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ARTICLE

Interaction among *SOX10*, *PAX3* and *MITF*, three genes altered in Waardenburg syndrome

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Waardenburg syndrome (WS) is an autosomal dominant disorder with an incidence of 1 in 40 000 that manifests with sensorineural deafness and pigmentation defects. It is classified into four types depending on the presence or absence of additional symptoms. WS1 and WS3 are due to mutations in the *PAX3* gene whereas some WS2 cases are associated with mutations in the microphthalmia-associated transcription factor (*MITF*) gene. The WS4 phenotype can result from mutations in the endothelin-B receptor gene (*EDNRB*), in the gene for its ligand, endothelin-3 (*EDN3*), or in the *SOX10* gene. PAX3 has been shown to regulate *MITF* gene expression. The recent implication of SOX10 in WS4 prompted us to test whether this transcription factor, known to cooperate *in vitro* with PAX3, is also able to regulate expression from the *MITF* promoter. Here we show that SOX10, in synergy with PAX3, strongly activates *MITF* expression in transfection assays. Analyses revealed that PAX3 and SOX10 interact directly by binding to a proximal region of the *MITF* promoter containing binding sites for both factors. Moreover, SOX10 or PAX3 mutant proteins fail to transactivate this promoter, providing further evidence that the two genes act in concert to directly regulate expression of *MITF*. *In situ* hybridization experiments carried out in the dominant megacolon (*Dom*) mouse, confirmed that SOX10 dysfunction impairs *Mitf* expression as well as melanocytic development and survival. These experiments, which demonstrate an interaction between three of the genes that are altered in WS, could explain the auditory–pigmentary symptoms of this disease.

INTRODUCTION

Waardenburg syndrome (WS) is an autosomal dominant disorder with an incidence of 1 in 40 000 that manifests with sensorineural deafness and pigmentation defects of the hair, skin and iris (1). It is classified into four types depending on the presence or absence of additional symptoms. Type 1 WS (WS1; MIM 193500) and type 2 WS (WS2; MIM 193510) are distinguished by the presence or absence of dystopia canthorum, respectively. The presence of limb abnormalities distinguishes type 3 WS (Klein–Waardenburg syndrome, WS3; MIM 148820) from type 1. Type 4 WS, referred to as Shah–Waardenburg syndrome or Waardenburg–Hirschsprung disease (WS4; MIM 277580) is characterized by the presence of an aganglionic megacolon. WS1 and WS3 are due to mutations in the *PAX3* gene (2–4) whereas some WS2 cases are associated with mutations in the microphthalmia-associated transcription factor (*MITF)* gene (5). The WS4 phenotype can result from mutations in the endothelin-B receptor gene (*EDNRB*), and in the gene for its ligand, endothelin-3 (*EDN3*) (6–8). Recently, we have shown that mutations in *SOX10*, a co-transcription factor that functions during neural crest development, also results in WS4 (9).

MITF, a basic helix–loop–helix leucine zipper (bHLHZip) protein (10,11), is a transcription factor involved in several developmental processes. In particular, it is essential for the development and post-natal survival of melanocytes. Over 20 different *Mitf* mutations have been described in mice. They all result in a deficiency in skin or coat melanocytes ranging in severity from minor pigmentary defects with normal eyes to total lack of coat and eye pigmentation, small colobomatous eyes, deafness and in some instances osteopetrosis (12). MITF controls melanocyte development in regulating expression of a variety of melanocytic genes, in particular the pigmentation enzymes tyrosinase and tyrosinase-related protein 1 (TRP-1), by binding to a shared regulatory sequence, known as the M box, that contains a CATGTG motif (13–18).

PAX3 is a member of the paired class homeodomain family of transcription factors. In mice, it is expressed in the neural tube and developing brain, neural crest and their derivatives,

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the dermomyotome of the developing somites and the limb buds (19). In addition to playing a role in skeletal muscle formation, this factor has been shown to regulate *MITF* gene expression (20). *TRP-1* promoter activity has recently been shown to be up-regulated by PAX3 as well (21). Thus, PAX3 seems to be another factor important for melanocyte development.

SOX10, a protein that modulates other transcription factors (including PAX3) (22) belongs to the high mobility group (HMG) box superfamily of DNA-binding proteins (for reviews see refs 23,24). It is first expressed during development in cells of the neural crest that contribute to the forming peripheral nervous system, and can be detected in the sensory, sympathetic and enteric ganglia and along nerves (22,25,26). *SOX10* is also transiently expressed in melanoblasts (25). Its expression in the central nervous system occurs later, increasing in strength until adulthood where expression levels are maximal (22,26). The *Sox10* gene is mutated in the dominant megacolon (*Dom*) mouse, an animal model of neurocristopathy, whose phenotype (intestinal aganglionosis and pigmentation defects such as a white belly spot and white paws) is reminiscent of Waardenburg–Hirschsprung patients (25,27,28). The pigmentary phenotype also suggests that *Sox10* expression is essential for melanocyte development.

Common to the various WS types is a deficiency of melanocytes, which are neural crest derivatives. This deficiency is responsible for the pigmentation defects observed, but also for the high incidence of deafness, which is caused by their loss from the stria vascularis of the cochlea (29). Whereas the absence (or dysfunction) of MITF observed in WS appears to specifically affect survival, proliferation and differentiation of melanocytes, PAX3 defects affect other neural crest cell derivatives, resulting in the additional presence of craniofacial malformations.

The association of pigmentation defects, hearing loss and megacolon that characterize WS4 prompted us to test whether SOX10, known to cooperate *in vitro* with PAX3, is able to regulate expression from the *MITF* promoter. Here we show that this factor, in synergy with PAX3, indeed strongly activates *MITF* expression in transfection assays. In agreement with this finding, SOX10 or PAX3 mutant proteins fail to transactivate this promoter, providing further evidence that the two genes act in concert to directly regulate expression of *MITF*. Finally, *in situ* hybridization experiments carried out in the *Dom* mouse confirmed that *Sox10* dysfunction impairs *Mitf* expression as well as melanocytic development and survival.

RESULTS

Effects of SOX10 and PAX3 on the *MITF* promoter

To examine the possible interactions of SOX10 and/or PAX3 with the *MITF* promoter, transient co-transfection assays were carried out in HeLa cells, a cell line chosen because of absence of endogenous *MITF* (30), *PAX3* (20, and data not shown) and *SOX10* (data not shown) expression. Approximately 2.3 kb of the *MITF* melanocyte-specific promoter sequences (nucleotides –2253 to +97) were cloned upstream of the luciferase reporter gene (*pGL3 basic*). This construct was co-transfected with the human *SOX10* or/and *PAX3* cDNA. When *PAX3* was co-transfected with the *MITF* construct, an ~10-fold increase in luciferase activity was detected (Fig. 1A), a result in agreement with previously published data (20). Co-transfection of *SOX10* with the *MITF* reporter vector resulted in an ~500-fold increase in luciferase activity. As SOX10 was previously

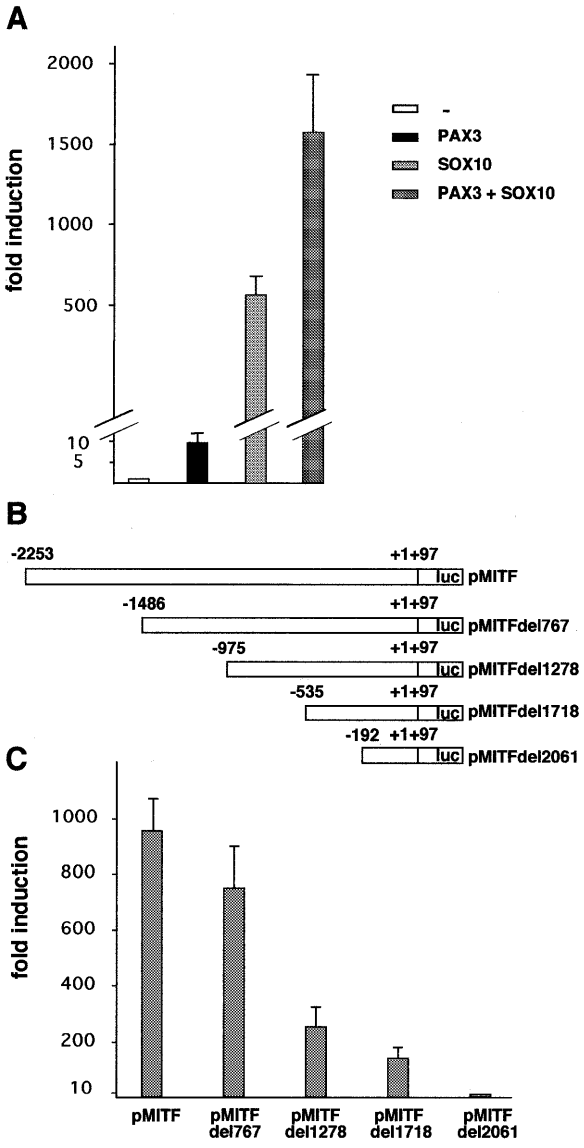


Figure 1. Effect of SOX10 and PAX3 on the *MITF* promoter and SOX10-responsive regions in this promoter. (**A**) The luciferase reporter plasmid containing 2.3 kb of the melanocyte-specific promoter *MITF* was transfected in HeLa cells in combination with empty pECE vector (–), pECE/PAX3 (PAX3) and/or pECE/SOX10 (SOX10), as indicated. Luciferase activity was normalized by measuring β-galactosidase activity. The basal level of induction was determined from values obtained from transfection with the *MITF* luciferase reporter and empty pECE plasmids. (**B**) Schematic representation of the various *MITF* promoter deletions used in (C). Numbers above each construct indicate, from left to right, the 5′ boundary (expressed in bp upstream of the initiation site), the transcriptional start site (+1) and the 3′ boundary (position +97). The designation of each deletion mutant is indicated on the right. (**C**) Succesive *MITF* promoter deletions fused to the luciferase reporter gene were transfected with empty pECE or pECE/SOX10 vectors. Data from all transfections in (A) and (C) are presented as fold induction above basal levels [determined as in (A)] and are means ± SE of three different experiments performed in triplicate.

shown to act as a modulator of PAX3, we next tested the possible cooperative activation of the *MITF* promoter by these factors. Indeed, transcriptional activation was enhanced 1500-fold (i.e. by a factor of three) compared with SOX10 activation, suggesting that PAX3 acts synergistically with SOX10 to activate the *MITF* promoter in this *in vitro* system.

SOX10-responsive regions in the *MITF* promoter

To determine which part of the *MITF* promoter mediates SOX10-dependent activation, we constructed a series of shortened versions of this promoter by deleting sequences of various lengths from the distal end (Fig. 1B). The promoter deletion mutants were co-transfected with the *SOX10* plasmid into HeLa cells to test their ability to be activated by this factor. As shown in Figure 1C, deletion of the distal-most 767 bp did not influence activation by SOX10, in contrast to larger deletions (del1278, del1718 and del2061 constructs) that resulted in a significant and progressive decrease in luciferase activity [4-, 8- and 80-fold, respectively (Fig. 1C)]. SOX10 responsiveness was almost completely lost on removal of 2061 bp. These results indicate that several SOX10 activation regions are scattered along the *MITF* promoter. The one closest to the transcriptional start site of the *MITF* gene is located between positions –535 and –192. This region is therefore responsible for the SOX10 responsiveness of the proximal promoter.

SOX10 binding sites in the *MITF* promoter

We next undertook to determine whether the action of SOX10 on the *MITF* promoter was direct or indirect. To this end, we searched for potential SOX10 binding sites in the region shown to mediate the major SOX-dependent promoter stimulation, making use of the published consensus sequences for SOX binding sites (31,32). These sequences consist of a core binding element of 7 bp and allows both adenosine and thymidine at three of seven positions. However, *SOX10* and other *SOX* genes have been shown to act on promoters or enhancer sequences which do not completely match this consensus binding site (33,34). Seeking sequences matching the consensus SOX binding site in allowing mismatches on the bases that flank the CAAT/A core sequence, we identified eight potential SOX10 binding sites between positions –535 and –192. Only the site located between positions –199 and –193 fully conformed with the published consensus binding site (Fig. 2A and B). The six more proximal SOX sites of the del1718 construct were mutated in such a way that the consensus was replaced by a GC-rich element. This resulted in a dramatic decrease in transcriptional activity identical to that observed with the del2061 mutant promoter (Fig. 2C). The same result was obtained when all eight putative SOX10 binding sites were mutated (data not shown). This result indicates that, in this context, and for the promoter region tested, SOX10 acts directly by binding to one or several of these sites.

To find out whether SOX10 would indeed bind to the six more proximal sites, we next performed a gel shift analysis using extracts from COS cells producing a shortened SOX10 version. This shortened SOX10 version shows DNA binding characteristics identical to full-length SOX10. Among the potential sites tested, only one (site 5) exhibited strong binding of SOX10 (Fig. 2D). The other sites, including the one that conformed to the published consensus, exhibited only weak binding (sites 4, 6 and 7/8) or no binding at all (site 3).

Assuming that the binding site with the highest affinity would also be the functionally most important one, we next mutated site 5 in the context of the del1718 construct by replacement with a GC-rich element. This resulted in a decrease in transcriptional activity almost comparable to that observed for the deletion of the *MITF* promoter in which we mutated the six putative SOX binding sites (Fig. 2C). The remaining difference in SOX10 responsiveness between both promoter constructs might be due to the contribution of the other weak SOX10 binding sites present in this region. Indeed, when we mutated site 7/8 or site 6 alone in the context of the del1718 construct, no significant decrease (site 7/8) or a 25% decrease (site 6) in transcriptional activity was observed (data not shown). In conclusion, these results indicate that, in this context, and for the promoter region tested, SOX10 acts directly by binding.

SOX10 acts synergistically with PAX3 on the *MITF* promoter

Previous work established that PAX3 acts directly by binding to the *MITF* promoter (20). Indeed, the effect of PAX3 on transcription activation was abolished in 624-mel cells when the specific binding site that lies between positions –260 and –244 (Fig. 2A) is destroyed by mutagenesis. Having demonstrated that PAX3 acts synergistically with SOX10 on the *MITF* promoter, we next tested to what extent this effect was maintained when shortened versions of the *MITF* promoter were assayed. We studied the effects of SOX10 and PAX3, alone or in combination, on the wild-type promoter and on the two most shortened versions of this promoter (del1718 and del2061 constructs) (Fig. 3A). SOX10-dependent transcription activation from the del1718 mutant promoter was decreased but still present (60- versus 500-fold activation compared with the intact promoter). This promoter truncation did not alter the responsiveness towards PAX3, and the synergistic cooperation of SOX10 and PAX3 was still observed. We expected that the 2061 bp deletion would drastically impair both SOX10- and PAX3-dependent activation, resulting in the loss of the synergistic effect of the two partner factors. Indeed, the PAX3 binding site previously described (P1) is removed from this construct. Surprisingly, in our *in vitro* model (i.e. in HeLa cells) no diminution of the PAX3-dependent stimulation was observed, whereas the synergistic effect of SOX10 and PAX3 seemed to be preserved.

To explain the discrepancies between our data and those of Watanabe *et al.* (20), we searched for a second PAX3 binding site, making use of the observation of Phelan and Loeken (35) who identified a new paired box consensus motif, GTTAT, downstream of the homeobox binding motif ATTA. A careful analysis of the *MITF* promoter sequence revealed the presence of this other PAX3 binding site (P2) between positions –40 and –26 (Fig. 2A). The two recognition elements for the paired domain and the homeodomain are separated by 6 nucleotides, a proximity that allows PAX3 to interact with high affinity. In light of these observations, we looked at PAX3-dependent transcription stimulation from the del1718 construct in which each PAX3 binding site, or both, are mutated (Fig. 3B).

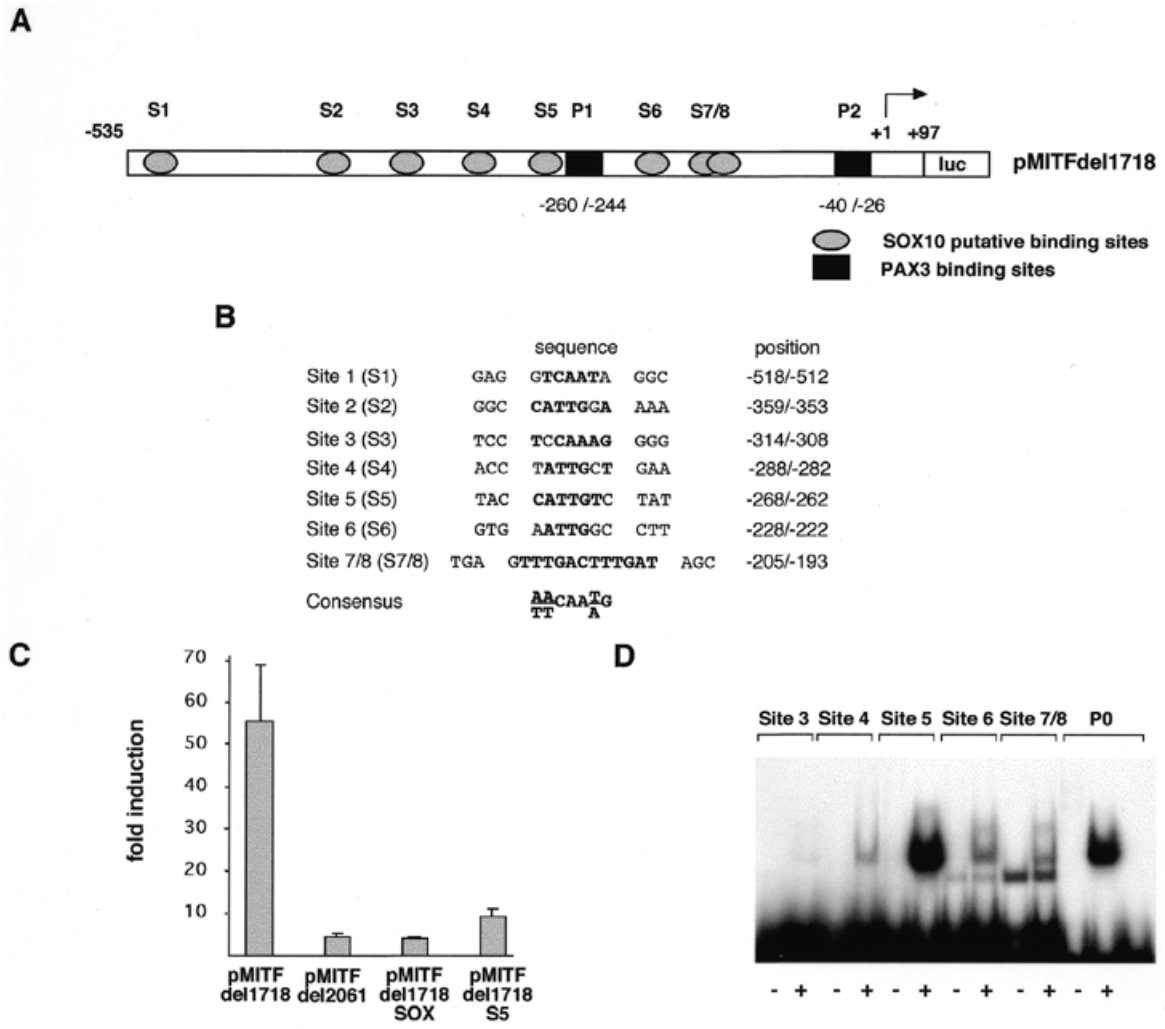


Figure 2. SOX10 binding sites in the *MITF* promoter. (**A** and **B**) Sequence of potential SOX10 binding sites (sites 1–8) and their relative localization in the relevant region of the *MITF* promoter (–535 to –192). Localization of PAX3 binding sites are also indicated in (A). In (B), the consensus SOX binding site (Consensus) is shown in bold. The agreement with the consensus sequence for each potential SOX10 binding site is also shown in bold. Most SOX proteins recognize this motif or its complement. (**C**) *MITF* promoter mutant pMITFdel2061, pMITFdel1718 and the same mutant in which the six putative SOX10 binding sites (pMITFdel1718SOX) or the binding site 5 (pMITFdel1718S5) only are mutated were transfected with empty pECE vector or pECE/SOX10. Data from all transfections are presented as fold induction above basal levels (Fig. 1A) and are means ± SE of three different experiments performed in duplicate. (**D**) Double strand oligonucleotides containing the potential SOX10 binding sites 3–8 or a high affinity binding site from the myelin glycoprotein gene *P0* (*P0*) (33) were analyzed for their ability to bind SOX10 in gelshift experiments using as protein source nuclear extract from COS cells transfected with the SOX10 mutant E189X (+). Extract from mock-transfected COS cells (–) served as control. Several oligonucleotides showed binding to a factor already present in mock-transfected COS cells.

When the enhancing effect of PAX3 alone was tested on the del1718 mutant promoter with P1 altered, no decrease in activity was observed, as with the del2061 construct. Alone, the P1 binding site does not seem sufficient to drive PAX3-dependent stimulation of the *MITF* promoter in HeLa cells. In contrast, when P2 or both binding sites (P1 and P2) were destroyed, PAX-dependent stimulation of transcription was abolished. A possible explanation is that the binding site that is mainly used by PAX3 to exert its effect would depend on the cell context (mel-624 or HeLa). As the P2 binding site is close to the TATA box, it was important to prove that its mutation does not disrupt any element critical for the function of the *MITF* promoter. The fact that SOX10 is able to stimulate transcription from this construct to the same extent as from the intact construct confirmed that the promoter integrity was maintained (data not shown).

Finally, we tested the effects on transcription activation of removing the six SOX10 and the two PAX3 binding sites from the del1718 mutant promoter (Fig. 3A). The dramatic decrease of SOX10-dependent stimulation that resulted was similar to that observed with the del2061 promoter mutant, and with the del1718 promoter in which six or eight binding sites for SOX10 were mutated. PAX3-dependent stimulation was also abolished as expected. Finally, we observed a complete loss of the synergistic effect between SOX10 and PAX3 with this construct. All these results confirmed that PAX3 and SOX10 act directly and synergistically on the *MITF* promoter.

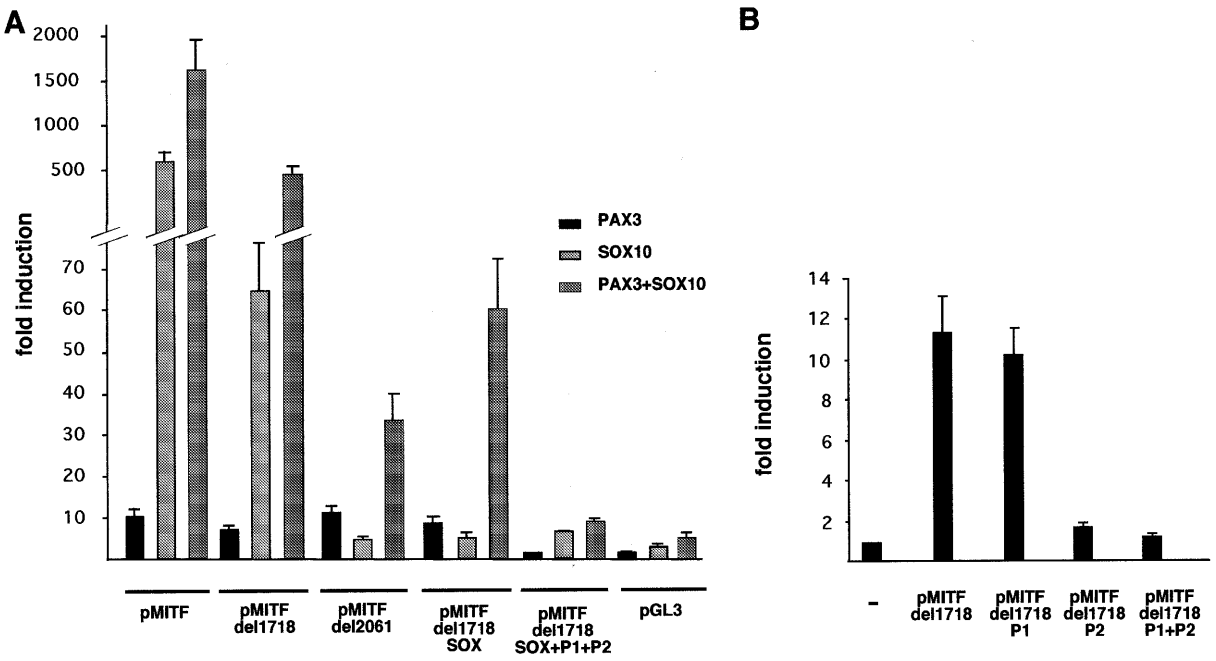


Figure 3. SOX10 acts synergistically with PAX3 on the *MITF* promoter. (**A**) The *MITF* promoter luciferase reporter plasmid (pMITF), two truncations of this promoter (pMITFdel1718 and pMITFdel2061) in wild-type or mutant version (pMITFdel1718SOX, six potential SOX10 binding sites removed; pMITFdel1718 SOX+P1+P2, SOX and the two PAX3 binding sites mutated) as well as the luciferase reporter plasmid alone (pGL3) were transfected in HeLa cells in combination with empty pECE vector, pECE/PAX3 (PAX3) and/or pECE/SOX10 (SOX10), as indicated. Data from all transfections are presented as fold induction above basal levels (determined as in Fig. 1A) and are means ± SE of three different experiments performed in triplicate. (**B**) Transfection of wild-type pMITFdel1718 or versions with mutated PAX3 sites P1 and P2 (pMITFdel1718P1, pMITFdel1718P2 and pMITFdel1718P1+P2) were transfected in HeLa cells along with empty pECE vector or pECE/PAX3. Data from all transfections are presented as indicated in Figure 1.

Consequences of PAX3 and SOX10 mutations on *MITF* promoter induction

Several mutations of the transcription factors PAX3 and SOX10 have been described in WS1, WS3 and WS4. In order to test the effect of these reported mutations on the MITF promoter, we analyzed several SOX10 and PAX3 mutant factors for their ability to transactivate this promoter, alone or in synergy with the other partner.

Three PAX3 mutants were generated and tested (36,37): (i) P50L is a substitution that alters the paired domain and impairs DNA binding; (ii) Q282X truncates the protein after the two DNA binding domains, thus resulting in the loss of the C-terminus transactivation domain; and (iii) 1185insTGA introduces a stop codon which truncates the protein, removing a part of the transactivation domain. The PAX3 effect on *MITF* promoter activation of the first two mutants (P50L and Q282X) was shown to be completely lost (Fig. 4A). The induction was reduced from 9- to 3-fold when the 1185insTGA mutant was tested. Moreover, the synergistic effects of PAX3 mutants with wild-type SOX10 were almost completely lost in all cases (Fig. 4B).

The seven SOX10 mutants constructed were as follows (Fig. 5A) (9,38–40): (i) 482ins6 is located in the HMG domain and prevents DNA binding; (ii) E189X, S251X, Y313X and Q377X are stop mutants that result in factors of various lengths containing the HMG domain; (iii) 1076delGA is a frameshift mutation that produces a premature stop codon and a loss of the transactivation domain; and (iv) 1400del12 removes the stop codon and thus elongates the protein, adding 82 amino acids. The SOX10-dependent activation of *MITF* promoter was lost almost completely (<1% of wild-type) for all mutants (Fig. 5B). The picture is more complex when synergistic activation of the *MITF* promoter with wild-type PAX3 is analyzed. Here, the SOX mutants fall into three classes: (i) the synergistic effect is completely abolished (482ins6, E189X, S251X); (ii) this effect remains partially (Y313X, 1076delGA and Q377X); and (iii) PAX3 activity appears to be decreased (42% of the PAX3 fold induction) when assayed in the presence of the mutant SOX10 1400del12 (Fig. 5C). Although behaving differently with respect to their synergistic effect, all mutants were defective in activation of the *MITF* promoter, demonstrating the usefulness of our *in vitro* model.

A spontaneous mouse model, the *Dom* mouse, carries a mutation in the *Sox10* gene (25,27). This mutation, an insertion of an additional G after position 579, results in an altered reading frame, which leaves the first 193 amino acids of SOX10 including the HMG domain intact, but replaces the remaining 273 residues by a divergent C-terminus of unrelated amino acids. We tested the effect of this mutation in the context of the human SOX10 cDNA in our *in vitro* assay. In this assay, the SOX10-dependent activation as well as the synergistic activation of the *MITF* promoter with wild-type PAX3 was lost (Fig. 5).

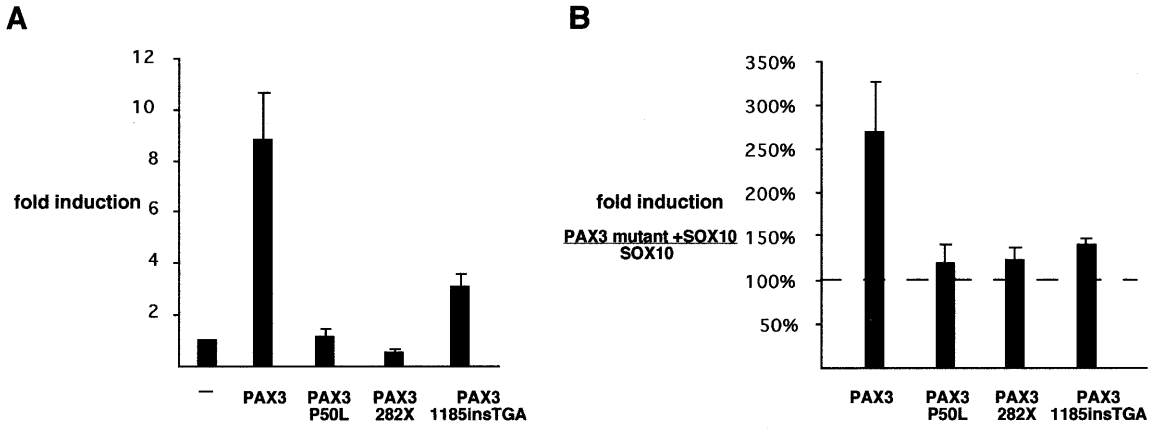
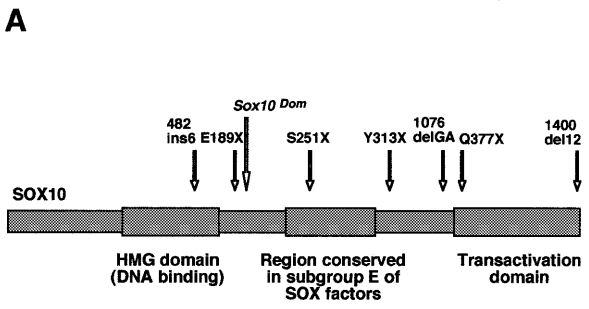


Figure 4. Consequences of PAX3 mutations on *MITF* promoter induction. (**A**) The luciferase reporter plasmid containing 2.3 kb of the *MITF* melanocyte specific promoter was transfected in HeLa cells in combination with empty pECE vector (–), pECE/PAX3 (PAX3), pECE/PAX3 mutants (PAX3P50L, PAX3Q282X, PAX3 1185insTGA) as indicated. Luciferase activities were determined in three independent experiments, each performed in triplicate. Values from transfections with reporter vector and empty expression plasmid were set to 1. Data for other transfections are represented as fold induction above this level. (**B**) The reporter plasmid was transfected in combination with empty pECE vector, pECE/PAX3, pECE/PAX3 mutants (A) and pECE/SOX10 vector. Data are means ± SE of three different experiments performed in triplicate. The synergistic effect of PAX3 or PAX3 mutants with SOX10 is represented as the ratio of PAX3 or PAX3 mutants and SOX10 co-activation over SOX10 activation alone. This ratio is expressed as a percentage. The dotted line indicates the level above which synergy exists between the two factors.



B

	482 ins6	E189X	*Sox10Dom*	S251X	Y313X	1076 delGA	Q377X	1400 del12
SOX10 mutant residual activity: SOX10 mutant / SOX10	0,86% ± 0,13	0,07% ± 0,01	0,57% ± 0,05	0,11% ± 0,03	0,50% ± 0,09	0,42% ± 0,09	0,41% ± 0,11	0,08% ± 0,02

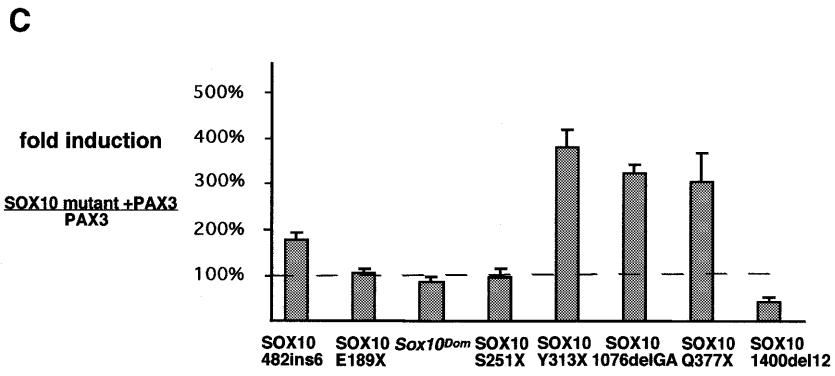


Figure 5. Consequences of SOX10 mutations on *MITF* promoter induction. (**A**) Schematic representation of the SOX10 factor. The DNA binding domain (HMG domain), the transactivation domain and a region that is conserved between group E SOX proteins are indicated by boxes. The positions of the *SOX10* mutations studied in (B) and (C), 482ins6, E189X, *Sox10*^{Dom}, S251X, Y313X, 1076delGA, Q377X and 1400del12 are indicated by arrows. (**B**) Table showing SOX10 mutant residual activity. The luciferase reporter plasmid containing 2.3 kb of the melanocyte specific *MITF* promoter was transfected in HeLa cells in combination with empty pECE vector, pECE/SOX10, pECE/SOX10 mutants [see (A)]. Luciferase activities were determined in three independent experiments, each performed in triplicate. SOX10 mutant residual activity was expressed as a percentage of the ratio of SOX10 mutant induction to SOX10 wild-type induction. (**C**) The reporter plasmid containing the 2.3 kb *MITF* promoter was transfected in combination with the empty pECE, pECE/SOX10, or pECE/SOX10 mutants [as in (A)] and pECE/PAX3 vector. The synergistic effect of SOX10 mutants with PAX3 is represented as ratio of SOX10 mutants and PAX3 co-activation to PAX3 activation alone. This ratio is expressed as a percentage. Data in (B) and (C) are means ± SE of three different experiments performed in triplicate.

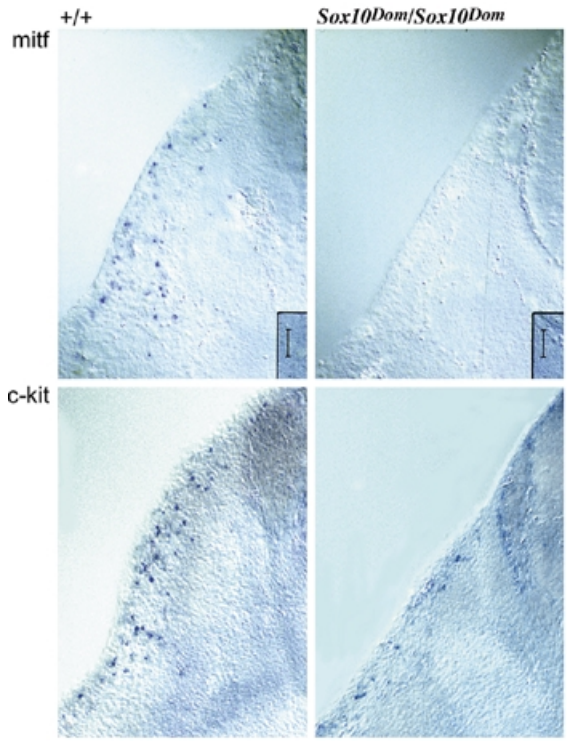


Figure 6. *In vivo* analysis of SOX10 effect on *MITF* expression. *In situ* hybridization analyses on transverse sections of 12.5-day-old wild-type (+/+) and homozygous *Dom* ($Sox10^{Dom}/Sox10^{Dom}$) embryos at the level of the hindlimbs using an antisense riboprobe specific for *c-Kit* (c-kit) and *Mitf* (mitf).

In vivo analysis of the effect of SOX10 on *MITF* expression

We have shown that, *in vitro*, SOX10 up-regulates directly the *MITF* promoter. We next examined whether there is an influence of SOX10 on *MITF* expression *in vivo*. For that purpose we performed *in situ* hybridization studies on the *Dom* mouse. For simplicity we confined our analysis to homozygous embryos and their wild-type littermates at 12.5 days of embryogenesis. Using an antisense riboprobe specific for *c-Kit*, an early marker of the melanocyte lineage, we detected many cells in transverse sections at a position typical of melanocytes (Fig. 6). These cells were strongly reduced in number in the homozygous *Dom* embryos confirming the overall importance of *Sox10* for melanocyte development. However, few *c-Kit*-positive cells remained present. All or some of these cells are probably mast cells. When *in situ* hybridizations were carried out on wild-type embryos with an *Mitf*-specific riboprobe, similar numbers of cells were detected in equivalent positions as with the *c-Kit* probe. However, contrary to what we observed for *c-Kit*, we failed to detect any remaining *Mitf*-positive cells in the homozygous *Dom* embryos. This shows that SOX10 influences *Mitf* expression *in vivo* during embryonic development.

DISCUSSION

Previous studies have identified MITF as a major transcriptional regulator of the genes for the pigment enzymes tyrosinase and TRP-1, placing this transcription factor in a key position in melanocyte development (13–18). Recently, it was shown that PAX3 is also involved in this process through its ability to transactivate the *MITF* promoter (20). This demonstration of an epistatic relationship between two of the genes whose dysfunction results in Waardenburg syndrome provides a link between the pigmentary–auditory symptoms that are common to the various forms of this syndrome (1). More recently, SOX10 was shown to be another player among the genes defective in this syndrome (9). Its spatial and temporal pattern of expression supports an important function in early neural crest development in humans and in mice (25–27). In keeping with this observation, mutations of *SOX10* result in a combination of defects affecting neural crest derivatives, such as pigmentation abnormalities, hearing loss and colonic aganglionosis in mice and in humans (WS4). The molecular mechanism of SOX10 action during melanogenesis is currently unknown. Nevertheless, considering that, using an artificial promoter containing SOX and PAX3 binding sites, SOX10 modulates *in vitro* the action of other transcription factors such as PAX3 (22), we sought to test its possible involvement in the regulation of *MITF* transcription, in concert with PAX3.

Molecular dissection of the *MITF* promoter

To examine the ability of SOX10 and PAX3 to regulate *MITF* transcription, we cloned ~2.3 kb of the promoter region of the *MITF* gene upstream of a luciferase reporter gene. This construct was co-transfected with SOX10 and/or PAX3 plasmids into HeLa cells, a cell line initially chosen because of its missing expression of the two factors. Although this cell line is physiologically distant from pigment cells, previous molecular studies of genes involved in melanogenesis showed comparable data in HeLa cells and in a melanoma cell line (MeWo) (15).

When assayed with SOX10, the promoter region showed a strong increase (~500-fold) in promoter activity. In contrast, when PAX3 was used as the transcription factor in this assay, a smaller but significant increase in promoter activity (10-fold) was observed. However, co-expression of SOX10 and PAX3 resulted in a much stronger promoter activity than that obtained in each of the separate transcription assays (>1500-fold). This observation establishes that the two transcription factors act in synergy to transactivate the *MITF* promoter.

The creation of a series of deletion mutants of the *MITF* promoter allowed us to show that it contains several DNA regions responsive to SOX10. Among them, a SOX10-responsive region was found lying between positions –535 and –192. We also tested whether SOX10 and PAX3 act by binding to the *MITF* promoter. A PAX3 binding site (P1), previously identified by Watanabe *et al.* (20), was shown to directly mediate *MITF* regulation in melanoma (624-mel) cells. Our study identified another PAX3 binding site (P2, located at positions –40 to –26) that seems to be stronger than P1 in HeLa cells. This discrepancy with the previous report may be related to the cell line used (melanoma versus HeLa cells), as other differences in site usage between one cell line and another have already been reported (15,41). Further experiments are needed to understand the role of this site in melanoma cells. Eight potential SOX10 binding sites were identified between positions –535 and –192 on the basis of

their homology with the SOX binding site consensus sequence. Mutation of all these binding sites resulted in a dramatic reduction of SOX10 induction, demonstrating that this transcription factor acts directly on the *MITF* promoter. We then tested the affinity of SOX10 for these SOX binding sites by gel shift analysis and found that one site (site 5) exhibited strong binding of SOX10 whereas the other sites exhibited weak or no binding. The *in vitro* analysis (mutation of site 5) confirmed these results. Interestingly, the site that displays strong binding of SOX10 does not fully conform to the consensus for SOX binding sites, thus indicating that binding of SOX proteins to DNA seems to be influenced by additional factors such as the exact flanking sequences or the DNA structure. This latter assumption is also supported by the observation that one of the other potential sites identified in the *MITF* promoter by sequence inspection binds SOX10 only with low affinity despite the fact that it fully conforms to the consensus.

We then analyzed the behavior of the various deletion mutants to test whether SOX10 and PAX3 had maintained the synergistic effects observed with the wild-type promoter. The synergistic effect was present in the del1718 promoter mutant. With the del2061 construct, and with the del1718 construct in which we mutated the SOX binding sites, synergy was preserved despite the fact that SOX10 induction was dramatically reduced. It is possible that some weak SOX10-responsive elements are located in the segment still present in the del2061 construct; alone, they might be unable to strongly activate the *MITF* promoter in HeLa cells, but they allow synergistic activation in the presence of PAX3. Another explanation could be that PAX3 and SOX10 can physically interact in such a way that only one or the other needs to bind DNA in order to recruit the entire complex. Nevertheless, this last hypothesis is in disagreement with the results obtained by Kuhlbrodt *et al.* (22), who showed that SOX10 and its cofactor must bind independently to a synthetic promoter DNA to function synergistically.

Whether SOX10 binds first to the promoter sequence in order to facilitate the binding of PAX3 or vice versa is presently unknown. However, Kamachi *et al.* (42) recently pointed out that in an *in vivo* situation where DNA is in a chromatin structure the HMG domain of SOX is not sufficient to form a stable protein–DNA complex at a SOX site, although its DNA binding is demonstrable *in vitro*. They postulate that a SOX protein would form a stable complex with the target DNA only in the presence of a partner factor, which interacts with the SOX protein and binds to a nearby DNA site.

Effects of PAX3 and SOX10 mutations on *MITF* promoter induction

To confirm the data obtained with the wild-type factors, we sought possible deleterious effects on *MITF* promoter activation of some of the PAX3 and SOX10 mutations identified in patients with WS. Watanabe *et al.* (20) previously showed that some of the PAX3 mutant factors failed to transactivate the *MITF* promoter. We confirmed these results in testing, in addition to P50L, two other mutant PAX3 factors. Interestingly, the 1185insTGA mutant retained a weak activity, probably because only the distal part of the transactivation domain of PAX3 is lost. In all cases, the mutant PAX3 proteins lost their ability to synergistically enhance wild-type SOX10-mediated transactivation.

Similarly, we tested several SOX10 mutants. All the mutants studied resulted in an almost complete loss of the transactivation effect. However, based on the results of co-transfection experiments with PAX3, the mutant factors belong to three categories. The 482ins6, E189X and S251X mutations result in a complete loss of synergistic effect, as opposed to Y313X, 1076delGA and Q377X, which do not drastically perturb this effect. These results agree with those of Kuhlbrodt *et al.* (22) who, using another co-factor (TST1/OCT6/SCIP) in their transfection assays, showed that the mutant factors tested lost the autonomous transactivation effect; however, some of them kept their ability to act in synergy (43). Mutant factors that have lost their ability for synergistic interaction with PAX3 are those that lead to complete loss of DNA binding or to very short proteins. Nonsense or frameshift mutations that lead to more distal truncations, on the other hand, retain their capacity for synergy. It is interesting to note that the ability of a truncation mutant to synergize correlates with the presence or absence of a region that is conserved between group E SOX proteins (Fig. 5A). Thus, it is tempting to speculate that this conserved region is a protein–protein interaction module involved in mediating the synergy between SOX10 and PAX3. The result obtained when the $Sox10^{Dom}$ mutation in the context of the human cDNA was tested confirmed this observation. However, whether these *in vitro* effects mimic those that produce the phenotypes observed in patients is presently unknown.

1400del12 represents a third category of SOX10 defects. This mutation induces a very particular phenotype, with demyelination of the central and peripheral nervous systems (39). When the mutant *SOX10* cDNA is co-expressed with the wild-type *PAX3* cDNA, PAX3 activity is reduced. A puzzling observation is that this patient is the only one, among those carrying a *SOX10* mutation, who manifests dystopia canthorum, an abnormality of the inner canthi of the eyes that is strikingly associated with *PAX3* mutations. A titration effect of PAX3 by the mutant SOX10 1400del12 could account for such an observation. In fact, Inoue *et al.* (39) suggested that this peculiar phenotype could be explained by a dominant negative effect of the mutation and by the remarkable proline-rich structure of the elongated protein resulting from deletion of the stop signal. Although more investigations are needed to unravel this mechanism, our observation supports this hypothesis. It is notable that mutations Y313X and S251X (40), which are also associated with a neurological phenotype, do not show such an effect when tested in our *in vitro* system. The analysis of SOX10 transcriptional targets involved in the development of the nervous system, when they are identified, could point to a similar mechanism.

With all *in vitro* data pointing to a role for SOX10 in the transcriptional regulation of the *MITF* gene, it was of interest to find out whether there would also be an indication for regulation of *Mitf* expression by Sox10 *in vivo*. *In situ* hybridization results with *Dom* mice seem to be in agreement with this assumption. These mice carry an inactivating mutation in the *Sox10* gene. It has been shown that this mutation leads to multiple defects in several neural crest-derived lineages with the effect being dose dependent such that defects are more severe in homozygous than in heterozygous *Dom* mice. One of

the neural crest lineages which is clearly affected in these mice is the melanocyte lineage. In homozygous *Dom* mice there is a severe defect in this lineage such that already at day 12.5 of embryogenesis most melanocytes are missing as judged by *in situ* hybridization with both *c-Kit* and *Mitf* probes. Indeed, few *c-Kit*-positive cells and no *Mitf*-positive cells were detected at this stage. One possible explanation for these observations would be to assume that *Mitf* expression *in vivo* is genetically downstream of *Sox10*. Previous analysis also revealed the absence of dopachrome tautomerase (Dct) expression in homozygous *Dom* mice at day 11.5 (25). We thus confirmed the importance of SOX10 in proliferation, differentiation and survival of melanocytes. It is also noticeable that no gross alteration in *Pax3* expression was detected in homozygous *Dom* mouse. Further analysis of *Mitf* and *Pax3* mouse mutants would be useful to determine whether the interactions seen *in vitro* between these three genes occur *in vivo*.

Recently, another HMG-containing protein (Lef/Tcf-Lef1) has been shown to regulate *MITF* and the zebrafish *MITF* gene (*Nacre*) expression *in vitro* and *in vivo* (44,45). It was suggested that Wnt-3a signaling recruits β-catenin and LEF1 to LEF1/Lef1 binding sites of the *MITF* promoter. The Lef1 binding sites of the *Nacre* promoter described are not conserved in the human *MITF* promoter. The LEF1 binding site of the human *MITF* promoter corresponds to the binding site 8, described in this paper, which does not bind SOX10 efficiently, thus suggesting that both LEF1 and SOX10 are able to bind the *MITF* promoter on different specific binding sites, i.e. sites 8 and 5, respectively. All these data suggest that the regulation of *MITF* is complex and could involve different pathways.

In conclusion, our analysis of the interplay between SOX10, PAX3 and MITF during development sheds light on the molecular bases of the auditory–pigmentary abnormalities that are common to the various forms of Waardenburg syndrome.

MATERIALS AND METHODS

Construction of mutant cDNAs

MITF promoter isolation. The ~2.3 kb melanocyte-type promoter sequence of *MITF* was amplified by PCR using primers 5′-CCGGTACGCCAGATCTTACTAATGTATAAT-CGTGTG-3′ and 5′-GACGTCCAGGAGATCTGACTTATC-CCTCCCTCTACT-3′, which were designed from the previously reported sequence (GenBank accession no. AF034755). PCR products were cloned in the pGL3-basic luciferase reporter vector (Promega, Madison, WI) following *Bgl*II digestion. Nucleotide sequence was verified using BigDye terminator cycle sequencing on a 373 ABI apparatus (PE Applied Biosystems, Les Ulis, France). Sequence comparison with the reported sequence of GenBank accession no. AF034755 shows nine differences at positions 334delA, 443insC, 952delT, 1024delG, 1036delG, 1077insT, 1535delT, 2254C→A and 2273A→C. The last two differences are in agreement with the sequence of GenBank accession no. D82874. PCR amplification from three different control DNAs identified the same differences.

MITF constructs. Deletion constructs pMITFdel767, pMITFdel1278, pMITFdel1718 and pMITFdel2061 were generated by creating enzyme restriction sites by directed mutagenesis using the Quick Change mutagenesis kit (Stratagene, Amsterdam, The Netherlands). Constructs pMITFdel1718P1, pMITFdel1718P2, pMITFdel1718P1+P2, pMITFdel1718SOX, pMITFdel1718–8SOX, pMITFdel1718S5 and pMITFdel1718-SOX+P1+P2 were also generated by sequential site-directed mutagenesis steps making use of the same kit. The nucleotide sequence of each construct was verified. Each construct corresponds to the pMITFdel1718 sequence except as follows: (i) in pMITFdel1718P1, the sequence between positions –260 and –244 is substituted with the mutant sequence AgcgATAC-TACTcGAAg as previously described (20); (ii) in pMITFdel1718P2, the sequence between positions –40 and –26 is substituted with the sequence gcgAGCTTAGcTTcT; (iii) pMITFdel1718P1+P2 includes the two sequence substitutions described above; (iv) in pMITFdel1718SOX sequences between –314 and –308, –288 and –262, –228 and –222 and –205 and –193 are substituted with sequences TCCggAG, TAccGCTGAAAGAGAAATACCAccGTC, AAccGGC and GTccGACTccGAT, respectively; (v) pMITFdel1718–8SOX includes the above sequence substitutions; in addition, sequences between –518 and –512 and –359 and –353 are substituted with sequences GTCggTA and CAccGGA, respectively; (vi) in pMITFdel1718S5, the sequence between –268 and –262 is substituted with TAccGCT; and (vii) pMITFdel1718-SOX+P1+P2 includes the sequence substitutions of pMITFdel1718P1+P2 and pMITFdel1718SOX.

PAX3 constructs. The human *PAX3* cDNA was amplified as described previously (20). The PCR product was cloned in the pECE vector (46) following *Bam*HI–*Xho*I digestion. The three mutations P50L, Q282X and 1185insTGA of *PAX3* cDNA correspond to reported WS1 mutations and were generated by directed mutagenesis using the Quick Change mutagenesis kit (Stratagene) (36,37). The nucleotide sequence of each construct was verified.

SOX10 constructs. The human *SOX10* cDNA in pCMV/Hu SOX10 (GenBank accession no. AJ001183) was subcloned in the pECE vector at the *Eco*RI site. The previously identified *SOX10* mutations, 482ins6, E189X, $Sox10^{Dom}$, S251X, Y313X, 1076delGA, Q377X and 1400del12 (9,25,27,38–40) were introduced by directed mutagenesis as described. The nucleotide sequence of each construct was verified.

Cell culture, transfection and reporter assays

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and transfected using Lipofectamine PLUS reagents (Gibco BRL, Life Technologies, Gaithersburg, MD) in six-well plates. Cells were plated at 3×10^5/well and were transfected 1 day later with 0.350 μg of each effector and reporter plasmid. The total amount of plasmid was kept constant by addition of empty pECE vector. The plasmid pCH110, which contains the SV40 promoter driving expression of a *LacZ* reporter, was used as an internal control to assess transfection efficiency (0.150 μg per transfection) as previously described (47). Twenty-four hours after transfection, cells were washed twice with phosphate-

buffered saline, lysed and extracts were assayed for luciferase activity using the Luciferase assay system (Promega).

Gel shifts

^{32}P-labeled probe (0.5 ng) was incubated with protein for 20 min on ice in a 20 µl reaction mixture containing 10 mM HEPES (pH 8.0), 5% glycerol, 50 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 4 µg of bovine serum albumin and 2 µg of poly(dGdC) as unspecific competitor. Nuclear extracts from COS cells transfected with the E189X mutant of SOX10 served as protein source. As probes we used double-stranded oligonucleotides each containing one or two potential SOX10 binding sites from the region between –535 and –192 of the *MITF* promoter as indicated in Figure 2A and B. After incubation, samples were loaded onto native 4% polyacrylamide gels and electrophoresed in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) at 120 V for 1.5 h. Gels were dried and exposed for autoradiography.

In situ hybridization

Mouse embryos at embryonic day 12.5 were fixed overnight at 4°C in 4% paraformaldehyde, dehydrated, bleached and embedded in 20% gelatine. After overnight fixation in 4% paraformaldehyde, transverse sections (100 µm) were prepared on a vibratome. Digoxigenin (DIG)-labeled antisense riboprobes for *c-Kit* and *Mitf* were produced with a DIG-RNA labeling kit (Roche Diagnostics, Mannheim, Germany). The 1.2 kb *Mitf* riboprobe corresponded to positions 177–1399 of the published mouse *Mitf* sequence (GenBank accession no. NM008601). Whole-mount *in situ* hybridization of vibratome slices was performed essentially as described (48,49) with 68°C as hybridization temperature.

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REFERENCES

1. Read, A.P. and Newton, V.E. (1997) Waardenburg syndrome. *J. Med. Genet.*, **34**, 656–665.
2. Baldwin, C.T., Hoth, C.F., Amos, J.A., da-Silva, E.O. and Milunsky, A. (1992) An exonic mutation in the HuP2 paired domain gene causes Waardenburg's syndrome. *Nature*, **355**, 637–638.
3. Tassabehji, M., Read, A.P., Newton, V.E., Harris, R., Balling, R., Gruss, P. and Strachan, T. (1992) Waardenburg's syndrome patients have mutations in the human homologue of the Pax-3 paired box gene. *Nature*, **355**, 635–636.
4. Hoth, C.F., Milunsky, A., Lipsky, N., Sheffer, R., Clarren, S.K. and Baldwin, C.T. (1993) Mutations in the paired domain of the human PAX3 gene cause Klein-Waardenburg syndrome (WS-III) as well as Waardenburg syndrome type I (WS-I). *Am. J. Hum. Genet.*, **52**, 455–462.
5. Tassabehji, M., Newton, V.E. and Read, A.P. (1994) Waardenburg syndrome type 2 caused by mutations in the human microphthalmia (MITF) gene. *Nature Genet.*, **8**, 251–255.
6. Puffenberger, E.G., Hosoda, K., Washington, S.S., Nakao, K., deWit, D., Yanagisawa, M. and Chakravarti, A. (1994) A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. *Cell*, **79**, 1257–1266.
7. Edery, P., Attié, T., Amiel, J., Pelet, A., Eng, C., Hofstra, R., Martelli, H., Bidaud, C., Munnich, A. and Lyonnet, S. (1996) Mutation of the endothelin-3 gene in the Waardenburg-Hirschsprung phenotype (Shah-Waardenburg syndrome). *Nature Genet.*, **12**, 442–444.
8. Hofstra, R.M., Osinga, J., Tan-Sindhunata, G., Wu, Y., Kamsteeg, E.J., Stulp, R.P., van Ravenswaaij-Arts, C., Majoor-Krakauer, D., Angrist, M., Chakravarti, A. *et al.* (1996) A homozygous mutation in the endothelin-3 gene associated with a combined Waardenburg type 2 and Hirschsprung phenotype (Shah-Waardenburg syndrome). *Nature Genet.*, **12**, 445–447.
9. Pingault, V., Bondurand, N., Kuhlbrodt, K., Goerich, D.E., Prehu, M.O., Puliti, A., Herbarth, B., Hermans-Borgmeyer, I., Legius, E., Matthijs, G. *et al.* (1998) SOX10 mutations in patients with Waardenburg-Hirschsprung disease. *Nature Genet.*, **18**, 171–173.
10. Hughes, M.J., Lingrel, J.B., Krakowsky, J.M. and Anderson, K.P. (1993) A helix-loop-helix transcription factor-like gene is located at the mi locus. *J. Biol. Chem.*, **268**, 20687–20690.
11. Hodgkinson, C.A., Moore, K.J., Nakayama, A., Steingrimsson, E., Copeland, N.G., Jenkins, N.A. and Arnheiter, H. (1993) Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. *Cell*, **74**, 395–404.
12. Moore, K.J. (1995) Insight into the microphthalmia gene. *Trends Genet.*, **11**, 442–448.
13. Yasumoto, K., Yokoyama, K., Shibata, K., Tomita, Y. and Shibahara, S. (1995) Microphthalmia-associated transcription factor as a regulator for melanocyte-specific transcription of the human tyrosinase gene. *Mol. Cell Biol.*, **15**, 1833.
14. Bentley, N.J., Eisen, T. and Goding, C.R. (1994) Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. *Mol. Cell Biol.*, **14**, 7996–8006.
15. Yasumoto, K., Yokoyama, K., Takahashi, K., Tomita, Y. and Shibahara, S. (1997) Functional analysis of microphthalmia-associated transcription factor in pigment cell-specific transcription of the human tyrosinase family genes. *J. Biol. Chem.*, **272**, 503–509.
16. Bertolotto, C., Bille, K., Ortonne, J.P. and Ballotti, R. (1996) Regulation of tyrosinase gene expression by cAMP in B16 melanoma cells involves two CATGTG motifs surrounding the TATA box: implication of the microphthalmia gene product. *J. Cell Biol.*, **134**, 747–755.
17. Bertolotto, C., Busca, R., Abbe, P., Bille, K., Aberdam, E., Ortonne, J.P. and Ballotti, R. (1998) Different cis-acting elements are involved in the regulation of TRP1 and TRP2 promoter activities by cyclic AMP: pivotal role of M boxes (GTCATGTGCT) and of microphthalmia. *Mol. Cell Biol.*, **18**, 694–702.
18. Aksan, I. and Goding, C.R. (1998) Targeting the microphthalmia basic helix-loop-helix-leucine zipper transcription factor to a subset of E-box elements in vitro and in vivo. *Mol. Cell Biol.*, **18**, 6930–6938.
19. Goulding, M.D., Chalepakis, G., Deutsch, U., Erselius, J.R. and Gruss, P. (1991) Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *EMBO J.*, **10**, 1135–1147.
20. Watanabe, A., Takeda, K., Ploplis, B. and Tachibana, M. (1998) Epistatic relationship between Waardenburg syndrome genes MITF and PAX3. *Nature Genet.*, **18**, 283–286.
21. Galibert, M.D., Yavuzer, U., Dexter, T.J. and Goding, C.R. (1999) Pax3 and regulation of the melanocyte-specific tyrosinase-related protein-1 promoter. *J. Biol. Chem.*, **274**, 26894–26900.
22. Kulhbrodt, K., Herbarth, B., Sock, E., Hermans-Borgmeyer, I. and Wegner, M. (1998) Sox10, a novel transcriptional modulator in glial cells. *J. Neurosci.*, **18**, 237–250.
23. Pevny, L.H. and Lovell-Badge, R. (1997) Sox genes find their feet. *Curr. Opin. Genet. Dev.*, **7**, 338–344.
24. Wegner, M. (1999) From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res.*, **27**, 1409–1420.
25. Southard-Smith, E., Kos, L. and Pavan, W. (1998) *Sox10* mutation disrupts neural crest development in *Dom* Hirschsprung mouse model. *Nature Genet.*, **18**, 60–64.
26. Bondurand, N., Kobetz, A., Pingault, V., Lemort, N., Encha-Razavi, F., Couly, G., Goerich, D.E., Wegner, M., Abitbol, M. and Goossens, M. (1998) Expression of the SOX10 gene during human development. *FEBS Lett.*, **432**, 168–172.
27. Herbarth, B., Pingault, V., Bondurand, N., Kuhlbrodt, K., Hermans-Borgmeyer, I., Puliti, A., Lemort, N., Goossens, M. and Wegner, M. (1998) Mutation of the Sry-related Sox10 gene in Dominant megacolon, a

mouse model for human Hirschsprung disease. *Proc. Natl Acad. Sci. USA*, **95**, 5161–5165.
28. Lane, P.W. and Liu, H.M. (1984) Association of megacolon with a new dominant spotting gene (*Dom*) in the mouse. *J. Hered.*, **75**, 435–439.
29. Steel, K.P. and Barkway, C. (1989) Another role for melanocytes: their importance for normal stria vascularis development in the mammalian inner ear. *Development*, **107**, 453–463.
30. Fuse, N., Yasumoto, K., Suzuki, H., Takahashi, K. and Shibahara, S. (1996) Identification of a melanocyte-type promoter of the microphthalmia-associated transcription factor gene. *Biochem. Biophys. Res. Commun.*, **219**, 702–707.
31. Harley, V.R., Lovell-Badge, R. and Goodfellow, P.N. (1994) Definition of a consensus DNA binding site for SRY. *Nucleic Acids Res.*, **22**, 1500–1501.
32. Mertin, S., McDowall, S.G. and Harley, V.R. (1999) The DNA-binding specificity of SOX9 and other SOX proteins. *Nucleic Acids Res.*, **27**, 1359–1364.
33. Peirano, R.I., Goerich, D.E., Riethmacher, D. and Wegner, M. (2000) Protein zero gene expression is regulated by the glial transcription factor Sox10. *Mol. Cell Biol.*, **20**, 3198–3209.
34. Zhou, G., Lefebvre, V., Zhang, Z., Eberspaecher, H. and de Crombrugghe, B. (1998) Three high mobility group-like sequences within a 48-base pair enhancer of the Col2a1 gene are required for cartilage-specific expression in vivo. *J. Biol. Chem.*, **273**, 14989–14997.
35. Phelan, S.A. and Loeken, M.R. (1998) Identification of a new binding motif for the paired domain of Pax-3 and unusual characteristics of spacing of bipartite recognition elements on binding and transcription activation. *J. Biol. Chem.*, **273**, 19153–19159.
36. Baldwin, C.T., Hoth, C.F., Macina, R.A. and Milunsky, A. (1995) Mutations in PAX3 that cause Waardenburg syndrome type I: ten new mutations and review of the literature. *Am. J. Med. Genet.*, **58**, 115–122.
37. Hol, F.A., Geurds, M.P., Cremers, C.W., Hamel, B.C. and Mariman, E.C. (1998) Identification of two PAX3 mutations causing Waardenburg syndrome, one within the paired domain (M62V) and the other downstream of the homeodomain (Q282X). *Hum. Mutat.*, **12** (Suppl. 1), S145–S147.
38. Southard-Smith, E.M., Angrist, M., Ellison, J.S., Agarwala, R., Baxevanis, A.D., Chakravarti, A. and Pavan, W.J. (1999) The Sox10(Dom) mouse: modeling the genetic variation of Waardenburg-Shah (WS4) syndrome. *Genome Res.*, **9**, 215–225.
39. Inoue, K., Tanabe, Y. and Lupski, J.R. (1999) Myelin deficiencies in both the central and the peripheral nervous systems associated with a SOX10 mutation. *Ann. Neurol.*, **46**, 313–318.
40. Touraine, R.L., Attie-Bitach, T., Manceau, E., Korsch, E., Sarda, P., Pingault, V., Encha-Razavi, F., Pelet, A., Auge, J., Nivelon-Chevallier, A. *et al.* (2000) Neurological phenotype in Waardenburg syndrome type 4 correlates with novel SOX10 truncating mutations and expression in developing brain. *Am. J. Hum. Genet.*, **66**, 1496–1503.
41. Bertolotto, C., Abbe, P., Hemesath, T.J., Bille, K., Fisher, D.E., Ortonne, J.P. and Ballotti, R. (1998) Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. *J. Cell Biol.*, **142**, 827–835.
42. Kamachi, Y., Uchikawa, M. and Kondoh, H. (2000) Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet.*, **16**, 182–187.
43. Kuhlbrodt, K., Schmidt, C., Sock, E., Pingault, V., Bondurand, N., Goossens, M. and Wegner, M. (1998) Functional analysis of Sox10 mutations found in human Waardenburg-Hirschsprung patients. *J. Biol. Chem.*, **273**, 23033–23038.
44. Dorsky, R.I., Raible, D.W. and Moon, R.T. (2000) Direct regulation of nacre, a zebrafish MITF homolog required for pigment cell formation, by the Wnt pathway. *Genes Dev.*, **14**, 158–162.
45. Takeda, K., Yasumoto, K., Takada, R., Takada, S., Watanabe, K., Udono, T., Saito, H., Takahashi, K. and Shibahara, S. (2000) Induction of melanocyte-specific microphthalmia-associated transcription factor by Wnt-3a. *J. Biol. Chem.*, **275**, 14013–14016.
46. Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A. and Rutter, W.J. (1986) Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell*, **45**, 721–732.
47. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 16.66–16.67.
48. Opdecamp, K., Nakayama, A., Nguyen, M.T., Hodgkinson, C.A., Pavan, W.J. and Arnheiter, H. (1997) Melanocyte development in vivo and in neural crest cell cultures: crucial dependence on the Mitf basic-helix-loop-helix-zipper transcription factor. *Development*, **124**, 2377–2386.
49. Qiu, F.H., Ray, P., Brown, K., Barker, P.E., Jhanwar, S., Ruddle, F.H. and Besmer, P. (1988) Primary structure of c-kit: relationship with the CSF-1/PDGF receptor kinase family—oncogenic activation of v-kit involves deletion of extracellular domain and C terminus. *EMBO J.*, **7**, 1003–1011.