OTX2 Activates the Molecular Network Underlying Retina Pigment Epithelium Differentiation*

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The retina pigment epithelium (RPE) is fundamental for the development and function of the vertebrate eye. Molecularly, the presumptive RPE can be identified by the early expression of two transcription factors, Mitf and Otx. In mice deficient for either gene, RPE development is impaired with loss of melanogenic gene expression, raising the possibility that in the eye OTX proteins operate either in a feedback loop or in cooperation with MITF for the control of RPE-specific gene expression. Here we show that Otx2 induces a pigmented phenotype when overexpressed in avian neural retina cells. In addition, OTX2 binds specifically to a bicoid motif present in the promoter regions of three Mitf target genes, QRN71, TRP-1, and tyrosinase, leading to their transactivation. OTX2 and MITF co-localize in the nuclei of RPE cells and physically interact, and their co-expression results in a cooperative activation of QRN71 and tyrosinase promoters. Collectively, these data suggest that both transcription factors operate at the same hierarchical level to establish the identity of the RPE.

In vertebrates, melanin is synthesized in specialized cells: the neural crest-derived melanocytes, the pigmented cells of the pineal organ, and the retinal pigmented epithelium (RPE). Melanin-producing cells share common features like the presence of the melanosome, a specialized organelle where transmembrane glycoproteins of the tyrosinase family synthesize melanin pigment (1). The RPE is a monolayer of cuboidal pigmented cells playing a key role in the maintenance and function of the vertebrate eye. In mice, genetic ablation of the developing RPE causes immediate arrest of eye growth, followed by its re-adsorption (2). In the adult, the specific functions of this tissue are turn-over of the photoreceptor outer segments, transepithelial transport, retinoid storage, and protection against light and free radicals (3). In vertebrate embryos, the RPE and neural retina (NR) are generated from common precursors that retain the capacity of trans-differentiate in each other cell type both in vivo and in vitro (4–7). During eye development, the presumptive RPE can be molecularly identified by the early expression of the Mitf and Otx genes (8, 9). Mitf encodes the microphthalmia-associated transcription factor of the basic helix-loop-helix and leucine zipper family (bHLH-LZ). Mitf is expressed as multiple isoforms termed Mitf-M, Mitf-A, Mitf-D, Mitf-C, and Mitf-H (10, 49). Mitf-M expression is restricted to neural crest-derived melanocytes, whereas those of Mitf-A and Mitf-D are enriched in the RPE (11, 49). Mutations in the human MITF are responsible for the Waardenburg syndrome type 2, a hereditary disorder causing deafness and pigmentation abnormalities (12). Functional inactivation of Mitf in mice impairs the development of the RPE, which becomes a laminated second NR (6, 13). Conversely, ectopic expression of this gene in avian NR cells induces a pigmented phenotype (4). In line with these observations, MITF has been shown to interact and transactivate the promoter regions of genes involved in the terminal differentiation of the RPE, including the melanosome glycoprotein QRN71, the melanogenic enzyme tyrosinase (Tyr), and the tyrosinase-related proteins TRP-1 and TRP-2, through specific binding to the hexameric motif CATGTG (M-box) present in all these promoters (reviewed in Ref. 11).

Otx genes, Otx1 and Otx2, are homeodomain-containing transcription factors known for their essential role in anterior head formation (14). A prominent feature of the Otx homeodomains is a lysine residue at position 9 of the recognition helix, which confers high affinity binding to the same functional target sequence motif (TAATC(C/T)) recognized by bicoid on DNA (15–18). Besides their DNA-binding properties, little is known about how Otx proteins function to activate target genes in selective regions of the embryo, and only a few direct downstream targets of Otx function have been identified so far (19). In the vertebrate eye, Otx genes are initially expressed in the entire optic vesicle, but their expression soon becomes restricted to the presumptive RPE, where it is maintained throughout adulthood (7, 9, 17). We have recently shown that mice deficient in Otx genes present clear defects in the patterning of the RPE, which is replaced by a NR-like territory. In Otx mutants, the expression of Mitf and Tyr is largely absent and maintained only in little patches of tissue where residual OTX2 expression is also localized (7). Conversely, in Mitf mutants the
expression of Otx2 is specifically down-regulated in those areas where RPE does not differentiate (6, 20).

These data provide genetic evidence that both Otx and Mitf are responsible for the development of the RPE (6, 7). However, it is not clear whether Otx and Mitf have a hierarchical relationship and act in a feedback loop, or whether they cooperate for the direct control of gene expression in the RPE. Here, we report a series of experiments that address these questions. We show that Otx2, as Mitf, is capable of inducing a pigmented phenotype in avian NR cells. OTX2 binds specifically to a bicoid motif present in the promoter regions of genes encoding melanosome glycoproteins, leading to their transactivation. This activity is enhanced by MITF. Furthermore, MITF and OTX2 co-localize in the nuclei of retinal pigmented cells and are capable of biochemical interaction. Because OTX2 and MITF do not appear to regulate each other’s expression, we propose that the two transcription factors operate at the same hierarchical level to establish the identity of the RPE.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The CMV-pC7 expression vectors containing the coding regions of the hOTX1, the mOtx2, and of its mutated K50Q version were a gift of Dr. A. D. Novitch (21). mOtx2 coding sequence was inserted in the CMV expression vector pC1neo (Promega) and pVNC3 (22); similar results were obtained when the two vectors were compared in transfection experiments. The in-frame fusion protein GST-mOtx2 was produced by insertion of the Xmal-XhoI full-length mOtx2 fragment from mOtx2-pCVC3 in the pGEX-OST vector (Amersham Biosciences). An EcoRl-XhoI fragment of 8.2 kb containing the region upstream of the first exon of mOtx2 cloned in the pKS vector (Dr. Mallamaci's generous gift) was analyzed by automated DNA sequencing (ABI 377, Applied Biosystems) and compared with the human sequence. The information derived from this analysis was used to determine the existence in human and mouse of two different Otx2 transcripts. The identification numbers of the EST sets corresponding to the two phylogenetically conserved Otx2 transcripts are the following: T0 human (National Institutes of Health Image: 5493541, 3868090, and 5547260); T0 mouse (Image: 5400892, H3008112, 5359966, G43006K08, 5365867, 528-5K1I, 5362013, and G430009N13); T1 human (Image: 6154186, 5495438, 3870868, 3870369, 3872745, and 3353521); T1 mouse (Image: 4527414). The vector pOTX2-luc-1219, containing the 1.8-kb promoter region of the mOtx2 T1 transcript inserted in the pXP2 luciferase vector, was a generous gift of Dr. S. Guazzi (23). To construct the pOTX2Luc-974 vector a 1.6 kb 5'-flanking fragment encompassing the putative initiation site of the mOtx2 T0 transcript was amplified using the following primers (5'-ggagtctcctgagagttgctgag-3'; 5'-atgtgcaagtcagggagaagag-3'). This fragment was then Sal/BlgII-digested and subsequently inserted into the pXP2 luciferase vector.

To express Mitf, the coding sequences of the isoform MitfA and Mitf-M were inserted in the pVNC vector. Truncations of the coding sequence surrounding the coding region of MitfA and the Mitf-M were inserted in the pVNC vector. The coding sequence containing the coding region of MitfA and the Mitf-M were inserted in the pVNC vector. The coding sequence containing the coding region of MitfA and the Mitf-M were inserted in the pVNC vector.

Luciferase- and fluorescent markers were kindly provided by Dr. S. Shibahara (24) and Dr. R. Balletti (25). CAT-coupled quiall QNR71 promoter details have been previously published (26). To generate the QNR71 2/4 mutant, we used the Chameleon double-stranded site-directed mutagenesis kit (Stratagene) using the QNR71 promoter as a template and oligonucleotides 5'-gggcttcgacgccgaggacgccgaggc-3' and 5'-agggaagacaccgaggatccgccgccgccgcggacgccgccgccgcggc-3'. The QNR71 promoter mutant in the Mitf-BS binding site has been previously described (26).

Cell Cultures, Transfections, and Luciferase and CAT Assays—Baby hamster kidney (BHK-21) cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 1% minimal essential medium vitamins x100, and 10 μg/ml ascorbic acid, and transfected by the calcium phosphate method as described previously (4).

Immunocytochemistry—Transfected cells were fixed for 20 min with 4% paraformaldehyde in PBS, and incubated with a polyclonal anti-serum anti-MITF (4) or anti-OTX2 (9). Primary antibodies were detected with Cy3-labeled goat anti-rabbit immunoglobulin secondary reagent (Jackson Immunoresearch). Electroplasmodial Mobility Shift Assay—Mitf-A, Otx2, and luciferase (control) proteins were synthesized by using the Tnt coupled wheat germ extract system (Promega). 1–2 μl of protein translation extracts was incubated for 15–30 min at room temperature with 1.5 × 10^4 cpm corresponding [32P]-labeled double-stranded oligonucleotide (5 μl of 20 nM Heps, pH 7.9, 60 mM KCl, 10% glycerol, 0.1 mM EDTA, 2 mM MgCl2, 1 mM dithiorthreitol, and 0.1% bovine serum albumin. For Otx2 binding to TRP-1 sequences, the concentration of EDTA was 5 mM and 1 μg of poly(dI-dC) was added. The reaction mixture was analyzed by electrophoresis and visualized by autoradiography. In competition assays a 500-fold excess of cold oligonucleotide was preincubated at room temperature for 15–30 min before the addition of the labeled oligonucleotide.

MitfT is a sequence of NYT to known to bind Mitf, and mMitfT is its version with a mutation in the M-box that prevents the binding (wt2 and mt2) (29). "Cons." is a sequence of the rat GnRH gene previously shown to bind Otx2 (17). OTX2 and "mCons" is its corresponding mutated oligonucleotide (rGnRH Otx and rGnRH mut) (29). Mitf1, OtxA, OtxB, and OtxC correspond to sequences of the human TRP-1 promoter (GenBank accession number D38305), whereas Mitf2, OtxD, OtxE, and OtxF are sequences of the mouse tyrosinase promoter (GenBank accession number D00439). They are underlined in Figs. 3A and 4A. mOtxA, mOtxB, and mOtxD are versions of OtxA, OtxB, and OtxD, respectively, where the consensus bicoid binding sequence was mutated from GAGTAT to GGGCCC. mMitf2 is identical to Mitf2, but the consensus M-box CATGTG is mutated to ACTGTG.

For the QNR71 gene promoter, gel retardation assays were performed as previously described (27) with 100 ng of bacterially expressed GST-mOtxA protein. The DNA fragment used as probe was the 1.8-kb promoter region of the rat Otx2 in Retina Pigment Epithelium Differentiation.

GFP and DoRed Fusion Protein Constructs and Wide Field Optical Sectioning Fluorescence Microscopy—The full coding region of mOtx2 was amplified using the BigDye®-terminal deoxynucleotide (T) D32uEGFP to make the pVNC EGFP-Otx2. pVNC MiRed and pVNC EGFP-Myc were already published (22, 30). pVNC-Mitf-Red and pVNC-EGFP-Otx2 or pVNC EGFP-Myc were cotransfected in quail RPE cells. To determine the localization of the chimerical proteins into the nuclei, cells were fixed for 20 min at room temperature in 3% paraformaldehyde in HPM 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 (Sigma) for 5 min. After a rinse in PBS, coverslips were mounted in 50% PBS/glycerol containing anti-fading reagent 1,4-diazabicyclo[2.2.2]octane (Sigma) at 100 μg/ml. Cells were imaged with the BioRev software (Universal Imaging), using a three-dimensional deconvolution imaging system.

In Situ Hybridization—The 5′-divergent regions of the T0 and T1 transcripts were amplified by RT-PCR from E10.5 mouse embryos using the following primers (T0: 5′-gaacatcctgagcaacagag-3′ and 5′-gggtgcatgggagagcaggagc-3′). The resulting 152- and 200-bp amplicons were cloned into the pGEM®-T Easy vector (Promega). Digoxigenin-labeled antisense probes were generated to recognize specifically the divergent 5′-ends of the mOtx2 T0 and T1 transcripts. A digoxigenin probe covering the entire coding sequence of Otx2 was used as control. Whole-mount in situ hybridization assays were performed as previously described (27). RT-PCR Assays—Poly(A)⁺ RNA was prepared from the indicated tissues, using the Quick-prep-Micro mRNA purification kit (Amersham Biosciences, UK). Reverse transcription was performed by random priming with the First-Strand-cDNA Synthesis kit (Amersham Bio-
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RESULTS

Expression of Otx2 in Quail Neural Retina Cells Induces a Pigmented Phenotype—The ocular phenotypes of Otx- and Mitf-deficient mice indicate that both factors are required for RPE differentiation (6, 7). Overexpression of the melanocytic isoform of Mitf (Mitf-M) in neural retina cells is sufficient to induce a pigmented phenotype, suggesting that this protein has, on its own, the capability of activating the genetic network that triggers RPE differentiation (4). To assess whether OTX2 has a similar capability, we stably transfected dissociated cells derived from E6 quail NR with the Otx2 vector. In parallel dishes, we transfected a vector containing one of the retina isoforms of Mitf (Mitf-A) (31) as well as Mitf-M and the empty vector as positive and negative controls. After G418 selection, pigmented foci were evident not only in Mitf-M- and Mitf-A-transfected dishes but also in cells expressing OTX2 (Fig. 1, B–D). No pigmented foci were ever observed in cells transfected with the empty vector (Fig. 1A). This result demonstrates that Otx2 overexpression can drive NR cells to a pigmented phenotype, although the overall morphology of the cells was different from that observed after Mitf transfection (Fig. 1, compare staining patterns in B and F). Interestingly, immunolocalization of MITF was observed in cell nuclei of some, but not all, Otx2-induced pigmented foci (Fig. 1P). Conversely, OTX2 was immunodetected in some of the pigmented foci induced by Mitf transfection (Fig. 1E).

OTX2 but Not MITF Regulates the Promoter Region of a Newly Identified Alternative Otx2 Transcript—These data underscore the importance of both Mitf and Otx2 in the induction of a pigment phenotype, but do not clearly point to the hierarchical relationship between Otx2 and Mitf. In fact they are consistent both with the existence of a regulatory loop between the two factors and with the possibility that OTX2 initiates melanogenic gene expression in the absence of Mitf. To distinguish between these two possibilities, we first asked whether Mitf could regulate Otx2 promoter region and vice versa.

In vertebrates the proposed genomic structure of the Otx2 gene encompasses three different exons and does not include any identified alternative splicing forms. However, sequencing of an 8.2-kb fragment upstream of the mOtx2 coding region, alignment with its human counterpart, and comparison with the EST databases revealed the presence of two phylogenetically conserved sets of clones corresponding to two distinct Otx2 mRNAs (Fig. 2A). These mRNAs are splicing variants of the Otx2 gene, both encoding for the same protein but differing in their 5′ untranslated region and consequently in their promoter region. We termed these transcripts T0 and T1, the last corresponding to the previously proposed unique mOtx2 mRNA (17). A schematic representation of the relative position (~4688 from ATG) of the newly identified alternative exon (E0) is shown in Fig. 2A. RT-PCR amplification with specific primers confirmed the presence of both transcripts in E9.5 mouse embryo as well as in postnatal and adult eyes. However, the relative abundance of the two transcripts seemed different, with a stronger expression of the T0 form (Fig. 2B). The trend of these results was further supported by in situ hybridization comparative analysis using specific digoxigenin-labeled probes directed against either the full coding sequence of the protein or the specific 5′ untranslated region of either T1 or T0. At E10.5, both T0 and T1 were expressed, although with different intensities, according to the previously reported distribution, including the developing fore- and midbrain and the presumptive RPE region of the eye (Fig. 2C) (9, 17).

A 1.8-kb DNA sequence upstream of the putative T1 transcription initiation site of Otx2 has been previously analyzed, showing that HOXB proteins are capable of activating this region when fused to luciferase (pOTX2Luc-1219) (23). We compared the response properties of this pOTX2Luc-1219 construct with those of a 974-bp 5′-flanking fragment encompassing the putative initiation site of the T0 transcript (as deduced from the 5′-end of the EST clones). To obtain comparable results, the 974-bp T0 fragment was cloned into the same vector (pXP2-Luciferase) used for the pOTX2Luc-1219, and the final construct was named pOTX2Luc-974. When the two constructs were transfected in BHK-21, they both showed basal activities, as compared with the empty pXP2-luciferase vector (Fig. 3A), indicating that the two Otx2 upstream fragments had, on their own, promoter capabilities. Interestingly, OTX1 and OTX2 itself were able to induce respectively a 3- and 4-fold increase in
the basal luciferase activity driven by the T0 but not by the T1 promoter (Fig. 3B). In contrast MITF was unable to modify the basal promoter activity of the two constructs, even at relatively high concentrations (Fig. 3B). Similar experiments designed to analyze the effect of OTX2 or MITF itself on the Mitf-A promoter indicated that both classes of proteins have no significant capability of modifying Mitf-A basal promoter activity (Fig. 3C). Together these data suggest that cross-regulation between Otx2 and Mitf may either require the intercalation of yet unidentified additional factor(s) or may rely on regulatory elements located outside of the promoter regions considered here.

**Otx2 Transactivates the Promoters of Genes Encoding Melanosome Glycoproteins**—Because the data described above did not support the idea that OTX2 induces a pigment phenotype through the activation of Mitf, we asked whether OTX2 could directly regulate the expression of RPE genes. To verify this hypothesis we focused on three genes: QNR71, Tyr, and TRP-1. The first encodes a transmembrane glycoprotein specifically targeted to the melanosomes (26), whereas the other two are enzymes involved in melanin biosynthesis (32), whose promoter regions have been extensively characterized both in vitro and in vivo (11).

The BHK21 cell line did not express Otx2 and Mitf as judged by PCR and immunostaining analysis (not shown) and therefore was used for transactivation assays. Thus, luciferase-coupled QNR71 promoter constructs, as well as CAT-coupled QNR71 promoter constructs were co-transfected in BHK21 cells either alone or in combination with constructs containing the coding sequence of Otx2, Otx1, and Mitf, under
the control of CMV promoters. Two days after transfection, cell lysates were collected and the levels of luciferase or CAT activity determined. OTX2 strongly enhanced the basal activity of Tyr, TRP-1, and QNR71 promoters in a dose-dependent manner (Fig. 4A). A mutated version of OTX2, where the lysine in position 50 of the homeodomain is substituted by a glutamine (K50Q), preventing the binding to bicoid consensus sequences (15, 21), was unable to transactivate these promoters (Fig. 4A), supporting the specificity of these interactions. OTX1, albeit at lower level, has an activity similar to that of OTX2 on both the Tyr and TRP-1 promoters (Fig. 4A). However, when assayed in combination, the two proteins did not display synergic activity on either promoter (Fig. 4A), suggesting that both factors may bind to the same DNA sequences but with different efficiency.

As shown in previous reports (reviewed in Ref. 11), MITF has a similar action on melanogenic promoters (Fig. 4B). A mutated version of OTX2, where the lysine in position 50 of the homeodomain is substituted by a glutamine (K50Q), preventing the binding to bicoid consensus sequences (15, 21), was unable to transactivate these promoters (Fig. 4A), supporting the specificity of these interactions. OTX1, albeit at lower level, has an activity similar to that of OTX2 on both the Tyr and TRP-1 promoters (Fig. 4A). However, when assayed in combination, the two proteins did not display synergic activity on either promoter (Fig. 4A), suggesting that both factors may bind to the same DNA sequences but with different efficiency.

As shown in previous reports (reviewed in Ref. 11), MITF has a similar action on melanogenic promoters (Fig. 4B). Interestingly, when assayed together, OTX2 and MITF behaved in a cooperative fashion, which was particularly evident for the Tyr and the QNR71 promoter regions (Fig. 4B), supporting the idea that their coordinated activity may be necessary for the full activation of these genes.

**Otx2 Binds to the Melanogenic Promoters through Otx/Bicoid Sites**—OTX proteins bind to DNA through a bicoid consensus sequence (15, 17, 33). Sequence analysis of the promoters of TRP-1, Tyr, and QNR71 indicated that several bicoid elements (Figs. 5A, 6A, and 7A) were present in the proximity of the already reported M-boxes (CATGTG) (24, 34) as binding sites for MITF. Thus, we asked whether these sites could mediate OTX2 transactivation capabilities.

In the hTRP-1 promoter, there are three bicoid/Otx consensus binding sites: OtxA (position −595), OtxB (−142), and OtxC (−82), in addition to an Mitf site (−194, named Mitf1; Fig. 5A). Using EMSA assays, it has been previously shown that MITF forms a complex with sequences of the Tyr promoter that are competed by sequences of the TRP-1 promoter (24). Here we confirm that MITF-A directly binds to the TRP-1 promoter. A specific complex was observed when MITF-A was incubated with the Mitf1 oligonucleotide (Fig. 5B). The binding was competed by an excess of cold Mitf1 as well as by an excess of the Mitf1 oligonucleotide, which includes the hTYR promoter M-box, but not by an excess of mMitf1, which harbors a mutation in the M-box.

With similar experiments, we next asked whether OTX2 could also bind directly to the TRP-1 promoter. Initial competition assays were performed using a labeled oligonucleotide designed on the already characterized OTX2 consensus binding site present on the GnRH gene promoter (29). When this oligonucleotide was incubated with OTX2, a retarded complex was originated. This complex was specifically competed by oligonu-
cleotides containing OtxA and OtxB, but not by an oligonucleotide containing OtxC, neither by the mutated oligonucleotides mOtxA and mOtxB (harboring mutations in the bicoid site, Fig. 5C), indicating that OTX2 specifically interacts with the OtxA and OtxB sites of the TRP-1 promoter. This result was further confirmed by similar competition assays where the labeled probes were either oligonucleotide OtxA or OtxB (Fig. 5D).

A similar sequence analysis of the mTyr promoter showed the presence of several bicoid/Otx consensus binding sites (Fig. 6A), including OtxD (position −1943), OtxE (−1246), and OtxF (−413), in the proximity of one (Mitf2) of the three binding sites described for Mitf (35). We confirmed by EMSA assay that MITF-A specifically binds to the Mitf2 site of this promoter (Fig. 6B). In addition, a specific complex was formed when OTX2 was incubated with a labeled oligonucleotide containing an OtxD site (Fig. 6C). This complex was specifically competed by the oligonucleotide containing OtxF but not by the one with OtxE (Fig. 6C). These data indicate that OTX2 can interact with the OtxD and OtxF sites of the mTyr promoter.

We also searched the quail QNR71 promoter for bicoid/Otx binding sites. Four potential binding sites were found in the QNR71 promoter fragment (Fig. 7A). Three of them, named otx-2, otx-3, and otx-4 (positions −79, −62, and −26, respectively), were located in close proximity to an M-box (position −51). EMSA analysis indicated that OTX2 could interact with sequences of the QNR71 promoter through the Otx consensus binding sites, because the complex is competed by an excess of the cold wild type oligonucleotide QNR71. The specificity of the binding is supported by the partial or total lack of competition observed in the presence of an excess of the same oligonucleotide harboring mutations in two (QNR71 2/4m) or three (QNR71 2/3/4m) of the Otx consensus sequences (Fig. 7B, compare lanes 9–11 for a 100-fold excess).

To support further the functional significance of OTX2 binding to the QNR71 promoter, we asked whether mutations in the bicoid/Otx sites, which abolish OTX2 binding (Fig. 7A), cause functional consequences. Thus, we introduced mutations in the Otx sites of the QNR71 promoter reporter vector, and this construct was co-transfected in BHK-21 cells together with the vectors expressing Otx2, Mitf, or both. Analysis of the reporter activity showed that OTX2-transactivating properties were lost, without affecting MITF-transactivating capacity (Fig. 7C). In contrast, mutation in the Mitf binding sites of the QNR71 promoter (Mitf-BS m) affected the response of the reporter in the presence of MITF alone but did not prevent the synergistic activation observed in the presence of both Mitf and

Fig. 5. Binding of OTX2 to its potential target sequences on the human hTRP-1 promoter. A, partial sequence of the hTRP-1 promoter according to GenBank™ accession number D83059, numbered with +1 according to the mRNA in GenBank™ accession number X51420. The oligonucleotides used in the assays are underlined and noted as OtxA, Mitf1, OtxB, and OtxC. Consensus binding sequences for OTX2 and MITF are highlighted in boldface. B–D, EMSA assays. The indicated labeled oligonucleotides (Probe, marked with an asterisk) were incubated with the in vitro translated protein (Prot.) MITF-A, OTX2, or luciferase (luc) as control and separated in polyacrylamide gels. An excess of 500× molar cold oligonucleotide (Comp.) was included in the binding reaction when indicated. MitfT and mMitfT are, respectively, wild type and mutated Mitf-binding sequences of the human tyrosinase gene; Cons. and mCons. are, respectively, wild type and mutated OTX2-binding sequences of the rat GnRH gene; mOtxA and mOtxB correspond to OtxA and OtxB sequences (see A) where the Otx/bicoid site is mutated (see “Experimental Procedures”).
This result clearly indicates that OTX2 transactivates the QNR71 promoter by directly binding to its bicoid/Otx binding sites and suggests that Mitf may contribute to QNR71 promoter transactivation also by interacting with OTX2.

OTX2 Interacts Physically with MITF through the b-HLH-LZ Domain—The cooperative effect of MITF and OTX2 on the Tyr and QNR71 promoter activity (Figs. 4 and 7) and the relative proximity of Otx and Mitf binding sites in the melanogenic promoters (Figs. 5–7) opened the possibility that the two proteins may form a complex. To test this hypothesis, we performed pull-down experiments. Affinity columns containing a glutathione-Sepharose matrix coupled to GST-OTX2 or GST-MITF were used to pull down in vitro radiolabeled Mitf and OTX2, respectively. The percentages of the labeled proteins that bound the columns were determined after SDS-polyacrylamide gel electrophoresis by PhosphorImager measurement. Similar levels of labeled OTX2 (63%) and MITF (55%) retention were observed using GST-Mitf and GST-Otx2 columns, respectively, whereas no retention was evident when GST alone was used (data not shown) indicating a strong interaction between MITF and OTX2 (Fig. 8).

To identify the regions of MITF involved in the interaction with OTX2, different radiolabeled Mitf deletion constructs were analyzed for their ability to bind GST-OTX2. A high level of retention was observed only in the constructs containing the b-HLH-LZ domain (Fig. 8), indicating that this region of MITF is responsible of the interaction with OTX2.

MITF and OTX2 Co-localize in the Nucleus of Retinal Pigmented Epithelial Cells—Because MITF and OTX2 physically interact in vitro, we asked whether they could also do so in retinal pigmented epithelial cells. Thus, we analyzed the nuclear localization of the two proteins in quail RPE cells using three-dimensional microscopy. We transfected quail RPE cells with vectors expressing either Mitf-A (Fig. 9, A–C) or Mitf-M (Fig. 9, D–F) tagged with the DsRed together with a vector expressing Otx2 tagged with the green fluorescent protein (EGFP; Fig. 9, A–F). After transfection, the fused proteins were observed in the RPE cell nuclei, which were stained for heterochromatin (4',6-diamidino-2-phenylindole staining, data not shown).
shown). Fig. 9 (C and F) shows the overlap of the two fluorescent proteins by maximal pixel intensity projections of a representative nucleus. In both cases, a significant amount of the isoforms of Mitf-DsRed and EGFP-Myc (a protein that has been shown not to interact with Mitf in vitro; Fig. 9, G and H) co-localize in common areas of the nucleus in vivo. As a control, we determined also the subnuclear localization of Mitf-DsRed and EGFP-Myc (a protein that has been shown not to interact with Mitf in vitro; Fig. 9, G and H). NO substantial co-localization of these two proteins was observed in the cell nuclei (Fig. 9).

**DISCUSSION**

Genetic and molecular analyses in different species have shown that Mitf has a conserved key function in the development of melanin-producing pigment cells, including the RPE (11). Recent genetic evidence suggested that Otx genes are also crucial for the development of the RPE (7). This study provides molecular evidence that Otx2 regulates the differentiation of the RPE. First, Otx2 is able to induce a pigmented phenotype in transfected retina cells. Second, Otx2 binds and activates the promoter regions of the QNR71, Tyr, and TRP-1 genes, synergizing with Mitf. Third, Mitf and Otx2 are capable of biochemical interaction through the bHLH domain of Mitf and the two factors co-localize within the nuclei of RPE cells.

The subdivision of the optic vesicle neuroepithelium into presumptive NR and RPE is critical for vertebrate eye development. How RPE and NR progenitor cells acquire their identity is still unclear. However, the regulation of a number of transcription factors, such as Mitf and Otx2, which are initially co-expressed throughout the optic vesicle, seems to be crucial to this process. These genes are initially expressed in the entire optic vesicle, but their expression becomes restricted to the presumptive RPE during optic cup formation. When either Mitf or Otx are functionally inactivated in mice, the identity of the RPE is lost and replaced by a laminated second NR that also loses the reciprocal expression of the other gene (6, 7, 20), suggesting that both genes are required for the normal development of the RPE. Here, we show that Otx2 can cooperate with Mitf to activate the molecular network underlying pigmentation and differentiation of the RPE. This would explain why these processes are impaired, in both Mitf and Otx mutant mice, even though one gene is still functional, indicating that interaction between the two proteins may play an important role in the establishment of RPE identity.

Gain-of-function studies in avian NR cultures had previously shown that Mitf-M is sufficient to induce a pigmented phenotype in the cultures (4). Now we show that both Mitf-A, an RPE-enriched isoform of Mitf, and Otx2 have the same capability, although the morphology of the Otx2-induced pigmented foci were different from that induced by Mitf, possibly because the two genes may differentially modify the expression of ad-
the QNR71 promoter were sufficient to abolish the transactivation capability of OTX2 on melanogenic genes.

Although the list of the identified, direct target genes of OTX2 is still short, most of the included genes are effector genes falling in the category of cytoskeletal, extracellular matrix, or secreted proteins (19). This has led to the proposition that Otx2 coordinates the activity of unrelated genes with overlapping functions without the need of additional intermediate regulatory molecules (19). Our results support this view. QNR71 is unrelated to the Tyr and TRP-1 genes, but the three molecules contribute to the formation of the melanosome. OTX2 acts directly on the promoters of these genes and does not require MITF for this activity, although we have observed a positive functional cross-talk between the two molecules. Indeed, the two proteins interacted physically, through the bHLH-LZ domain of MITF, and had a synergistic activity in the transactivation of the Tyr and QNR71 promoters. A similar, bHLH-LZ-mediated interaction between MITF and PAX6, another homeodomain containing transcription factor, resulted in the inhibition of both DNA binding and PAX6-mediated transcriptional activation (22), suggesting that the cooperative interaction between MITF and OTX2 is not common to all homeodomain containing proteins.

Other examples of interaction between transcription factors of the bHLH and homeodomain classes have been reported (22, 38–40). In the particular case of Pitx (a bicoïd-type homeodomain protein) and Pan1 (40), protein interaction resulted in a synergistic transactivation of the pro-opiomelanocortin target promoter. Removal of the Pitx1 binding site from this promoter indicated that binding of Pitx1 to the DNA is not required for a synergistic activation with Pan1. This is different from the results we have obtained in a transient transfection assay using the QNR71 promoter, which suggest that binding of the homeodomain protein (OTX2) to the DNA is sufficient to mediate the cooperative activity of both factors. Indeed, mutation of the OTX2 binding sites abolished both its response to OTX2 activation and the cooperative effect with Mitf, without affecting the response to MITF alone. In contrast, mutations in the QNR71 promoter abrogating Mitf transcriptional activity (26) did not affect the synergistic activation of the QNR71 promoter observed in the presence of both MITF and OTX2, indicating that MITF contributes to QNR71 promoter transactivation also by interacting with the OTX2 protein.

Formation of a protein complex on the DNA has been demonstrated for the case of the homeodomain protein PDX-1 and bHLH E47/Pan1 factors on the insulin promoter (39). Super-shift experiments to demonstrate an OTX2-MITF complex on the Tyr, TRP-1, and QNR71 promoter regions were unsuccessful in this case (not shown). Protein complex instability during electrophoresis is a possible cause of the absence of a new, slowly migrating band in our experiments. In vivo, other proteins may contribute to the complex formation. These additional proteins could alter DNA binding of either OTX2 or MITF, stabilize their interaction, or modify the structure of the promoter region (41). Recently, it has been shown that Mitf-F interacts with the lymphoid enhancing factor 1, a mediator of Wnt signaling, and their functional cooperation results in the synergistic transactivation of the TRP-2 gene promoter (42). Although lymphoid enhancing factor 1 is not expressed in the RPE, TCP-1, another member of the same family of transcription factors has been detected in this tissue (42) and is a possible candidate for protein complex formation with MITF and OTX2.

Here we have described that mammalian Otx2 gene has at least two evolutionary conserved alternative initiation start sites, which we named T0 and T1, the last corresponding to the
previously proposed unique mOtx2 mRNA (17). The newly identified T0 isofrom is activated by OTX2 itself and to a lower extent by OTX1, in line with the idea that Otx2 expression depends on an autoregulatory loop (43). However, despite the analysis of Otx and Mitf null mice and the transfection experiments reported in Fig. 1 (E and F), which suggested a possible interdependence between Otx2 and Mitf, we were unable to detect a mutual regulation of the expression between the two factors. Our results, however, do not rule out that this cross-regulation may exist, involving other promoters or enhancers. Although Mitf-A, whose promoter region was used in the transient transfection experiments, is one of the most abundant isoforms in the RPE, we cannot exclude that other Mitf isoforms (i.e. Mitf-D), which are under the control of distinct promoters (10), may be expressed in the eye in response to Otx2. Conversely, elements regulated by Mitf may exist outside of the two regulatory regions we have considered for Otx2, because the expression of this gene may involve start sites other than the two reported here (44). Alternatively, the transcriptional control between Otx2 and Mitf may be indirect and require the intercalation of yet unidentified additional factors.

The analysis of the network underlying the determination of the pigmented cells in Tunicates, a parallel branch in the phylum Chordata, may provide useful information to deduce the origins of the different prototypes of pigmented cells present in vertebrates. In the ascidian larvae there are two types of pigment cells, located in the cerebral vesicles: a single-celled otolith, involved in geotactic responses, and a photo-sensing ocellus, which functions as a simple eye. Both cells express tyrosinase family members with structural characteristics highly similar to those of their vertebrate counterparts (45). Surprisingly, the relevant regulatory regions of the ascidian tyrosinase gene (HrTyr) do not contain the characteristic M-box motifs specific for MITF binding present in their vertebrate homologues. Rather, HrTyr appears under the control of Hr-Pax3/7, an ascidian homologue of Pax3 (45). Furthermore, a very recent report (46) has demonstrated that the single ascidian Otx gene, Hroth, is capable of transactivating the ascidian TRP (HrTRP) gene promoter binding to Otx consensus sites and that interference with Hroth translation by morpholino oligonucleotides impairs the development of the sensory pigmented cells. This striking result confirms the data presented here and indicates that Otx genes have an ancestral regulatory function in the determination of the pigmented lineage among chordates. In a simplistic view, it can be speculated that originally the melanogenic cascade might have been under the direct control of members of the Pax and Otx families, as proposed for the ancestral opsin (47). Ancestral eyes may derive from photoreceptor cells that used intracellular pigment redistribution for their light-dependent functions (48). The addition of Mitf as a further regulatory factor may have allowed the diversification of the control of the melanogenic cascade and the definition of RPE.

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Note Added in Proof—A recent paper by the group of Shigeki Shibahara shows that OTX2 binds and activates also the DOPAchrome tautomerase gene (TRP2) promoter, adding further evidence to the idea that Otx2 is required for the differentiation of the RPE (Takeda, K., Yokoyama, S., Yasumoto, K., Suito, H., Udono, T., Takahashi, K., and Shibahara, S. (2003) Biochem. Biophys. Res. Commun. 300, 908–914).

REFERENCES

1. Marks, M. S., and Seabra, M. C. (2001) Nat. Rev. Mol. Cell. Biol. 2, 738–748
2. Raymond, S. M., and Jackson, I. J. (1995) Curr. Biol. 5, 1296–1295
3. Boulton, M., and Dayhaw-Barker, P. (2001) Eye 15, 384–389
4. Planque, N., Turque, N., Opdecamp, K., Bailly, M., Martin, P., and Saule, S. (1999) Cell Growth Differ. 10, 525–536
5. Pittack, C., Gruenwald, G. B., and Roh, T. A. (1997) Development 124, 805–816
6. Nguyen, M-T. T., and Arnheiter, H. (2000) Development 127, 3581–3591
7. Martinez-Morales, J. R., Signore, M., Acampora, D., Simeone, A., and
8. A. Simeone, personal communications.
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Bovolenta, P. (2001) Development 128, 2019–2030
8. Hodgkinson, C. A., Moore, K. J., Nakayama, A., Steingrimsson, E., Copeland, N. G., Jenkins, N. A., and Arneiter, H. (1993) Cell 74, 395–404
9. Bovolenta, P., Mallamaci, A., Briata, P., Corte, G., and Boncinelli, E. (1997) J. Neuroscience 17, 4243–4252
10. Udono, T., Yasumoto, K., Takeda, K., Amae, S., Watanabe, K., Saito, H., Fuse, N., Tachibana, M., Takahashi, K., Tamai, M., and Shibahara, S. (2000) Biochim. Biophys. Acta 1491, 205–219
11. Goding, C. R. (2000) Genes Dev. 14, 1712–1728
12. Tassabehji, M., Newton, V. E., and Read, A. P. (1994) Nat. Genet. 6, 253–255
13. Nakayama, A., Nguyen, M. T., Chen, C. C., Opdecamp, K., Hodgkinson, C. A., and Arneiter, H. (1998) Mech. Dev. 70, 155–166
14. Simeone, A., Puelles, E., and Acampora, D. (2002) Curr. Opin. Genet. Dev. 12, 409–415
15. Hanes, S. D., and Brent, R. (1989) Cell 57, 1275–1283
16. Driever, W., and Nusslein-Volhard, C. (1989) Nature 337, 138–143
17. Simeone, A., Acampora, D., Mallamaci, A., Sternmarko, A., D’Apice, M. R., Nigro, V., and Boncinelli, E. (1990) EMBO J. 9, 2735–2747
18. Briata, P., Ilengo, C., Bobola, N., and Corte, G. (1999) FEBS Lett. 445, 160–164
19. Boncinelli, E., and Morgan, R. (2001) Trends Gen. 17, 633–636
20. Bumsted, K. M., and Barnstable, C. J. (2000) Invest. Ophthal. Vis. Sci. 41, 903–908
21. Panne, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G., and Boncinelli, E. (1995) Development 121, 707–720
22. Planque, N., Leconte, L., Coquelle, F. M., Martin, P., and Saule, S. (2001) J. Biol. Chem. 276, 28330–28337
23. Guazzi, S., Pintonello, M. L., Vigano, A., and Boncinelli, E. (1998) J. Biol. Chem. 273, 11092–11099
24. Yasumoto, K., Nakayama, A., Takahashi, K., Tomita, Y., and Shibahara, S. (1995) J. Biol. Chem. 272, 563–569
25. Bertolotto, C., Bille, K., Ortonne, J. P., and Ballotti, R. (1996) J. Cell Biol. 134, 747–755
26. Turque, N., Denhez, F., Martin, P., Planque, N., Bailly, M., Begue, A., Stelahin, D., and Saule, S. (1996) EMBO J. 15, 3338–3350
27. Plaza, S., Dozier, C., and Saule, S. (1993) Cell Growth Diff. 4, 1041–1050
28. Mahalingam, H., Watanabe, A., Tachibana, M., and Niles, R. M. (1997) Exp. Cell Res. 237, 85–92
29. Kelley, C. G., Lavergne, G., Clark, M. E., Boncinelli, E., and Mellon, P. L. (2000) Mol. Endocrinol. 14, 1246–1256
30. Planque, N., Leconte, L., Coquelle, F. M., Benkhemef, S., Martin, P., Felder-Schmittbuhl, M. P., and Saule, S. (2001) J. Biol. Chem. 276, 35751–35760
31. Amae, S., Fuse, N., Yasumoto, K., Saito, S., Yajima, I., Yamamoto, H., Udono, T., Durui, Y. K., Tamai, M., Takahashi, K., and Shibahara S. (1998) Biochim. Biophys. Res. Commun. 247, 710–715
32. del Marmol, V., and Beermann, F. (1996) FEBS Lett. 381, 165–168
33. Gehring, W. J., Affolter, M., and Burglin, T. (1994) Annu. Rev. Biochem. 63, 487–526
34. Yasumoto, K., Mahalingam, H., Suzuki, H., Yoshisawa, M., Yokoyama, K., and Shibahara S. (1995) J. Biochem. 118, 674–881
35. Aksan, I., and Goding, C. R. (1998) Mol. Cell. Biol. 18, 6930–6938
36. Bobola, N., Briata, P., Ilengo, C., Rosatto, N., Craft, C., Corte, G., and Ravazzolo, R. (1999) Mech. Dev. 82, 165–169
37. Morgan, R., Hooiveld, M. H., In der Reiden, P., and Durston, A. J. (1999) Mech. Dev. 85, 97–102
38. Kosepfiller, P. S., Bergstrom, D. A., Uetsuki, T., Dae-Korytoko, I., Sun, Y. H., Wright, W. E., Tappolt, S. J., and Kamps, M. P. (1999) Nucleic Acids Res. 27, 3752–3761
39. Ohneda, K., Mirmira, R. G., Wang, J., Johnson, J. D., and German, M. S. (2000) Mol. Cell. Biol. 20, 900–911
40. Poulin, G., Lebel, M., Chamberland, M., Paradis, F. W., and Drouin, J. (2000) Mol. Cell. Biol. 20, 4826–4837
41. Kim, T. K., and Maniatis, T. (1997) Mol. Cell 1, 119–129
42. Yasumoto, K., Takeda, K., Saito, H., Watanabe, K., Takahashi, K., and Shibahara, S. (2002) EMBO J. 21, 2703–2714
43. Gammill, L. S., and Sive, H. (1997) Development 124, 471–481
44. Courtois, V., Chatelain, G., Han, Z.-H., Le Nove, R., Brun, G., and Lamonerie, T. (2003) J. Neurochem. 84, 840–853
45. Sato, S., and Yamamoto, H. (2001) Pigmented Cell Res. 14, 428–436
46. Wada, S., Toyoda, R., Yamamoto, H., and Saiga, H. (2002) Dev. Dyn. 225, 46–53
47. Sheng, G., Thouvenot, E., Schmucker, D., Wilson, D. S., and Desplan, C. (1997) Genes Dev. 11, 1122–1131
48. Arnheiter, H. (1998) Nature 391, 632–633
OTX2 Activates the Molecular Network Underlying Retina Pigment Epithelium Differentiation
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