Retinal pigmented epithelium determination requires the redundant activities of Pax2 and Pax6

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SUMMARY

The transcription factors Pax2 and Pax6 are co-expressed in the entire optic vesicle (OV) prior and concomitant with the establishment of distinct neuroretinal, retinal, pigmented-epithelial and optic-stalk progenitor domains, suggesting redundant functions during retinal determination. Pax2; Pax6 compound mutants display a dose-dependent reduction in the expression of the melanocyte determinant Mitf, accompanied by transdifferentiation of retinal pigmented epithelium (RPE) into neuroretina (NR) in Pax2+/−; Pax6+/− embryos, which strongly resembles the phenotype of Mitf-null mutants. In Pax2−/−; Pax6−/− OVs Mitf fails to be expressed and NR markers occupy the area that usually represents the Mitf+ RPE domain. Furthermore, both, Pax2 and Pax6 bind to and activate a MITF RPE-promoter element in vitro, whereas prolonged expression of Pax6 in the Pax2-positive optic stalk leads to ectopic Mitf expression and RPE differentiation in vivo. Together, these results demonstrate that the redundant activities of Pax2 and Pax6 direct the determination of RPE, potentially by directly controlling the expression of RPE determinants.

Key words: Eye development, Pigmented retina, Regionalization, Pax2, Pax6, Mitf

INTRODUCTION

The vertebrate eye is a highly specialized neurosensory organ. In the mouse, eye development starts on embryonic day 8.5 (E8.5) when the OV grows out from the anterior forebrain. After contacting the surface ectoderm by E9.5, both the OV and the surface ectoderm invaginate. This forms the two-layered optic cup from the OV and the lens pit and subsequently the lens vesicle from the surface ectoderm (reviewed by Chow and Lang, 2001). Although the very proximal part of the OV will narrow to surround the optic nerve, the outer layer of the optic cup will become the RPE and the inner layer will differentiate into the NR with its different cell types (Young, 1985).

Initially, the cells of the early OV are bipotent because they can differentiate into either NR or RPE cells. The first indication for the determination of different areas within the developing eye is apparent at the OV stage. Here, the microphthalmia-associated transcription factor Mitf, which belongs to the family of basic helix-loop-helix (bHLH)/leucine zipper transcription factors, is first expressed throughout the entire OV (Bora et al., 1998; Nguyen and Arnheiter, 2000). Subsequently, the Mitf expression is downregulated in the distal portion of the OV to mark the region of the presumptive RPE in the proximal-dorsal portion of the OV (Nguyen and Arnheiter, 2000). In the adjacent, distal OV, the presumptive NR region (Burmeister et al., 1996; Liu et al., 1994), the paired-like homeodomain transcription factor Chx10 starts to be expressed at the same time. Later on Mitf and Chx10 continue to be expressed in adjacent regions of the eye, suggesting a reciprocal regulation between these two factors (Nguyen and Arnheiter, 2000). Interestingly, in Mitf-mutant eyes, portions of the outer RPE layer transdifferentiate into NR instead of RPE, reflecting the important roles of Mitf in promoting the RPE differentiation and suppressing NR characteristics (Mochii et al., 1998; Bumsted and Burnstable, 2000; Nguyen and Arnheiter, 2000). Chx10-mutant NRs are generally hypocellular and lack bipolar cells (Ferda Perkin et al., 2000; Burmeister et al., 1996).

The paired and homeodomain transcription factor Pax6 is assumed to be a ‘master regulator’ of eye development (Gehring and Ikeo, 1999; Ashery-Padan and Gruss, 2001) because forced expression of Pax6 alone is sufficient to induce ectopic eyes in fly and frog embryos (Halder et al., 1995; Chow et al., 1999). When Pax6 is lacking no functional eye structures
form in organisms as different as mouse, human, rat, frog and fly (reviewed by Gehring and Ikeo, 1999). Mice heterozygous for Pax6-null mutations exhibit a small eye phenotype, which is characterized by multiple ocular abnormalities, such as microphthalmia, lens cataracts and iris defects, and human Pax6 mutations lead to Aniridia, as well as multiple lens and corneal defects (Hill et al., 1991; Glaser et al., 1992; Glaser et al., 1994). Pax6 is expressed highly throughout the early OV and the surface ectoderm, and remains expressed in all eye components at the optic-cup stage, including lens vesicle, outer and inner optic cup layers, and optic stalk (Walther and Gruss, 1991). Later, Pax6 expression becomes restricted to the lens, corneal and conjunctive epithelia, iris and inner portion of the NR (Walther and Gruss, 1991). Conditional inactivation of Pax6 in the surface ectoderm leads to a specific ablation of the lens (Ashery-Padan et al., 2000). Furthermore, the conditional elimination of Pax6 in the distal NR causes a complete failure of differentiation of all NR cell types except amacrine cells (Marquardt et al., 2001). However, the function of Pax6 during the early phase of OV genesis has not been studied extensively (Grindley et al., 1995).

At early stages of eye development, the Pax family member Pax2 is co-expressed with Pax6 in the OV, but is absent in the surface ectoderm (Nornes et al., 1990) (this study). During optic nerve formation at ~E12.5, Pax2 expression becomes restricted to the ventral NR that surrounds the closing optic fissure and the presumptive glia cells of the optic nerve (Nornes et al., 1990; Torres et al., 1996). After E12.5, Pax2-deficient mice exhibit a severe retinal coloboma – a failure to close the choroid fissure. Furthermore, eyes are achiasmatic and the optic nerve formation at ~E12.5,

**MATERIALS AND METHODS**

**Transgenic and targeted mice**

Pax6+/-; Pax6fluc2/+ (Pax6+/-); lens-Cre and Pax6flox mice were generated previously (see Torres et al., 1995; St-Onge et al., 1997; Ashery-Padan et al., 2000). In the pPax2Neo mice (Schwarz et al., 2000), a 9.3 kb genomic fragment of the 5' region of the first exon of Pax2 was fused Norl (blunt) to full-length Pax6 cDNA in the vector pBSKII+.

DNA from either the yolk sac or tail of Pax6+/- embryos was genotyped by PCR as described (Bäumer et al., 2002). DNA from either the yolk sac or tail of Pax2-/- embryos was genotyped using two PCRs. The following primers were used to identify the mutant allele: Neo-f4, 5'-CTTCTATCGCCCTTCTT-GACG-3'; Pax2-r3, 5'-TCCACGCTATTGTAAGC-3'. A band of 600 bp indicated the existence of a Pax2-mutant allele. Each PCR assay contained 1 µg of DNA, 1/10 vol of HotStarTag buffer (Qiagen), 200 µM dNTP mix, 10 µM of Neo-f4 primer, 23 µM of Pax2-r3 primer and 1 U HotStarTag polymerase (Qiagen). Cycling conditions were 95°C for 15 minutes, followed by 34 cycles of 94°C for 45 seconds, 58°C for 30 seconds, 72°C for 1 minute, and a final extension of 72°C for 5 minutes.

For the identification of the wild-type allele, the following primers were used: Pax2-f, 5'-CCGGGCTGCTGTTGACGCTG-3'; Pax2-r, 5'-GCTTTGACGTGCATCCATGC-3'. A band of 300 bp indicated the existence of a Pax2 wild-type allele. Each PCR assay contained 1 µg of DNA, 1/10 vol of PCR buffer (Biotherm), 200 µM dNTP mix, 0.33 pmol µl-1 of each primer and 1.5 U Taq polymerase (Biotherm). Cycling conditions were 94°C for 2 minutes, 80°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 30 seconds, then 72°C for 5 minutes.

**Immunohistochemistry**

The embryos were fixed for 30 minutes in 4% PFA/PBS (pH 7.8), washed with PBS, incubated in cold 30% Sucrose/PBS over night and frozen in Tissue Freezing Medium (Jung). Sections of 6-10 µm were air-dried and stored at ~80°C.

For antibody staining, the sections were washed in PBS (3 X 5 min), blocked with 1% BSA (IgG-free, Sigma), 0.05% Tween-20 in PBS for 30 minutes at room temperature. Primary antibodies were diluted in blocking solution and incubated at 4°C overnight. Primary antibodies: 1:300 rabbit anti-β-Gal (Cappel); 1:20 monoclonal mouse anti-Pax6 (DSHB); 1:200 rabbit anti-Pax2 (Babco); 1:150 rabbit anti-Mitf (gift of H. Amheriter); 1:500 rabbit anti-Chx10 (gift of R. McInnes); 1:2000 rabbit anti-Otx2 (gift of F. Vaccarino); 1:75 goat anti-Bmp4 (Santa Cruz). After three, 5-10 minute washes in PBS, the secondary antibody was applied in blocking solution for 1 hour [1:500 Alexa 568 goat anti rabbit (Molecular Probes); 1:60 FITC goat anti mouse (Southern Biotechnology)]. After three washes with PBS, counterstaining was performed with Dapi and the sections were embedded with Mowiol.

Hematoxylin-Eosin (HE) stainings were performed using standard protocols.

**Bandshift assays**

Pax6 and Pax2 proteins were overexpressed using SP6 promoter-coupled Pax2 and Pax6 cDNA in the TNT in vitro transcription and translation system (Promega) according to the manufacturer's protocol.

Double-stranded oligonucleotides (see Fig. 5B for sense sequences) were end-labeled using polynucleotide kinase and γ-32P-ATP. The binding reaction was performed for 1 hour on ice in retard buffer (40 mM HEPES-NaOH, pH 7.6, 8% Ficoll, 10 mM MgCl2, 80 mM NaCl, 0.2 mM EDTA, 1 mM DTT) with 5 µg poly-d-dC. 25,000 cpm of the double-stranded oligonucleotide and 2-10 µl of either Pax2 or Pax6 TNT protein. To test the binding specificity, either Pax6 or Pax2 polyclonal rabbit antibodies (Babco) were preincubated 1:10 in retard
buffer with poly-dI-dC and protein for one hour on ice. The probes were run over an 8% polyacrylamide gel. The gel was exposed to a Kodak Biomax film over night.

**Cell culture**

Co-transfection experiments of Cos-7 cells were performed as described (Marquardt et al., 2001). The cDNAs of Pax2 (Chalepakis et al., 1990), Pax6 (Walther and Gruss, 1991) and Pax1 (Chalepakis et al., 1990) were cloned to a hCMV promoter. The promoter of the MITF exon A (Udono et al., 2000) was a generous gift of S. Shibahara.

**RESULTS**

**Pax2 and Pax6 are co-expressed in the early OV with both RPE and NR markers**

Although several reports showed that Pax2 and Pax6 are expressed in the early OV (Nornes et al., 1990; Walther and Gruss, 1991), whether their expression domains overlap tightly has not been addressed in detail. We found that Pax2 and Pax6 are co-expressed at high levels in virtually all cells of the early OV at E9.5 (Fig. 1A-D; Fig. 4A) and E10.0 (Fig. 1E-H), (I-L) At E12.5, Pax2 is restricted mainly to the optic stalk but Pax6 is still expressed in all distal eye components. (M-P) At E9.5, the segregation of RPE markers, such as Mitf (M) and Otx2 (N) is detectable, in contrast to the NR marker Chx10 (O). The adjacent section (P), stained for Pax2, reveals the overlap of Pax2 and Pax6 (see also A and B) with both RPE and NR markers.

**The activity of Pax2 or Pax6 is dispensable for proximo-distal patterning of the early OV**

To elucidate the early function of Pax2 and Pax6 during OV formation, we examined the patterning of Pax6+/− and Pax2+/− OVs. At E12.5 the RPE determinant Mitf is completely restricted to the RPE in the wild-type eye (Fig. 2A), whereas Chx10 remained confined to the NR (Fig. 2C). Remarkably, expression of both Mitf and Chx10 is initiated in the Pax6+/− OV (Fig. 2E,G), although neither retinal neurogenesis nor RPE
differentiation is initiated in Pax6-null mutants (Grindley et al., 1995; data not shown). At E12.5, Mitf expression is confined to the RPE and some cells in the dorsal optic stalk (A, arrowhead). By contrast, Otx2 is expressed in the RPE and the dorsal optic stalk, with a sharp, distal-proximal gradient in the distal NR arrowheads, (B). Chx10 is restricted to the NR (C) and Pax2 mainly to the optic stalk (D, arrowhead). (E) At E12.5, Mitf expression in the Pax6<sup>+/−</sup> mutant eye is confined to the distal tip of the OV. Otx2 is expressed highly in the same region (F) but less in the medial portion of the OV where Chx10 is expressed at a high level (G). (H) The proximal region of the Pax6<sup>+/−</sup> OV expresses Pax2 strongly (p), expression in the medial region is at a lower level (m) and the distal region is Pax2 negative (d). (F, inset) In the Pax6<sup>lacZ/lacZ</sup> OV, the β-gal expression is restricted to the distal and the medial regions, which reflects the distribution of β-gal expression in the RPE and NR in Pax6<sup>+/−</sup> eyes (B, inset). Broken lines in E-H indicate the borders between d, m and p regions. The comparison of the patterning of E10.5 wild-type (I-L) and Pax2<sup>−/−</sup> eyes (M-P) revealed in both genotypes the unchanged expression of Mitf (I and M) in the RPE and optic stalk, of Otx2 (J and N), which is found in both phenotypes in the RPE and optic stalk, gradually in the NR and some cells of the optic stalk, as well as the unchanged expression of Chx10 in the NR (K and O) and of Pax6 throughout all eye components (L and P). (I, inset) Expression of Pax2 in the wild-type optic disc and the missing Pax2 expression in the Pax2<sup>−/−</sup> eye (M, inset). le, lens; os, optic stalk; nr, neural retina; rpe, retinal pigmented epithelium.
but was largely absent from the optic stalk (Fig. 2B, inset). The E12.5 *Pax6lacZ/lacZ* (*Pax6–/–*) OV had high levels of β-gal expression in the distal and the medial (‘RPE’ and ‘NR’), but much lower levels in the proximal ‘optic-stalk’ domain (Fig. 2F, inset), which is comparable to the β-gal expression in *Pax6 lacZ/+* eyes (Fig. 2B, inset). In situ hybridization with a *Pax6* riboprobe revealed essentially the same localization of *Pax6* transcripts in the distal OV of *Pax6 Sey/Sey* embryos (Grindley et al., 1995; data not shown). Therefore, the distribution of *Pax6* transcripts detected in *Pax6*-null mutants reflects the largely undisturbed proximo-distal patterning of the OV.

*Pax2*–/– eyes display retinal and optic nerve coloboma, which are visible by E12.5 (Torres et al., 1996) (data not shown). Morphologically, these eyes can be identified after this stage by elongation of the NR towards the optic stalk (Torres et al., 1996; Schwarz et al., 2000) (Fig. 3G; data not shown). We further studied the putative function of early Pax2 expression during OV regionalization of *Pax2*–/– OVs and eyes from E9.5 to E12.5 (Fig. 2I-P; data not shown). Interestingly, the expression domain of Mitf in the RPE (Fig. 2I,M), Otx2 expression in the RPE, NR and a subpopulation of optic-stalk cells (Fig. 2J,N), and Chx10 in the NR (Fig. 2K,O) were identical in wild-type and *Pax2*–/–; *Pax6*+/– eyes (N, il). Mitf is still expressed the forming RPE in the wild-type E11.5 eye (L), whereas in the *Pax2*–/–; *Pax6*+/– optic cup, it is only found in regions between the newly formed, folded, NR regions and in the proximal RPE remnant (O, arrows). In the lens-cre/Pax6flx/flx eye, Mitf-positive RPE surrounds the folded NRs, similar to wild-type RPE (P).
Pax2–/–; Pax6–/– eyes transdifferentiate RPE into a second, inverted NR and display reduced Mitf expression

To study the putative redundant function of Pax2 and Pax6 during early eye determination, we examined the patterning of developing eyes in Pax2; Pax6 compound-mutant embryos. Interestingly, in absence of Pax2, reduction of Pax6 activity led to a severe optic cup phenotype. At stages E10.5 and E11.5, the Pax2+/–; Pax6+/– optic cups were not an ordered, two-layered structure (Fig. 3N,O). Instead the inner layer (the presumptive NR) appeared folded, whereas the outer layer (the presumptive RPE) seemed to be reduced (compare Fig. 3K-L with Fig. 3N-O). Additionally, the lens vesicle was either reduced in size or completely absent (Fig. 3N,O; data not shown). Interestingly, at E18.5 the RPE in Pax2+/–; Pax6+/– eyes was severely reduced compared to wild type (Fig. 3A,D), and, in the region of the outer layer, a second, multilayered NR had transdifferentiated, which is not found in the Pax2–/– eye (Fig. 3B,F,G-I). This second NR in Pax2+/–; Pax6+/– eyes contained all the neural retina cell types and progenitors associated with the inner NR and the wild-type and Pax2–/– NR (Fig. 3C,F,I; data not shown). However, cell layering in the newly transdifferentiated NR was inverted, as indicated by the expression of the ganglion-cell marker Brn3b in the outer part of the mutant NR rather than innermost layer of the wild-type NR (Fig. 3C,F,I, arrows).

Similar defects have been described following loss of function of Mitf in eyes of the mi/mi mice (Scholtz and Chan, 1987; Mochii et al., 1998; Bumsted and Burnstabl, 2000). Therefore, we examined the expression of Mitf in Pax2–/–; Pax6+/–embryos. At E9.5, immunoreactivity of Mitf in the Pax2–/–; Pax6+/– and wild-type OV was comparable. Notably, at E10.5 and E11.5, the outer layer of the wild-type optic cup was Mitf–positive (Fig. 3K-L), but Mitf expression was reduced in Pax2+/–; Pax6+/– optic cups (Fig. 3N-O).

To determine whether the RPE transdifferentiation observed in the Pax2–/–; Pax6+/– mutants reflected a secondary effect of either loss or reduction of the lens (West-Mays et al., 1999; Ashery-Padan et al., 2000), we studied Mitf expression in lens-CRE/Pax6 flx/flx mice (Scholtz and Chan, 1987; Mochii et al., 1998; Bumsted and Burnstabl, 2000). Therefore, we examined the expression of Mitf in Pax2–/–; Pax6+/–embryos. At E9.5, immunoreactivity of Mitf in the Pax2–/–; Pax6+/– and wild-type OV was comparable. Notably, at E10.5 and E11.5, the outer layer of the wild-type optic cup was Mitf–positive (Fig. 3K-L), but Mitf expression was reduced in Pax2+/–; Pax6+/– optic cups (Fig. 3N-O).

Using the consensus sequence of optimal Pax2 and Pax6 binding to DNA established by Epstein and co-workers (1994) (Fig. 5B), five potential binding sites, A1-A5, were identified in the Pax2–/–; Pax6+/– optic cups and in the Pax6–/– mutant eyes show multiple NR domains (Ashery-Padan et al., 2000), the position of the RPE and the level of Mitf expression at E11.5 were comparable to wild type (compare Fig. 3L with Fig. 3P). Together, these results indicate that Mitf expression is initiated but not maintained at a sufficient level in the optic cups of the Pax2–/–; Pax6+/– embryos, which leads to a transdifferentiation of a second NR at the expense of RPE.

Mitf expression and OV regionalization depend on cooperative Pax2 and Pax6 activity

Expression of Chx10 and Otx2 were also initiated in mutant eyes (Fig. 4J,N,L,P). Therefore, we conclude, that the lack of Mitf expression in the OV of the double-null mutants is a specific defect rather than a general failure of OV development. Similar to the wild type (Fig. 4I), the expression of Chx10 at E9.5 was mainly confined to the distal portion of the Pax2+/–; Pax6+/– OV (Fig. 4J). Some additional Chx10-positive cells were also detected in the more proximal part of the Pax2+/–; Pax6+/– OV that is usually occupied by the Mitf-positive RPE domain (Fig. 4J, arrow). Although in the absence of Pax6, Chx10 expression was regionalized mostly within the ‘m’ domain of the OV remnant (Fig. 2G), in Pax2+/–; Pax6+/– OV, Chx10 immunoreactivity was confined to the very distal tip (region ‘d’) of the vesicle, occupying the normally Mitf-positive progenitor domain of the RPE. By contrast, expression of Otx2 was detected first at E9.5 in both wild-type and Pax2+/–; Pax6+/– OVs (Fig. 4M,N) possibly slightly expanded proximally in the mutant OV (Fig. 4N, arrow). At E11.5, wild-type RPE expressed high levels and NR low levels of Otx2 (Fig. 4O). At this stage, the distal-most region of the Pax2+/–; Pax6+/– OV was Otx2 positive (Fig. 4P), which indicates that the distal tip of the Pax2+/–; Pax6+/– OV co-expressed the NR marker Chx10 and the RPE marker Otx2.

Accordingly, we conclude that, in absence of both Pax2 and Pax6 function, the bipotent, early OV cells could not be further specified into the determined NR and RPE domains, a process that possibly involves a direct control on Mitf-gene activity.

Pax2 and Pax6 can bind and activate a MITF RPE promoter in vitro

The specific loss of Mitf expression in Pax2/Pax6 double-null mutants raised the question of whether Mitf was a direct target gene of Pax2 and Pax6. The Mitf gene produces at least four different splice variants (see Fig. 5A). Three of them, Mitf-A, Mitf-H and Mitf-D are expressed in RPE cells (Hallisson et al., 2000; Udono et al., 2000; Takeda et al., 2002). The recently identified MITF-A promoter drives expression in RPE and melanocyte cell lines (as expected by the expression pattern of this splice variant) as well as in HeLa cells, which might indicate a more widespread activity caused by a missing repressor element in this promoter fragment (Udono et al., 2000). However, our results suggested a role of Pax2 and Pax6 in activation, rather than in repression of Mitf. Therefore, we studied the effect of the binding of Pax2 and Pax6 proteins on the human MITF-A promoter. The 2.2 kb MITF-A promoter sequence is 66.3% identical to the homologous mouse sequence, in some regions 79-91%, compared with sequence information by Celera (data not shown). The MITF-A promoter, which is located upstream of the first exon, was fused to the luciferase reporter gene (Fig. 5A) (Udono et al., 2000).

Using the consensus sequence of optimal Pax2 and Pax6 binding to DNA established by Epstein and co-workers (1994) (Fig. 5B), five potential binding sites, A1–A5, were identified (Fig. 5B). Bandshift assays using in vitro overexpressed Pax2 and Pax6 proteins and 32P-labelled oligonucleotides (for the sequences see Fig. 5B) revealed that A5 represented a MITF-A promoter sequence that can bind both Pax2 and Pax6 (Fig. 5C, red arrow). The other four oligonucleotides only bound unspecific components of the assay, as shown by controls without overexpressed proteins (Fig. 5C, –Pax). The binding specificity of the A5 site was verified by preincubation of Pax2
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and the Pax6 proteins with specific antibodies, which impaired formation of the binding complex (Fig. 5D, lanes 2 and 5). By contrast, addition of the Pax6 antibody to the Pax2 protein, and vice versa, did not perturb the binding (Fig. 5D, lanes 3 and 6). Therefore, we concluded that the A5 sequence in the MITF-A promoter represents a specific binding site for Pax2 and Pax6.

To examine the potential transactivation of this promoter element by Pax2 and/or Pax6, we co-transfected Cos-7 cells with the luciferase-coupled MITF-A promoter, CMV-Pax2-cDNA and/or CMV-Pax6-cDNA. CMV-Pax1-cDNA was used as a control (Fig. 5E). These co-transfections showed that the basal level of luciferase activity driven by the MITF-A promoter was increased ~13-fold by the addition of Pax6, ~40-fold by addition of Pax2, and ~12-fold by an equimolar mixture of Pax2 and Pax6 (Fig. 5E). Pax1 increased the luciferase activity only fivefold (Fig. 5E).

These results demonstrate that both Pax2 and Pax6 can bind to and activate the MITF-A promoter. The fact that the mixture of Pax2 and Pax6 did not surpass the activation obtained by either Pax2 and Pax6 alone accords with the single binding site for both factors identified in this promoter sequence (Fig. 5D). It is likely that both factors compete for this binding site and that the double-mutant phenotypes result from the requirement of a specific concentration of either protein for sufficient activation of Mitf.

A similar competitive relationship has been reported for the segmentation genes kreisler (Mafb – Mouse Genome Informatics) and Krox20, which control the expression of the Hoxb3 gene in rhombomere 5 (Manzanares et al., 2002).

Ectopic expression of Pax6 in the Pax2-positive optic nerve results in the development of ectopic, Mitf-expressing RPE

To test if Pax2 and Pax6 can direct the expression Mitf in vivo, we took advantage of a previously generated transgenic mouse line (pPax2Pax6) that expresses Pax6 under the control of a Pax2-upstream promoter fragment. This drives expression of
Pax6 in the optic stalk (Schwarz et al., 2000) (Fig. 6A). At E13.5, Mitf expression was confined to the developing iris (Fig. 6C,G, ir) and to some cells in the RPE (Fig. 6E, arrows).

Interestingly, pPax2/Pax6 mice displayed ectopic RPE in the region of the distal optic nerve after E13.5, possibly caused by prolonged Pax6 activity in the Pax2-positive optic stalk (compare Fig. 6F,H with Fig. 2D). The appearance of ectopic RPE was accompanied by elevated Mitf immunoreactivity in the optic nerve itself, which was even stronger than Mitf immunoreactivity in the RPE in the normal optic cup (compare Fig. 6E,I, arrows).

In summary, these results indicate that (1) the expression of Mitf is not initiated in the absence of both Pax2 and Pax6, (2) both Pax2 and Pax6 can bind to and activate the MITF-A promoter in vitro, and (3) the forced co-expression of Pax2 and Pax6 in vivo leads to RPE development and ectopic expression of Mitf.

**DISCUSSION**

**Determination of distinct progenitor domains in the retinal primordium**

The cells of the early OV are bipotent neuroepithelial cells...
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Pax2 and Pax6 in pigmented retina determination from which both NR and RPE can differentiate. Classical embryological experiments indicate that the microenvironment of the developing eye determines the decision of these bipotent cells to enter one of these two fates (reviewed by Chow and Lang, 2001). The surface ectoderm is thought to be one of the major sources of signaling factors, such as fibroblast growth factor 1 (FGF1) and FGF2, that are necessary for regionalization of the eye (Guillemot and Cepko, 1992; Pittack et al., 1997; Hyer et al., 1998; Nguyen and Arnheiter, 2000; Araki et al., 2002).

Probably because of these signaling events, the neuroepithelium of the distal OV becomes patterned into distinct NR-progenitor and RPE-progenitor domains, as indicated by the segregation of the expression domains of Chx10 and Mitf (see Fig. 2). Cross-repressive interactions between Chx10 and Mitf are thought to mediate the sharpening and stabilization of the boundary between NR-progenitor and RPE-progenitor domains (Nguyen and Arnheiter, 2000), which might, therefore, mark a switch from exogenous to OV-autonomous patterning mechanisms.

It was unclear how the expression of progenitor factors such as Chx10 and Mitf is initiated in the OV neuroepithelium because both factors are activated, even in the absence of the surface ectoderm (see below) (Nguyen and Arnheiter, 2000). Recently, we demonstrated that the expression of the naso-temporal axis markers BF1 (Foxg1 – Mouse Genome Informatics) and BF2 (Foxd1 – Mouse Genome Informatics) is dependent on Pax6 activity (Bäumer et al., 2002). This provides a link between broadly expressed retinal determinants and retinal-axial patterning. In Pax6-null mutants, however, proximo-distal patterning of the OV into distinct NR, RPE and optic-stalk-progenitor domains appears to be unaffected (Fig. 2E-H). This rules out a ‘master’ function of Pax6 during the specification of murine retinal identity. The remarkable inverted orientation of the Pax6-mutant OV, in which the presumptive RPE domain faces the surface, might contribute to the failure of specification of the mutant surface ectoderm, and strongly resembles the results of ablation experiments (Nguyen and Arnheiter, 2000). Therefore, the surface ectoderm in Pax6 mutants might lack signaling factors that are required for the activation of either RPE-repressing or NR-activating factors.

In the present study we provide evidence that the combined action of Pax2 and Pax6 directly mediates the initiation of Mitf expression in the OV and, thereby, determines the RPE-progenitor domain. Subsequently, the extraocular and OV-autonomous patterning events mentioned above (Araki et al., 2002) restrict the initial, broad Mitf-expression domain to the future RPE-progenitor domain, thereby assuring that RPE differentiation is confined to the future outer layer of the optic cup.

**Redundant and distinct functions of Pax2 and Pax6 in regionalization of the OV**

In contrast to the distinct regionalization of Pax2+/− and Pax6+/− OVs into NR and RPE-progenitor domains, the Pax2+/−; Pax6+/− OV is incorrectly patterned in this respect. The RPE marker Mitf fails to be expressed in the Pax2+/−; Pax6+/− OV and a second RPE marker, Otx2, largely colocalizes with Chx10, a NR marker (Fig. 4). The expansion of Chx10 into the region usually occupied by the RPE-progenitor domain probably reflects the failure to express the NR repressor Mitf in this region. However, co-localization of Otx2 and Chx10 indicates that the normal determination of the NR domain is also affected, which indicates additional, redundant roles of Pax2 and Pax6 in establishing this progenitor domain. Therefore, the determination of both NR and RPE from bipotent OV cells appears to be dependent on redundant Pax2 and Pax6 function.
Consequently, in Pax6 mutants Pax2 activity is sufficient to directly form the RPE-progenitor domains, but Pax2 activity alone is not sufficient to induce optic-cup formation (Fig. 2) (Grindley et al., 1995). Conversely, Pax6 activity is sufficient to direct optic-cup formation in Pax2+/– mutants, but not to maintain the sharp border between optic cup and optic nerve after E12.5, and the differentiation of the optic nerve itself (Fig. 3G) (Torres et al., 1996). These observations imply distinct, nonredundant functions of these two Pax proteins at later stages of OV development. Interestingly, Pax2+/–; Pax6+/– mutants have reduced RPE differentiation as well as more severe optic-nerve defects than Pax2+/– mutants (Fig. 3D), which might indicate additional functions of Pax6 in optic-nerve formation.

The involvement of Pax2 and Pax6 in the regionalization of other tissues has already been implied. For example, Pax6 can restrict the expression of Pax2 at the boundary between the diencephalon and mesencephalon in chick embryos (Matsumana et al., 2000) and this border is affected in absence of Pax6 in mice (Stoykova et al., 1996; Mastick et al., 1997; Pratt et al., 2000). Pax6 is, furthermore, required for the specification of ventral-progenitor-cell identity in the spinal cord and hindbrain (Ericson et al., 1997; Takahashi and Osumi, 2002), and the lack of Pax6 function causes a prominent ventralization of the molecular patterning and morphogenesis of the embryonic forebrain (Stoykova et al., 2000; Yun et al., 2001).

In the OV, after E10, the expression of Pax6 and Pax2 increasingly segregates and becomes mutually exclusive after E12.5. During these later stages both factors might acquire distinct functions in the further specification of the optic cup and the optic nerve domains, where they might now repress each other (Macdonald et al., 1995; Schwarz et al., 2000). Taken together, these observations suggest that, during eye development, Pax2 and Pax6 initially function redundantly in the OV during the determination and patterning of the RPE, NR and, possibly, optic-stalk-progenitor domains. During later stages, a switch appears to occur that brings out the distinct functions of both factors, so that they now mediate the differentiation of discrete tissue compartments of the eye in a mutually exclusive manner (Macdonald et al., 1995; Schwarz et al., 2000). Interestingly, redundant as well as distinct functions have been implied for other Pax-family members, such as Pax3 and Pax7 in spinal cord and somite development (Borycki et al., 1999; Mansouri and Gruss, 1998), Pax1 and Pax9 in sclerotome development (Neubüser et al., 1996; Peters et al., 1999), and Pax2 and Pax5 in developing organs (Schwarz et al., 1997; Urbanek et al., 1997; Bouchard et al., 2000). The recruitment of the same factor to drive distinct processes during sequential stages in the development of the same tissue or organ is starting to become a recurring theme in developmental biology (reviewed by Marquardt and Pfaff, 2001).

Based on observations that members of the same subgroup of the Hox-gene family can, to a large extent, substitute for each other, it has been proposed recently that the quantity rather than the quality of a required factor might be decisive for some developmental mechanisms (Duboule, 2000; Greer et al., 2000). The dose-dependence of the eye phenotype on the Pax6 concentration in Pax2–/–; Pax6+/– compound mice, as shown in this study, corroborates this idea. Moreover, the Pax2 dose dependence of Mitf expression can be observed in OVs of Pax6+/–; Pax2+/– embryos that are phenotypically identical to Pax6–/–; OV. These Pax6+/–; Pax2+/– OVs express less Mitf in the distal presumptive RPE region than the Pax6–/–; OV, although the medial presumptive NR region is highly Chx10 positive in both genotypes (data not shown; Fig. 2).

**Mitf as a putative target gene of Pax2 and Pax6**

Mitf was the only OV determinant identified that failed to be expressed in Pax2–/–; Pax6–/– OV (Fig. 4). Maintained expression of another RPE marker, Otx2, in mutant OVs implied a specific loss of Mitf activity rather than a complete failure in the specification of RPE characteristics. As Otx2 is co-expressed with the NR marker Chx10, we assume that the bipotent character of the OV cells is maintained, possibly due to the absence of Mitf.

Transdifferentiation of the RPE in Pax2+/–; Pax6–/– eyes closely resembles the ocular phenotype of Mitf-deficient mice (Scholtz and Chan, 1987; Bumsted and Burnstable, 2000; Nguyen and Arnheiter, 2000). Because RPE differentiation appears to be normal in the lens-ablated lens-CRE; Pax6flo/flo mutants (Ashery-Padan et al., 2000) (this study) this defect is unlikely to be due to the partial loss of lens tissue in these mutants.

Interestingly, our results indicate that both Pax2 and Pax6 can specifically bind to and activate the MITF-A promoter in vitro (see Fig. 5). This might indicate that one aspect of the complicated regulation of the Mitf gene might involve indirect binding of Pax2 and Pax6, although the in vitro results are indirect because a transgenic approach to study the in vivo function of the binding has not yet been examined. However, the fact that the pan-specific Mitf-antibody did not detect Mitf activity in Pax2–/–; Pax6–/– OV might indicate a general requirement of Pax2 and Pax6 function for the expression of all Mitf isoforms in the RPE. To follow this hypothesis, it will be necessary to further characterize Mitf regulatory elements, such as the MITF-H, MITF-D and MITF-A promoters. Although the H-form of Mitf also occurs at low level in the RPE, we were unable to identify binding sites for Pax2 and Pax6 in the MITF-H promoter (Udono et al., 2000), and did not detect activation of this promoter in cell-culture experiments (data not shown). Because the promoter region of MITF-H is less well conserved than the MITF-A promoter between humans and mice (data not shown), it is also possible that the MITF-H promoter did not adequately represent the mouse promoter in the in vitro analysis. Alternatively, the MITF-A promoter region could act as an enhancer to control the expression of the other isoforms. Furthermore, because expression driven by the MITF-A promoter is more widespread than wild-type Mitf expression (Udono et al., 2000) (N.B. and D.S., unpublished), the existence of other regulatory elements that restrict Mitf expression, is likely. This issue requires further intensive studies, including mutational analysis of potential transcription-factor binding sites.

To date, more detailed information is available concerning the regulation of Mitf activity during melanocyte development (reviewed by Tachibana, 2000). Mutations in the MITF gene in humans cause type II Waardenburg syndrome, a severe disease that specifically affects melanocyte function (Tassabehji et al., 1994; Hodgkinson et al., 1998; Lee et al., 2000). Intriguingly, in melanocyte cell lines the human MITF-
An essential role of Pax6 in RPE differentiation has been suggested previously. Quinn and coworkers established chimeric mice from Pax6-deficient and wild-type ES cells to examine the influence of Pax6 deficiency in different compartments of the eye. When these mutants had incorporated a high percentage of Pax6-deficient cells in the outer layer of the optic cup, the eyes had a disorganized optic cup with a folded NR and reduced RPE differentiation (Quinn et al., 1996). This resembles the phenotype of Pax2/–; Pax6/– eyes. Further studies, possibly involving tissue-specific inactivation of Pax6 in the RPE, should specifically address the direct function of Pax6 in RPE differentiation.

Another hint for the possible function of Pax6 in inducing RPE differentiation came from experiments in which Pax6 was ectopically expressed. In frog embryos, ectopic expression of Pax6 leads to the formation of complete ectopic eye structures outside the optic system, but after overexpression within the optic system it induces only RPE, not NR, along the (Pax2-positive) optic nerve (Chow et al., 1999). In transgenic mice that express Pax6 under the control of a Pax2-regulatory element, differentiation of ectopic RPE (again without NR differentiation) was observed in the distal optic nerve (Schwarz et al., 2000) (this study). Although, initially, we interpreted this phenotype as an indication of the retinal differentiation potential of Pax6 in a region of the optic stalk that expresses lower concentrations of Pax2 (Schwarz et al., 2000), we had to refine this interpretation following our more recent results showing the important role of the redundant function of Pax2 and Pax6 in RPE development (see below). The region of ectopic RPE formation in these mice appears to correlate with the region that endogenously expresses Otx1 in the dorsal optic stalk (see Fig. 2). This indicates that Otx1 is required to allow Pax6; Pax2-mediated RPE differentiation. Because Otx1 and Otx2 are thought to be essential for RPE development (Martinez-Morales et al., 2001), this would reflect the situation during normal RPE determination. In this case, Otx2 is co-expressed with Pax2 and Pax6 in the RPE-progenitor domain (see Fig. 2) (Martinez-Morales et al., 2001), and Otx1, which can take over various functions of Otx2 in different tissues (Acampora et al., 1999; Martinez-Morales et al., 2001) in the ectopic situation of the transgenic optic stalk.

In summary, Pax6 activity appears to be sufficient to induce RPE development, whereas both Pax2 and Pax6 are necessary and sufficient to activate the RPE determinant Mitf in a competent tissue.

Redundant and divergent roles of Pax2 and Pax6 in eye development

To summarize, initially Pax2 and Pax6 carry out redundant functions in setting up the RPE progenitor domain in the OV neuroepithelium. This shared role is demonstrated clearly by their entirely overlapping expression domains in the OV neuroepithelium and by their redundant function in mediating Mitf expression. Later, during optic-cup stages, the distribution of Pax2 and Pax6 segregate to give their well-documented, mutually exclusive patterns in optic stalk and optic cup, respectively. At these stages Pax2 is necessary for oligodendrocyte differentiation in the optic stalk and for the closure of the choroid fissure (Torres et al., 1997), whereas Pax6 is required for normal retinal neurogenesis (Marquardt et al., 2001) and iris morphogenesis (Glaser et al., 1992). Therefore, the later segregation of Pax2 and Pax6 activities reflects their divergent functions during later eye development and might be necessary for them to carry out their functions in different tissues of the eye.

In this respect it might be significant that the late retinal enhancer of Pax6 (‘alpha’) (Kammandel et al., 1999; Báumer et al., 2002), which is repressed by Pax2 (Schwarz et al., 2000), is excluded from the Pax2-positive choroid fissure (see Báumer et al., 2002). In this light, the original model in which the mutual repression of Pax2 and Pax6 was assumed to direct the spatial segregation of territories in the early eye (Schwarz et al., 2000) might only apply to later aspects of eye development, such as optic-cup morphogenesis. Such mutual repression might assure the spatial exclusion of their diverging functions in optic nerve/choroid fissure and optic cup/retina. It remains to be shown how the switch from coordinate expression and function to divergent activities and mutual exclusion is achieved at the level of gene regulation.

Pax transcription factors as regulators of bHLH transcription factors in cellular determination

Mitf is not the first bHLH transcription factor that is known to be regulated by Pax-family transcription factors. During muscle development, Pax3 is involved in the activation of MyoD, a myogenic bHLH transcription factor (Maroto et al., 1997; Tajbakhsh et al., 1997), although it is unclear whether activation is direct or indirect (Borycki et al., 1999). Similarly, direct activation of the bHLH factors Mef5 and MyoD by Pax7 during the differentiation of pluripotent muscle-stem cells into satellite cells was assumed (Rudnicki et al., 1993; Megenary et al., 1996; Seale et al., 2000) (reviewed by Tajbakhsh and Buckingham, 2000). Recently, a direct requirement of Pax6 activity for the activation of another bHLH factor, Ngn2, in the spinal cord (Scardigli et al., 2001) and the developing neuroretina (Marquardt et al., 2001) was demonstrated. We previously found that Pax6 normally binds to and activates Ngn2-specific enhancers (Marquardt et al., 2001; Scardigli et al., 2003) that are not activated in Pax6-deficient embryos (Scardigli et al., 2001). Furthermore, after specific inactivation of Pax6 in the distal NR in α-Cre/Pax6fl/fl mice, bHLH factors Mash1 and Math5 are not expressed (Marquardt et al., 2001). Another bHLH transcription factor, NeuroD1 (previously NeuroD), is still expressed in the distal NR of this Pax6 mutant (Marquardt et al., 2001), but is absent in Pax6–/– OV, which might indicate its dependence on early Pax6 expression (data not shown).

Pax transcription factors are often required for the determination of a specific cell fate from multipotent cells (Nutt et al., 1999; Marquardt et al., 2001; Marquedt and Gruss, 2001; Seale et al., 2000; Borycki et al., 1999) and bHLH transcription factors function in the differentiation of determined progenitor cells (Cepko, 1999; Kageyama et al., 1997). Taken together, several lines of evidence indicate that the presence of particular Pax transcription factors in different progenitor cells might be a general requirement for the initiation of a number of specific differentiation pathways.
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