 is required to specify retinal identity in optic vesicle cells. Ectopic expression of both mouse *Pax6* and *Drosophila eyeless* can re-specify imaginal disc cells to form ectopic eyes on legs, wings and antennae (Quiring et al., 1994; Halder et al., 1995). We asked the question of whether Pax6 can also specify retinal identity in cells outside the normal retinae in vertebrates, in order to demonstrate that the failure to form retina in homozygous *Pax6* mice is attributable to a requirement of Pax6 within presumptive retinal cells.

If Pax6 was a potent inducer of retinal development and the expanded retina in the *Pax2* mutant mice was due to the expansion of Pax6 expression, then ectopic expression of Pax6 in proximally located cells of the stalk should result in the differentiation of retinal cells. In order to test this hypothesis, we expressed *Pax6* cDNA ectopically under the control of the *Pax2* optic stalk enhancer. By way of this experiment, we demonstrate that Pax6 indeed triggers the development of retinal compartments: RPE appears in the dorsal region of the optic stalk. Our experiments suggest that Pax6 is sufficient to induce a developmental cascade, which in turn leads to the differentiation of presumptive optic stalk cells into RPE. Interestingly, we were only able to detect RPE but no neural retina, suggesting that Pax6 is not sufficient to initiate the development of neural retina. Alternatively, the responding proximal stalk tissue has only the capacity to differentiate along the pigmented retinal lineage. It is noteworthy that the proximal eye defects caused by Pax6 ectopic expression also resemble those in *Xenopus* embryos misexpressing *Pax6* or *Rx*, where extensions of RPE towards the midline have been observed (Chow et al., 1999; Andreazzoli et al., 1999).

The expansion of Pax6 expression domain and the differentiation of RPE do not reach the proximal end of the optic nerve. This was expected as we showed that Pax6 could bind to the *Pax2* promoter, leading to the repression of *Pax2* transcription. The transgene would start to repress its own transcription, following a first transcriptional activation. However, as the mechanism of reciprocal inhibition depends crucially on the equilibrium between those transcription factors, we subsequently shifted the Pax6-expressing domain in a proximal direction by disrupting this equilibrium. The effect is most apparently visible in the dorsal stalk, the domain of lowest Pax2 expression, suggesting the existence of a dosage effect of Pax2 in its repressional activity. The phenotypical appearance points to a disruption of the optic cup/optic stalk boundary, underlining the importance of Pax2 and Pax6 in the correct partitioning of the eye into optic cup and optic stalk. This model is further strengthened by the heterozygous phenotypes because of the haploinsufficiency of *Pax2* and *Pax6* (Hill et al., 1991; Sanyanusin et al., 1995). According to our model, the appearance of smaller eyes in heterozygous *Pax6* mice should be due to the expansion of the Pax2-positive domain on the expense of the Pax6 expression domain. In

contrast, the *Pax2* heterozygous mice should show a proximal expansion of retinal cells, similar to the expansion we observe in our transgenics. Indeed, we were able to confirm a proximal expansion of RPE in heterozygous *Pax2* animals (data not shown), pointing again to the necessity of the exact regulation of the *Pax2* and *Pax6* gene dosage in eye regionalization.

Molecular interactions between Pax2/Pax6 proteins and their reciprocal enhancers

In order to show a direct interaction between Pax2 protein and *Pax6* enhancer and vice versa at the molecular level, we characterized the tissue-specific enhancers by generation of transgenic reporter strains using genomic DNA fragments and subsequent sequence analysis (Kammandel et al., 1999). The molecular characterization of reporter gene constructs, used to generate transgenic animals, serves as an ideal tool with which to study possible molecular interactions. We detected one relevant Pax2- and one relevant Pax6-binding site on the *Pax2* promoter, which enabled *lacZ* expression within the optic stalk. On the *Pax6* retina-specific element, we identified two relevant Pax2- and Pax6-binding sites.

We confirmed our in vitro findings and showed in *cos-7* cells that Pax6 protein could bind to the *Pax2* element thereby repressing its transcriptional activity. The same effect was also shown for Pax2 protein on the *Pax6* enhancer. We showed repression of the endogenous *lacZ* activity (driven by the tissue-specific enhancers) by up to 90% upon co-transfection with the corresponding repressor proteins. This strongly suggests that Pax2 and Pax6 proteins are potent repressors on their reciprocal enhancers in living cells. As a control for the specificity of our co-transfections we performed competition experiments with the oligonucleotides identified in the gel shift assays. Upon co-transfection with the oligonucleotides, we restored the transcription of the reporter construct up to 60% of the endogenous level. We thus provide a strong in vivo demonstration that Pax2 and Pax6 proteins can bind to their corresponding reciprocal enhancers and repress transcription. Furthermore, we have shown that Pax6 can activate by autoregulation its own retinal element.

The capability of the Pax6 protein to regulate the *Pax2* promoter negatively but to regulate the *Pax6* retinal element positively in our system can be explained by the presence of co-factor(s) in the *cos-7* cell system that play a co-operative positive role (together with Pax6) on P6e. The basal level of P6e transcription observed in the control experiment, which is slightly higher than the corresponding P2e activity, could be accounted for by the presence of such putative factors (see Fig. 6B, controls). Alternatively, we could attribute to the *Ret²* binding site on P6e, which shows a very high affinity for Pax6 (Fig. 5B), an intrinsic positive activity. Nucleotide differences between the *Ret²* element on P6e and the b site on P2e (Fig. 5C) could account for the absence of Pax2 autoregulation (Fig. 6B).

A molecular model for the spatial specification of the mammalian visual system

We propose the following molecular model for visual system regionalization (see Fig. 7). The signaling molecule sonic hedgehog, which establishes the midline of the brain and subdivides the eye domain, is presumably the initial activator of Pax2 (Macdonald et al., 1995; Macdonald and Wilson,

1996). Strong evidence comes from analysis in mice lacking sonic hedgehog function (Chiang et al., 1996). With regard to eye patterning, it has been demonstrated that sonic hedgehog is required for Pax2 expression and optic stalk formation (Chiang et al., 1996). These results are consistent with previous evidence from zebrafish studies suggesting that sonic hedgehog activity from the ventral midline normally stimulates expression of Pax2 in the adjacent optic stalk precursors and represses expression of Pax6 (Macdonald et al., 1995). This in turn restricts Pax6 expression to distal portions of the optic vesicle. Unfortunately, no candidate gene activating Pax6 in the prosencephalic portion of the neural plate has been described. A possible candidate directly or indirectly acting on Pax6 activation, based on the spatial-temporal expression pattern could be the homeobox protein Otx2 (Simeoni et al., 1992) or the cell-signalling molecule Notch1 (W. Gehring, personal communication).

After their initial activation, the Pax2 and Pax6 expression would become independent of the activating factors, owing to their autocatalytic enhancement. Pax6 protein can bind its own enhancer and has the potential to stimulate transcription. The Pax2 protein, in turn, has the capacity to bind to the *Pax6* enhancer and represses transcription. The Pax6 protein has the same function on the *Pax2* enhancer. Although *Pax2* promoter region contains one site for Pax2 protein binding, we did not observe Pax2 autocatalytic enhancement in the cell culture assays. As discussed above, sonic hedgehog could be hypothesized to be the Pax2 activator, since the sonic hedgehog gradient always reaches the Pax2-expressing territory; alternatively we could speculate that the promoter region we used in the transfection experiment does not contain the element(s) that are essential for Pax2 self-activation.

Consequently, in a region where the activity of these genes overlaps, reciprocal inhibition establishes a boundary, finally leading to a steady state where no cells express both proteins at the same time (Meinhardt, 1982). This in consequence leads to the formation of the optic stalk and optic cup boundary. However, we do not know whether or not the Pax2 and Pax6 boundary and therefore the division of a Pax2- and a Pax6-positive domain (which follow very different developmental fates) is the first separation generating positional information for the eye field.

In *Drosophila*, similar to the vertebrate system where Pax2 expression is restricted to the optic stalk and Pax6 expression to the optic cup and the lens, *sparkling* and *eyeless* are expressed in the homolog structures (Fu et al., 1997). *sparkling* expression is found in the precursors of cone and primary pigment cells, whereas *eyeless* expression is restricted to regions anterior to the morphogenetic furrow in the undifferentiated part of the eye disc epithelium (Fu et al., 1997). In addition, the phenotype of the *eyeless* or the *sparkling* mutation is comparable to the *Pax6^{-/-}* and *Pax2^{-/-}* phenotypes in the mouse. In conclusion, Pax2 and Pax6 and their *Drosophila* homologues *sparkling* and *eyeless*, play important and strikingly conserved roles in the morphogenesis and regional specification of the vertebrate or insect eyes (Fu et al., 1997).

Finally, we would like to mention that the visual system is most probably not the only example of reciprocal inhibition between Pax genes in the regionalization of the body plan. These genes are expressed in adjacent territories in other

embryonic structures (Walther and Gruss, 1991; Rowitch and McMahon, 1995). We have previously shown that the regionalization of the midbrain/forebrain boundary is crucially depending on Pax6 expression in the prosencephalon, and on Pax2 and Pax5 expression in the mesencephalon (Schwarz et al., 1999). In the spinal cord, Pax1/9 and Pax3/7 could be good candidates for such a regionalization mechanism in more caudal parts of the neuroectoderm. Therefore, the molecular mechanism we describe for the eye may be of more general importance.

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