Specific Pax-6/Microphthalmia Transcription Factor Interactions Involving Their DNA-binding Domains and Inhibit Transcriptional Properties of Both Proteins*

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Pax-6 and microphthalmia transcription factor (Mitf) are required for proper eye development. Pax-6, expressed in both the neuroretina and pigmented retina, has two DNA-binding domains: the paired domain and the homeodomain. Mice homozygous for Pax-6 mutations are anophthalmic. Mitf, a basic helix-loop-helix leucine zipper (b-HLH-LZ) transcription factor associated with the onset and maintenance of pigmentation, identifies the retinal pigmented epithelium during eye development. Loss of Mitf function results in the formation of an ectopic neuroretina at the expense of the dorsal retinal pigmented epithelium. In the present study, we investigated the interaction between Pax-6 and Mitf. In transient transfection-expression experiments, we found that transactivating effects of Pax-6 and Mitf on their respective target promoters were strongly inhibited by co-transfection of both transcription factors. This repression was due to direct protein/protein interactions involving both Pax-6 DNA-binding domains and the Mitf b-HLH-LZ domain. These results suggest that Pax-6/Mitf interactions may be critical for retinal pigmented epithelium development.

Eye development proceeds from two principal tissue components: the neural ectoderm, which buds from the forebrain to form the optic vesicle, and the surface ectoderm, which forms the lens. The optic vesicle invaginates, producing the optic cup with an inner layer that forms the neural retina (NR)† and an outer layer that forms the retinal pigmented epithelium (RPE), which can be related to melanocytes on the basis of its melamin content. RPE and NR embryonic cells have a common precursor and share the characteristic of remaining plastic after they have reached their terminal differentiation stage. Indeed, cells from the RPE have the potential to transdifferentiate into lens or neuronal cells (1–4), and conversely, cells from the NR have the potential to transdifferentiate into pigmented cells (5). Cell differentiation is the product of differential gene expression, and transcription factors regulate such events.

Recent progress in the genetics of vertebrate eye development is linked to the discoveries of genes encoding transcription factors, such as Pax-6, which is able to initiate an entire cascade of gene activity sufficient to generate a complete eye (6). Pax-6, a member of the paired domain family of transcription factors, has a specialized homeodomain, which is downstream of a DNA-binding paired domain and upstream of a C-terminal activation domain (7, 8). Pax-6 encodes five proteins through alternative splicing and internal initiations (9). Three proteins of 48, 46, and 43 kDa contain a paired domain, but two proteins of 33 and 32 kDa are devoid of this DNA-binding domain. Pax-6 is known to be critical for eye development (10). Mutations in the Pax-6 gene cause the aniridia syndrome in humans (11) and the small eye phenotype in mice (12). Pax-6 is expressed in presumptive eye tissues, and both the semi-dominant pattern of inheritance of the Pax-6 mutant phenotype (10, 12) and experimental manipulations using transgenes based on the Pax-6 locus (13) indicate that achieving the correct level of Pax-6 is important for development of a normal eye.

Mutation of the microphthalmia-associated transcription factor (Mitf, a b-HLH-LZ family member) (14) induces the human Waardenburg syndrome type 2, a hereditary disorder associated with melanocyte abnormalities (15, 16). This gene is of special interest in relation to the NR and RPE development and transdifferentiation phenomenon because it has been shown to play a critical role in RPE development. Mutation of Mitf in mi mice results in microphthalmia associated with an abnormal development of the RPE, which appears unpigmented and often pluristratified (17–19). Spontaneous transdifferentiation of quail RPE in vitro is accompanied by a mutation in this gene (20), suggesting a role for Mitf in both cellular differentiation and control of cell division. We have previously found that chicken or quail NR cells expressing Mitf are responsive to FGF2 and that these cells become rapidly pigmented in vitro, in contrast to the control cells transfected with the empty DNA vector (21). Mitf regulates the expression of genes involved in melanogenesis, including QNR-71, which encodes a melanosomal protein (5), and several enzyme-encoding genes controlling the melanin synthesis. These genes include the tyrosinase (22, 23) and tyrosinase-related peptide 1 (24). All of these genes are regulated by Mitf through a direct binding at the CATGTG hexameric motif (E-box) present in their promoter regions (5, 25, 23).

Experiments have shown that Pax-6 can interact with homeodomain-containing transcription factors (27–29), CBP/p300 co-factors (30), and with members of the tumor suppressor retinoblastoma protein (pRB) (31). Mitf also interacts with pRB (24) and CBP/p300 (33). Thus, in view of the importance of both
Pax-6 and Mitf for eye development, and because the two proteins are simultaneously present in the developing RPE, we tested whether they could interact with each other. This interaction would be of particular interest for RPE differentiation because Mitf-deficient mice or quails exhibit spontaneous neuronal transdifferentiation of pigmented epithelium in vivo (20).

The present results showed that both DNA-binding domains of Pax-6 interact with the b-HLH-LZ domain of Mitf. This interaction strongly reduces transactivation of Pax-6 as well as Mitf-responsive promoters. No inhibition is observed with the mi mutant form of Mitf. Based on these data, we consider that Pax-6 and Mitf interaction may be required for normal retinal pigmented epithelium differentiation in vivo.

**EXPERIMENTAL PROCEDURES**

**Mitf and Pax-6 Constructs**—The pSFCV-LE-Mi (see Ref. 21) was digested with EcoRI to produce construct pSG5-Mitf (amino acids 1–413). pCRII-Mitf (193–413) (see Ref. 21) was digested with BamHI to produce construct pSG5-Mitf (193–413) using a modified pSG5 including an AUG upstream of the BamHI site. The pSG5-Mitf (1–413) plasmid was digested with PstI, blunt-ended with mung bean nuclease, and ligated to produce construct pSG5-Mitf (1–318). pSG5-Mitf (193–413) was digested with AgeI, blunt-ended with Dl I, and ligated to make construct pSG5-Mitf (193–297). pSG5-Mitf was digested with XbaI. The resulting vector was then digested with SpeI, blunt-ended with mung bean nuclease, and ligated to obtain pSG5-Mitf (1–140). pSG5-Mitf (193–413) was digested with SpeI/XhoI, end-filled with Klenow, and ligated. The resulting plasmid was then digested with BamHI/EcoRI, end-filled with Klenow, and ligated, giving construct pSG5-Mitf (297–413). The pEG-BOS-Mitf wild type and mi mutant constructs have been described previously (34) and were kindly provided by Dr. S. Nomura. A Mitf cDNA fragment corresponding to the full-length protein was amplified by polymerase chain reaction using oligonucleotides 5′-CAC GAA TTC CAT GCT GGA AAT GCT AGA A-3′ and 5′-CAA AAC CCC ATT ATT TAC-3′ (position +45) and 5′-TGT GGG GCC GCC TAA CAC GCA TGC TCC GTG-3′ (position +1270). The amplification product was cloned in pCRII and sequenced. The Mitf cDNA was then inserted between the EcoRI-XhoI sites of pGEX-4T3. pCRII-Mitf (193–413) was digested with EcoRI and cloned in the EcoRI site of pGEX-1A. These two subclones resulted in the in-frame fusion of the desired sequence to the GST protein.

**pSG5-p46** encodes a Pax-6 p46 isoform containing three point mutations in the paired domain (S93A, F95N, and E98Q). To generate this mutant, we used the Chameleon double-stranded site-directed mutagenesis kit (Stratagene) using pSG5-p46 (8) as a template and oligonucleotides 5′-CGA GAG TGC CTC GAG GGA TC-3′, 5′-GGA TGC AAT TTC ACG-3′, and 5′-CCA AAC CCC ATT ATT TAC-3′. The DNA probe used was the P6CON double-stranded oligonucleotide (5′-GGA TGC AAT TTC ACG-3′) (5′-PstI labeled with the PolyNucleotide kinase T4, G1–S1 (5′-CAA ACC CCC ATT ATT TAC AGA TGA GAA ATT TAT ATT GTC AGA GTA ATA TCT-3′) is a wild type glucagon promoter element that has been shown to bind Pax-2 (28). Gel retardation assays were performed as described previously (36) with 50 ng of bacterially expressed proteins.

**Immunocytochemistry**—Transfected cells cultured on 16-mm microcoscope coverslips were fixed for 1 h with 2% paraformaldehyde in PBS and then treated with serum Mic (see Ref. 21). Anti-Mi-reactive proteins were detected with CY3-labeled goat anti-rabbit immunoglobulin secondary reagent (Jackson ImmunoResearch). EGFP and DsRed Fusion Protein Constructs—To visualize both Pax-6 and Mitf proteins, enhanced green fluorescent protein (EGFP, Clontech) and DsRed (CLONTECH) were used as fusions partners of p46 and Mitf, respectively. The following plasmids were constructed: a BglII-NotI EGFP fragment was ligated to the BglII and Bsp1201 sites of pVNC3 (modified from pVM116 (38)) to produce pVNC3EGFP. A BamHI-Bsp1201 Pax-6 fragment was inserted into the NotI and BglII sites of pVNC3EGFP to make pVNC3Pax-6-EGFP, which was then digested by HindIII and BamHI and Klenow-treated, giving pVNC3PaxEGFP. A Bg/II-NorI DsRed fragment was ligated to the BamHI and Bsp1201 sites of pVNC3 producing pVNC3Red. A HindIII-SalI fragment encompassing the whole Mitf coding region was inserted in the HindIII-SalI sites of pVNC3Red to produce pVNC3 MiRed. An EGFP-myc fusion was used as a negative control. An EcoRI-Bsp1407 Klenow-treated EGFP fragment (from pGM1.8) was ligated to an EcoRI-SphI Klenow-treated y-Myc fragment (from MC29) and inserted into the EcoRI and BglII Klenow-treated sites of pVNC7 (which is the pVNC plasmid with an inverted multiple cloning site) to produce pVNC7EGFPmyc. pVNC3MiRed was co-transfected with either pVNC3PaxEGFP or pVNC7EGFPmyc into E8 qual retinal pigmented cells. To determine the two chimera localization, cells were fixed for 20 min at room temperature in 3% paraformaldehyde and 0.1% PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 1 mM magnesium acetate, pH 6.9). Cells were washed three times in PBS and permeabilized for 25 min in 0.1% Triton X-100 in PBS. Chromosomes were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 5 min. After a rinse in PBS, coverslips were mounted in 50% PBS-glycerol containing anti-fading reagent 1.4 di-azabicyclo-(2,2,2)-octane (DABCO, Sigma) at 100 mg/ml.

**Wide Field Optical Sectioning Fluorescence Microscopy**—Pictures of fixed cells were collected using a three-dimensional deconvolution imaging system, the detailed description and validation of which will be published elsewhere. Briefly, it consisted of a Leica DM RXA microscope equipped with a piezoelectric translator (PIFOC, Physik Instruments, Waldbronn, Germany) placed at the base of a 100X P-FLA objective, and a 5-MHz Micromax 1300Y interline CCD (charge-coupled device) camera (Roper Instruments, France). For the acquisition of Z-series, the camera was operated at full speed and controlled the Piezo translator at the start of each CCD chip read out. Stacks containing fluorescence images were collected automatically at 0.2 μm Z-intervals (Metamorph software, Universal Imaging). Wavelength selection was achieved by switching to the corresponding motorized selective Leica filter block before each stack acquisition. Tests using 50-nm Tetraspec beads (Molecular Probes) showed that the system did not generate x-y pixel shifts, and that Z-plane shifts between colors, due to chromatic aberrations, were reproducible and could be corrected for. Exposure times were adjusted to provide ~3000 gray levels at sites of strong labeling. Automated batch deconvolution of each Z-series was achieved using a measure and spread function technique followed by an iterative deconvolution with a custom-made software package. The point spread function of the optical system was extracted from three-dimensional images of fluorescent beads 0.1 μm in diameter (Molecular Probes) collected at each wavelength.

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Mains (Fig. 1, compare interacted simultaneously with both of these DNA-binding domains. Efficient binding to Mitf using the GST-Prd46-Hom (4.6%) than isoforms interacted with Mitf. In addition, we observed a more similar results were obtained with the p48 paired domain interacted directly with both the paired and homeodomains of Pax-6. (Fig. 2). Binding results obtained with the GST-p46 truncated proteins were synthesized in the reticulocyte lysate (amino acids 1–140, both the N terminus of the protein (amino acids 224–284), GST-Prd46-Hom (amino acids 3–270) and GST-p46 containing the full-length Pax-6 product. We also used GST as a negative control. In vitro radiolabeled Mitf protein was loaded onto these columns and washed, and bound proteins were eluted by boiling and analyzed on SDS-polyacrylamide gel electrophoresis. The percentage of bound radioactivity was calculated using a PhosphorImager. A comparable amount of radiolabeled Mitf was recovered on GST-Paired (1.7% of the input, Fig. 1, lane 3) and GST-Hom (3.7% of the input, Fig. 1, lane 4). These results indicated that Mitf associated directly with both the paired and homeodomains of Pax-6. Similar results were obtained with the p48 paired domain (0.75% of the input; Fig. 1, lane 2), suggesting that both Pax-6 isoforms interacted with Mitf. In addition, we observed a more efficient binding to Mitf using the GST-Prd46-Hom (4.6%) than with the paired or homeodomain alone, suggesting that Mitf interacted simultaneously with both of these DNA-binding domains (Fig. 1, compare lanes 3, 4, and 5). Stronger retention was not observed with the full-length p46 (3.9%), indicating that the p46 C terminus is not a Mitf-interacting domain (Fig. 1, lane 6).

To identify the regions in Mitf required for physical interaction with the Pax-6 p46, we carried out in vitro binding experiments by using various deletion mutants of Mitf (Fig. 2A). As shown in Fig. 2B, both the N terminus of the protein (amino acids 1–140, lane 5) and the b-HLH-LZ domains (193–297, lane 4) were able to bind to Pax-46 with weak and strong affinities, respectively. The C terminus of Mitf (amino acids 298–413, lane 6) was unable to bind, whereas similar amounts of the truncated proteins were synthesized in the reticulocyte lysate (Fig. 2C, lane 6). Binding results obtained with the GST-p46 (Fig. 2B) were also observed with GST-Paired and GST-Hom (data not shown), indicating that the b-HLH-LZ domain of Mitf interacts with both of the Pax-6 DNA-binding domains.

Mitf Recognizes the p46 Paired Domain through the C-terminal Subdomain and Can Inhibit the DNA Binding Activity of Pax-6—It has been shown that the Pax-5 paired domain can recruit Ets DNA-binding domains to the Pax-5 C-terminal subdomain DNA-binding site to form ternary complexes on a B-cell-specific promoter (39). The structure and docking of Pax-5 should be nearly identical to Pax-6, because their C-terminal subdomains are 75% identical and all DNA-contacting residues are conserved (40). Modeling the C-terminal subdomain of paired with the Max b-HLH shows that residues of the second helix of the paired C-terminal subdomain (Ser-93, Phe-95, and Glu-98; see Fig. 5A) can pack against the loop of Max HLH and interact with the Asp-236, Asp-238, and Arg-240 (equivalent amino acids in Mitf). To test the possibility that these residues are involved in the Pax-6/Mitf interaction, we replaced by directed mutagenesis S93A, F95N, and E98Q in the p46 protein and D236N, D238N, and R240N in the Mitf protein. Wild type Pax-6 (Fig. 3 lane 5) or mutagenized Pax-6 proteins (Fig. 3, lane 6) synthesized in vitro, were loaded onto GST columns. The percentage of bound radioactivity was calculated using a PhosphorImager. 23% (Fig. 3, lane 3) of the wild type p46 Pax-6 protein recognized the GST-Mitf (193–413), but only a 3% residual binding (Fig. 3, lane 4) was detected using the mutagenized p46 (p46*) protein. This residual binding could be observed because of the presence of the homeodomain in the p46*, since this DNA-binding domain can also bind the Mitf protein (Fig. 1). However, the paired-less proteins (p32–33)
synthesized in vitro (Fig. 3, lane 5) were not recognized by Mitf protein (Fig. 3, lanes 3 and 4, compare with lanes 5 and 6). However, no difference was observed in the amount of radiolabeled Mitf protein recovered on the GST-Prd46 column between the wild type (12.5% of the input) or the Mitf triple mutant (D236N,D238N,R240N, 12.8% of the input) showing that the modifications introduced into the loop of the HLH domain still allow protein/protein interaction with Pax-6 (data not shown).

Because Mitf and Pax-6 interacted through the paired domain, we tested whether Mitf binding to the p46 protein was able to modulate the DNA binding activity of this Pax-6 product by using the p46 ability to bind an oligonucleotide bearing a high affinity paired binding site named Pax6CON (41). As shown in Fig. 4A, when GST-Mitf-(193–413) or GST-Mitf-(1–413) proteins were co-incubated with GSTPrd46, a marked decrease in binding to Pax6CON oligonucleotide was observed (Fig. 4A, compare lane 2 with lanes 3 and 4). However, because the Pax6CON binding site was not a real binding site present in a Pax-6 target promoter, we performed a similar experiment using an oligonucleotide containing the Pax-6 binding site of the G1 element present in the glucagon promoter. As for the Pax6CON, co-incubation with GST-Mitf-(193–413), which is unable to bind this probe (lane 3), reduced significantly the Pax-6 binding on this oligonucleotide (Fig. 4B, lane 4).

Effects of Pax-6/Mitf Protein Interaction on Their Transactivating Properties—The Mitf factor is essential for melanocyte differentiation and is able to activate the promoter of several genes involved in melanogenesis (5, 14, 22, 24, 42). We tested for whether the Pax-6/Mitf interaction observed was able to regulate Mitf-dependent QNR-71 and tyrosinase promoter expression (Fig. 5B). The QNR-71 promoter (pPΔ71–220; 0.2 μg) or tyrosinase constructs (1 μg) were co-transfected into RPE cells with the vector expressing the murine Mitf protein and increasing amounts of the pSG5-Pax-6 vector expressing either the p46 or the p48 isoforms (Fig. 5A). Cell lysates were collected 2 days after transfection, and the levels of CAT and luciferase activities present in the lysates were determined. Co-transfection of the pPΔ71–220 with the vector expressing the murine Mitf protein resulted in an 8-fold increase of CAT activity relative to the vector control. A strong increase in luciferase activity (40-fold) was also observed with the tyrosinase promoter in response to the Mitf protein expression (Fig. 5C).

Co-transfection of the Mitf-responsive promoters with the vectors expressing the Pax-6 isoforms alone (Fig. 5A) did not modify their activity level relative to the control vector (Fig. 5C). Co-transfection of either QNR-71 or tyrosinase promoter with the vectors expressing the Mitf and p46 Pax-6 proteins resulted in an 80–90% reduction of the Mitf transactivational effect in a dose-dependent manner (Fig. 5C). Similar results were obtained with the p48 isoform (Fig. 5C). This Pax-6 inhibition was specific to Mitf transcription factor, because no repression was observed on the 4xGal4 thymidine kinase promoter activated by the Gal4VP16 protein co-transfected with the p46 encoding vector (data not shown).

Because the Pax-6 proteins were able to repress Mitf activity, we investigated whether Mitf was able to repress the Pax-6 transactivating properties. For that purpose, we used as a target the glucagon promoter linked to the CAT gene, which is strongly activated by the p46 protein (28). To demonstrate that in the same cells we could obtain both positive and negative effects on transcriptional regulation, we introduced the tyrosinase promoter linked to the luciferase reporter gene (activated by Mitf) together with the glucagon promoter linked to the CAT gene (activated by Pax-6). Luciferase and CAT activities present in the lysates were determined, and both were found to have decreased strongly when compared with the activities obtained with Pax-6 or Mitf transcription factor alone (Fig. 5D). No effect of Mitf or Pax-6 (alone or together) was observed on the β-galactosidase activity of a cytomegalovirus promoter in-
cluded as a control in the experiment. When we used instead in this assay the p46* (which interacted poorly with Mitf, Fig. 3), this protein activated the glucagon promoter as efficiently as the wild type protein but was 2-fold less repressed by Mitf (Fig. 5E). This result confirms that the C terminus of the paired domain is an important domain of interaction between the two proteins.

Because Pax-3, a Pax family member expressed in the neural crest-derived melanocytes, activated the Mitf promoter (43, 44), we tested whether PAX-3 was able to repress Mitf activity. As shown in Fig. 5F, Pax-3 was able to reduce Mitf activity on the QNR-71 promoter but to a lesser extend than Pax-6.

Next we asked whether a mutation abrogating the Mitf activity (deletion of one amino acid in the DNA-binding domain (34)) and resulting in transdifferentiation of dorsal retinal pigment epithelium into neural retina (19) could have any effect on Pax-6 transactivation. This was indeed the case, as shown in Fig. 6. The mutant p46* is as efficient as the wild type p46, but its transactivation effect on the glucagon promoter is only marginally repressed by Mitf. F, co-expression of PAX-3 and Mitf inhibits their transactivation effects on QNR-71 promoter. BHK21 cells were transiently transfected with 10 ng of pP2P−220 and 100 ng of pSG5-Mitf and 500 ng or 1 μg of PAX3-encoding vector pSRα MSV/PAX-3. The total amount of transfected DNA was kept constant by adding pSG5 empty vector to the reaction.

**Fig. 5.** Co-expression of Pax-6 and Mitf inhibits their transactivation effects on their target promoters. A, representation of two Pax-6 isoforms used in this study, namely p46 and p48. Both isoforms contain two DNA-binding domains: the paired box and the homeodomain. p48 contains an additional 42-base pair exon (4A) located in the paired domain. The numbers refer to amino acids. B, reporter gene promoter constructs used in the transfection assays. DNA-binding sites for Mitf (mi) or Pax6 are indicated in the promoters, which drive either luciferase (Luc) or CAT gene expression. C, tyrosinase and QNR-71 promoter transactivation assays. E5 quail RPE cells were transiently transfected with 1 μg of pMT2.2 (pGL2tyrosinase) or 200 ng of pP2P−220. The total amount of transfected DNA was kept constant by adding pSG5 empty vector to the reaction. B, tyrosinase and glucagon promoter transactivation assays. BHK21 cells were transiently transfected with 500 ng of pMT2.2 (pGL2tyrosinase) and 200 ng of pG3−138 (pBLCAT3 glucagon). The results were normalized to β-galactosidase activity derived from a co-transfected pcDNA3-LacZ expression plasmid. E, effect of paired domain mutations in Pax-6/Mitf interactions. 50 ng of pSG5-Mitf was transiently co-transfected into BHK21 cells with 1 μg of a plasmid encoding either the wild type Pax6 (pSG5-p46) or the p46* mutated in the paired domain and 200 ng of a CAT reporter glucagon promoter construct (pG3−138) CAT activities were expressed relative to the value for the pG3−138 with the empty vector (set at a value of 1). Thin lines indicate standard deviations calculated from transfections performed in duplicate. The mutant p46* is as efficient as the wild type p46, but its transactivation effect on the glucagon promoter is only marginally repressed by Mitf. F, co-expression of PAX-3 and Mitf inhibits their transactivation effects on QNR-71 promoter. BHK21 cells were transiently transfected with 10 ng of pP2P−220 and 100 ng of pSG5-Mitf and 500 ng or 1 μg of PAX3-encoding vector pSRα MSV/PAX-3. The total amount of transfected DNA was kept constant by adding pSG5 empty vector to the reaction.
The interaction of Pax-6 and Mitf is a significant aspect of eye development. Mitf interacts with the C terminus of the p46 paired domain. First, the p46 and p48 paired domains differ only in their N terminus (45) and are both efficient in Mitf binding and trans-inhibition. In addition, specific mutagenesis of 3 amino acids in the C terminus (Ser-93, Phe-95, and Glu-98; see Fig. 5A) of the p46 paired domain strongly reduced both the interaction with and the repression by Mitf of a Pax-6-responsive promoter. Interestingly, Pax-3 repressed Mitf activity to a lesser extent than Pax-6. Close examination of the Pax-3 paired domain reveals that if Phe-95 and Glu-98 are conserved in the paired domains of both proteins, Ser-93 of Pax-6 corresponds to a Gly in Pax-3 (46). Therefore, this amino acid may be important for protein interaction with Mitf. In contrast to the homeodomain alone (Fig. 1, lane 4), the paired-less proteins (p32–33) synthesized in vitro in Fig. 3, compare lanes 5 and 3) were not recognized by the Mitf protein. That the paired domain may be able to modulate the function of the homeodomain intramolecularly (47) explains why the Mitf protein may bind the homeodomain in the context of p46, explaining the residual interaction observed with the p46* containing the mutagenized paired domain (Fig. 3, lane 4). Indeed, it has been shown recently that C-terminal amino acid 422 is also critical for the DNA binding activity of the homeodomain in the p46 protein (48). These data suggest that folding of the homeodomain is controlled by both the N and C termini of the p46. Therefore, the folding of the homeodomain in paired-less p32–33 proteins may be different from that of the p46. However, it remains to be formally demonstrated that the homeodomain in the p46 binds Mitf. Such an inhibitory interaction has already been observed between paired domain transcription factors of the Pax-2/5/8 subfamily with a HLH Id protein (49). This binding occurred through the paired domain and resulted in the disruption of DNA-bound complexes. Thus, the HLH Id protein dissociated the complex formed between the Ets family member Elk1 and Pax-2/5/8 proteins (49). As for Mitf, the paired C terminus was involved in the interaction between Pax-5 and Ets family members (40). Amino acids involved in this interaction (Trp-97, Arg-100, and Val-117) are very close to those involved in the interaction between Pax-6 and Mitf, suggesting that the HLH Id protein may dissociate the Pax-5/Elk1 complex by directly competing for the same protein/protein interaction domain.

In contrast to the inhibitory effect of the Pax-6/Mitf interaction, activatory interactions between homeodomain-containing factors and b-HLH proteins have already been reported (50–52). The presence of many other factors and co-factors in a cell nucleus in vivo may influence the DNA-binding and transactivation properties of these proteins. In this respect, it is interesting to note that the p300 co-factor, which is coupled to the basal transcription machinery, is able to bind both Pax-6 and Mitf. This interaction enhances the synergistic effect of Pax-6 and homeodomain-containing protein cdx2 on the glucagon promoter (30). Such a factor could modulate the interaction between Pax-6 and Mitf and dictate the inhibitory or activatory nature of the interaction. Inhibition of Pax-6 DNA binding is not restricted only to Mitf, because we previously reported that homeodomain containing Engrailed 1 and 2 factors inhibited Pax-6 DNA binding and transactivation (27).

What could be the consequence of the inhibitory interaction between Pax-6 and Mitf for eye development? It is well established that the division of the optic neuroepithelium into two domains, the presumptive neuroretina and the pigmented retina, is critical for the development of the vertebrate eye. The notion that the early optic epithelium is bipotential may be reflected by the fact that distinct transcription factors that are later important separately for NR or RPE are initially coexpressed. For example, in cultured optic vesicles, Mitf expres-

**DISCUSSION**

This study provided evidence that Pax-6 interacts with the Mitf transcription factor and that this interaction suppresses the function of these molecules as transactivators. Furthermore, we observed that this interaction involved their respective DNA-binding domains. Because Pax-6 and Mitf form a protein-protein complex, they are no longer able to bind DNA and to transactivate their target promoters. We then investigated which regions of Pax-6 and Mitf DNA-binding domains may be required for their interaction. The paired domain of Pax-6 can be divided into two DNA-binding subdomains (45): the N-terminal domain (PAI) and the C-terminal domain (RED). Several data indicate that Pax-6/Mitf interactions imp-
sion was restricted to the RPE layer, whereas Pax6 staining was found in both RPE and NR (53). Implantation of FGFl-coated beads resulted in down-regulation of Mitf and up-regulation of Pax-6 and Chx10. Then RPE thickened and differentiated along the neuroretinal pathway (53), suggesting that Mitf down-regulation is required to initiate neuronal differentiation. Introduction of Mitf-expressing vectors into the avian neuroretina in vivo induced the differentiation of pigmented cells in the culture (21) showing that Mitf is dominant over neuroretinal factors to induce a pigmented phenotype. Consistent with these observations, when a functional Mitf was missing in vivo, as in mi/mi mice, the RPE (at least its dorsal part) lost the expression of RPE-specific genes, thickened, and then became a laminated second neuroretina (19). Therefore, if we expect the Pax-6/Mitf interaction to be a critical event in RPE differentiation, the mutant Mi protein should abolish such an interaction. This mutant (34) lacks an arginine in the basic domain of Mitf is indeed critical (directly or indirectly) for the interaction with Pax-6 (data not shown). That Pax-6, suppresed the Pax2 transactivating function (data not shown), suggesting that one function of Pax2 may be at least to restrict neuroretinal factors to induce a pigmented phenotype. Consistent with these observations, when a functional Mitf was missing in vivo, as in mi/mi mice, the RPE (at least its dorsal part) lost the expression of RPE-specific genes, thickened, and then became a laminated second neuroretina (19). Therefore, if we expect the Pax-6/Mitf interaction to be a critical event in RPE differentiation, the mutant Mi protein should abolish such an interaction. This mutant (34) lacks an arginine in the basic region of the nuclear localization signal, resulting in a cytoplasmic misrouting of an important part of the protein and lack of DNA-binding capability. In cotransfection experiments with both Pax6 and mi coding vectors, we detected a substantial part of the Mi protein in the nucleus of the transfected cells, but we noticed a complete lack of Pax-6 inhibition. In vitro pull-down experiments performed with the Mi protein translated in a reticulocyte lysate revealed a 33% interaction with the p46 protein when compared with Mitf set at 100%, suggesting that the basic domain of Mitf is indeed critical (directly or indirectly) for the interaction with Pax-6 (data not shown). That Pax-6, activated by the lack of interaction with mi mutant, is involved in the neuronal transdifferentiation of the RPE remains to be demonstrated. Several lines of evidence suggest a direct involvement of Pax-6 in neuronal differentiation of the retina. First, mice lacking Pax-6 function do not develop retina (12). In accordance with this finding, it has been shown that in zebrasfish with an experimentally reduced number of Pax6-expressing cells in the optic vesicle, retinal differentiation is restricted to cells that retain the Pax6 protein (54). Pax-6 ectopic expression in cells nonspecified to generate neurons, both in invertebrates and vertebrates, is able to specify retinal identity (6, 10). Finally, introduction of the Pax-6 coding vector into the cultured RPE cells, in contrast to the empty control vector, induced expression of HuD into the culture (data not shown). HuD is an RNA-binding protein specifically expressed in neurons. Mis-expression of HuD in cultured neural crest cells results in a dramatic increase in the proportion of cells exhibiting neuronal morphology, neuronal markers, and neurotrophin dependence (26). Thus, Pax-6 is able to induce expression of a neuronal marker into RPE cells. Pax-6 may therefore play a role in the in vivo neuronal transdifferentiation of RPE cells observed in the mi mutant, where an inhibitory interaction with Mitf is no longer possible.

Another Pax family member, Pax2, is expressed in the ventral optic vesicle, later being confined to the proximal region destined to contribute to the optic nerve. Pax2 null mutant mice show an extension of the RPE into the optic stalk and failure of the optic fissure to close (32). As for Pax-6, Mitf suppressed the Pax2 transactivating function (data not shown), suggesting that one function of Pax2 may be at least to restrict Mitf activity in the optic stalk area. Therefore, direct interaction of Pax family members with Mitf may be a general rule.

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Fig. 7. Expression of Pax6-EGFP and Mitf-DsRed fusion proteins in RPE cells shows co-localization in nuclei. Pax6-EGFP and Mitf-DsRed fusion proteins were transfected into quail retinal pigmented cells, and the subcellular localization of the fusion proteins was determined by three-dimensional fluorescence microscopy of fixed cells. A, Pax6-EGFP expression in cell nuclei. B, Mitf-DsRed expression in cell nuclei. C, overlay of these two expression patterns (A and B) showing co-localization of Pax6-EGFP and Mitf-DsRed fusion proteins in the cell nuclei (see yellow spots). As a control we determined the subcellular localization of v-Myc and Mitf. D, EGFP-myc expression in cell nuclei. E, Mitf-DsRed expression in cell nuclei. F, overlay of these two expression patterns (D and E) showing no co-localization of EGFP-myc and Mitf-DsRed fusion proteins in the cell nuclei (compare with panel C).
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Specific Pax-6/Microphthalmia Transcription Factor Interactions Involve Their DNA-binding Domains and Inhibit Transcriptional Properties of Both Proteins
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