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Pax6 is required for establishing naso-temporal and dorsal characteristics of the optic vesicle

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SUMMARY

The establishment of polarity is an important step during organ development. We assign a function for the paired and homeodomain transcription factor Pax6 in axis formation in the retina. Pax6 is a key factor of the highly conserved genetic network implicated in directing the initial phases of eye development. We recently demonstrated that Pax6 is also essential for later aspects of eye development, such as lens formation and retinogenesis. In this study, we present evidence that a highly conserved intronic enhancer, α, in the *Pax6* gene is essential for the establishment of a distal^{high}-proximal^{low} gradient of Pax6 activity in the retina. In the mature retina, the activity mediated by the α-enhancer defines a population of retinal ganglion cells that project to two sickle-shaped domains in the superior colliculus and lateral geniculate nucleus. Deletion of the α-enhancer in vivo revealed that retinal Pax6 expression is regulated in two complementary topographic domains. We found that Pax6 activity is required for the establishment, as well as the maintenance of dorsal and nasotemporal characteristics in the optic vesicle and, later, the optic cup.

Key words: Pax6, Enhancer, Retina, Autoregulation, Axon guidance, Superior colliculus, Axis formation, BF-1, BF-2, Mouse

INTRODUCTION

The establishment of topographic neuronal connections with precise spatial relationships is a key problem in neurobiology (O'Leary et al., 1999). The visual system serves as a paradigm for studying the axon guidance mechanisms underlying the formation of topographic neuronal maps (Goodhill and Richards, 1999). The projections of retinal ganglion cells (RGCs) to the primary visual centers precisely reflect the relative position of their cell bodies in the neuroretina (NR) (Lund et al., 1974). Axons originating from a specific point in the nasal or temporal NR (see schematic views in Fig. 1G, Fig. 4D) stereotypically project to a specific point within the caudal or rostral superior colliculus, and, in a tilted angle, into the caudal or rostral lateral geniculate nucleus (LGN), respectively (Godement et al., 1984; Simon and O'Leary, 1992). RGC axons therefore are connected to the primary visual centers in the mesen- and diencephalon in a point-to-point topographic manner, allowing the correct spatial representation of the visual environment in the brain.

Several lines of evidence indicate that the establishment of topography in the NR is already initiated in the optic vesicle, preceding the formation of the optic cup and the onset of neuronal differentiation in the NR. For example, retinoic acid and BMP signaling have been shown to ventralize the optic cup (Hyatt et al., 1996; Wagner et al., 2000; Sakuta et al., 2001), suggesting that localized extrinsic signaling implements a positional grid on the optic vesicle across the dorsoventral and possibly also the nasotemporal axis. The initially coarse positional grid later becomes refined by the graded expression of axon guidance molecules in RGCs across the retinal axes, most prominently of the Eph family of receptor tyrosine kinases (Drescher et al., 1995; Cheng et al., 1995). In outline, Eph receptors are thought to mediate axonal repulsion upon encountering their membrane-bound ligands, the ephrins (O'Leary and Wilkinson, 1999; Holder and Klein, 1999). The complementary expression gradients of Eph receptors in the NR and their ligands in the superior colliculus and LGN are thereby assumed to underlie the topographic mapping RGC axons (O'Leary and Wilkinson, 1999; Feldheim et al., 2000). Until recently, however, the regulation of these axon guidance molecules across the retinal axes remained obscure.

The winged-helix transcription factors BF-1 (Foxg1 – Mouse Genome Informatics) and BF-2 (Foxd2 – Mouse Genome Informatics) are among the first factors that mark the distinction between the presumptive nasal and temporal NR as they are already expressed before the onset of neuronal differentiation in the anterior and posterior optic vesicle, respectively (Hatini et al., 1994). Overexpression of BF-1 and BF-2 in chick embryos lead to targeting errors of RGC

projections to the tectum (Yuasa et al., 1996). In mice, the absence of BF-1 function severely affects the development of the ventral NR and appears to lead to a mirror-image duplication of the temporal axis (Huh et al., 1999). Similarly, the T-box and homeodomain transcription factors Tbx5 and Vax2 become expressed in the dorsal and ventral optic cup, respectively, prior to the onset of retinogenesis (Koshiba-Takeuchi et al., 2000; Barbieri et a., 1999). Overexpression of Tbx5 results in dorsalization of the NR and in misrouting of ventral axons (Koshiba-Takeuchi et al., 2000). Conversely, the absence of Vax2 leads to a dorsalization of the NR in the mouse (Mui et al., 2002; Barbieri et al., 2002), whereas misexpression of the orthologous Vax in chick embryos disturbed dorsal RGC projections (Schulte et al., 1999). Null mutants of *Vax1*, a mouse paralog of Vax2, show optic cup coloboma and optic nerve dysplasia with axon misguidance, which is due to its expression in the very ventral NR and in the optic stalk (Hallonet et al., 1998; Hallonet et al., 1999; Bertuzzi et al., 1999). Furthermore, the homeodomain factors SOHo1 and GH6 were implicated in mediating proper retinotectal projections from nasal RGCs in the chick embryo by controlling the expression of EphA3 (Schulte and Cepko, 2000).

The paired and homeodomain factor Pax6 is a key member of a highly conserved interactive network of transcription factors implicated in the initiation of eye development (Gruss and Walther, 1992; Gehring and Ikeo, 1999). Forced expression of Pax6 is sufficient to promote the formation of ectopic eye structures in insects and vertebrates (Halder et al., 1995; Altmann et al., 1997; Chow et al., 1999), while *Pax6* null mutations invariably lead to the absence of all functional eye structures (Glaser et al., 1990; Hill et al., 1991; Quiring et al., 1994; Grindley et al., 1995). Recently, we could provide a link between these generic mechanisms governing retinal formation and the cytogenetic potential of retinal progenitor cells (Marquardt et al., 2001), demonstrating a continued requirement for Pax6 activity in later retinal development. Moreover, the proper development of distal eye structures, iris, cornea and lens epithelium appears to be highly sensitive to variations in the level of Pax6 activity. Heterozygosity for *Pax6* mutations in human and mouse lead various degrees of iris hypoplasia and dysplasia, as well as lens and corneal defects (Jordan et al., 1992; Glaser et al., 1994; Hanson et al., 1994; Collinson et al., 2001), while mice overexpressing Pax6 display microphthalmia associated with retinal, corneal and iris dysplasia (Schedl et al., 1996). In the newly formed optic cup, Pax6 is soon observed to be expressed in a marked distal^{high}-proximal^{low} gradient, suggesting a role in optic cup patterning. The recent identification of a downstream target of Pax6 expressed in the distalmost region of the optic cup (Bernier et al., 2001) further supports such a role.

In this study, we demonstrate that the distal^{high}-proximal^{low} gradient of Pax6 activity in the optic cup is mediated by a highly conserved intronic enhancer ('α') (Kammandel et al., 1999) of the *Pax6* locus. In the mature NR, this enhancer drives expression in a subset of RGCs that stereotypically send their axonal projections to two concise stripes in both the superior colliculus and LGN. Intriguingly, in a targeted mouse line in which the α-enhancer is deleted (St-Onge et al., 1997), the expression of the remaining regulatory elements of *Pax6* can be mapped to RGCs that send their projections to the central region of the superior colliculus, precisely complementing the area targeted by the α-positive RGCs. Therefore, in the mature NR Pax6 expression appears to be regulated in distinct topographic domains that divide superior colliculus and LGN into two concise areas that receive axonal input from (1) the distal nasal and temporal NR, and (2) the proximal NR. In the optic vesicle and later the optic cup, we found that Pax6 activity was required for the establishment, as well as the maintenance, of the dorsal and nasotemporal characteristics, providing a link between generic mechanisms governing retinal development and the patterning of the optic cup, which ultimately underlie the topographic mapping of retinal axons to the brain.

MATERIALS AND METHODS

Transgenic and targeted mice

For the generation of *α-tau-lacZ* transgenic mice, a 1.8 kb *Acc*I fragment from intron 4 of murine *Pax6*, containing the *α*-enhancer (Kammandel et al., 1999), was fused to a 0.4 kb *Bgl*II-*Xba*I fragment containing the *Pax6* P0 promoter (P0). This *α-P0* enhancer-minimal promoter fragment was cloned as a 2.2 kb *Pac*I fragment upstream of *IRES-tau-lacZ* (pKS^{-}ETL) (Mombaerts et al., 1996). The resulting 8.2 kb construct was excised as a *Sal*I/*Xho*I fragment prior to microinjection following standard procedures (Hogan et al., 1994). *α-tau-lacZ* mice were genotyped by Southern analysis with a *Bam*HI bovine *tau* fragment of pKS^{-}ETL or PCR using *lacZ* primers. *Pax6^{lacZ}*, *Pax6^{flox}* and *α-Cre-gfp* mice were generated previously (St-Onge et al., 1997; Ashery-Padan et al., 2000; Marquardt et al., 2001). *Pax6^{lac}* were genotyped by PCR using the following primers: P6S, 5′-GCATATGGGGGCAAGACTATGTG-3′; AS2, 5′-CCAGAGAAAG-ACCTGAGACACT TAC-3′; and Lac1, 5′-CTGTTGGGAAGGGCG-ATCGGTG-3′. The final reaction mixture contained 1 μg of yolk sac DNA, 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 10 mM βME, 1.5 mM $MgCl_2$, 10% DMSO, 200 μM dNTP mix, 0.25 μM of each primer and two units of Taq-polymerase. Cycling conditions were 94°C for 2 minutes, hot start at 80°C for 2 minutes, 30 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds, final extension 72°C for 5 minutes. The wild-type allele produced a band of 250 bp, the *Pax6^{lacZ}* allele a band of 300 bp.

Immunohistochemistry on cryosections

The embryos were washed in phosphate-buffered saline (PBS), fixed for 30 minutes fresh in cold 4% PFA/PBS (pH 7.8), washed three times 20 minutes each with PBS, incubated in 30% sucrose/PBS for overnight at 4°C and shock frozen in tissue freezing medium (Jung). Sections of 6-10 μm were air-dried for 30 minutes and stored at –20°C or –80°C.

For the antibody staining, the sections were washed three times in PBS (5 minutes each), blocked with 1% BSA (IgG-free, Sigma), 0,05% Tween-20 in PBS 30 minutes at room temperature. The primary antibody was diluted in blocking solution and incubated at 4°C overnight (rabbit anti-β-gal, Cappel, 1:300; monoclonal mouse anti-Pax6, DSHB, 1:20). After three washes in PBS, 5-10 minutes each, the secondary antibody, diluted in blocking solution, was applied for 1 hour (Alexa 568 goat anti rabbit, Molecular Probes, 1:500; FITC goat anti mouse, Southern Biotechnology, 1:60). After again three washes with PBS, counterstaining was performed with Dapi and the sections were embedded with Mowiol.

Histochemical detection of β-galactosidase activity

For X-gal staining, embryos or tissues were rinsed in PBS, fixed for 0.5 to 1.5 hours in cold 1% formaldehyde, 0.2% glutaraldehyde in wash buffer (0.2% NP-40, 0.1% Na-desoxycholate in PBS), then

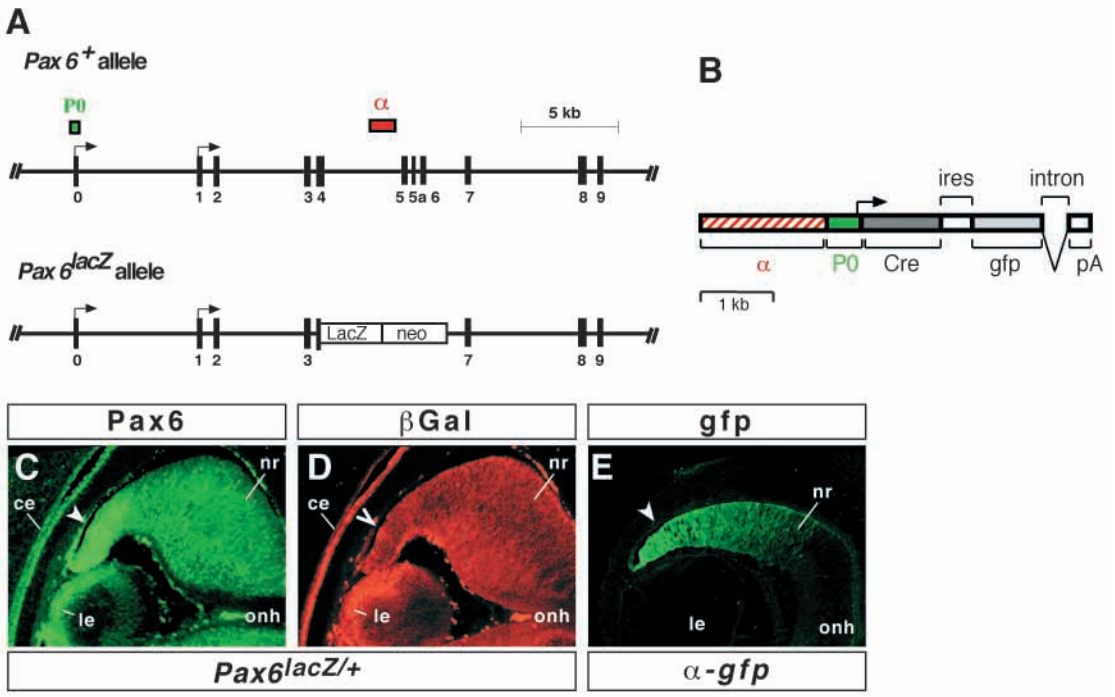

Fig. 1. The α-enhancer is required for establishing a distal^{high}-proximal^{low} gradient of Pax6 activity in the neuroretina (NR). (A, top) Wild-type *Pax6* genomic locus. Positions of the 1.8 kb fragment containing the α-enhancer and the 0.4 kb P0 promoter used in the transgene constructs shown in B and Fig. 3I are indicated. (A, bottom) In the *Pax6^{lacZ}* allele *lacZ/neo* was inserted in frame with the translation start, replacing exon 4 to exon 6 (St-Onge et al., 1997), thereby eliminating the α-enhancer. (B) Transgene driving gfp (and Cre) expression under control of α-enhancer via P0 (see Marquardt et al., 2001). (C) Immunohistochemical detection of Pax6 in an E15.5 *Pax6$^{lacZ/+}$* eye reveals normal distal^{high}-proximal^{low} gradient of Pax6 in the NR (arrowhead indicates distal high end of gradient). (D) In the same eye, β-gal is uniformly distributed throughout the NR (open arrowhead). Relatively higher β-gal levels are still found in corneal (ce) and lens epithelium (le), matching endogenous relative Pax6 levels in these tissues (see C). (E) gfp expression in transgenic mice carrying the construct shown in B. At E15.5, α drives a sharp distoproximal gradient of gfp expression in the NR, matching the high end of the Pax6 gradient (arrowheads). onh, optic nerve head.

washed three time for 20 minutes at room temperature in wash buffer and stained up to 3 days at 30°C in 1 mg/ml X-gal/5 mM $K_3Fe(CN)_6$/5 mM $K_4Fe(CN)_6$/2 mM $MgCl_2$ in wash buffer. The tissues then underwent the standard procedure for paraffin embedding, were sectioned at 7-10 μm and counterstained with Nuclear Fast Red.

For the staining of cryosectioned tissues, the 10 μm sections were washed twice in PBS, postfixed in cold 0.2% glutaraldehyde for 5 min and washed again in PBS. The X-gal staining was then performed at 37°C for 4 hours to overnight in 1 mg/ml X-gal/5 mM $K_3Fe(CN)_6$/5 mM $K_4Fe(CN)_6$/2 mM $MgCl_2$ in PBS.

In situ hybridization

In situ hybridization on paraffin wax-embedded sections using ^{35}S-labeled antisense RNA probes to detect the transcripts of *BF-1* and *BF-2* (Hatini et al., 1994) were carried out as previously described (Kessel and Gruss, 1991). The in situ hybridization of *Tbx5* (probe a gift of J. Johnson), *Vax1* (Hallonet et al., 1999) and *Vax2* (Barbieri et al., 1999) were performed as described previously (Marquardt et al., 2001).

RESULTS

A highly conserved regulatory element establishes a distal^{high}-proximal^{low} gradient of Pax6 activity in the neuroretina

Prior to optic cup formation, Pax6 is widely expressed during eye development in both the optic vesicle and the presumptive lens and corneal ectoderm (Walther and Gruss, 1991). In the newly formed optic cup, Pax6 expression is detected in a sharp distal^{high}-proximal^{low} gradient, with the highest activity in the region of the future inner iris epithelium (Fig. 1C, data not shown). Recently, it was shown that a conserved enhancer in intron 4 of the *Pax6* genomic locus (‘α’; Fig. 1A, top) drives reporter gene expression in the distal nasal and temporal neuroretina (NR), omitting the dorsal and proximal regions of the NR (Kammandel et al., 1999) (see Fig. 1E, Fig. 2A, Fig. 3A,B). The onset of the α-directed expression coincides with the appearance of the proximal-distal Pax6 expression gradient at E10.5 (Marquardt et al., 2001) (data not shown) and matches the region with the highest Pax6 expression in the distal neuroretina. Together, these observations suggested a potential role for this regulatory element in the establishment of a gradient of Pax6 activity in the optic cup.

To investigate the function of the α-enhancer in regulating retinal Pax6 expression, we took advantage of a previously generated *Pax6^{lacZ}* allele in which exon 4-6, including the α-enhancer, has been replaced by a *lacZ* reporter gene (St-Onge et al., 1997) (Fig. 1A, bottom). β-galactosidase (β-gal) expression in *Pax6$^{lacZ/+}$* embryos largely follows the wild-type Pax6 expression pattern in the head surface ectoderm and at later stages in lens and cornea (Fig. 1D, Fig. 5B). From E9.5 to E10.5, staining is also seen above the optic stalk, which becomes downregulated by E11.5 when compared with Pax6

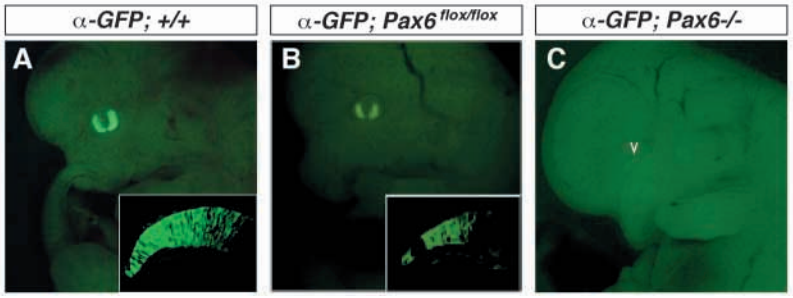

Fig. 2. Pax6 activity is required for initiating and upregulating, but not for maintaining α-enhancer-mediated expression. (A-C) Whole-mount views of E11.5 transgenic embryos in which the α-enhancer drives gfp expression. (A) Normal gfp expression in *α-Cre-gfp; Pax6*$^{+/+}$ (wild-type) embryo. Inset: 10 μm horizontal section revealing gfp expression in distal NR. (B) Reduced gfp levels in *α-Cre-gfp; Pax6*$^{flox/flox}$ where Pax6 is specifically eliminated from the distal (gfp^{+}Cre^{+}) NR. Note that the low fluorescence levels in B and inset, as well as in C were digitally enhanced to reveal expression. (C) No gfp expression can be detected in *α-Cre-gfp; Pax6*$^{-/-}$ (*Pax6*-null mutant) embryos. Arrowhead indicates eye rudiment in mutant embryo.

expression itself (data not shown). Additionally, *Pax6*$^{lacZ/+}$ embryos show β-gal expression in surface ectoderm derived eye structures (Fig. 1D). This expression is consistent with the recently identified regulatory element which drives Pax6 expression in these structures (Kammandel et al., 1999) and which is not deleted in the *Pax6*$^{lacZ/+}$ allele. Remarkably, despite the removal of the α-enhancer driven activity, *Pax6*$^{lacZ/+}$ embryos also display strong β-gal expression in the optic cup and earlier in the optic vesicle (Fig. 1D; data not shown). These results therefore indicate the presence of other regulatory elements within the *Pax6* locus that are sufficient to drive the early aspects of Pax6 expression in optic vesicle and later in the retina**.** This observation is supported by the recent identification of mutations in the human *PAX6* gene in individuals with *Aniridia*. These mutations were mapped downstream of the last exon of the *PAX6* gene, indicating that these new *Pax6* regulatory elements contribute to the β-galactosidase activity in the eyes of *Pax6*$^{lacZ/+}$ mice (Lauderdale et al., 2000; Kleinjan et al., 2001).

Remarkably, in contrast to the graded distribution of endogenous Pax6 (Fig. 1C), the deletion of the α-enhancer in the *Pax6*$^{lacZ/+}$ genotype results in uniform distribution of β-gal expression in the retina along the proximodistal axis (Fig. 1D). At the same time, the level of Pax6 expression outside the retina remained unaffected by the deletion of the α-enhancer as compared with the wild type (compare Fig. 1C,D), indicating that the activity of the α-enhancer is required for establishing a distal^{high}-proximal^{low} gradient of Pax6 expression in the developing NR. Consequently, the combined function of distinct regulatory elements including the α-enhancer appears to result in the full Pax6 expression pattern in the retina.

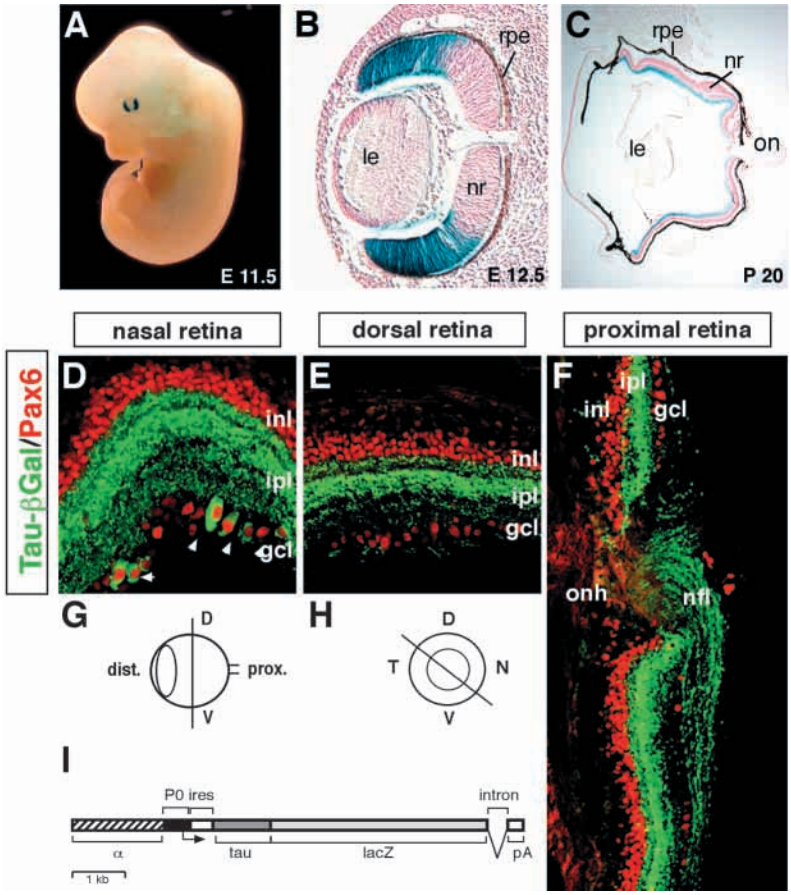

Fig. 3. The α-enhancer directs expression in a subset of Pax6 positive retinal ganglion cells (RGCs) in the distal ventronasal and ventrotemporal retina. (A) Whole-mount view of E11.5 X-gal stained embryo carrying the *α-tau-lacZ* transgene (see I) reveals specific tau-β-gal in the nasal and temporal retina (see Fig. 2A). (B) Horizontal section through E12.5 *α-tau-lacZ* eye (counterstained with neutral red) reveals tau-β-gal expression in the distal nasal and temporal NR (see Fig. 2A, inset). (C) Horizontal section of P20 *α-tau-lacZ* eye reveals tau-β-gal mainly in the distal ganglion cell layer (gcl), as well as tau-β-gal^{+} axons in the nerve fiber layer (nfl, see F). (D-F) Immunohistochemical detection of tau-β-gal (green) and Pax6 (red) in P20 *α-tau-lacZ* eye. (D) In the distal nasal NR, Pax6 is found in the nuclei of inl and gcl, while tau-β-gal labels the cytoplasm (arrows) and axons (see F) of RGCs, as well as dendritic processes of RGCs and amacrine cells (AC) in the inner plexiform layer (ipl). (E) In the dorsal NR, tau-β-gal is only detected in the ipl, representing long dendritic processes of nasal/temporal tau-β- gal^{+} ACs and RGCs. (F) Nasal tau-β-gal^{+} RGC fibers can be detected passing through the optic nerve head (onh). Note that proximal RGCs are tau-β-gal^{-}, but Pax6^{+}. (G) Plane of sections depicted in D,E. (H) Plane of section shown in F. (I) *α-tau-lacZ* transgene: α drives expression of *tau-lacZ* from the P0 promoter. inl, inner nuclear layer; D, dorsal; V, ventral; T, temporal; N, nasal.

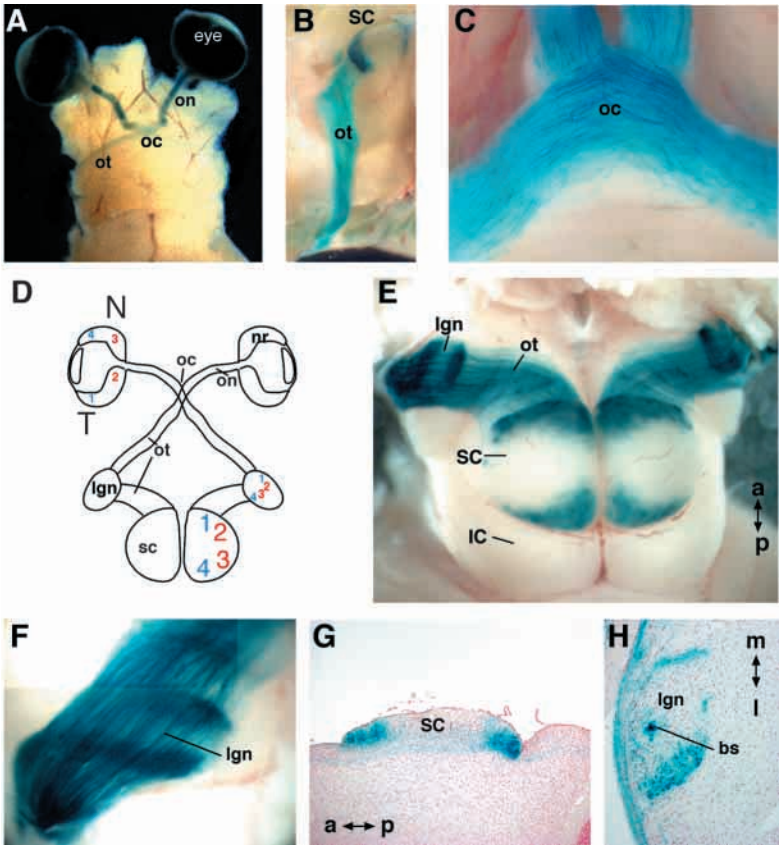

Fig. 4. The α-enhancer directs expression in RGCs that project to two concise domains in the lateral geniculate nucleus (LGN) and superior colliculus (SC). (A-C,E-F): Whole-mount views of P0 (A) and P20 (B-C,E-F) *α-tau-lacZ* brains stained for tau-β-gal activity. (A) At P0, tau-β-gal$^+$ axons can be followed from the eyes through the optic nerve (on), via the optic chiasm (oc) and optic tract (ot) up to the LGN (latter not shown in A). Ventral view of brain to reveal on, oc and ot. (B) At P20, tau-β-gal activity allows tracing of entire extent of the ot, up to the superior colliculus (SC; lateral view of brain; overlying brain tissue was removed). (C) Ventral view of chiasm showing tau-β-gal$^+$ fibers crossing to the ot (anterior towards the top; posterior towards the bottom). (D) Schematic view of the topographic map drawn by tau-β-gal$^+$ axons in the *α-tau-lacZ* line. tau-β-gal$^+$ axons project the distal nasal (4) and temporal (1) NR to the posterior (4) and anterior (1) LGN and superior colliculus, respectively. Proximal RGC axons (2,3) project to the remaining portions of LGN and superior colliculus. (E) Dorsal view on P20 *α-tau-lacZ* brain showing tau-β-gal$^+$ axons terminating in two sickle shaped domains in the superior colliculus and LGN. Note that central portion of superior colliculus is devoid of staining, as is the inferior colliculus (IC) and surrounding tissue. (F) Close-up lateral view of the LGN of a P20 brain *α-tau-lacZ* brain revealing tau-β-gal$^+$ axons terminating mainly in two domains in the ventral and dorsal LGN, respectively. (G) Sagittal section through the superior colliculus revealing tau-β-gal$^+$ axons terminating in the anterior and posterior regions, while omitting the central region (axons projecting to the posterior superior colliculus can be detected). (H) Transversal section through the *α-tau-lacZ* diencephalon revealing tau-β-gal$^+$ axons terminating in the ventral and dorsal LGN. The additional focus of termination presumably corresponds to the binocular segment (bs). Lateral of the LGN, tau-β-gal$^+$ axons can be seen traversing the optic tract.

Pax6 activity is required for initiating and upregulating, but not for maintaining retinal expression via the α-enhancer

Pax6 has previously been suggested to regulate its own expression. First, *Pax6* transcripts were shown to be absent from the head surface ectoderm of *Pax6*-null mutant embryos (Grindley et al., 1995). Second, gfp reporter gene expression driven by a lens-specific regulatory element of *Pax6* was observed to be lost after the Cre-mediated inactivation of *Pax6* (Ashery-Padan et al., 2000). Results from in vitro experiments indicated that Pax6 directly binds and activates the α-enhancer (Schwarz et al., 2000). However, the relevance of Pax6 activity for the α-enhancer-mediated expression in vivo remained unaddressed. To elucidate whether the α-enhancer becomes activated in the absence of Pax6 activity we crossed a transgene, *α-Cre-gfp*, expressing gfp and Cre under the control of the α-enhancer (Fig. 1B) (Marquardt et al., 2001), to mice carrying a targeted *Pax6*-null mutation (Fig. 1A) (St-Onge et al., 1997). We failed to detect gfp expression in *α-gfp; Pax6*$^{-/-}$ embryos (Fig. 2C), indicating that the initiation of α-enhancer mediated expression depends on the presence of Pax6 activity.

We next analyzed the impact of Cre-mediated inactivation of *Pax6* in the optic cup stage retina on α-enhancer mediated activity in *α-Cre-gfp; Pax6*$^{flox/flox}$ embryos (Marquardt et al., 2001; Ashery-Padan et al., 2000). In *α-Cre-gfp; Pax6*$^{flox/flox}$ embryos Pax6 is eliminated from virtually all cells of the distal nasal and temporal NR at around E10.5 (Marquardt et al., 2001). In *α-Cre-gfp; Pax6*$^{flox/flox}$ embryos, gfp expression was detectable in the distal NR up to the day of birth (P0), that is for at least 8 days after loss of Pax6 activity (Fig. 2B) (Marquardt et al., 2001) (data not shown). However, the gfp expression is significantly reduced in the *α-Cre-gfp; Pax6*$^{flox/flox}$ NR (Fig. 2B). Interestingly, gfp expression ultimately becomes extinguished in most of the distal NR during the first postnatal days (data not shown). Together, these results indicate that an early phase of Pax6 activity appears to be essential for the initiation of α-enhancer-mediated expression, while Pax6 activity appears not to be strictly required for maintaining α-enhancer activity. However, the significantly reduced gfp expression levels in the *α-Cre-gfp; Pax6*$^{flox/flox}$ (Fig. 2B) and the *α-Cre-gfp; Pax6*$^{+/-}$(data not shown) NR indicate that positive Pax6 autoregulation via direct interaction with the α-enhancer might contribute to the steep distal^{high}-proximal^{low} gradient of Pax6 activity in the optic cup.

α-enhancer directed expression defines a subset of Pax6 positive retinal ganglion cells projecting to two concise areas in the primary visual relay centers

The *Pax6* α-enhancer continues to direct expression in the postnatal and adult eye, most prominently in the retinal ganglion cells (RGCs) of the distal nasal and temporal NR (Fig. 3C-F). This observation prompted us to map the projections of this subset of Pax6$^+$ RGCs. To this end we created a transgenic mouse line in which the axonal marker gene *tau-lacZ* (Callahan

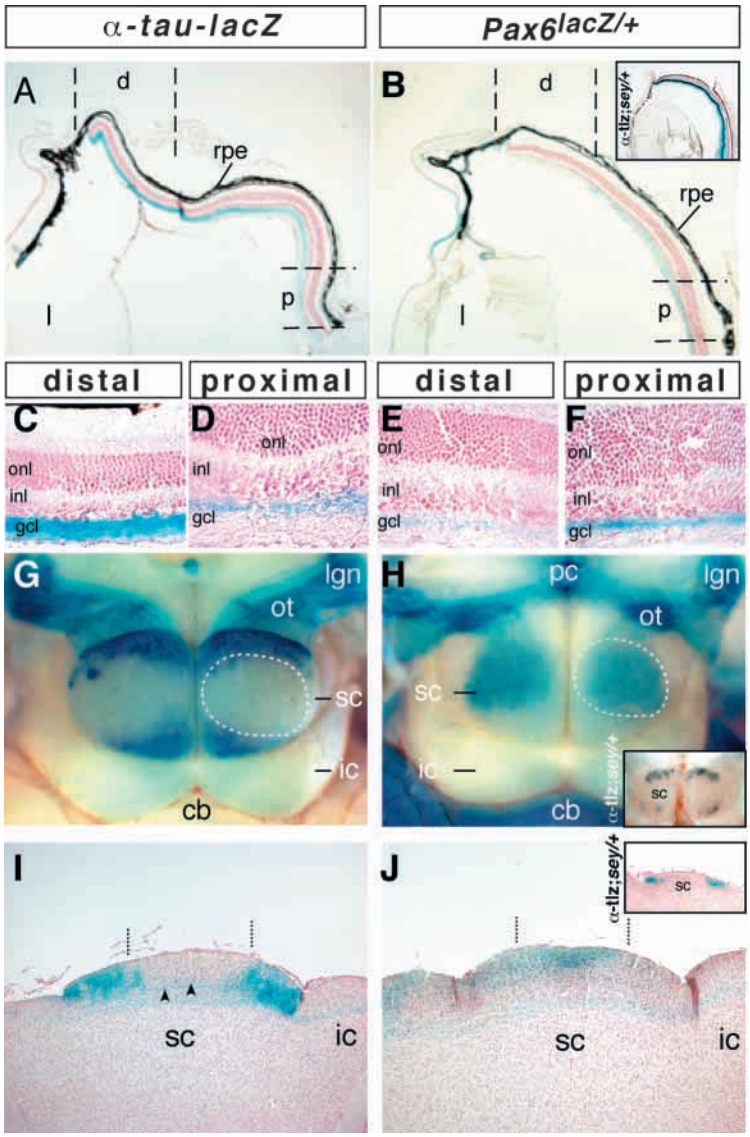

Fig. 5. Retinal Pax6 expression is regulated in two complementary topographic domains. Staining for tau-β-gal/β-gal activity of P20 *α-tau-lacZ* (A,C,D,G,I) and *Pax6*$^{lacZ/+}$ (B,E,F,H,J) eyes and brains. (A) Horizontal section of a *α-tau-lacZ* eye reveals steep distal-to-proximal gradient of expression in the gcl, while *Pax6*$^{lacZ/+}$ eyes display a precisely complementary pattern of β-gal activity (B). (C-F) Higher magnifications of the distal (C,E) and proximal (D,F) NR of *α-tau-lacZ* and *Pax6*$^{lacZ/+}$ eyes shown in A and B, respectively. The *α-tau-lacZ* NR displays strong expression in distal (C), but not in proximal (D) RGCs, while the *Pax6*$^{lacZ/+}$ shows strong expression in proximal (F), but no expression in distal (E) RGCs. (G-H) Dorsal view of the superior colliculus and ot of *α-tau-lacZ* (G) and *Pax6*$^{lacZ/+}$ (H) brains. (G) In the *α-tau-lacZ* brain, tau-β-gal^{+} axons omit the central region of the superior colliculus (dotted circle), which is innervated by β-gal^{+} axons in the *Pax6*$^{lacZ/+}$ brain (H, dotted circle), while the sickle-shaped domains stained in *α-tau-lacZ* are devoid of β-gal^{+} axons (compare G with H). (I,J) Sagittal sections through the of *α-tau-lacZ* (I) and *Pax6*$^{lacZ/+}$ (J) mesencephalon reveals complementary innervation of the superior colliculus by tau-β-gal^{+} and β-gal^{+} axons. Arrowheads in I indicate single stained axons. *α-tlz*, *α-tau-lacZ*; cb, cerebellum; l, lens; onl, outer nuclear layer; pc, posterior commissure.

and Thomas, 1994; Mombaerts et al., 1996) was placed under the control of the α-enhancer (Fig. 3I, referred to as *α-tau-lacZ*). Tau-β-galactosidase (tau-β-gal) expression directed by the *α-tau-lacZ* transgene displays a gradient in the distal NR (Fig. 3B,C) corresponding to the Cre/gfp activity directed by *α-Cre-IRES-gfp* (see Fig. 1E, Fig. 2A) (Marquardt et al., 2001).

In newborn (P0) *α-tau-lacZ* mice, the histochemical detection of tau-β-gal allowed the visualization of axons projecting from distal nasal and temporal NR through the optic chiasm up to the lateral geniculate nucleus (LGN) (Fig. 4A). Around postnatal day 15 (P15), tau-β-gal was detectable in RGC axons growing into the superior colliculus (SC, Fig. 4B,E,G). In the superior colliculus of *α-tau-lacZ* mice, the tau-β-gal^{+} axons terminated in a highly stereotypic fashion within two concise areas, forming two sickle-shaped domains in the rostral and caudal superior colliculus, respectively (Fig. 4E,G). Virtually no tau-β-gal^{+} RGC axons could be observed to terminate within the central region of the superior colliculus in all *α-tau-lacZ* analyzed individuals (Fig. 4E,G) and the labeled axons invariably followed the projection pattern outlined above. In the LGN, tau-β-gal^{+} RGC projections were detected to terminate in a similar fashion, marking two stripes in the rostroventral and caudodorsal LGN, respectively (Fig. 4E,F,H). In coronal sections through the LGN, an additional condensed area with terminating projections were detected in the central LGN (Fig. 4H: 'bs'), possibly corresponding to the binocular segment which receives projections from ipsilateral ventrotemporal RGCs (Dräger and Olsen, 1980; Godement et al., 1984). Thus, the *α-tau-lacZ* transgene line provides a valuable tool to study retinal projection defects in mutant models.

Additionally, the highly ordered pattern of projections of distal Pax6^{+} RGCs marked by the *α-tau-lacZ* transgene revealed an intriguing complexity in the regulation of retinal Pax6 expression, which was further explored as described below. The *α-tau-lacZ* transgene furthermore provides a valuable transgenic tool to study projections in mutant models for axonogenesis in the optic tract, potentially complementing limitations inherent to conventional antero- and retrograde dye tracings.

Pax6 expression is regulated in two complementary topographic domains in the mature retina

The sustained expression of tau-β-gal in the postnatal and adult retina of *α-tau-lacZ* animals suggested a role of the α-enhancer in regulating Pax6 expression in mature RGCs of the distal NR. To examine the late function of this regulatory element, we analyzed the expression of β-gal in the postnatal and adult *Pax6*$^{lacZ/+}$ retina, in which the α-enhancer is deleted by the insertion of *lacZ* as mentioned above (see Fig. 1A). Like the normal distribution of endogenous Pax6 expression in the adult retina, β-gal expression in the P20 *Pax6*$^{lacZ/+}$ retina was mainly confined to the ganglion cell layer (Fig.

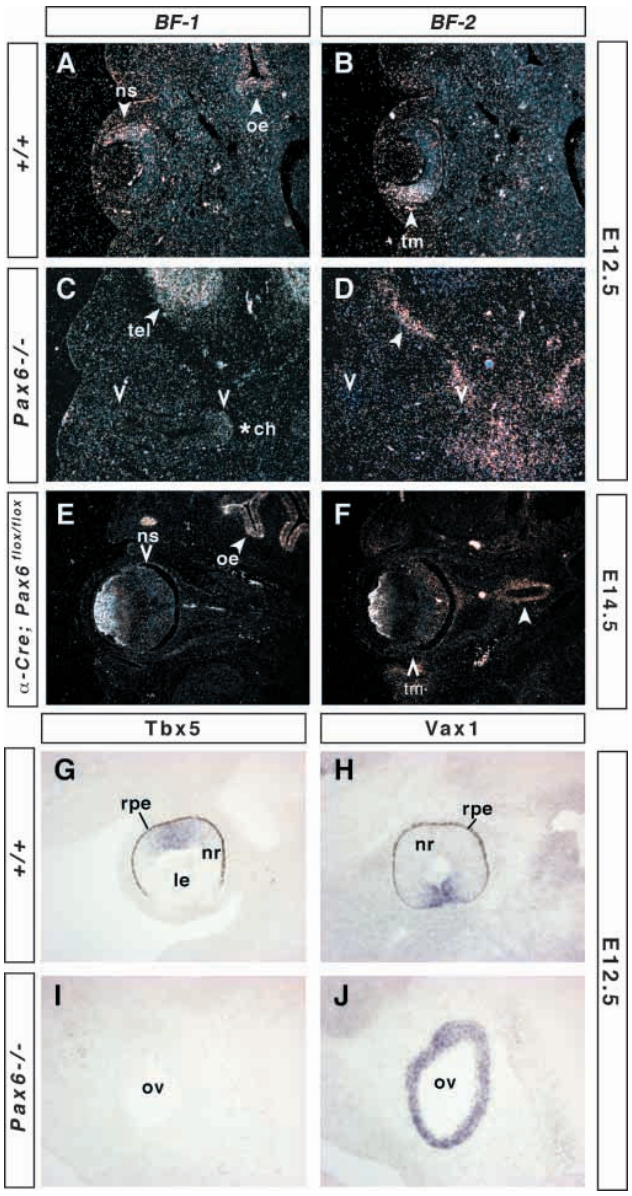

Fig. 6. Pax6 is required for initiating and maintaining the expression of the nasal and temporal retinal determinants *BF-1* and *BF-2*. Radioactive situ hybridization of 6 μm horizontal sections. (A) In wild type at E12.5, *BF-1* is expressed in the nasal (ns) NR and in the olfactory epithelium (oe), as well as the telencephalon (see C), while *BF-2* is detected in the temporal (tm) NR (B). (C) In $Pax6^{-/-}$ embryos, no *BF-1* can be detected in the optic vesicle (open arrowheads), but normal levels in the telencephalon (tel) and low levels in the neuroepithelium around the chiasm (asterisk). (D) Likewise, no *BF-2* expression is detectable in the $Pax6^{-/-}$ optic vesicle, while normal levels are found in the surrounding mesenchyme (white arrowhead). (E,F) Absence of retinal *BF-1* (E) and *BF-2* expression (open arrowheads) after conditional Pax6 inactivation in the distal NR of E14.5 *α-Cre;* $Pax6^{flox/flox}$ embryos. Normal levels of *BF-1* are still detected in the oe (E), as well as normal *BF-2* levels in the dura mater surrounding the optic nerve (F, white arrowhead). Note that in E and F, signal in the lens is due to reflection of lens fibers in dark-field microscopy. (G) In the wild-type embryo, *Tbx5* is expressed specifically in the dorsal NR, while *Tbx5* fails to be expressed in the $Pax6^{-/-}$ optic vesicle (I). *Vax1* whose expression at E12.5 is found in the very ventral NR (H), is expressed throughout the whole $Pax6^{-/-}$ optic vesicle (J). le, lens; nr, neural retina; ov, optic vesicle; rpe, retinal pigmented epithelium; ch, chiasm.

5B,F). However, unlike endogenous Pax6, β-gal expression in the P20 $Pax6^{lacZ/+}$ retina was virtually absent in distal RGCs (Fig. 5B,E) compared with the proximal NR (Fig. 5B,F). This expression pattern was precisely complemented in the retina of *α-tau-lacZ* animals (Fig. 5A,B, inset), where high levels of tau-β-gal expression were present in the distal retina (Fig. 5C), while the expression sharply decreased towards the optic nerve head (Fig. 5D).

The high levels of β-gal activity in the retina of $Pax6^{lacZ/+}$ animals allowed us to follow axonal projections from RGCs up to the superior colliculus (Fig. 5H,J). Interestingly, the β-gal^+ axons in $Pax6^{lacZ/+}$ animals terminated into the central region of the superior colliculus, leaving out the most anterior and posterior regions (Fig. 5H,J). The comparison of the labeled optic tract projections in P20 $Pax6^{lacZ/+}$ and *α-tau-lacZ* transgenic animals revealed that the β-gal^+ axons in $Pax6^{lacZ/+}$ terminate exactly within the gap left by the sickle-shaped tau-β-gal^+ stripes observed in the anterior and posterior superior colliculus of *α-tau-lacZ* animals (compare Fig. 5G,H with I,J).

To examine whether the absence of β-gal^+ projections in the anterior and posterior superior colliculus in the $Pax6^{lacZ/+}$ mice is not a consequence of possible misrouting of the distal RGC axons, we studied the β-gal staining pattern of *α-tau-lacZ* in the background of *Pax6* heterozygous *small eye* mutant mice (*α-tau-lacZ*; $Pax6^{Sey/+}$). As illustrated in the insets of Fig. 5B,H,J, the tau-β-gal^+ axons in *α-tau-lacZ*; $Pax6^{Sey/+}$ embryos essentially displayed the same innervation pattern as in *α-tau-lacZ;* $Pax6^{+/+}$ mice. However, a significant reduction in the number, but not in the intensity of staining of the tau-β-gal^+ axons could be observed (Fig. 5H, inset), which might be attributable to the hypocellular appearance of the distal $Pax6^{Sey/+}$ retina (data not shown). We conclude therefore that Pax6 activity in the retina is specifically regulated in distinct non-overlapping RGC subpopulations that parcellate the primary visual centers into two complementary topographic domains.

Establishment and maintenance of nasotemporal and dorsal patterning of the retina requires Pax6

The presence of a regulatory element promoting high levels of Pax6 activity in the distal nasal and temporal NR prompted us to address whether Pax6 is required for patterning of the nasotemporal axis in the retina. BF-1 and BF-2 are among the first factors to mark the distinction between the future nasal and temporal retina and become expressed in the nasal and temporal optic vesicle at around E9, respectively (Hatini et al., 1994). We first examined the expression of *BF-1* and *BF-2* in *Pax6*-null mutant ($Pax6^{-/-}$) embryos by in situ hybridization. Both *BF-1* and *BF-2* fail to be expressed in the optic vesicles of $Pax6^{-/-}$ embryos (Fig. 6C,D), while high levels could still be observed in tissues outside the eye, such as telencephalon and head mesenchyme (Fig. 6C,D). By contrast, in E12.5 wild-type embryos *BF-1* and *BF-2* expression can be detected in the nasal and temporal NR, respectively (Fig. 6A,B). This observation therefore suggests that Pax6 activity in the optic

vesicle is required for initiating the expression of determinants of the nasotemporal axes of the retina.

To address whether the maintenance of *BF-1* and *BF-2* requires Pax6 function, we analyzed the expression of BF-1 and BF-2 upon Cre-mediated *Pax6* inactivation in the optic cup stage NR in *α-Cre; Pax6$^{flox/flox}$* embryos (see above). In the wild-type situation, both *BF-1* and *BF-2* continue to be expressed at high levels in the E12.5 and E14.5 nasal and temporal retina (Fig. 6A,B; data not shown). However, in the *α-Cre; Pax6$^{flox/flox}$* retina at E14.5, virtually no expression of *BF-1* or *BF-2* can be detected (Fig. 6E,F). At the same time normal levels of *BF-1* expression could still be detected in the olfactory epithelium and forebrain (Fig. 6E), as well as normal *BF-2* expression in head mesenchyme and the dura mater surrounding the optic nerve (Fig. 6F). In the E12.5 *α-Cre; Pax6$^{flox/flox}$* embryos only very low levels of *BF-1* or *BF-2* could be detected in the distalmost tip of the retina (data not shown), indicating that expression of both factors becomes rapidly and progressively extinguished upon loss of Pax6 activity.

These results indicate that Pax6 is required, first, for initiating the expression of factors instrumental in establishing the nasotemporal axis of the retina and, second, for maintaining the expression of nasal and temporal determinants in the NR.

In order to determine the specificity of the failure in establishment of nasotemporal characteristics that is due to the loss of Pax6 activity, we then also examined the expression of dorsoventral markers in the NR. Interestingly, *Tbx5*, which is normally at E12.5 only expressed in the dorsal NR (Fig. 6G) (Koshiba-Takeuchi et al., 2000), was not detectable in the *Pax6* mutant optic vesicle (Fig. 6I). Furthermore, the ventral NR marker *Vax1* (Fig. 6H) (Hallonet et al., 1998) displayed strong expression throughout the dorsoventral extent of the *Pax6$^{-/-}$* optic vesicle. Additionally, another ventral NR determinant, *Vax2*, was weakly expressed in the entire optic vesicle (Barbieri et al., 1999) (data not shown). Together, these data suggest that in the optic vesicle of *Pax6*-null mutants, ventral retinal characteristics expand at the expense of dorsal and nasotemporal characteristics, consequently leading to a failure in the specification of the nasotemporal and dorsoventral retinal axes.

The *Pax6* null mutant optic vesicle expresses markers, such as Chx10, Mitf and Trp2 in regionally defined areas that indicate the appropriate specification of NR and pigmented epithelium progenitor domains (N. B., T. M. and P. G., unpublished) (Grindley et al., 1995). Furthermore, in the *α-Cre; Pax6$^{flox/flox}$* retina, retinal progenitor cell characteristics are maintained and neuronal differentiation occurs (Marquardt et al., 2001). Therefore, in both cases the loss of nasal, temporal (as well as dorsal) retinal determinants is apparently not due to a general failure in retinal development, but rather reflects a direct requirement for Pax6 activity.

DISCUSSION

Different levels of Pax6 in the developing eye are controlled by multiple regulatory elements

Owing to its central and evolutionary conserved role in the molecular mechanisms governing eye development, Pax6 has been termed ‘master control factor’ of eye development (Gehring and Ikeo, 2000). In vertebrates, the requirement for Pax6 activity can now stepwise be assigned to different spatial and temporal levels of eye development. Together with a set of other transcription factors, Pax6 appears to participate in a regulatory network initiating the formation of the retinal anlage from the anterior neural plate, as well as the lens placode from the head surface ectoderm (Oliver et al., 1996; Chow et al., 1999; Zuber et al., 1999; Bernier et al., 2000; Ashery-Padan et al., 2000). In the absence of Pax6 activity, the initial establishment of the retinal anlage occurs, because in *Pax6*-null mutants the optic vesicle is formed (Grindley et al., 1995). However, further development of the optic vesicle is arrested and actual retinal pigmented cell differentiation and retinal neurogenesis fail to be initiated (Grindley et al., 1995) (T. M., R. A. P. and P. G., unpublished). At optic cup stage, Pax6 appears to control the full range of retinal cell types to be generated via activation of retinogenic basic helix-loop-helix transcription factors (Marquardt et al., 2001) (reviewed by Vetter and Brown, 2001; Marquardt and Gruss, 2002). In the present study, we reveal the requirement for Pax6 activity in the establishment and maintenance of distal nasal and temporal characteristics in the optic vesicle and later in the NR.

The dynamic expression of Pax6 during embryogenesis necessarily requires complex regulatory mechanisms. Recently, several distinct regulatory elements have been identified that are able to recapitule Pax6 expression in ectodermally derived eye structures, in the distal NR (Kammandel et al., 1999; Xu et al., 1999), as well as in the telencephalon and endocrine pancreas (Kammandel et al., 1999); together revealing an intriguing modular organization of the *Pax6* locus. The function of the evolutionary highly conserved retinal α-enhancer (Kammandel et al., 1999; Xu et al., 1999) provided a puzzle, as its activity starting E10.5 is initiated well after the onset of endogenous Pax6 and only in a subdomain of retinal Pax6 expression.

In this study, we provide evidence that Pax6 activity controlled by the α-enhancer contributes to the establishment of the distal^{high}-proximal^{low} gradient of Pax6 expression in the NR and for maintaining Pax6 expression in the distal NR (Fig. 1C-E, Fig. 2), while the remaining regulatory element/s of Pax6 continue to drive expression in the proximal region of the optic cup (see Fig. 5). Recently, a regulatory element residing downstream of the *Pax6* gene has been isolated, apparently driving the early aspects of Pax6 expression from E8.5 in the optic vesicle and later the neural and pigmented retina (Lauderdale et al., 2000; Kleinjan et al., 2001). Further small regulatory sequences that drive specific aspects of retinal Pax6 expression have been identified, one of them driving expression in the developing photoreceptor cells (Xu et al., 1999). Therefore, a complex interplay of distinct modularly organized regulatory elements scattered across the entire Pax6 locus appears to underlie the highly dynamic pattern of Pax6 activity during optic vesicle and retinal development.

Assuring sufficient Pax6 levels in the distal retina

The presented results strongly suggest that a main function of the α-enhancer consists in mediating high levels of Pax6 activity in the distal NR (Fig. 7A). The highest level of the Pax6 gradient in the NR marks the future iris epithelium (see Fig. 1). The iris appears to be especially sensitive to variations in Pax6 levels, as both reduction (Jordan et al., 1992; Glaser et

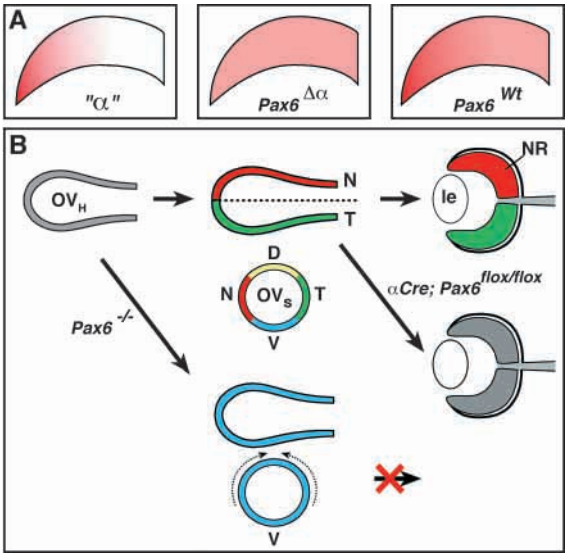

Fig. 7. (A) Schematic representation of the NR (distal is towards the left). The α-enhancer (α) alone drives gradient expression in the distal half of the NR (red), while upon deletion of α (*Pax6*$^{\Delta\alpha}$ =*Pax6*^{lacZ}) the remaining *Pax6* elements mediate evenly distributed expression throughout the proximodistal extent of the NR (pink). In the wild type NR, the composite activity of both regulatory systems lead to the full retinal expression pattern of Pax6 (*Pax6*^{WT}). (B) Nasal (red), temporal (green) and ventral (blue) characteristics are established around E9 in the wild-type optic vesicle (see OV_H) and maintained in the optic cup (E12), while dorsal characteristics (yellow) are established slightly later (shown in OV_S). In *Pax6*-null mutants (*Pax6*$^{-/-}$) the distinction between nasal and temporal optic vesicle fails to be established, while after Cre/loxP mediated inactivation (*α-Cre; Pax6*$^{flox/flox}$) at E10.5 nasal and temporal patterning of the optic cup is lost. In addition, in the *Pax6*$^{-/-}$ optic vesicle ventral characteristics (Vax1, Vax2) expand dorsally (dotted arrows) and, possibly as a consequence, dorsal determinants (Tbx5) are lost. See discussion for details. D, dorsal; le, lens; N, nasal; NR, neuroretina; OV_H, horizontally sectioned optic vesicle; OV_S, sectioned sagittally optic vesicle; T, temporal; V, ventral.

al., 1994) or increase (Schedl et al., 1996) in Pax6 dose can result in severe iris malformations or absence of iris structures. These observations appear to reflect a cell-autonomous requirement for Pax6 function.

Our previous study has indicated that Pax6 directly stimulates expression via the α-enhancer (Schwarz et al., 2000). Our present data confirm the requirement for sufficient Pax6 levels for full activation and maintenance of α-enhancer driven expression in vivo. It is tempting to speculate that such an autoregulatory mechanism might underlie the particular sensitivity of the iris to the Pax6 dose, possibly via amplifying small decreases in Pax6 levels. Positive autoregulation via direct interaction with the α-enhancer might therefore contribute to the steep distal^{high}-proximal^{low} gradient of Pax6 activity in the optic cup, which in turn assures sufficiently high Pax6 levels for the proper development of distal retinal structures.

A link between retinal determination and the establishment of retinal axes

The jigsaw puzzle like organization of Pax6 expression in distal and proximal territories of the postnatal NR (Fig. 5A-F) suggested a more specific role of Pax6 in retinal ganglion cell (RGC) axonogenesis. However, the experimental shallowing of the distal^{high}-proximal^{low} Pax6 gradient, by conditional ablation of one Pax6 allele in the distal NR, did not appear to result in gross defects in the targeting of distal RGC axons to the superior colliculus and lateral geniculate nucleus (data not shown). Similarly, the general ability of distal RGC axons to project to their correct target positions is not impaired in *Pax6* heterozygotes (Fig. 5B,H,J, insets). Therefore, in contrast to the development of the iris, neither absolute nor relative high Pax6 levels in the distal NR appear to be required for the proper targeting of distal RGC axons. Nevertheless, a hypocellular appearance of the distal NR could be observed in *Pax6* heterozygotes. This observation, however, might reflect the improper specification of distalmost NR into inner iris epithelium, resulting in an incomplete separation of both tissues. A significant change in the cellular composition of the distal NR could not be observed in adult *Pax6*$^{lacZ/+}$ eyes (data not shown). Further reductions in Pax6 levels are unlikely to reveal a specific impact on RGC axonogenesis, owing to the central function of Pax6 in retinal progenitor cells (Marquardt et al., 2001). The precise relevance of the division of the postnatal optic cup in two complementary Pax6 expression domains with distinct targeting fields in the primary visual center requires further investigation. Nevertheless, these observations underline the complex interconnected nature of the regulatory mechanisms governing retinal development, apparently coordinating diverse processes, such as retinogenesis and axon guidance.

However, it remained unclear how the action of such factors is coupled to the generic mechanisms of retinal determination. We provide evidence that one of the central early retinal determinants, Pax6, mediates the regulation of factors that display restricted localization across the retinal axes. We observed that the expression of *BF-1* and *BF-2* depends on Pax6 activity (see Fig. 6), implicating a direct role of Pax6 in establishment of the naso-temporal axes (Fig. 7B). The concomitant failure to initiate *Tbx5* expression in the absence of Pax6 might be indirectly mediated by the dorsal extension of *Vax2*, which has recently been shown to be a potent repressor of Tbx5 in the chick retina (Koshiba-Takeuchi et al., 2000). *Tbx5* overexpression appears to specifically affect dorsoventral, but not naso-temporal retinal axon pathfinding (Koshiba-Takeuchi et al., 2000), while *BF-1* expression seems to be unaffected in *Tbx5*-null mutants (Mui et al., 2002; Barbieri et al., 2002). Therefore, the absence of *BF-1* and *BF-2* expression in the *Pax6* null mutant optic vesicle seems to be directly due to the lack of Pax6 rather than indirectly via repression by the expanded domain of *Vax2* activity. These results are summarized schematically in Fig. 7B. The relevance of Pax6 for the maintenance of dorsal and/or ventral retinal characteristics, i.e. *Tbx5* or *Vax2* expression, remains to be elucidated, as in our *α-Cre;Pax6*$^{flox/flox}$ model the ventral and dorsalmost retinal domains are omitted by Cre activity (Marquardt et al., 2001). Remarkably, a similar ventralization in the absence of Pax6 activity was already observed in several neural tissues, including the telencephalon (Stoykova et al., 2000), diencephalon (Grindley et al., 1997; Warren and Price, 1997), spinal cord (Ericson et al., 1997) and pituitary gland (Kioussi

et al., 1999), indicating a general role of Pax6 in dorsoventral patterning of the neural tube.

These observations moreover provide an intriguing link between the generic mechanisms of retinal determination, as well as retinogenesis and the establishment of topographic organization in the visual system. Such a link, however, raises the issue of how a transcriptional activator expressed throughout the entire optic vesicle and later the NR might control the expression of factors that have to be confined to only one particular axis. The inactivation of *BF-1* has been reported to result in ectopic expression of the temporal marker *BF-2* in the nasal NR (Huh et al., 1999). This observation suggests that BF-1, presumably via its function as a transcriptional repressor (Yao et al., 2001), mediates nasal NR characteristics by actively suppressing temporal NR determinants that otherwise would become activated throughout the optic cup. The mechanisms leading to the initial restriction of these factors, however, remain to be elucidated, but presumably involves signaling originating from periocular tissue.

These findings furthermore highlight the fact that during embryonic development, the same transcription factor is commonly used during different sequential stages, often contributing to vastly different developmental outcomes. Although initial steps have recently been accomplished in elucidating how a single factor can sequentially govern the specification of two different cell fates (reviewed by Marquardt and Pfaff, 2001), the deciphering of the underlying transcriptional mechanisms remains a major problem in developmental biology.

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Note added in proof

Note the name change of Nicole Andrejewski to Nicole Bäume.

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