Direct Regulation of the Microphthalmia Promoter by Sox10 Links Waardenburg-Shah Syndrome (WS4)-associated Hypopigmentation and Deafness to WS2*

Melanie Lee, Jane Goodall, Carole Verastegui‡, Robert Ballotti‡, and Colin R. Goding§

From the Eukaryotic Transcription Laboratory, Marie Curie Research Institute, The Chart, Oxted, Surrey RH8 OTL, United Kingdom and §INSERM U385, Biologie et Physiopathologie de la Peau, Faculté de Médecine, Avenue de Valombreuse, Nice, 06107 Cedex, France

The transcription factor Sox10 is genetically linked with Waardenburg syndrome 4 (WS4) in humans and the Dominant megacolon (Dom) mouse model for this disease. The pigmentary defects observed in the Dom mouse and WS4 are reminiscent of those associated with mutations in the microphthalmia (Mitf) gene, which encodes a transcription factor essential for the development of the melanocyte lineage. We demonstrate here that wild type Sox10 directly binds and activates transcription of the Mitf promoter, whereas a mutant form of the Sox10 protein genetically linked with WS4 acts as a dominant-negative repressor of Mitf expression and can reduce endogenous Mitf protein levels. The ability of Sox10 to activate transcription of the Mitf promoter implicates Sox10 in the regulation of melanocyte development and provides a molecular basis for the hypopigmentation and deafness associated with WS4.

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EXPERIMENTAL PROCEDURES

Plasmid Constructs—The human Mitf promoter from −395 to +128 was isolated by PCR using appropriate primers that generated 5’ SstI

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and 3' HindIII sites and was cloned between the SstI and HindIII sites of the pGgL3 (Promega) firefly luciferase reporter plasmid. The Pax3-binding site within the MITF promoter was mutated from the wild type sequence ATTAAACTACTGGAACT to the mutant sequence AGCAGAGAGAGAGAGAGAGAGAG. The fusion protein was expressed and purified from the bacterial strain X-TL21(De3) p-LysS, the Sox10-binding sites A, B, C, D, B + C/D, and F in the MITF promoter were mutated using a PCR-based strategy, generating plasmids pMITF-Δmut, pMITF-Bmut, pMITF-Cmut, pMITF-B+C/Dmut, and pMITF-Fmut, respectively (details of all sub-cloning is available on request). The sequence of the wild type Sox10-binding sites and the mutations introduced are described below. The pCMV-Sox10 and pGEX-KG.Sox10HMG expression plasmids were a gift from Dr Michael Wegner and have been described previously (16). The pCMV-Sox10 HMG domain mutant construct (pCMV-Sox10HMGdom.mt) was generated by replacing nucleotides 482–503 inclusive with an oligonucleotide of the sequence 5’-ATGCGTTTTTTTGCTGCGATCCGGAG-3’. The MIC mutant of SOX10 (6) was generated by PCR-mediated mutagenesis and introduced into pcDNA3.1 (Invitrogen) to produce the pcDNA3.1-Sox10.MIC construct. All mutant constructs were verified by sequencing. The Sox10 in vitro transcription/translation/translation products were subcloned by subcloning the Sox10 HMG domain cDNA or the full-length Sox10 cDNA downstream from a β-globin leader sequence p7Plink in which Sox10 expression is controlled by the bacteriophage T7 promoter. The C terminus of Sox10 (amino acids 249–466) was sub-cloned into the plasmid pGEX2TKP to produce pGEX.Soxt10CTerm, which was used to express the GST-Sox10CTerm fusion protein. The transfection control plasmid consists of the G3PDH promoter used previously by us (18) cloned into the Renilla luciferase reporter (Promega). The GFP expression plasmid, pEGFP-C1, was obtained from CLONTECH.

Cell Culture—501 melanoma cells (501mel cells) were maintained in RPMI supplemented with 10% fetal calf serum and penicillin/streptomycin, and NIH-3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and penicillin/streptomycin.

Transient Transfections and Reporter Assays—Cells were seeded at a density of 1.5 × 10^5 cells/cm^2 in a 24-well plate the day before transfection. 25 ng of reporter-plasmid construct were transfected with increasing amounts of plasmid-expressing activators using Fugene (Roche Molecular Biochemicals) according to the manufacturer’s instructions. An equal total amount of DNA was maintained by compensation with empty expression vector DNA. Cells were harvested 48 h after transfection, assayed for firefly luciferase activity, and the results normalized to the Renilla luciferase activity expressed from the pRL-G3PDH plasmid to control for transfection efficiency.

Transient Transfections and Immunofluorescence—Cells were seeded on coverslips at a density of 3.75 × 10^4 cells/well in a 6-well plate the day before transfection. 100 ng of the pEGFP-C1 plasmid and increasing amounts of the pcDNA3.1-Sox10.MIC construct were transfected using Fugene according to the manufacturer’s instructions, with the total amount of DNA being maintained using empty expression vector DNA. 48 h after transfection, cells were fixed with 10% paraformaldehyde, permeabilized with 0.2% Triton X-100, and washed with PBS. The signal was detected using a rabbit anti-MITF primary antibody diluted 1:100 (gift from Simon Saule, Paris, France), followed by a Texas Red-conjugated anti-rabbit secondary antibody diluted 1:100 (Sigma). Cells were mounted using Vectashield mounting medium and examined by confocal microscopy.

Raising Antiserum and Western Blotting—A GST-Sox10 C-terminal fusion protein was expressed and purified from the bacterial strain BL21(DE3) p-LysS and used to raise rabbit polyclonal antiserum. Cell extracts were prepared by lysis in Laemmli sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membrane. The membrane was blocked in 10% milk, PBS, 0.1% Triton X-100 for 1 h at room temperature and then incubated overnight at 4 °C with Sox10 antibody diluted with 0.2% Triton X-100. MITF protein was visualized using a rabbit anti-MITF primary antibody diluted 1:100 (gift from Simon Saule, Paris, France), followed by a Texas Red-conjugated anti-rabbit secondary antibody diluted 1:100 (Sigma). Cells were mounted using Vectashield mounting medium and examined by confocal microscopy.

DNA-binding Bandshift Assays—Full-length Sox10 and Sox10 HMG domain proteins were produced in in vitro transcription/translation using the TNT T7-coupled Reticulocyte Lysate System kit (Promega). GST-Sox10 C-terminal protein was expressed and purified from the bacterial strain BL21(DE3) p-LysS, and the Sox10HMG protein was recovered by cleavage with thrombin. Oligonucleotide probes used are as follows with the mutated sequences underlined: consensus SOX10 binding site, 5’-ctagaATTAACCTATTGCTGAAAGAGt-3’; WT site A, 5’-ctagaATTAACCTATTGCTGAAAGAG-3’; WT site B, 5’-ctagaATTAACCTATTGCTGAAAGAG-3’; WT site C, 5’-ctagaATTAACCTATTGCTGAAAGAG-3’; WT site D, 5’-ctagaATTAACCTATTGCTGAAAGAG-3’. The Sox10 expression is controlled by the bacteriophage T7 promoter. The C terminus of Sox10 (amino acids 249–466) was sub-cloned into the plasmid pGEX2TKP to produce pGEX.Soxt10CTerm, which was used to express the GST-Sox10CTerm fusion protein. The transfection control plasmid consists of the G3PDH promoter used previously by us (18) cloned into the Renilla luciferase reporter (Promega). The GFP expression plasmid, pEGFP-C1, was obtained from CLONTECH.

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RESULTS

Given the importance of Mitf in the genesis of the melanocyte lineage and the relative timing of Sox10 and Mitf expression during mouse development, we wished to investigate the possibility that the hypopigmentation and deafness associated with mutations in the SOX10 gene reflected an ability of SOX10 to regulate the expression of MITF. As a first step, we assayed the ability of a Sox10 protein to activate transcription of a luciferase reporter driven by the melanocyte-specific human MITF promoter (MITF-M) (22) extending from −395 to +128 using a transient transfection assay in 501 mel human melanoma cells that express endogenous MITF (23). The results (Fig. 1) demonstrate that transfection of a Sox10 expression vector resulted in up to 11-fold activation of the MITF promoter. Activation was dependent on Sox10 binding to DNA since mutating the HMG domain to inhibit DNA binding abolished the ability of Sox10 to stimulate transcription (data not shown).

If SOX10 were able to target the MITF promoter, mutations in SOX10 linked to WS4 would be anticipated to impair MITF expression. The SOX10 MIC mutant, which has been genetically linked with WS4, arises due to a G to T change at position 565 which results in the expression of a protein which is truncated shortly after the HMG domain (6). This mutant lacks the C-terminal transcription activation domain, while retaining the ability to bind DNA (24), and would therefore be predicted to act in a dominant-negative fashion by competing with wild type SOX10 for binding to the MITF promoter. Consistent with
this, expression of a Sox10 MIC mutant protein in the 501 melanoma cell line resulted in around a 10-fold decrease in expression from the MITF-luciferase reporter (Fig. 2A). Thus in contrast to the WT Sox10 protein which activates the MITF promoter efficiently, the Sox10 MIC mutant that is expressed from the CMV promoter at similar levels (Fig. 2B and Ref. 24) acts as an effective repressor. To verify that the Sox10 MIC protein could also reduce endogenous MITF expression, 501 melanoma cells were transfected with either an empty expression plasmid or the Sox10 MIC expression construct, and endogenous MITF levels were examined by immunofluorescence using an anti-MITF antibody. Cells transfected with the Sox10 MIC expression vector were identified using a cotransfected GFP expression vector. All GFP-positive cells showed severely reduced levels of endogenous MITF protein compared with either untransfected cells (Fig. 2C, upper panel) or control cells expressing GFP alone (Fig. 2C, lower panel), consistent with Sox10 MIC suppressing endogenous MITF expression. The most likely mechanism to account for the repression of the MITF promoter by Sox10 MIC would be that expression of the mutant protein lacking a transcription activation domain would compete with endogenous SOX10 for recognition of the MITF promoter. It was therefore important to verify that the 501mel cells indeed express endogenous SOX10. Preliminary results using reverse transcriptase-PCR indicated that the 501mel cell lines expressed SOX10 mRNA, whereas 3T3 cells did not (data not shown). To determine whether SOX10 protein was expressed, we raised a specific anti-Sox10 antibody that recognized SOX10 but not the related transcription factor SOX4, and we used this antibody in a Western blot together with extracts from 501 melanoma cells. The result revealed a band with a molecular weight corresponding to SOX10 in 501mel cells but not in 3T3 cells which are Sox10-negative (Fig. 2D).

Combined with the results obtained using the MITF promoter assays, these data provide compelling evidence that one consequence of the SOX10 MIC mutation associated with WS4 would be reduced MITF expression. Given the critical requirement for MITF in melanocyte development, the reduction in MITF expression arising as a consequence of mutations in the SOX10 gene would be sufficient to account for the hypopigmentation and deafness associated with WS4 and the Dom mouse.

Although we have provided firm evidence that Sox10 regulates MITF expression, there remained a possibility that the requirement for SOX10 was indirect. However, examination of the MITF promoter revealed numerous potential partial and complete SOX protein consensus binding sites (19). To determine whether any of these sites were bound by Sox10, labeled fragments derived from the MITF promoter (Fig. 3A) were tested in a bandshift assay together with in vitro transcribed/translated (ITT) Sox10. The results revealed that Sox10 binds well to three of the four fragments tested (Fig. 3B). Putative consensus binding sites within these fragments were identified using cold competitor oligonucleotides against smaller regions of the promoter (not shown) and designated A–F (Fig. 3A); sites C and D overlap to such an extent that they have been treated as a single element in our analysis. Labeled oligonucleotides specific to each of sites A–F and the Sox consensus binding site sites (19) were next tested in the bandshift assay. The results (Fig. 3C) indicate that Sox10 binds strongly to sites B and C/D, less well to site F, and poorly to sites A and E. Binding to sites B and C/D was specific, with competition being impaired by introducing mutations into the putative Sox10 recognition elements (Fig. 3, D and E). Elements F and A competed between 5- and 25-fold less efficiently than either elements B or C/D, consistent with the results shown in Fig. 3C. No competition

**Fig. 2.** The WS4-associated Sox10 MIC mutant protein is a dominant-negative repressor of MITF expression. A, the MITF promoter-luciferase reporter (25 ng) was transfected into 501mel cells with 500 ng of either control empty expression plasmid (−) or the Sox10 MIC expression plasmid (+). The cells were assayed for luciferase activity 48 h post-transfection. A cotransfected G3PDH promoter reporter was used as transfection control, and the experiment was repeated three times with similar results. B, wild type Sox10 and the Sox10 MIC mutant are expressed at similar levels from the CMV promoter in melanoma cells. Cells in 24-well plates were transfected with 40 ng of the Sox10 or Sox10 MIC expression plasmid as indicated, and extracts were prepared 48 h later from these and mock-transfected cells. Expression of the Sox10 and Sox10 MIC proteins was then detected by Western blotting of an equivalent amount of total cell extract using an anti-Sox10 antibody provided by Micheal Wegner. Overexpression of the blot revealed a low level of endogenous Sox10 in the untransfected cells (not shown). C, Sox10 MIC mutant protein represses expression from the endogenous MITF gene. 501mel cells were transfected with the Sox10 MIC mutant expression plasmid (upper panel) or an empty activator expression plasmid (lower panel) together with a vector expressing the green fluorescent protein. 48 h after transfection cells were fixed, and MITF protein was detected by immunostaining and examined by confocal microscopy. Transfected cells (GFP-positive) are shown in green (left-hand sections), and MITF protein is shown in red (middle sections). Merged images are shown in the right-hand sections. D, Sox10 is expressed in 501mel cells. ITT Sox10 and SOX4 proteins and cell extracts were resolved by SDS-PAGE, and Sox10 expression was detected by Western blotting. Similar amounts of 3T3 and 501mel extracts were loaded, and the translation of both Sox10 and SOX4 protein was confirmed by [35S]methionine labeling of the translated proteins.
was observed using an MITF-binding site, the M box (20), as a heterologous competitor.

To determine the relevance of these binding sites for activation of the MITF promoter by Sox10 in vivo, we introduced point mutations corresponding to those used in the DNA binding assays into each individual Sox10-binding site (A, B, C/D, and F) and also into the two strongest binding sites B and C/D in combination. We then determined the effects of these mutations on Sox10-mediated activation of the MITF promoter activity in a transient transfection assay. The results (Fig. 4) demonstrate that mutation of any single site alone had little effect on the ability of Sox10 to activate expression from the MITF promoter, whereas mutation of sites B and C/D in combination severely impaired the ability of Sox10 to stimulate transcription. Thus, whereas Sox10 could activate the WT and single site mutant promoters between 10- and 12-fold, only a 4-fold activation was observed with the site B and C/D mutant.

The failure to impair the ability of Sox10 to regulate the MITF promoter by mutation of either of the strong Sox10-binding sites B or C/D alone, together with the reduced activation observed upon mutation of both sites B and C/D in combination, suggested to us that these sites may act together to regulate MITF expression. Consistent with this, the Sox10 MIC mutant failed to repress transcription from an MITF promoter in which both the B and C/D sites are mutated (data not shown).

Previous reports have indicated that artificial promoter-re-
reporter constructs can be activated by Sox10 in partnership with Pax3, Krox-20, or Tst-1/Oct6/SCIP in a synergistic manner in glial cells (16). Within the MITF promoter we have identified a strong Sox10-binding site (site B) that is immediately adjacent to the Pax3-binding site (see Fig. 3A). We therefore felt it was important to determine whether Sox10 requires Pax3 for the activation of the MITF promoter and also whether Sox10 and Pax3 can synergistically activate MITF expression. We first performed a bandshift assay using a probe containing both the Sox10 site B and the Pax3-binding sites from the MITF promoter. The results (Fig. 5A) indicate that both Sox10 and Pax3 can bind this probe, but we were unable to observe any cooperative DNA binding under the conditions used. This is consistent with our observation that Sox10 and Pax3 do not interact in vitro in a GST-pulldown assay under conditions when another Sox protein can efficiently interact with Pax3, suggesting that Sox10 and Pax3 do not directly interact (data not shown). To demonstrate that binding of one protein could not preclude binding of the second protein to this region of the MITF promoter, we performed a similar assay using an excess of bacterially expressed Sox10HMG domains such that the probe would become limiting. Under these conditions a ternary complex between the probe, the Sox10HMG domain protein, and the Pax3 protein was observed (Fig. 5B). Thus although Sox10 and Pax3 can bind simultaneously to their respective sites within this region of the MITF promoter, we have been unable to observe any evidence for cooperativity. Consistent with this, we have not observed any synergistic activation of the MITF promoter by Sox10 and Pax3 in transient transfection assays in numerous cell types under conditions where Sox10 or Pax3 can activate transcription individually (data not shown). To verify that the ability of Sox10 to activate the MITF promoter is independent of Pax3, we used an MITF promoter in which the Pax3-binding site has been mutated. This mutation has been demonstrated previously to prevent Pax3 from binding to and activating the MITF promoter (25). As expected, the basal level of MITF promoter activity was reduced by mutation of the Pax3-binding site (Fig. 5C), but this had no significant effect on the ability of Sox10 to activate the promoter, confirming that Sox10 activation of MITF expression can occur independently of Pax3.

**DISCUSSION**

Our data provide several lines of evidence that the product of the SOX10 gene implicated in WS4 regulates the MITF promoter. First, the MITF promoter is up-regulated by Sox10 in co-transfection assays. Second, the Sox10 MIC mutant protein that lacks the C-terminal activation domain acts to suppress expression from a transfected MITF promoter luciferase reporter and significantly also reduces expression from the endogenous MITF gene. Third, the MITF promoter contains multiple functional Sox10-binding sites. Our results further suggest that the hypo-pigmentation and deafness associated with the dominant SOX10 MIC mutation may arise as a result of SOX10 MIC competing with WT SOX10 for binding to the MITF promoter. Taken together with the genetic evidence implicating SOX10 in the development of the melanocyte lineage, our data provide a significant insight into how the onset of *Mitf* expression in development may be established.

*Mitf* plays a pivotal role in melanocyte development. In the absence of a functional *Mitf* protein, mice exhibit a complete...
loss of neural crest-derived melanocytes (10, 11), as well as a failure of the retinal pigment epithelium to differentiate (26). Mutations in the MITF gene in humans give rise to WS2 (12), and in the zebrafish a null mutation in an MITF gene, 

nacre, results in loss of melanophores (15). Given the importance of Mitf for melanocyte development, a key question is how the initial expression of Mitf is established in the neural crest. To date, in addition to the requirement for Sox10 reported here, three other transcription factors have been implicated in Mitf expression. These are as follows: the paired homeodomain transcription factor Pax3 (25), with mutations in the Pax3 gene giving rise to WS1 and WS3 (27–29); Lef1/Tcf transcription factors that have been shown in the zebrafish and in mammalian cells to confer regulation of the Mitf promoter in response to Wnt signaling via β-catenin (30, 31); and factors such as cAMP-response element-binding protein that regulate Mitf expression in response to cAMP levels (32, 33). Since Sox10, Pax3, the Lef1/Tcf family, and factors able to target the CRE are all widely expressed in the neural crest during development, why is Mitf expression restricted to a subset of cells destined for the melanocyte lineage? One attractive possibility is that the initial onset of Mitf expression is a stochastic process, requiring an array of transcription factors acting coordinately to overcome the nucleosomal barrier at the Mitf promoter. Only in the few cells where such a combination of factors is assembled and active will Mitf expression be established. The idea that stochastic events can play a critical role in commitment or differentiation has been discussed previously (34) and is likely to be related to the “stabilization” concept described by Bennett (35) in the study of B16 melanoma cell differentiation using single cell assays. Although the onset of Mitf expression would be dependent on those factors such as Sox10 and Pax3 that are genetically implicated in melanocyte development, we can find no evidence that these proteins act synergistically at this promoter despite their ability to cooperate effectively in activating an artificial reporter in glial cells (16). That is not to say that these proteins do not act synergistically at certain times in development, but simply that in the cell types we have tested we can find no evidence for anything other than an independent ability of each protein to activate the melanocyte-specific MITF promoter. Although we have not been able to demonstrate any cooperativity in melanocytes between Pax3 and Sox10, it is possible that cooperativity may take place between Lef1 and Sox10, for example through interactions mediated by β-catenin that is known to interact both with Lef1 factors (36–38) and with some members of the Sox family (39). In addition, once Mitf expression is established there is no reason to suppose that there would need to be a continued requirement for the same factors later in development, and it is possible that the role of Sox10 might well be undertaken by other members of the Sox family later in development. Equally, the activity of any individual factor binding to the Mitf promoter will be dependent on its regulation and its interaction with other factors or cofactors. Finally, given the regulation of Lef1 by Wnt signaling and the responsiveness of the CRE by cAMP, one purpose of the array of factors targeted to the Mitf promoter will be to integrate the output from signal transduction pathways acting to regulate Mitf expression as melanoblasts migrate away from the neural crest to their final destinations in the epidermis and hair follicles. How Sox10 is regulated and how the DNA binding resulting from Sox10 DNA binding contributes to the regulation and architecture of the Mitf promoter will be key questions that will need to be addressed if the controls operating on Mitf expression are to be understood.

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