FEBS Letters 556 (2004) 236–244 FEBS 27974

Melanocyte-specific expression of dopachrome tautomerase is dependent on synergistic gene activation by the Sox10 and Mitf transcription factors

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Received 27 October 2003; revised 2 December 2003; accepted 4 December 2003

First published online 12 December 2003

Edited by Ned Mantei

Abstract Sox10 regulates melanocyte development at least partly through its stimulatory effect on Mitf gene expression. Here, we characterize the gene for dopachrome tautomerase (Dct/Trp2) as the second direct Sox10 target in melanocytes, arguing for the existence of Sox10 functions in melanocytes that are independent of its epistatic relationship to Mitf. Sox10 responsiveness was mediated by multiple binding sites within the proximal Dct/Trp2 promoter which display varying affinities and bind Sox10 monomers or dimers. Mitf synergistically enhanced Sox10-dependent activation of the Dct/Trp2 promoter. Synergy appears mechanistically complex and requires both direct binding of Sox10 to the promoter and the protein's transactivation domain.

Key words: Tyrosinase-related protein 2; Dopachrome tautomerase; Sox; Sry; High-mobility group

1. Introduction

Sox10 is a member of the Sox family of transcription factors [1,2]. Unlike other proteins that contain a high-mobility-group box as their DNA binding domain, many Sox proteins, including Sox10, are capable of binding to DNA in a sequence-specific manner and of recognizing regulatory sequences in distinct target genes. Sequence-specific DNA binding and a well defined spatial and temporal expression pattern are both prerequisites for the function of Sox proteins as important developmental regulators [1,2].

Sox10 has been identified as a crucial transcription factor for the development of vertebrate neural crest cells in a variety of species including rodents, zebrafish, *Xenopus* and humans [3–11]. Neural crest cells represent a highly mobile, pluripotent population of ectodermal cells in the early embryo that give rise to many different cell types and thus contribute to numerous tissues throughout the body. Peripheral nervous system and melanocytes are prominent examples of neural crest derivatives. Interestingly, inactivation or deletion of Sox10 has been reported to affect many processes in neural crest cells, including multipotency, proliferation, apoptosis, survival and commitment to defined neural crest-derived lineages [3,12–14]. Even further roles of Sox10 during later phases of development, in particular during terminal differentiation, are strongly suggested by target gene analyses in Sox10-deficient mice [15,16].

Neural crest development is sensitive to Sox10 gene dosage. In mice and humans, those neural crest cells which give rise to melanocytes or the enteric nervous system are most sensitive. As a consequence, heterozygous loss of Sox10 already results in partial loss of melanocytes and a missing innervation of the distal colon [4,7]. The resulting pigmentation deficits and aganglionosis of the distal colon give rise to a combined Waardenburg–Hirschsprung syndrome in most patients carrying heterozygous Sox10 mutations [9]. Homozygous Sox10 mutation obliterates melanocytes and the enteric nervous system completely [3,5,7,17]. The importance of Sox10 for melanocyte development has also been confirmed in *Xenopus* where ectopic overexpression of Sox10 generated increased numbers of melanocytes [10,11].

Recent studies have started to address the regulatory mechanisms that underlie Sox10 function by identifying target genes. In melanocytes, Sox10 has been shown to regulate expression of the gene for the transcription factor Mitf [3,18–20]. In vitro studies indicate that Sox10 functions through direct activation of the melanocyte-specific promoter of the Mitf gene leading to a selective production of the melanocyte-specific Mitf-M isoform [18–22]. Mitf-M in turn is a key player in melanocyte development, as it influences melanocyte survival through the antiapoptotic Bcl-2 [23], and regulates expression of many melanocyte markers including tyrosinase, tyrosinase-related protein 1 and dopachrome tautomerase (tyrosinase-related protein 2, Dct/Trp2) (for review, see [24]). With Mitf being the critical regulator of melanocyte development [24], it is possible to explain the melanocyte defect in Sox10-deficient mice solely through Mitf activation. In support of such a model, forced expression of Mitf can rescue development of at least some melanocytes in Sox10-deficient zebrafish with restoration of marker gene expression in these cells [19].

However, this does not exclude that there are additional target genes for Sox10 in cells of the melanocyte lineage. The gene for Dct/Trp2 is a good candidate for such a gene. The encoded protein is an essential component of the final phases of eumelanin synthesis in melanocytes as it converts dopachrome to 5,6-dihydroxyindole-2-carboxylic acid [25,26]. It appears to be important for melanocyte detoxification and is already expressed long before the start of melanin synthesis [27]. By comparison of Dct/Trp2 expression with that of other melanocyte markers, a transient loss of Dct/Trp2 expression has previously been detected in $Sox10^{+/-}$ mouse embryos between embryonic days 10.5 and 12.5 [3,28]. Additionally,

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0014-5793/03/$30.00
doi:10.1016/S0014-5793(03)01446-7

Sox10 was able to activate reporter gene expression from the 5′ flanking regions of both mouse and human Dct/Trp2 genes [3,28]. These studies stopped short of analyzing whether Sox10 influenced Dct/Trp2 expression directly through interaction with the Dct/Trp2 promoter. Given its influence on Mitf expression, Sox10 could have functioned through Mitf which also activates the Dct/Trp2 promoter [29,30]. Here, we have analyzed the effect of Sox10 on the Dct/Trp2 promoter. Our studies indicate that Sox10 regulates Dct/Trp2 expression both directly and indirectly via upregulation of Mitf with both effects reinforcing each other in the synergistic activation of Dct/Trp2 expression by Sox10 and Mitf.

2. Materials and methods

2.1. Plasmids

Eukaryotic expression plasmids for human Sox10 and various mutants have been described before [31]. pCMV-Mitf was generated by inserting a V5-epitope tagged version of the cDNA for mouse M-Mitf (gift of C. Goding) between *Hin*dIII and *Sma*I sites of pCMV5.

3.7 Trp2 luc, the luciferase reporter plasmid containing positions −3240 to +443 of the mouse Dct/Trp2 gene [3], was used to generate several deletion mutants (2.2 Trp2 luc, 1.1 Trp2 luc, 0.9 Trp2 luc, 0.8 Trp2 luc, 0.75 Trp2 luc, 0.7 Trp2 luc, 0.5 Trp2 luc, see Fig. 1A) by polymerase chain reaction-directed or enzymatic successive shortening from the distal end. The replacement of potential Sox binding sites within the Dct/Trp2 promoter by GC-rich sequences (1.1 Trp2 luc construct, see Fig. 2A) was through the use of the QuickChange Mutagenesis Kit (Stratagene).

For bacterial expression of Mitf, cDNA sequences corresponding to the complete open reading frame of Mitf and an amino-terminal V5 tag were inserted into pGEX-KG as a *Hin*dIII/*Sma*I fragment.

2.2. Cell culture and luciferase assays

N2A neuroblastoma, HMB2 melanoma and COS cells were maintained in Dulbecco's modified Eagle's medium containing 5–10% fetal calf serum. N2A and HMB2 cells were transfected using Superfect reagent according to the manufacturer's instructions (Qiagen), whereas the DEAE-dextran method was used for COS cells as previously described [32]. For luciferase assays, N2A and HMB2 cells were transfected in duplicate in 24-well plates with 500 ng of luciferase reporter plasmid and 50 ng of effector plasmid per well if not stated otherwise. Cells were harvested 48 h post transfection, and extracts were assayed for luciferase activity.

2.3. Proteins, cell extracts, Western blots and electrophoretic mobility shift assays

Mouse Mitf was produced in bacteria as a glutathione *S*-transferase (GST) fusion protein and purified according to standard procedures [33]. Extracts from transfected COS cells or HMB2 cells were prepared and checked for expression of the protein of interest in Western blots as described [34] using a polyclonal rabbit antiserum directed against Sox10 [6] or a monoclonal against MITF (NeoMarkers).

For electrophoretic mobility shift assays, 0.5 ng of ^{32}P-labeled probe (for sequences see Figs. 2A, 3B and 6A) were incubated with protein extracts from transfected COS cells for 20 min on ice in 20 µl reaction mixture containing 10 mM HEPES (pH 8.0), 5% glycerol, 50 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM EDTA, 4 µg of bovine serum albumin, and 1 µg of poly(dGdC) or poly(dIdC) as unspecific competitor. Where indicated, 0.5 µl GST antibody (Santa Cruz Biotechnologies) was added. 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.

3. Results

3.1. The Dct/Trp2 promoter is activated by Sox10

We have previously shown that Sox10 activates a reporter gene under the control of a 3.7-kb fragment from the 5′ regulatory region of the mouse Dct/Trp2 gene [3]. The setup used in the original studies (inducible Sox10 expression in a stably transfected cell line) yielded only modest activation rates and therefore did not allow reliable mapping studies. For detailed analysis of the 5′ flanking region of the Dct/Trp2 gene, we switched to transient co-transfection of N2A cells with Sox10 expression plasmid and luciferase reporter plasmid. We chose N2A cells as these mouse neuroblastoma cells are neural crest-derived, and should therefore possess an overall transcriptional repertoire not too distant from melanocytes. Unlike melanocytes, however, N2A cells do not express Sox10 [6] nor Mitf-M (data not shown) so that results from transient transfections are not complicated by endogenous background expression of these transcription factors. We optimized trans-

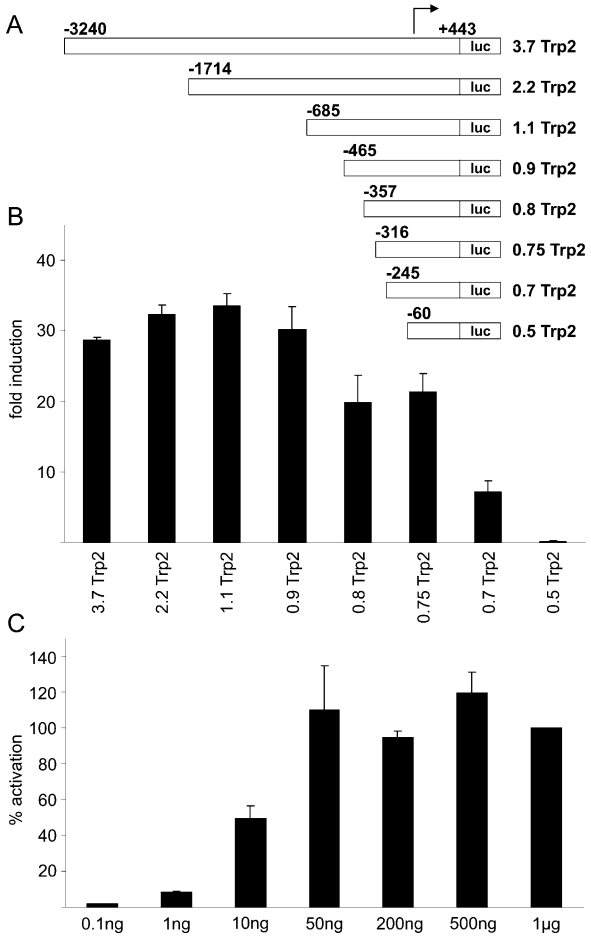

Fig. 1. The proximal Dct/Trp2 promoter is activated by Sox10 in N2A cells. A: Schematic representation of luciferase reporter plasmids carrying successively shortened 5′ flanking regions of the mouse Dct/Trp2 gene. Numbers indicate positions of the last base pairs still contained within the fragment on either side. The transcriptional start is marked by an arrow. Negative values were assigned to positions preceding the transcription start site, positive values to transcribed sequences. B: Transient transfections were performed in N2A cells with the luciferase reporters depicted in A in the absence or presence of Sox10. Data are presented as fold induction ± S.E.M. with the activity for each reporter in the absence of co-transfected Sox10 arbitrarily set to 1. C: Transient transfections with the 1.1 Trp2 luc plasmid and increasing amounts of Sox10 expression plasmid (0.1 ng–1 µg per well). Data are presented as relative activation rates, with the activation obtained with the highest Sox10 concentration arbitrarily set to 100%. Luciferase activities in B and C were determined in three independent experiments each performed in duplicate.

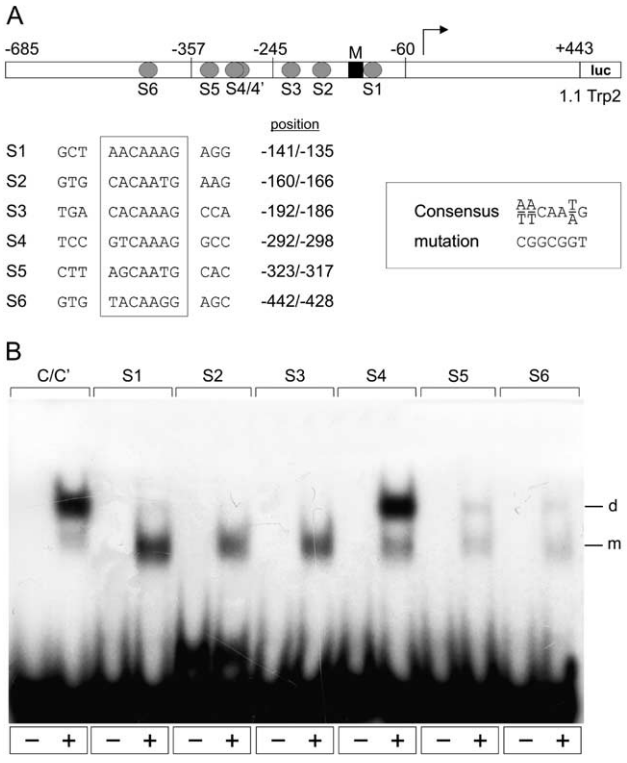

Fig. 2. Multiple Sox10 binding sites are present within the Dct/Trp2 promoter. A: Localization and sequence of Sox10 binding sites between positions −685 and +443 of the proximal Dct/Trp2 promoter. B: Electrophoretic mobility shift assay with extracts from mock-transfected COS cells (−) or COS cells expressing the MIC variant [31] of Sox10 (+). Oligonucleotides with sequences S1–S6 depicted in A and C/C′ which contained a dimeric Sox10 binding site from the Protein zero promoter [15] were used as probes as indicated above the lanes. m, bound monomer; d, bound dimer.

fection conditions such that we obtained on average a 29-fold Sox10-dependent induction of reporter gene expression for the same 3.7 kb Dct/Trp2 promoter fragment, for which we previously detected a three- to five-fold induction rate (Fig. 1B).

To map the Sox10-responsive elements within the 3.7-kb fragment which includes 3240 bp preceding the transcriptional start site of the Dct/Trp2 gene and 443 bp following it, we generated a series of deletion mutants by successively shortening the 5′ flanking region from the distal end (Fig. 1A). The 1.1 Trp2 luc construct (encompassing positions −685 to +443) retained full inducibility, indicating that the proximal promoter of the Dct/Trp2 gene contains the *cis*-acting elements which mediate Sox10 activity (Fig. 1B). While there was still no change for the 0.9 Trp2 luc construct (encompassing positions −465 to +443), activation rates dropped in a stepwise fashion with further shortening, from a 30-fold to a 20-fold induction after removal of sequences between −465 and −357, followed by a further reduction to seven-fold activation rates after deletion of sequences between −316 and −245. Sox10-dependent activation was completely lost in the 0.5 Trp2 luc construct (Fig. 1B) which corresponds to the minimal promoter of the Dct/Trp2 gene (positions −60 to +443). Thus we conclude that the proximal part of the Dct/Trp2 promoter between positions −465 and −60 contains several response elements which contribute to Sox10-dependent promoter activation.

Titration experiments revealed that the amounts of expression plasmid used for our study was in the range where Sox10 yielded maximal activation. A further increase of Sox10 levels left activation rates unchanged (Fig. 1C). Mutant Sox10 proteins previously identified in human patients [9,31] failed to activate the Dct/Trp2 promoter (Fig. 9, see below).

3.2. The Dct/Trp2 promoter contains multiple functionally important binding sites for Sox10

The proximal Dct/Trp2 promoter (positions −685 to −60) was analyzed for potential Sox10 binding sites. Based on the 7-bp consensus for Sox binding sites (A/T)(A/T)CAA(A/T)G [2], we identified six putative sites if we allowed for no more than one mismatch that furthermore did not alter the conserved CAA core (Fig. 2A). Using oligonucleotides carrying each of these sites embedded in their natural environment, electrophoretic mobility shift analyses were carried out (Fig. 2B). These studies confirmed that Sox10 is able to bind to all of these sequences in principle, albeit with highly varying affinities. With constant amounts of Sox10 proteins and comparable specific activities of all radiolabelled oligonucleotides, sites 1 and 4 were bound most strongly, followed by sites 2 and 3. Sites 5 and 6 exhibited only weak binding. Complexes between Sox10 and the various sites exhibited one of two

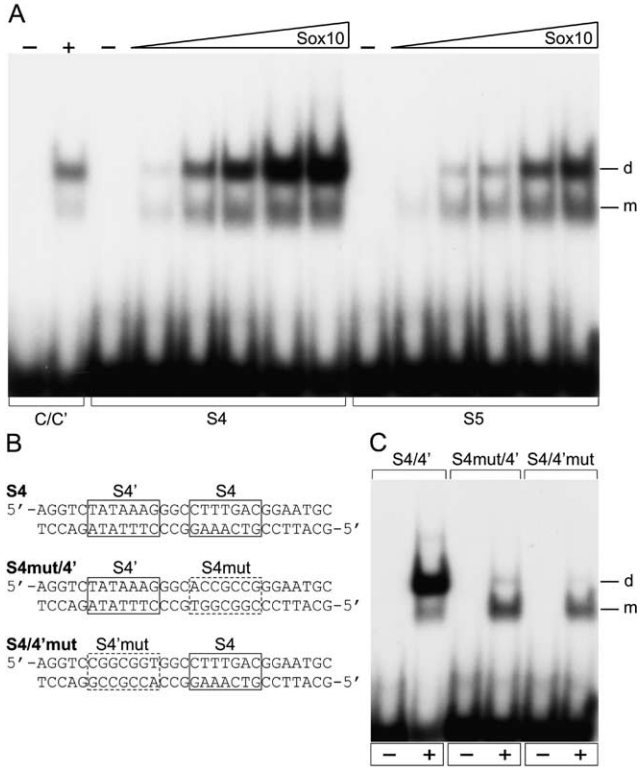

Fig. 3. Dimeric Sox10 binding to the Dct/Trp2 promoter. A: Electrophoretic mobility shift assay with increasing amounts of extracts from COS cells expressing Sox10 MIC using sites 4 (S4) and 5 (S5) from the proximal Dct/Trp2 promoter as probes as indicated below the lanes. C/C′ from the Protein zero promoter served as positive control for a dimeric site. (−) extract from mock-transfected COS cells; (+) extract from Sox10 MIC expressing COS cells. m, bound monomer; d, bound dimer. B: Sequence of oligonucleotides S4 and mutant versions S4mut/4′ and S4/4′mut. C: Electrophoretic mobility shift assay with the oligonucleotide probes shown in B using extracts from mock-transfected COS cells (−) or COS cells expressing Sox10 MIC (+) as protein source. m, bound monomer; d, bound dimer.

mobilities (compare site 1 and site 4 in Fig. 2B). By comparison to oligonucleotides of identical length which contained Sox10 sites with well known binding modes (e.g. C/C′ from the Protein zero promoter in Fig. 2B), these two mobilities were identified as corresponding to the monomeric and the dimeric binding mode originally described for Sox10 and confirmed for the related Sox9 [35–37]. Thus monomeric binding was strongest to site 1, whereas dimeric binding appeared to be preferred on site 4. Dimeric binding was already prevalent on site 4 at low Sox10 concentrations as expected when binding of the two participating Sox10 molecules is cooperative (Fig. 3A). To further confirm that site 4 is a site that supports binding of two Sox10 molecules to adjacent sites, we generated mutant oligonucleotides in which the originally predicted Sox10 binding site or adjacent regions were replaced by GC-rich sequences (Fig. 3B) previously found to be incompatible with Sox10 binding [15]. The predicted Sox10 binding site differs in one position from the consensus motif. Replacement of this site led to a strong reduction in Sox10 binding and a concomitant shift from preferentially dimeric to monomeric binding as evidenced by the increased mobility of the Sox10–DNA complex (Fig. 3C). Mutation of the adjacent upstream region caused a similar reduction in binding affinity and a switch to monomeric binding (Fig. 3C). Closer inspection revealed that this region contains a motif that deviates in two positions from a consensus Sox binding site. Therefore, the composite site 4 consists of two adjacent non-consensus Sox10 binding sites in a head-to-tail orientation separated by 3 bp with one and two mismatches, respectively. The general configuration is typical for many dimeric sites [16,35,38]; the exact spacing and orientation has not been found so far in Sox10 response elements, but is highly similar to Sox9 binding sites D and E in the enhancer of the collagen 11a2 gene [37].

Site 5 and site 6 also seem to bind two molecules of Sox10. Because of the low affinity and the low amounts of bound Sox10, it was difficult to distinguish whether binding of the two Sox10 molecules was cooperative (Fig. 2B). We therefore repeated electrophoretic mobility shift experiments with site 5 using Sox10 over a wide concentration range (Fig. 3A). With increasing amounts of Sox10 protein, mobility of the protein–DNA complex switches from a high-mobility complex to a low-mobility complex. As the low-mobility complex becomes predominant already under conditions where probe saturation has not been achieved, we infer that binding of the first molecule of Sox10 to site 5 increases the likelihood of a second molecule to bind to the same probe indicating that there is some degree of Sox10 cooperativity on this site.

To analyze whether these binding sites contribute to Sox10-dependent activation of the Dct/Trp2 promoter, we mutated each site in the context of the 1.1 Trp2 luc reporter plasmid (Fig. 4A) and assayed the consequences of each mutation on Sox10-dependent reporter gene induction by transient transfections (Fig. 4B). Compatible with a stepwise reduction of Sox10 responsiveness in consecutively shortened Dct/Trp2 promoters, mutations of several sites led to lowered activation. The strongest effect was observed following mutation of site 1 which reduced Sox10-dependent induction by 60%. Mutation of site 4 and site 5 decreased Sox10-dependent activation rates by 37% and 32%, respectively (Fig. 4B). For all other sites, we were unable to reproducibly detect altered activation rates in our assay system. We like to point out that deletion and mutagenesis studies came to slightly different

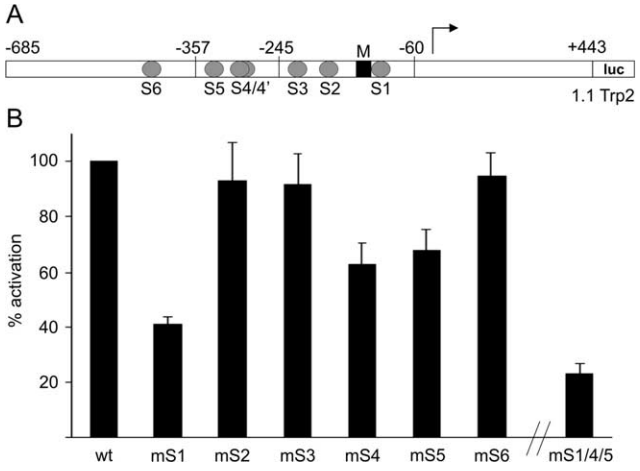

Fig. 4. Sox10-dependent activation of the Dct/Trp2 promoter is mediated by several Sox10 binding sites. A: Localization of Sox10 binding sites 1–6 (S1–S6) between positions −685 and −60 of the proximal Dct/Trp2 promoter. B: Transient transfections were performed in N2A cells in the absence or presence of Sox10 with the wildtype 1.1 Trp2 luciferase reporter (wt) or mutants in which Sox10 binding to sites 1–6 (S1–S6) was abrogated. (mS1–mS6), single site mutants. (mS1/4/5), triple mutant of sites 1, 4 and 5. Luciferase activities in extracts from transfected cells were determined in three independent experiments each performed in duplicate. The data for mS1/4/5 were from separate transfection series which yielded induction rates for the wildtype and mS1 constructs comparable to the ones shown. Data are presented as relative activation rates, with the Sox10-dependent activation obtained for the wildtype construct arbitrarily set to 100%.

conclusions (compare Figs. 1 and 4), as the contribution of site 5 to Sox10-dependent activation of Dct/Trp2 became evident only in the mutagenesis study, whereas the importance of site 6 can only be inferred from the deletion study. The underlying reason is currently unknown, but may result from the respective presence or absence of modulatory transcription factor sites in the reporter constructs. These minor inconsistencies notwithstanding, both the high-affinity monomeric site 1 and the high-affinity dimeric site 4 were always among the sites with effects on Sox10-dependent activation of the Dct/Trp2 promoter, indicating a reasonable correlation between Sox10 binding affinity and Sox10-mediated effect.

To confirm that sites 1, 4 and 5 are indeed the Sox10 binding sites with the biggest contribution, we generated a triple mutant in which all three sites were rendered inactive in the context of the 1.1 Trp2 luc reporter. As expected, Sox10 responsiveness of the triple mutant is further reduced to residual 23% of wildtype levels (Fig. 4B).

We also investigated potential Sox10 effects on the Dct/Trp2 promoter in a human melanoma. In HMB2 cells, Sox10 stimulated expression of the 0.75 Trp2 luc reporter (encompassing positions −316 to +443 of the Dct/Trp2 gene) 3.5-fold and left activity of the Dct/Trp2 minimal promoter unaltered (Fig. 5B). Although present, Sox10-dependent stimulatory effects were thus less pronounced than in N2A cells, probably due to the strong endogenous Sox10 expression in HMB2 cells (Fig. 5A). To study the influence of endogenous Sox10, we compared the activity of the wildtype 1.1 Trp2 luc reporter with that of the triple mutant in sites 1, 4, and 5. As shown in Fig. 5C, expression was reduced by 88% in the absence of the major Sox10 binding sites in the Dct/

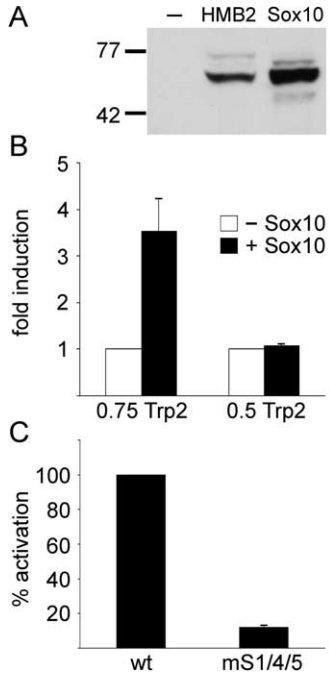

Fig. 5. The proximal Dct/Trp2 promoter is activated by Sox10 in melanoma cells. A: Western blot analysis of HMB2 cell extract (HMB2) using a previously described polyclonal rabbit anti-Sox10 antiserum [6]. Extract from transiently transfected COS cells expressing ectopic Sox10 (Sox10) served as control. Molecular weight markers in kDa are indicated on the left. B: Transient transfections were performed in HMB2 cells with the 0.75 Trp2 luc and 0.5 Trp2 luc reporters in the absence (−Sox10) or presence (+Sox10) of ectopic Sox10. Data are presented as fold induction ± S.E.M. with the activity for each reporter in the absence of co-transfected Sox10 arbitrarily set to 1. C: Transient transfections of HMB2 cells with the 1.1 Trp2 luc plasmid in wildtype or triple mutant (mS1/4/5) version. Data are presented as relative activation rates, with the activation obtained for the wildtype construct arbitrarily set to 100%. Luciferase activities in B and C were determined in two independent experiments each performed in duplicate.

Trp2 promoter. Thus, both ectopic and endogenous Sox10 activate the Dct/Trp2 promoter in melanoma cells.

3.3. *The Dct/Trp2 promoter is synergistically activated by Sox10 and Mitf*

Sox10 binding site 1 is in the immediate vicinity of an M-box that has previously been shown to confer most of the Mitf responsiveness to the Dct/Trp2 promoter [29]. This M-box bound Mitf with an affinity comparable to the M-box from the mouse tyrosinase promoter (Fig. 6A,B). The presence of Mitf in the obtained complex was verified by addition of a specific antibody and the resulting supershift (Fig. 6B). Furthermore, Mitf-dependent activation of the Dct/Trp2 promoter was roughly comparable in N2A cells to Sox10-dependent activation and followed similar kinetics with maximal induction rates being stable over a wide range of concentrations (Fig. 6C).

When the 3.7 Trp2 luc reporter construct was co-transfected with expression plasmids for both Sox10 and Mitf, a clear synergistic activation of the Dct/Trp2 promoter was observed (Fig. 7B). Whereas Sox10 or Mitf increased expression 44-fold and 57-fold, respectively, their combination led to a dramatic 311-fold stimulation. We never observed comparable stimulation rates for either protein alone. Activation rates in the presence of both proteins were more than three-fold higher than the sum of single activation rates (Fig. 7C). Synergistic activation was similarly achieved with the 1.1 Trp2 luc construct. Upon further shortening of the Dct/Trp2 promoter to positions −316 to +443 (0.75 Trp2 luc in Fig. 7A), absolute induction rates dropped significantly in the presence of both Sox10 and Mitf, due to the lowered responsiveness of this construct to each transcription factor (Fig. 7B). Reduction of Mitf-dependent stimulation was probably caused by removal of an E-box that has been shown to bind Mitf with low affinity [29]. However, induction was still synergistic (Fig. 7C). In contrast, no synergistic activation was observed for the minimal Dct/Trp2 promoter. These results indicate that

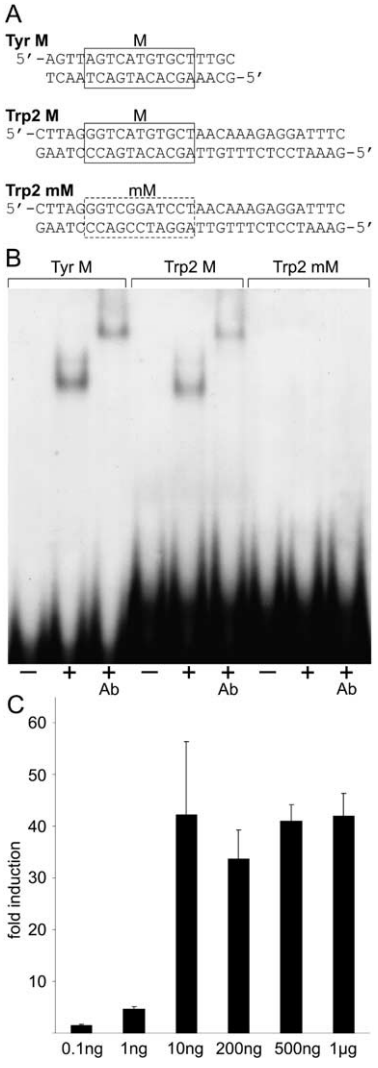

Fig. 6. Mitf binds to and activates the Dct/Trp2 promoter. A: Sequence of oligonucleotide probes containing the M-boxes from the mouse tyrosinase (Tyr M) and Dct/Trp2 promoters in wildtype (Dct/Trp2 M) and mutant (Dct/Trp2 mM) version. B: Electrophoretic mobility shift assay with (+) or without (−) GST-Mitf and oligonucleotide probes from A. Antibodies directed against the GST moiety (Ab) were added to the reaction resulting in a supershift of the protein–DNA complex. C: Transient transfections with the 1.1 Trp2 luc plasmid and increasing amounts of Mitf expression plasmid (0.1 ng–1 µg per well). Luciferase activities in extracts from transfected cells were determined in three independent experiments each performed in duplicate. Data are presented as fold induction ± S.E.M. with the activity for each reporter in the absence of co-transfected Mitf arbitrarily set to 1.

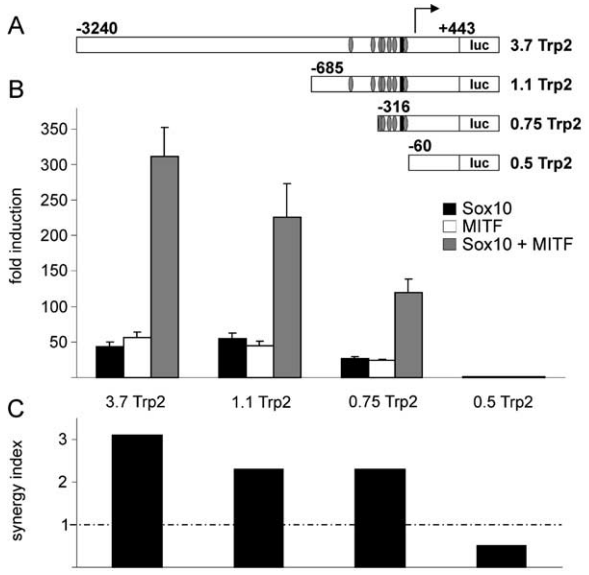

Fig. 7. Sox10 and Mitf synergistically activate the Dct/Trp2 promoter. A: Schematic representation of Dct/Trp2 promoter deletion constructs used to study synergy between Sox10 and Mitf. M-box (black bar) and Sox10 binding sites (gray ellipses) mapped in the Dct/Trp2 promoter are shown. B: Transient transfections were performed in N2A cells with the luciferase reporters depicted in A in presence of Sox10 (black bars), Mitf (white bars) or both proteins (gray bars). Luciferase activities in extracts from transfected cells were determined in three independent experiments each performed in duplicate. Data are presented as fold induction ± S.E.M. with the activity for each reporter in the absence of co-transfected transcription factor arbitrarily set to 1. C: Synergy indices were determined for each luciferase reporter shown in B by dividing the activation rate obtained with the combination of Sox10 and Mitf by the sum of the two activation rates obtained with either transcription factor alone. An index of 1 (broken line) equals additive activation.

cis-acting elements minimally required for synergistic activation of the Dct/Trp2 promoter are located in its proximal part between positions −316 and −60. Sequences between −685 and −316 furthermore seem to modulate this effect.

To analyze whether the observed synergy requires direct binding of Sox10 to the Dct/Trp2 promoter, we tested Dct/Trp2 promoter constructs with mutations in various Sox10 binding sites for their ability to be synergistically activated by Sox10 and Mitf. All mutant constructs exhibited comparable Mitf-dependent activation rates (Fig. 8A,C). Mutation of Sox10 binding site 1 reduced Sox10-dependent and overall induction rates, but the residual activation remained synergistic (Fig. 8A,B). Dct/Trp2 promoter constructs carrying mutations in either Sox10 binding site 4 or site 5 exhibited induction rates virtually identical to those obtained for the wildtype promoter (Fig. 8A). Synergy remained unaltered (Fig. 8B). Thus, none of the Sox10 binding sites alone mediated synergy which was lost only when a Dct/Trp2 promoter was used with combined mutations in all three major Sox10 binding sites (Fig. 8C,D). This argues that these three sites are involved in the synergistic activation of the Dct/Trp2 promoter, but can compensate for each other in this function.

A further set of Dct/Trp2 promoter mutants was generated to address the role of M-box and Mitf binding to the Dct/Trp2 promoter in synergy (Fig. 8E). Mutation of the M-box in the context of the 1.1 Trp2 luc construct led to a drastic reduction of Mitf responsiveness. Instead of a 49-fold induction, we now only observed an 11-fold induction of the Dct/Trp2 promoter by Mitf. It is unlikely that the remaining Mitf-dependent induction stems from residual binding to the M-box, as the introduced mutation abolished Mitf binding completely (Fig. 6B). The already mentioned E-box further upstream in the Dct/Trp2 promoter [29] is probably responsible. Sox10-dependent activation remained unaltered.

Despite the strong reduction of Mitf-dependent reporter gene activation, we still observed a significant synergistic stimulation of the Dct/Trp2 promoter by Sox10 and Mitf (Fig. 8F). This indicates that synergy can be achieved without Mitf binding to the M-box. Synergy was lost, however, when mutation of Sox10 binding site 1 was combined with mutation of the immediately adjacent M-box.

To further analyze the molecular mechanism underlying the observed synergy, Dct/Trp2 promoter activation studies were carried out with mutant Sox10 proteins (Fig. 9A). If synergy between Sox10 and Mitf is mediated mainly at the level of DNA binding, Sox10 mutants still capable of DNA binding should show at least some degree of synergy. This, however, was not the case as evidenced for three different Sox10 proteins with carboxy-terminal truncations, but intact HMG domain (Q377X, Y207X and MIC in Fig. 9B). Instead, removal of 90 amino acids from the carboxy-terminal transactivation domain already sufficed to abolish synergy completely (Fig. 9B). Activation rates were reduced even below the level obtained with Mitf alone. We also failed to obtain synergistic activation in the presence of a Sox10 variant, that is incapable of DNA binding due to an insertion of two amino acids in the DNA binding HMG domain, but is otherwise identical to the wildtype (095 in Fig. 9B). Thus we conclude that although not sufficient by themselves, both DNA binding and the presence of a transactivation domain are important prerequisites for synergy.

4. Discussion

4.1. Dct/Trp2 as a Sox10 target gene in melanocytes

Since its identification, Sox10 has been found to be an important transcriptional regulator in several cell types. Apart from oligodendrocytes within the central nervous system [16,39], all other Sox10-dependent cell types are derived from neural crest cells and include glial cells of the peripheral nervous system, chromaffin cells of the adrenal medulla, enteric neural crest and the pigment producing melanocytes [3–5,7].

We have previously studied the function of Sox10 in peripheral glia, where Sox10 regulates at least two types of genes, namely those responsible for early specification events [3] and those conveying glial identity during terminal differentiation [15,38]. Target genes with functions in different processes and stages of glial development are compatible with proposed multiple roles of Sox10 in survival, proliferation, specification and terminal differentiation of glial cells [3,12,13].

The present study throws light on the nature of Sox10 target genes in melanocytes. It has previously been shown that Sox10 directly regulates expression of the basic helix-loop-helix leucine zipper protein Mitf which is involved both in melanocyte specification and terminal differentiation in a wide variety of different species, and thus constitutes a critical regulator of melanocytes [18–22]. The epistatic relationship between Sox10 and Mitf could in principle account for most of

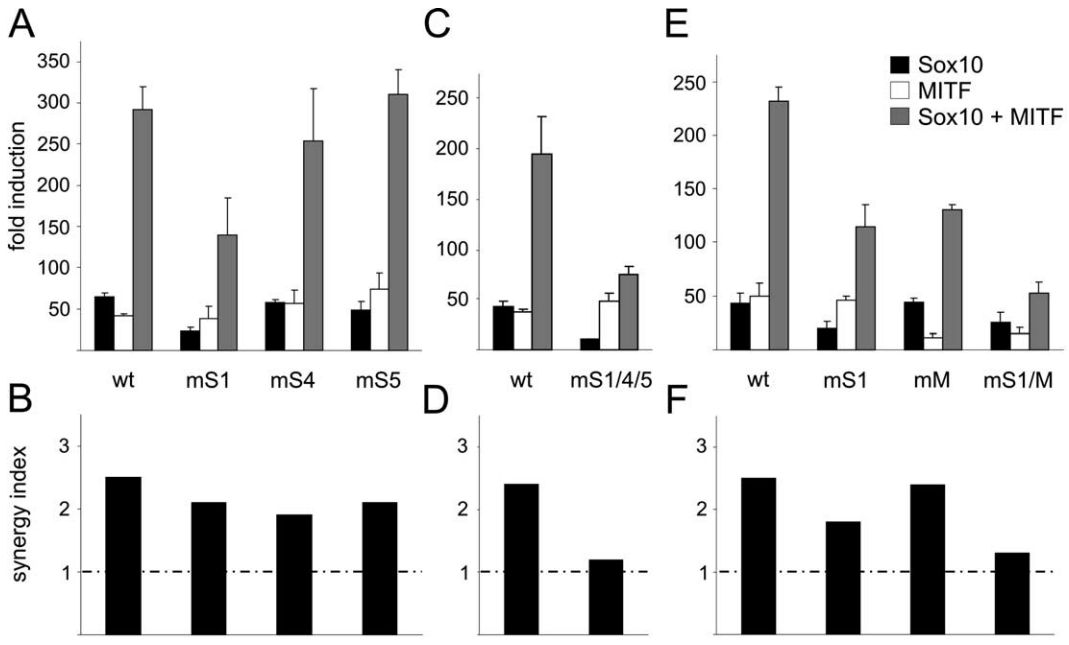

Fig. 8. Binding site requirements for synergistic activation of the Dct/Trp2 promoter. A,C,E: Transient transfections were performed in N2A cells with the 1.1 Trp2 luc reporter in wildtype (wt) or mutant versions in the presence of Sox10 (black bars), Mitf (white bars) or both proteins (gray bars). Mutations abrogated Sox10 binding to site 1 (mS1, mS1/M), site 4 (mS4), site 5 (mS5), or all three of these sites (mS1/4/5) as well as Mitf binding to the M-box (mM, mS1/M). Luciferase activities in extracts from transfected cells were determined in three independent experiments each performed in duplicate. Data are presented as fold induction ± S.E.M. with the activity for each reporter in the absence of co-transfected transcription factor arbitrarily set to 1. B,D,F: Synergy indices were determined for each luciferase reporter shown in A,C,E by dividing the activation rate obtained with the combination of Sox10 and Mitf by the sum of the two activation rates obtained with either transcription factor alone. An index of 1 (broken line) equals additive activation.

the Sox10-dependent effects on the melanocyte lineage [19]. In agreement with such an assumption, forced expression of Mitf in Sox10-deficient zebrafish rescued at least some melanocytes [19]. Impressive as they are, these results might not necessarily be transferable on a one to one scale from fish to mammals. Secondly, unphysiologically high Mitf levels during ectopic expression could easily override additional signal transduction pathways which are under Sox10 control, Mitf independent and needed for melanocyte development under normal conditions.

Here, we show that Dct/Trp2 is at least one additional direct target gene for Sox10 in the melanocyte lineage, thus strengthening the view that Sox10 might have functions in melanocytes different from Mitf regulation. Dct/Trp2, the target identified in this study belongs to the group of genes that define the melanocyte phenotype and is thus comparable to several previously identified Sox10 targets which define a glial phenotype such as the genes for Protein zero, connexin-32, myelin basic protein and proteolipid protein [15,16,38].

4.2. *Direct versus indirect regulation of Dct/Trp2 expression by Sox10*

Expression of Dct/Trp2 has previously been shown to be under the control of the Mitf transcription factor [29]. Thus Sox10 can influence Dct/Trp2 expression in two separate ways, (i) indirectly by stimulating Mitf expression, and (ii) directly by binding to the Dct/Trp2 promoter. This double mode of action appears to be particularly important during early embryonic development, as studies on mouse embryos have revealed a transient Sox10-dependence of Dct/Trp2 expression from embryonic day 10.5–12.5 [28]. Mitf levels might be initially below the threshold needed to activate Dct/Trp2

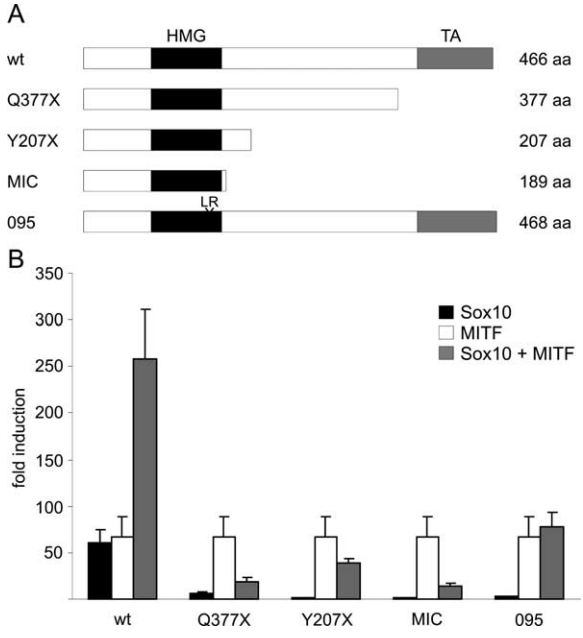

Fig. 9. Requirements of Sox10 domains for synergistic activation of the Dct/Trp2 promoter. A: Schematic representation of wildtype Sox10 and various mutant versions of the protein previously identified in human patients [9,31]. B: Transient transfections were performed in N2A cells with the 1.1 Trp2 luc reporter and expression plasmids for Mitf and the Sox10 proteins depicted in A either alone or in combination. Luciferase activities in extracts from transfected cells were determined in three independent experiments each performed in duplicate. Data are presented as fold induction ± S.E.M. with the activity for each reporter in the absence of co-transfected transcription factor arbitrarily set to 1.

expression. Alternatively, additional signalling pathways that will assist Mitf in the activation of the Dct/Trp2 promoter at later times might not yet be active during this early period so that Sox10 has to pitch in. Both elevated cAMP levels and Wnt signalling present candidates for such signalling pathways as they have been shown to activate the Dct/Trp2 promoter in tissue culture systems [29,30].

Direct and indirect pathways for Sox10-dependent activation of the Dct/Trp2 promoter converge in the synergistic activation of the Dct/Trp2 promoter by Mitf and Sox10. This synergy might allow Sox10 to potentiate Mitf function on the Dct/Trp2 promoter from day 10.5 to day 12.5 of embryonic development. It is poorly understood how Sox10 manages to regulate different sets of target genes in different Sox10-expressing cell types. Synergy between Sox10 and other transcription factors with cell type-restricted expression might be one of the mechanisms by which Sox10 function is modulated in a cell specific manner.

4.3. Mode of action of Sox10 on the Dct/Trp2 promoter

Analysis of the Dct/Trp2 promoter also reveals important aspects about the mode of action of Sox10. In agreement with findings on other target gene promoters, Sox10 exerts its function through multiple binding sites [15,16,18]. The contribution of each binding site to the overall activation rate differs significantly, with one or few sites usually accounting for most of the Sox10-dependent effects. Six binding sites were identified in the proximal promoter of the Dct/Trp2 gene. Of these sites, binding sites 1 and 4 were most important, as their influence was strongest and detected both in deletion and mutagenesis studies. Next were sites 5 and 6, which scored in only of these studies. The contribution of each binding site to Sox10-dependent activation of the Dct/Trp2 promoter roughly correlated with the affinity of each site for Sox10. Accordingly, site 1 and site 4 exhibited the highest affinity for Sox10. The presence of multiple Sox10 binding sites, each of which mediates part of the Sox10 response and binds the protein with different affinity, should make a promoter exquisitely sensitive to Sox10 dosage effects. This is indeed the case for Dct/Trp2.

Many target gene promoters contain binding sites for single Sox10 molecules as well as closely spaced sites that allow binding of two Sox10 molecules in a cooperative manner [15,16]. This ability for dimeric binding is a general feature of Sox10 and its relatives Sox9 and Sox8 [35], and is essential for their function both in vitro and in vivo [36,37]. The Dct/Trp2 promoter also contains a high-affinity dimeric site (site 4) as well as a high-affinity monomeric site (site 1) which both contribute significantly to the overall activation of the Dct/Trp2 promoter by Sox10. Site 4 conforms to the overall structure of these dimer sites with at least one of the 'half-sites' being a non-consensus site with weak affinity for single Sox10 molecules.

Some of the high-affinity monomeric sites in other target gene promoters are localized in close proximity to binding sites for other transcription factors that also regulate the target gene in question. Thus, the major Sox10 binding site within the Mitf promoter is in close proximity to a Pax3 binding site, and both of these sites contribute significantly to synergistic activation of the Mitf promoter by Pax3 and Sox10 [18,20]. As shown here, the monomeric Sox10 binding site 1 in the Dct/Trp2 promoter is immediately adjacent to the M-box, which mediates most of the Mitf-dependent activation of the Dct/Trp2 promoter [29].

4.4. Mechanism of synergy between Sox10 and Mitf

Given the close proximity of Sox10 binding site 1 and M-box, it might have been expected that it is this composite element that mediates synergistic activation of the Dct/Trp2 promoter by Sox10 and Mitf. However, this was not the case. Surprisingly, synergy persisted both in the absence of a functional M-box and in the absence of a functional Sox10 binding site 1. Synergy was only lost upon mutation of both sites. Several explanations exist for this observation. If Sox10 and Mitf were to interact directly, binding of one of the partners to the composite element might be sufficient to keep both proteins tethered to the Dct/Trp2 promoter. Such a mechanism seems plausible as both Mitf and Sox10 have each been shown to interact directly with other transcription factors [30,40,41]. However, we failed to detect any direct interaction between both proteins in GST pulldown experiments and co-immunoprecipitation assays under various experimental setups (data not shown). Similarly, no cooperative binding of Mitf and Sox10 to the composite element was observed in electrophoretic mobility shift assays (data not shown). While all of this may be due to technical difficulties, there is at least no strong evidence at present for a direct protein–protein interaction between both transcription factors.

How can synergy be explained in the absence of a direct protein–protein interaction between Sox10 and Mitf? Binding of Sox10 to its many sites might help arrange the Dct/Trp2 promoter into a three-dimensional enhanceosome configuration of which Mitf is as much a part as other transcription factors previously shown to bind or activate the Dct/Trp2 promoter such as CREB or Lef-1 [29,30]. Synergy could then result from the combined interaction of enhanceosome components with the transcription machinery or chromatin remodeling activities. While minor interferences with Sox10 binding to the Dct/Trp2 promoter could be tolerated, substantial binding loss would be incompatible with enhanceosome formation and result in a loss of synergy. This could explain why mutation of sites 1, 4 or 5 alone did not interfere with synergy, whereas combined mutation of all three sites abolished synergy completely. In the presence of full Sox10 binding activity, mutation of the M-box might be tolerated, because Mitf could still be recruited into the enhanceosome through interaction with enhanceosome components other than Sox10 such as Lef-1 [30,40] and through binding to an E-box within the Dct/Trp2 promoter to which Mitf displays weak affinity [29]. Upon partial loss of Sox10 binding, however, the enhanceosome would be too much destabilized already to tolerate additional mutation of the M-box. Mitf would no longer be recruited and as a consequence synergy between Sox10 and Mitf is lost.

Whatever the exact mechanism through which synergy is mediated, analyses of Sox10 mutants confirmed that synergy is critically dependent on direct binding of Sox10 to the Dct/Trp2 promoter. However, these studies also prove that DNA binding alone is not sufficient. Additionally, the carboxy-terminal transactivation domain of Sox10 is needed for synergistic activation. In the context of the proposed enhanceosome model, these results could mean that the transactivation domain of Sox10 provides interfaces with transcription or chromatin remodeling machineries. It strengthens our view that

the observed synergy is not so much a result of increased transcription factor binding to the Dct/Trp2 promoter, but a result of improved crosstalk with transcriptional components at a step subsequent to transcription factor binding.

Acknowledgements: This work was supported by a grant from the Deutsche Forschungsgemeinschaft to M.W. (We1326/7-2). C. Goding, I. Jackson and A. Bosserhoff are acknowledged for the gift of Mitf cDNA, Dct/Trp2 promoter and HMB2 cells, respectively.

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