Regulation of the Microphthalmia-associated Transcription Factor Gene by the Waardenburg Syndrome Type 4 Gene, SOX10*

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The absence of melanocytes from the cochlea and epidermis is responsible of deafness and hypopigmentation, two symptoms shared by the four Waardenburg syndrome (WS) subtypes. Microphthalmia-associated transcription factor (MITF) controls melanocyte survival and differentiation. Mutations, which impair MITF function or expression, result in an abnormal melanocyte development leading to the WS2. WS1 and WS3 are caused by mutation in the gene encoding the transcription factor Pax3, which regulates MITF expression. Recently, mutations in SOX10, a gene encoding a SRY-related transcription factor, have been reported in patients with WS4. However, the molecular basis of the defective melanocyte development in these patients remained to be elucidated. In the present report, we demonstrate that Sox10 is a strong activator of the MITF promoter, and we identify a Sox10 binding site between −264 and −266 of the MITF promoter. Finally, we show that three SOX10 mutations found in WS4 abolish the transcriptional activity of the resulting Sox10 proteins toward the MITF promoter. Taken together, our observations bring new and meaningful information concerning the molecular process that leads to a defective melanocyte development in WS4 patients with SOX10 mutations.

Microphthalmia-associated transcription factor (MITF)1 is a basic helix-loop-helix, leucine zipper transcription factor that plays a pivotal role in survival and differentiation of melanocytes, the cells that produce melanin pigments. In mice, mutations in the microphthalmia gene cause pigmentation disorders because of the absence of melanocytes, supporting the involvement of MITF in melanocyte survival (1, 2). MITF has been also demonstrated to up-regulate the expression of the genes involved in melanin synthesis, such as tyrosinase, TRP1, and TRP2 (3). Further MITF is thought to be a master gene in melanocyte differentiation, because its forced expression in fibroblasts leads to the expression of melanocyte-specific enzymes required for melanin synthesis (4). In humans, mutations of MITF are responsible for Waardenburg syndrome (WS) type 2a, characterized by pigmentation abnormalities and sensorineural deafness due to the absence of melanocytes from the stria vascularis of the inner ear (5, 6).

In humans, at least four MITF isoforms with different amino termini have been described. The existence of four alternative promoters allows a specific expression pattern of these isoforms (7). A melanocyte-specific promoter controls melanocyte-restricted expression of the MITF-M isoform. Analysis of this promoter has identified several potential cis-acting regulatory elements. Among these regulatory sequences, a canonical cAMP-responsive element is involved in the regulation of MITF expression by cAMP-elevating agents such as alpha melanocyte-stimulating hormone (aMSH) and forskolin (8). Wnt proteins have been shown to play a key role in the differentiation of neural crest precursors toward melanocytes (9). Wnt proteins mediate their biological effects through the binding to frizzled, a seven-transmembrane receptor, leading to the subsequent nuclear accumulation of β-catenin. β-catenin in turn cooperates with lymphoid enhancer factor (LEF1)/T cell factor (10) transcription factor to regulate the expression of the target genes (11). Very recently, Wnt 3a has been shown to stimulate MITF expression, and a LEF1 site has been identified in the MITF promoter (12). This site appears to play a pivotal role in pigment cell development in vertebrates, because a LEF1 site is present and functional in the promoter of nacre, a zebra fish MITF homologue (13).

Mutations in PAX3, which encodes a paired homeodomain transcription factor, are responsible for Waardenburg syndrome 1 and 3 (14, 15). PAX3 was shown to bind and transactivate the MITF promoter, thereby demonstrating the role of PAX3 in the regulation of MITF expression. This observation supports an epistatic relationship between MITF and PAX3 and can explain the pigmentary disorders observed in WS1 and 3 (16). Taken together, these observations show that MITF is subjected to a complex array of transcriptional regulation that allows an accurate developmental expression of MITF.

WS4 is caused by mutations in either the endothelin B receptor, endothelin 3, or SOX10, a sex-determining gene (SRY)-related transcription factor (17, 18). How mutations in these genes lead to deafness and pigmentary abnormalities, shared by all the WS subtypes, was not elucidated. It was tempting to propose that the WS4 genes, mentioned above, are directly or indirectly involved in the regulation of MITF expression that is crucial for melanocyte development. In the present report, we have investigated the possible involvement of Sox10 in the regulation of the MITF gene expression. We show that Sox10 binds and transactivates the MITF promoter, whereas Sox10 mutants found in WS4 patients failed to stimulate the MITF promoter. Thus, we demonstrate an epistatic relationship between SOX10 and MITF, thereby giving a molecular basis for the audio-pigmentary defect in patients with WS4.
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**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium, bovine serum albumin, 4(2-aminoethyl)-benzene-sulfonyl fluoride (AEBSF), aprotinin, and leupeptin were purchased from Sigma. γ-[32P]ATP (3000 Ci/mmol) was from Amersham Pharmacia Biotech, and LipofectAMINE reagent was from Life Technologies, Inc. Escherichia coli DNA polymerase, Klenow fragment, T4 polynucleotide kinase, and T4 DNA ligase were from New England Biolabs, Inc. (Beverly, MA). Synthetic oligonucleotides was purchased from Life Technologies, Inc.

**Cell Culture**—B16/F10 murine melanoma cells, A293 cells, and NIH3T3 fibroblast cells were grown at 37 °C under 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 7% fetal calf serum and penicillin/streptomycin (100 units/ml/50 μg/ml). Human melanocytes were obtained from foreskins of Caucasian children by overnight digestion in phosphate-buffered saline containing 0.5% dispase grade II at 4 °C, followed by a 1-h digestion with trypsin/EDTA solution (0.05%/0.02% in phosphate-buffered saline) at 37 °C. Cells were grown in MCDB 153 medium supplemented with 2% fetal calf serum, 0.4 mg/ml hydrocortisone, 5 μg/ml insulin, 16 μg/ml dexamethasone, 13-aceate, 1 ng/ml b-fibroblast growth factor, and penicillin/streptomycin (100 units/ml/50 μg/ml) in a humidified atmosphere containing 5% CO₂ at 37 °C.

**Reporter Plasmids and Expression Vectors**—pMITF, pMITF-1512 (−1812/+136), pMITF-1580 (−1580/+136), pMITF-880 (−880/+136), pMITF-387 (−387/+136), and pMITF-177 (−177/+136) were previously described (8). pMITF-277 (−277/+136), pMITF-251 (−251/+136), pMITF-239 (−239/+136), and pMITF-217 (−217/+136) were constructed using the Transformersite-directed mutagenesis kit (CLONTECH Laboratories Inc.). pMITF-SXm and pMITF-SXm/880 contain deletions of 3 base pairs between −264 and −266 in the context of pMITF or pMITF-880, respectively. pMITF-Px3 contains mutations as described previously (16). These mutants were constructed using the Transformer™ site-directed mutagenesis kit. We thank M. Wegner for providing us with plasmid-encoding Pax3, Sox4, Sox10, Sox10–059 (19). Sox-MIC was constructed using the Transformer™ site-directed mutagenesis kit. All constructs were purified using the silica column from Qiagen.

**Western Blot Assays**—Cells were lysed in RIPA buffer, pH 7.5, containing 10 mM Tris-HCl, 1% sodium deoxycholate, 1% Nonidet-P40, 150 mM NaCl, 0.1% SDS, 5 μg/ml leupeptin, 1 mM AEBSF, 100 μM aprotinin. Proteins (30 μg) were separated on 10% SDS polyacrylamide gels and transferred to a nitrocellulose membrane. Sox10 protein was detected with a rat polyclonal antibody (20) at a 1/1000 dilution in saturation buffer and with a secondary peroxidase-conjugated anti-rabbit antibody at a 1/3000 dilution. Proteins were visualized using the ECL system from Amersham Pharmacia Biotech.

**Transfections and Luciferase Assays**—B16 melanoma cells and NIH3T3 fibroblast cells were seeded in 24-well dishes. 24 h later cells were transfected with 0.3 μg of the reporter plasmid, 0.02 and 0.04 μg of the expression vector, and 0.05 μg of pCMVβgal to control the transfection efficiency. Transfection of B16 cells and NIH3T3 fibroblast cells were performed using LipofectAMINE in 200 μl of OptiMEM™ for 6 and 3 h, respectively. Then the transfection medium was changed to fresh medium, and 40 h later cells were washed with a saline phosphate buffer and lysed with 25 μl Tris phosphate buffer (pH 7.8) containing 1% Triton X-100, 2 μl EDTA, and 2 μl dithiothreitol. Soluble extracts were harvested and assayed for luciferase and β-galactosidase activities. All transfections were repeated at least five times using different plasmid preparations and gave similar results.

**Gel Mobility Shift Assays**—Gel shift assays were performed with nuclear extracts from A293 cells transfected with a plasmid encoding Sox10. Double-stranded synthetic nucleotide, −250 5′-GCTGAAA-GAGAATACCTACTGCTATTATAACTACTCGG-3′ 270 was γ-[32P] end-labeled with the T4 polynucleotide kinase. Nuclear extracts (5 μg) were preincubated for 15 min in a binding buffer containing 10 mM Heps-NaOH, pH 8, 50 mM NaCl, 2 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, 25 ng poly(dI-dC) as unsppecific competitor, 2 μg of bovine serum albumin, 5 mM MgCl₂. Then, 50,000–100,000 cpm of [32P] probe were added to the reaction mixture for 30 min at room temperature. DNA-protein complexes were resolved by electrophoresis on a 5% polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) in TAE 0.25 mM Tris, 9 mM sodium acetate/acetic acid, 275 mM EDTA, pH 8) for 45 min at 100 V. When indicated, a 50-fold excess of cold competitor oligonucleotides was added during preincubation.

**RESULTS AND DISCUSSION**

**Sox10 Is Expressed in Melanocyte Cells and Stimulates the Transcriptional Activity of pMITF**—To investigate the possible involvement of Sox10 in the regulation of MITF expression, we first evaluated the expression of Sox10 in melanocyte cells. Western blot analysis using a polyclonal antibody against rat Sox10 showed that Sox10 was expressed in B16 melanoma cells and in human melanocytes but not in NIH3T3 fibroblasts (Fig. 1A). Then we analyzed the effect of Sox10 on the MITF promoter activity. A 2.2-kilobase pair fragment of the melanocyte-specific MITF promoter was cloned upstream of the luciferase gene (8). This construct (pMITF) was transfected alone or together with expression vectors encoding either Sox10, Sox4, or the catalytic subunit of PKA as a positive control. In B16 melanoma cells, Sox10 and PKA induced more than a 20-fold increase in the activity of pMITF, whereas co-transfection with Sox4 failed to increase the luciferase activity (Fig. 1B). In NIH3T3 fibroblasts, Sox10 increased significantly pMITF expression (8-fold), whereas PKA and Sox4 did not affect the MITF promoter activity (Fig. 1C). In these experiments, Sox4 stimulated the activity of a Sox reporter construct (20) demonstrating that Sox4 was indeed expressed (not shown).

Hence, Sox10, which is expressed in melanocytes and melanoma cells, transactivates the MITF promoter as efficiently as PKA or LIF (12), which are, to date, the most potent activators of MITF expression. Sox4, another SRY-related transcription factor that has been reported to have strong transcriptional activity (20), is unable to activate the MITF promoter. Interestingly, Sox10 transactivates pMITF in both fibroblasts and melanoma cells, whereas the effect of the CAMP pathway activation is restricted to melanoma cells as previously reported (8).

**Localization of a Sox10-responsive Element (−260/-266) in MITF Promoter**—In an attempt to localize the cis-acting elements accountable for the Sox10 response of the MITF promoter, we constructed a series of deletions in the 5′-region of pMITF (schematized in Fig. 2A) and evaluated their responsiveness to Sox10. In cells transfected with pMITF, pMITF-1812, or pMITF-1580, Sox10 induced a 20-fold increase in the luciferase activity. pMITF-880 and pMITF-387 constructs showed a slight but significant decrease in their responsiveness to Sox10 (14-fold). Then, using pMITF-177, we observed a more...
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FIG. 2. Localization of a Sox10-responsive element (−264/−266) in MITF promoter. The MITF promoter, the different deletions, and mutations are schematized in A. B and C, B16 cells were transfected with the indicated pMITF constructs (0.3 µg), pCMV-β-gal (0.05 µg), and an expression vector encoding Sox10 (0.04 µg). The luciferase activity was normalized by the β-galactosidase activity, and the results were expressed as -fold stimulation of the basal luciferase activity from cells transfected with an empty expression vector. Data are means ± S.E. of five experiments performed in triplicate. D, a probe containing the Sox10 binding site (−252/−283) was 32P-labeled and used in gel shift assays with nuclear extract from A293 cells transfected with an empty plasmid (mock) or with a plasmid encoding Sox10. For competition experiments, unlabeled homologous (SX) or mutated oligonucleotides (SXm) were added in 50-fold excess. 2 µl of non-immune serum (nis) or anti-sox10 (α-Sox) were added when indicated.

FIG. 3. Sox10 and PAX3 do not synergize to transactivate the MITF promoter. B16 melanoma cells (A) or NIH3T3 cells (B) were co-transfected with pMITF or pMITF mutated at the Pax3 site described by Watanabe et al. (16), pMITF-Px3 (0.3 µg), pCMV-β-gal (0.05 µg), and expression vectors encoding Sox10, Pax3, or both Sox10 and Pax3. The luciferase activity was normalized by the β-galactosidase activity, and the results were expressed as -fold stimulation of the basal luciferase activity from cells transfected with an empty expression vector. Data are means ± S.E. of five experiments performed in triplicate.

This first series of experiments pointed out to the existence of at least two regulatory regions that seem to confer to the MITF promoter its responsiveness to Sox10. One of these elements would be located between −387 and −177, and the other one should lay between −1580 and −880.

Then, a new series of deletions between −387 and −177 were performed to identify the key Sox10-responsive element. The following four additional deletions were constructed: pMITF-277, pMITF-251, pMITF-239, and pMITF-217. pMITF-277 showed the same Sox10 responsiveness (14-fold) as pMITF-387, whereas the responsiveness of pMITF-251, pMITF-239, and pMITF-217 was dramatically reduced (Fig. 2C). These experiments identify a Sox10-responsive element between −277 and −251. Within this region, we found a consensus binding sequence for Sox10 (−266/−260). A mutation of this site in pMITF-SXm moderately impaired the Sox10 responsiveness (13-fold). However, when this mutation was combined with the deletion of the region upstream −880 pMITF-SXm/880, we observed a complete loss of the Sox10 response (Fig. 2C).

Using a probe encompassing the Sox10 consensus binding sequence, we performed a gel shift assay (Fig. 2D). When using nuclear extracts from mock-transfected A293, we observed no complex. On the other hand, nuclear extracts from A293 cells expressing Sox10 protein formed a complex that was displaced by an excess of unlabeled probe, whereas a probe carrying the same mutation as pMITF-SXm failed to do so. Further, this complex was specifically supershifted by an anti-Sox10 antibody. This result demonstrates that the Sox10 binds to the sequence −264/−266 of the MITF promoter.

This Sox10 binding site cooperates with another cis-acting element between −1580 and −880. Mutation of Sox10 consensus binding sequences in this region, in the context of either the intact promoter or in pMITF-SX, did not affect the stimulation evoked by Sox10 (not shown). Thus, either the Sox10-responsive element between −1580 and −880 is a non-canonical Sox binding motif, or Sox10 acts indirectly on the MITF promoter through the up-regulation of another transcription factor, which needs to be identified in future studies.

FIG. 4. Mutations found in WS4 patients invalidate the transcriptional activity of Sox10. A, schematic representation of the Sox10 mutations used in the following experiments. B, B16 cells were transfected with pMITF (0.3 µg), pCMV-β-gal (0.05 µg), and expression vectors encoding Sox10 wild type (WT), Sox10−95 (95), Sox10−59 (659), and Sox10-MIC (MIC) (0.04 µg). The luciferase activity was normalized by the β-galactosidase activity, and the results were expressed as -fold stimulation of the basal luciferase activity from cells transfected with an empty expression vector. Data are means ± S.E. of five experiments performed in triplicate. C, a Western blot of the cell lysates was performed using the anti-Sox10 antibody to control the expression of the transfected proteins. An autoradiogram of a representative experiment is shown.
Sox10 and PAX3 Do Not Synergize to Transactivate the MITF Promoter—Noteworthy, the Sox10-responsive element of the MITF promoter, identified in this report, is located near a Pax3 binding site involved in the regulation of MITF expression (16). Because Sox10 has been reported to synergize the effect of Pax3 on a reporter gene containing adjacent Sox10 and PAX3 binding sites (20), it was tempting to propose that Sox10 would cooperate with the downstream PAX3 site to stimulate MITF transcription. To verify this hypothesis, we have studied, in both B16 melanoma and NIH3T3 cells, the combined effects of Sox10 and PAX3 on pMITF. Surprisingly, in B16 melanoma cells, Pax3 did not stimulate the activity of pMITF, and the effect of Sox10 plus Pax3 was similar to the effect of Sox10 alone. Further, the mutation of the Pax3 site in pMITF (pMITF-Px3) did not impair the Sox10 responsiveness of the promoter (Fig. 3A). In total agreement with the previous report of Watanabe et al. (16), we observed in NIH3T3 that Pax3 induces a 5-fold increase in MITF promoter activity. Combined action of Pax3 and Sox10 gave a 13-fold increase of the luciferase activity (Fig. 3B). In these experiments, Pax3 expression was verified by using a Pax3 reporter gene (20) (not shown). Thus, we did not observe any synergy between Pax3 and Sox10 but observed solely an additive effect.

Mutations Found in WS4 Patients Invalidate the Transcriptional Activity of Sox10—Finally, we wished to evaluate the functional consequences of Sox10 mutations found in WS4 patients (18). Three mutations schematized in Fig. 4A were studied. The WS095 mutant has an insertion of 6 nucleotides between 482 and 483 resulting in the addition of a leucine and an arginine in the high mobility group domain. In the mutant WS095, a deletion of two nucleotides at 1076 leads to frameshift and a premature stop codon. The resulting protein is WS059, a deletion of two nucleotides in 1076 leads to frame-shift and a premature stop codon. The resulting protein is Sox-MIC induced a very reproducible inhibition (55%) of the basal MITF promoter activity, suggesting that this mutant had invalidated the transcriptional activity of Sox10. Notewor-thy, Sox-MIC induced a 5-fold increase in MITF promoter activity. Combined action of Pax3 and Sox10 gave a 13-fold increase of the luciferase activity (Fig. 3B). In these experiments, Pax3 expression was verified by using a Pax3 reporter gene (20) (not shown). Thus, we did not observe any synergy between Pax3 and Sox10 but observed solely an additive effect.

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