Developmental Biology **237,** 245–257 (2001)
doi:10.1006/dbio.2001.0372, available online at http://www.idealibrary.com on IDEAL®

Analysis of SOX10 Function in Neural Crest-Derived Melanocyte Development: SOX10-Dependent Transcriptional Control of Dopachrome Tautomerase

S. Brian Potterf,* Ramin Mollaaghababa,* Ling Hou,* E. Michelle Southard-Smith,* Thomas J. Hornyak,† Heinz Arnheiter,‡ and William J. Pavan*,[1]

**Genetic Disease Research Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland 20892; †Henry Ford Health System, Dermatology Research, Detroit, Michigan 48202; and ‡Laboratory of Developmental Neurogenetics, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland, 20892*

SOX10 is a high-mobility-group transcription factor that plays a critical role in the development of neural crest-derived melanocytes. At E11.5, mouse embryos homozygous for the *Sox10^{Dom}* mutation entirely lack neural crest-derived cells expressing the lineage marker KIT, MITF, or DCT. Moreover, neural crest cell cultures derived from homozygous embryos do not give rise to pigmented cells. In contrast, in *Sox10^{Dom}* heterozygous embryos, melanoblasts expressing KIT and MITF do occur, albeit in reduced numbers, and pigmented cells eventually develop in nearly normal numbers both in culture and *in vivo*. Intriguingly, however, *Sox10^{Dom}*/+ melanoblasts transiently lack *Dct* expression both in culture and *in vivo*, suggesting that during a critical developmental period SOX10 may serve as a transcriptional activator of *Dct*. Indeed, we found that SOX10 and DCT colocalized in early melanoblasts and that SOX10 is capable of transactivating the *Dct* promoter *in vitro*. Our data suggest that during early melanoblast development SOX10 acts as a critical transactivator of *Dct*, that MITF, on its own, is insufficient to stimulate *Dct* expression, and that delayed onset of *Dct* expression is not deleterious to the melanocyte lineage.

Key Words: **neural crest; development; melanocyte; DCT; mouse embryo; pigmentation; MITF; KIT; SOX10.**

INTRODUCTION

The embryonic neural crest (NC) gives rise to multipotent cells originating in the dorsal part of the neural tube that are capable of differentiating into several lineages, including the peripheral nervous system (PNS), glia, bone and cartilage, adrenomedullary cells, and melanocytes of the skin, hair, and inner ears. Melanoblasts (nondifferentiated melanocyte precursors) appear at E10.5 and migrate along a dorsolateral pathway, moving through the dermis to the epidermis before taking up final positions in the hair follicles and epidermis, whereupon differentiation into pigment-producing melanocytes occurs.

Several mouse models have proven useful in studying NC-derived melanocyte development. For example, mutations in MITF (*microphthalmia*) can result in the complete lack of NC melanocytes due to early developmental loss (Nakayama *et al.*, 1998; Opdecamp *et al.*, 1997). PAX3 (*Splotch*) mutations result in melanocyte deficiencies, while mutations in KIT (*Dominant spotting*) do not apparently affect commitment to the melanocyte lineage but result in a subsequent failure of melanoblasts to survive past early stages (Hou *et al.*, 2000; Mackenzie *et al.*, 1997; Wehrle-Haller and Weston, 1995). We have determined that the hypopigmentary and enteric ganglion defects associated with the *Dominant megacolon* mouse are due to a muta-

[1] To whom correspondence should be addressed. Fax: (301) 402-2170. E-mail: bpavan@nhgri.nih.gov.

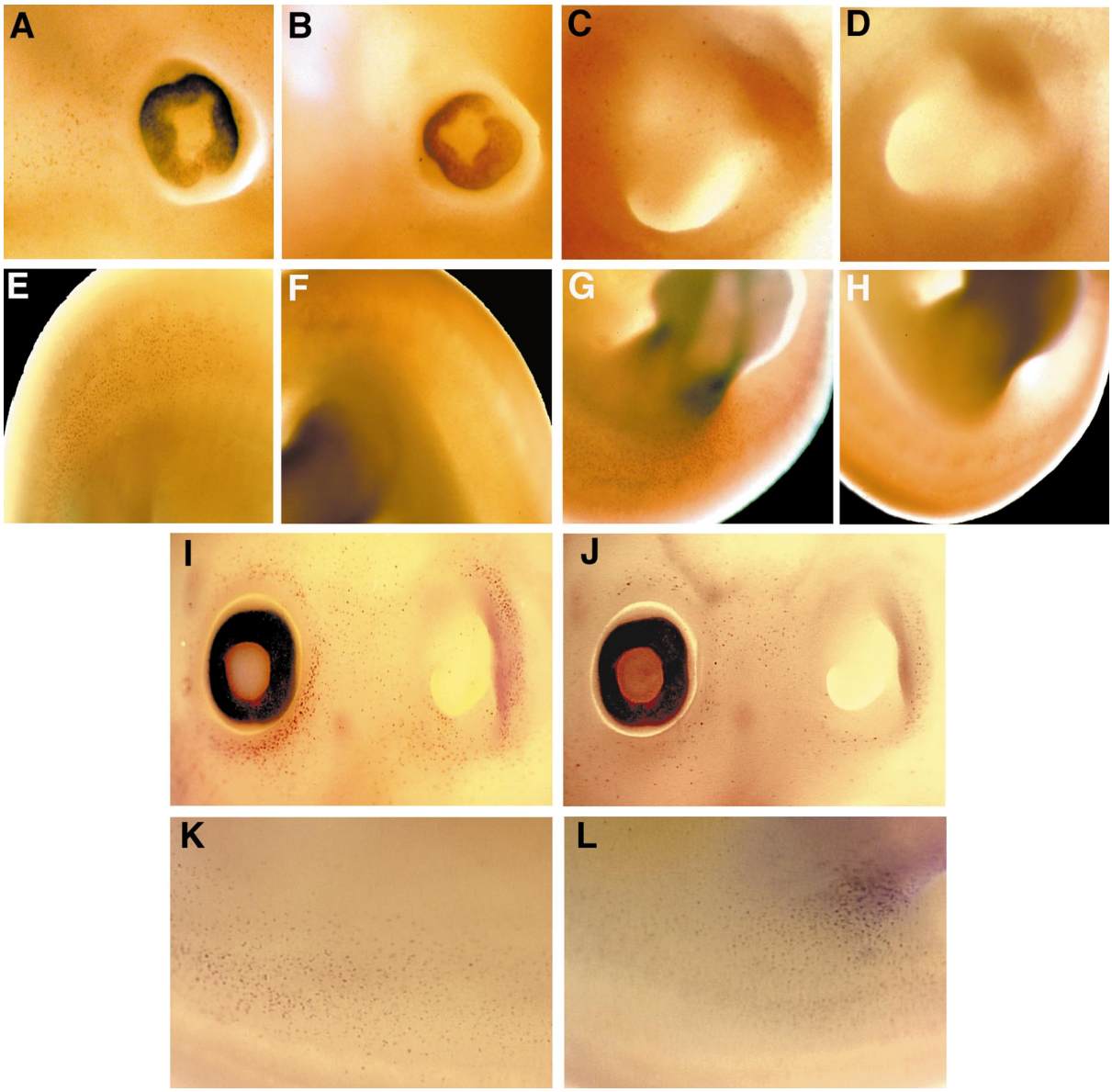

FIG. 1. *In situ* hybridization for *Dct* expression in wild-type and *Sox10*^{Dom}/+ E11.5–E13.5 mouse embryos. *Dct* expression is greatly reduced in *Sox10*^{Dom}/+ embryos. *Dct* expression pattern revealed by *in situ* for *Dct* in wild-type E11.5 head and trunk (A, E), E12.5 pinna and caudal trunk (C, G), and E13.5 head and caudal trunk (I, K). Same probe was used in age-matched *Sox10*^{Dom}/+ mutant embryos, E11.5 head and caudal trunk (B, F), E12.5 pinna and caudal trunk region (D, H), and E13.5 head and caudal trunk (J, L). E13.5 *Dct* expression is clearly apparent in *Sox10*^{Dom}/+ embryos.

tion within the *SOX10* gene (*Sox10*^{Dom} allele) (Southard-Smith *et al.*, 1998). SOX10 is a member of the SRY (sex-determining factor)-like, HMG (high-mobility group) DNA binding proteins (Wegner, 1999) that govern cell fate decisions during embryonic development. It has a highly restricted tissue distribution and plays a critical role in the development of NC derivatives (Herbarth *et al.*, 1998; Kuhlbrodt *et al.*, 1998a; Southard-Smith *et al.*, 1998). The *Sox10*^{Dom} mutation is a single base insertion downstream of the DNA binding region that results in premature truncation of the C-terminal transactivation domain, with the addition of 99 extraneous amino acids (Herbarth *et al.*, 1998; Southard-Smith *et al.*, 1998). In *Sox10*^{Dom}/*Sox10*^{Dom} embryos, NC development is severely disrupted (Southard-Smith *et al.*, 1998; Kapur, 1999). *Sox10*-expressing NC cells form and are located at the dorsal portion of the neural tube.

TABLE 1
Melanoblast/Melanocyte Marker Expression in Neural Tube Cultures

	MITF		DCT		Melanin	
Genotype	2 day	14 day	2 day	14 day	2 day	14 day
Wild type	4/4	5/5	4/4	5/5	0/4	5/5
Sox10^{Dom}/+	5/5	3/3	0/5	3/3	0/5	3/3
Sox10$^{Dom/Dom}$	0/2	0/3	0/2	0/3	0/2	0/3

[a] Melanocyte differentiation was determined by identification of melanin granules within cells and a typical dendritic morphology as visualized using phase-contrast and bright-field microscopy.
[b] Values represent number of cultures positive for respective marker per total number of cultures.

However, by E10.5, NC cells in rostral portions of the embryo are greatly reduced; by E11.5, *Sox10*-expressing cells are mostly gone, presumably due to apoptosis.

A complete lack of expression of the melanoblast marker dopachrome tautomerase (*Dct*) (Steel *et al.*, 1992) in E11.5 *Sox10*^{Dom}/*Sox10*^{Dom} embryos (Southard-Smith *et al.*, 1998) indicated that the melanocyte lineage is affected in *Sox10* mutants. In addition to *Sox10*, a number of mutations in other factors important to NC-derived melanocyte development have been found. Like *Sox10*, *Ednrb* and *Edn3* have been implicated in melanocyte malformations resulting in depigmented patches and sensorineural deafness in human Waardenburg syndrome (WSIV) (Hofstra *et al.*, 1996; Puffenberger *et al.*, 1994). Mutations in transcription factors PAX3 (WSI and III) and MITF (WSIIa) also result in the type of aberrant melanocyte development present in Waardenburg syndrome (Read and Newton, 1997; Tachibana, 1999). The molecular mechanisms that underlie the effects of these mutations on NC development are not fully understood. Recent reports have shown that SOX10, either alone (Bondurand *et al.*, 2000; Lee *et al.*, 2000; Potterf *et al.*, 2000; Verastegui *et al.*, 2000) or in a synergistic relationship with PAX3 (Bondurand *et al.*, 2000; Potterf *et al.*, 2000), can directly regulate *Mitf* gene expression, leading to the development of a model in which mutations in factors that affect *Mitf* expression or function are causal agents of the depigmented patches and sensorineural deafness common to WS patients. This suggests a transcriptional hierarchy headed by SOX10 in which potential downstream targets such as *Mitf* and others are dependent on functional SOX10 for proper NC-derived melanocyte development. However, a detailed analysis of melanoblast development in *Sox10* mutant embryos has not been performed, and the role of SOX10 in melanocyte development remains obscure.

In mammals, the role of MITF in the process of pigmentation (melanogenesis) in differentiated melanocytes involves regulating the expression of tyrosinase (*albino* locus) (Tanaka *et al.*, 1990), a melanocyte-specific enzyme responsible for the initial, rate-limiting steps in melanin synthesis from tyrosine (Hearing, 1993). Also important to this process are two melanocyte-specific enzymes responsible for regulating the type, quality, and amount of generated mela-

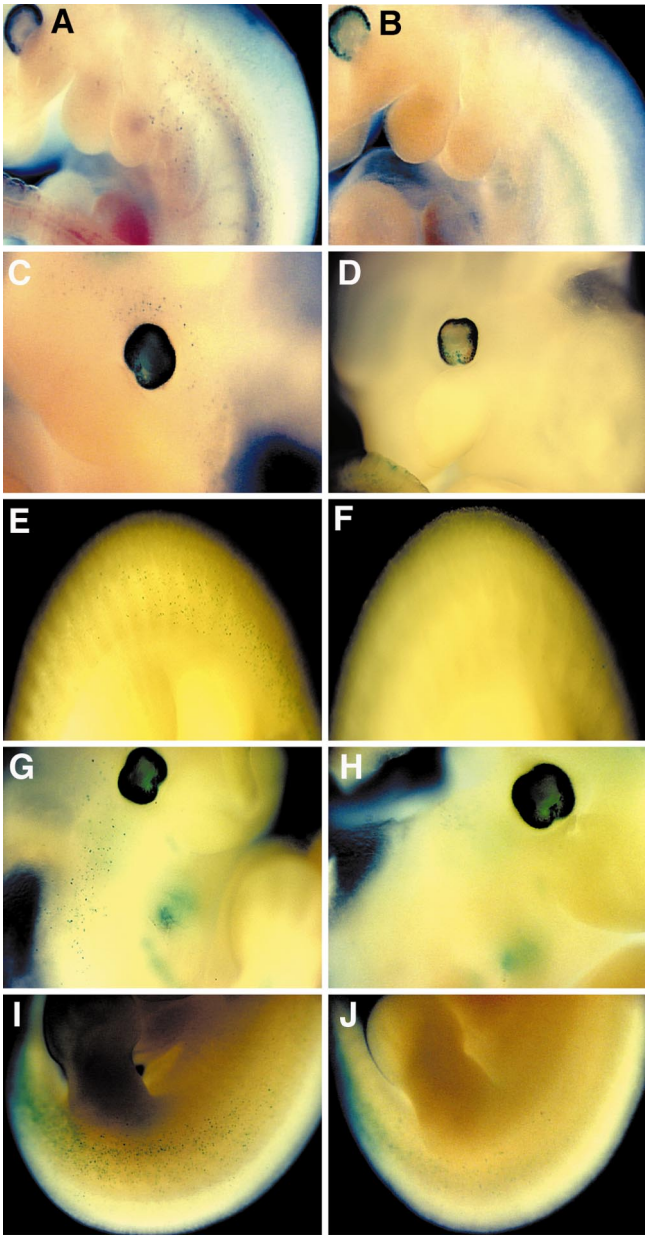

FIG. 2. *Dct* transgene expression in wild-type and *Sox10*^{Dom}/+ mutant E10.5–E11.5 mouse embryos. No DCT production is apparent in *Sox10*^{Dom}/+ embryos. *Dct* transgene expression in otic vesicle region of E10.5 wild-type embryo (A). Transgene expression in mutant E10.5 embryo (B). *Dct*-driven transgene expression in wild-type E11.0 (C, E) and E11.5 (G, I) head and trunk. *Dct* transgene expression in *Sox10*^{Dom}/+ E11.0 (D, F) and E11.5 (H, J) head and trunk regions. Note the variable levels of *Dct* transgene expression in the eye. Since *Sox10* is not expressed in the eye, it is not expected to be linked to *Dct* expression.

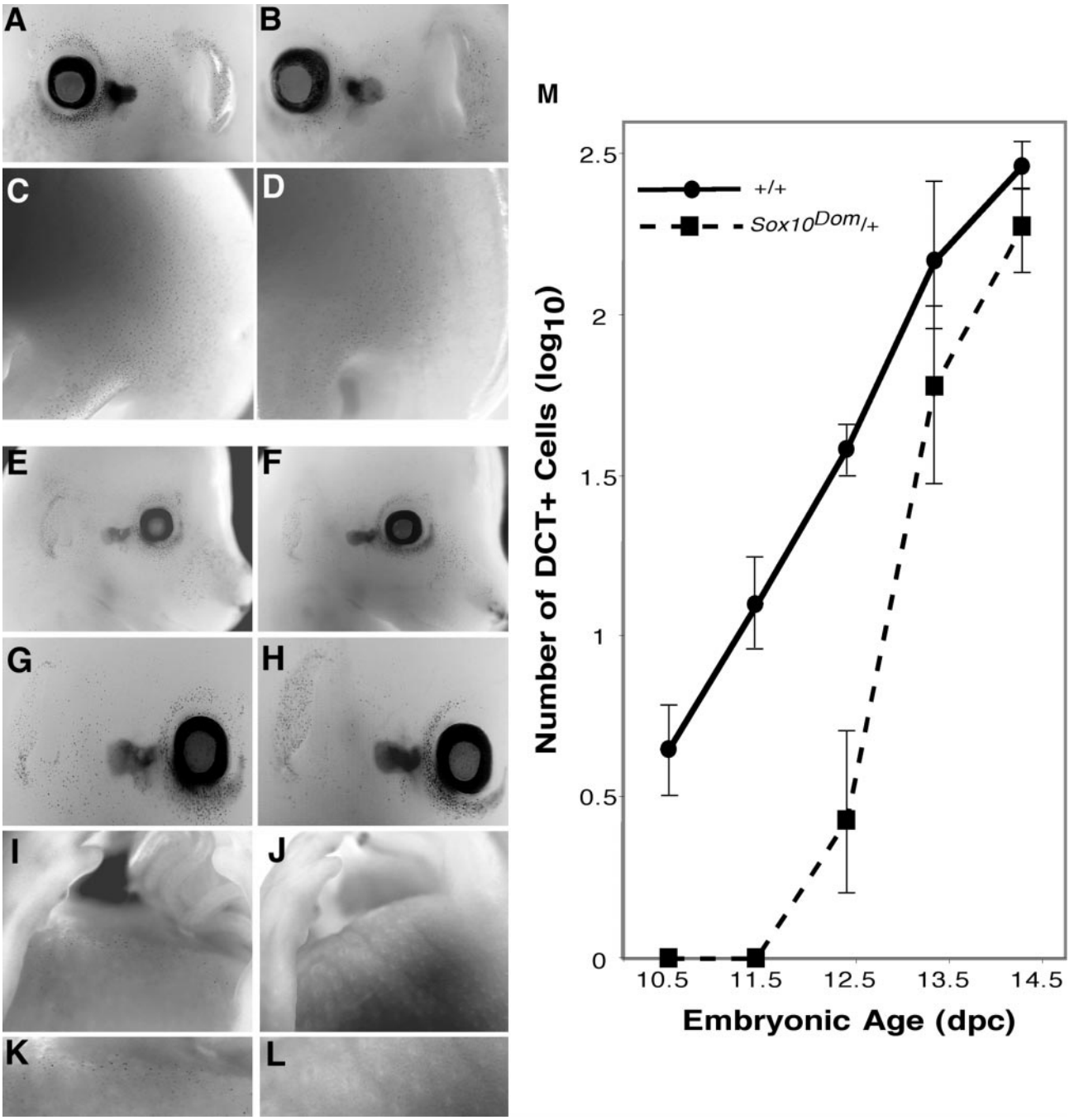

FIG. 3. E13.5 and E14.5 *Dct* transgene expression in wild-type and *Sox10*^{Dom}/+ embryos in head, ear, trunk, and ventrum. Wild-type *Dct* transgene expression in E13.5 head and ear regions (A) and caudal trunk (C). *Sox10*^{Dom}/+ *Dct* transgene expression in E13.5 head and ear regions (B) and caudal trunk (D). Wild-type transgene expression in E14.5 head (E); higher magnification of head (G); ventrum (I); and higher magnification of ventrum (K). *Sox10*^{Dom}/+ *Dct* transgene expression in E14.5 head (F); higher magnification of head (H); ventrum (J); and higher magnification of ventrum (L). Very few melanoblasts colonize the ventrum in mutant embryos at this stage of development. Comparison of the number of DCT+ cells (*y* axis) present directly above the eye in wild-type (filled circle and solid line) and *Sox10*^{Dom}/+ embryos at different embryonic ages (*x* axis) (M). The number of DCT+ cells was counted from three *Dct-LacZ* transgenic embryos for each data time point and is represented as the log of the mean values. Vertical bars represent the standard deviation of the mean.

nin: DCT (also known as TYRP2 or tyrosinase-related protein 2; *slaty* locus) (Jackson *et al.,* 1992; Tsukamoto *et al.,* 1992a), and TYRP1 (tyrosinase related protein 1; *brown* locus) (Jackson, 1988; Shibahara *et al.,* 1986). In the mouse, dopachrome tautomerase activity associated with DCT converts melanin biosynthetic intermediate dopachrome to a less toxic and more stable melanin precursor, 5,6-dihydroxyindole-2-carboxylic acid (DHICA), which serves as a substrate for TYRP1 prior to incorporation into melanin polymer (Jackson *et al.,* 1992; Tsukamoto *et al.,* 1992b). *Dct* is expressed early in E10.5 NC-derived melanoblasts (Steel *et al.,* 1992), appearing several hours after *Mitf* and *Kit* expression (Opdecamp *et al.,* 1997), near the dorsal midline, otic vesicle, and cranial regions, and has been a useful marker for analyzing early melanocyte development in mice. *In vivo,* utilization of DCT as a marker has been particularly important in elucidating the roles of melanoblast genes *Mitf* (Hornyak *et al.,* 2001; Nakayama *et al.,* 1998; Opdecamp *et al.,* 1997), *Ednrb* (Pavan and Tilghman, 1994), *Pax3* (Hornyak *et al.,* 2001) and *Kit* (Mackenzie *et al.,* 1997; Wehrle-Haller and Weston, 1995; Steel *et al.,* 1992) in melanocyte development. Accordingly, we have used these markers in the present study to analyze the roles of *Sox10* in NC-derived melanoblast development, both *in vivo* and *in vitro.* Our results suggest a critical function for SOX10 in initial melanoblast determination, showing that SOX10 transiently regulates *Dct* expression during early melanocyte development and that this transient regulation appears to be independent of MITF function.

MATERIALS AND METHODS

Mice and Genotyping

Dominant megacolon ($Sox10^{Dom}$/+) mice were obtained from the Jackson Laboratory and were maintained on a C57BL6/C3HeB/FeJLe-a/a background. $Mitf^{mi}$/+ mice were maintained on a C57BL6/C3Fe-a/a background; $Pax3^{Sp}$/+ mice were maintained on a C57BL/6 background and both were obtained from the Jackson Laboratory. $Kit^{W\text{-}lacZ}$/+ mice were obtained from J. J. Panthier (Bernex *et al.,* 1996), and were maintained on a mixed C57BL6/C3H129Sv background. In $Kit^{W\text{-}lacZ}$/+ mice, the β-galactosidase gene containing a nuclear localization signal was inserted into exon 1 of the *Kit* locus (Bernex *et al.,* 1996). Therefore, resultant β-galactosidase expression is indicative of KIT expression patterns in heterozygous (or haploinsufficient) animals. $Sox10^{Dom}$, $Mitf^{mi}$, and $Pax3^{Sp}$ genotypes were determined by PCR (Southard-Smith *et al.,* 1998; Hornyak *et al.,* 2001). *LacZ* genotyping with modifications was performed as previously described (Bernex *et al.,* 1996). NIH guidelines for animal care were followed.

Transgenic Construction

The transgene was constructed by insertion of a β-galactosidase coding sequence (*LacZ*) in front of approximately 3.5 kb of 5′ upstream genomic regulatory sequence (Budd and Jackson, 1995) into the plasmid pPDct (Hornyak *et al.,* 2000) to generate the plasmid pPDct/lacZ (Hornyak *et al.,* 2001). A linearized fragment containing the *Dct* promoter and *LacZ* was obtained by digestion with *Not*I, and subsequently linear DNA was purified and used in the construction of transgenic mice by standard techniques. Mice on an FVB inbred background were used for transgenic construction and were maintained either by brother–sister matings or by mating to FVB mice. Three founders were derived and found to have *LacZ* expression patterns comparable to endogenous *Dct* (Mackenzie *et al.,* 1997; Pavan and Tilghman, 1994; Steel *et al.,* 1992). Line "I" was chosen as having the most stable and reproducible *Dct* promoter-driven *LacZ* expression pattern of DCT throughout several generations. Previously published *Dct* transgenic mice (Hornyak *et al.,* 2001; Mackenzie *et al.,* 1997) have an extremely strong ectopic dorsal root ganglion (DRG) staining as well as staining in melanoblasts and have proven valuable for embryo whole-mount analysis. Line "I" (this study), in contrast, had little or no ectopic β-galactosidase in the DRG, with melanoblast staining still being readily detectable, thus establishing a system suitable for further study, particularly in neural tube explants and cell culture.

Whole-Mount in Situ Hybridization of Mouse Embryos and X-Gal Staining

Timed matings were set up to obtain staged mouse embryos, designating the noon of plug formation as E0.5. Embryos were fixed for 3 h to overnight in 4% paraformaldehyde/1× PBS. *In situ* hybridizations were performed as previously described (Kos *et al.,* 1999). For detection of β-galactosidase activity in whole mounts, embryos were fixed in 1% formaldehyde/0.2% glutaraldehyde/0.02% NP40 in PBS for 30–90 min on ice and visualized by using X-gal as previously described (Bernex *et al.,* 1996).

Neural Tube Culture, Antibodies, and Immunohistochemistry

E9.5 murine embryos were obtained from either $Sox10^{Dom}$/+ (X) $Sox10^{Dom}$/+ or $Sox10^{Dom}$/+ (X) *Dct-LacZI/Dct-LacZI* matings. Neural tube explants were prepared as described previously (Hou *et al.,* 2000). Resultant neural tubes were placed in fibronectin-coated wells containing DMEM, 10% FBS, and endothelin-3 (EDN3) (5 nM final). Neural tube cultures were grown for 2, 5, or 14 days with medium changes every other day prior to fixation. Cultures were fixed in 4% paraformaldehyde/1× PBS (pH 7.5) for 25 min at RT, followed by 0.1% Triton X-100 permeabilization for 5 min. For double indirect immunolabeling (Table 1 only), NC cultures were incubated with mouse primary antibodies against MITF (C5, Neo-Markers, Inc) or rabbit antibodies against DCT (αPEP8, courtesy of Vincent Hearing, NIH) for 60 min. The primary antibodies were revealed with RITC-coupled goat anti-rabbit $(Fab)_2$ and FITC-coupled goat anti-mouse $(Fab)_2$ (Molecular Probes). The samples were examined and photographed with a Reichert–Jung Polyvar microscope.

Polyclonal anti-SOX10 antibody was generated by using a highly purified peptide corresponding in sequence to the SOX10 amino acids 329–346 with an additional cysteine residue included at the N-terminal end (CDHRHPEEGSPMSDGNPEH-COOH). The antibody was purified by immunoaffinity chromatography with the purified peptide 329–346 immobilized on a SulfoLink column (Pierce) using its terminal cysteine. The purified antibody was tested by immunofluorescence and by Western analysis of extracts from melan-c (positive control) and NIH3T3 (negative control) cells (R.M. and W.J.P., unpublished observations).

Fixation and preparation of neural tube cultures for SOX10 and

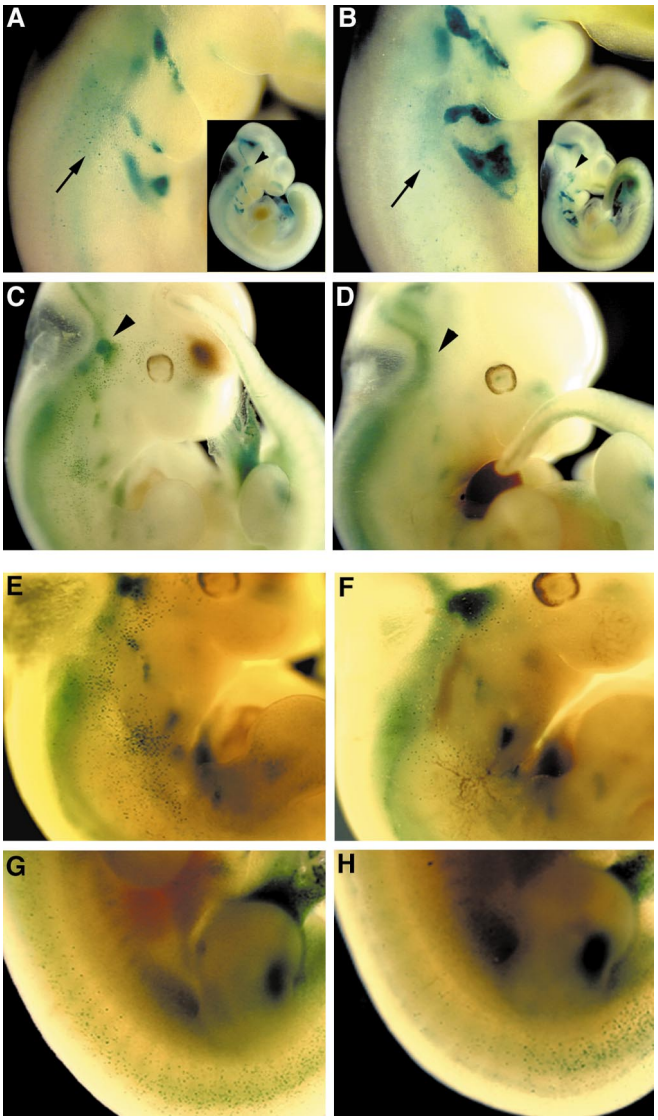

FIG. 4. KIT expression in E10.5–E11.5 embryos derived from *Sox10*^{Dom}/+; *Kit*$^{W\text{-}lacZ}$/+ matings. KIT expression in E10.5 otic vesicle region in wild-type (A) and *Sox10*^{Dom}/*Sox10*^{Dom} (B) embryos. Wild-type and *Sox10*^{Dom}/*Sox10*^{Dom} E11.5 whole embryo KIT expression (C, D). Head and ear KIT expression in wild-type (E) and *Sox10*^{Dom}/+ (F) E11.5 embryos. Rostrocaudal trunk KIT expression in wild-type (G) and *Sox10*^{Dom}/+ (H) E11.5 embryos. Arrows indicate melanoblasts; arrowheads indicate trigeminal ganglion. Melanoblast and trigeminal ganglion KIT expression is absent by E11.5 in *Sox10*^{Dom}/*Sox10*^{Dom} mutant embryos. Melanoblasts are still present on a *Sox10*^{Dom}/+ background, although decreased in number.

βgal double-labeling were carried out as described above. The cells were incubated with diluted, purified anti-SOX10 antibody for 1 h at 37°C. Anti-rabbit rhodamine-conjugated secondary antibody (Molecular Probes) was diluted 1:400 and added to the cells for incubation at 37°C for 1 h. Following removal of excess secondary antibody, cells were incubated in mouse anti-βgal antibody (1:150) (Promega), for 1 h at RT. Anti-mouse fluorescein-conjugated secondary antibody (Molecular Probes) was diluted 1:200 and added to the cells for 1 h, RT.

Cell Line Culture, Plasmid Construction, and Transient Transfections

Melan-a and melan-c (immortalized murine melanocyte cell lines, pigmented and albino, respectively) were obtained from D. Bennett (St. Georges Hospital, London) and were maintained in RPMI 1640, supplemented with 5% FBS, 10 U/ml penicillin/streptomycin, L-glutamine, sodium bicarbonate, sodium pyruvate, and 100 nM phorbol 12-myristate 13-acetate and kept at 10% CO_2. NIH3T3 and HeLa cells were maintained and grown in DMEM supplemented with 10% FBS and 10 U/ml penicillin/streptomycin and were kept in 5% CO_2. The cells were routinely passaged every 3–4 days and discarded after 15 passages. The murine SOX10 and SOX10 deletion expression vector constructions have been described previously (Potterf *et al.*, 2000). The *Dct* reporter was constructed by inserting the luciferase coding region from the plasmid pGL2-Basic (Promega) into the *Dct* expression vector pPDct (Hornyak *et al.*, 2000) to generate plasmid pPdct/luc (henceforth referred to as pMuDct). The human *Dct* promoter was generated by PCR (M. Furumura, unpublished observations) and was placed upstream of firefly luciferase to generate plasmid pHuDct. Transient transfections were conducted as published previously (Potterf *et al.*, 2000). Reporter assays were performed by using the dual luciferase assay system (Promega) with *Renilla* luciferase serving as an internal control. Luciferase activities were determined by using a Lumat LB 9507 luminometer (EG&G Berthold). All experiments were conducted at least three times on different days using different batches of cells.

RESULTS

Absence of Melanoblast Marker Expression in Mutant Neural Tube Cultures

To explore the functional role of SOX10 in NC-derived melanocyte development, we analyzed the expression of melanoblast markers in neural tube explants. E9.5 neural tubes were cultured for 2 or 14 days (corresponding to E11.5 and 4 days postnatal, respectively) and examined for pigmentation and expression of MITF and DCT.

Pigmenting melanocytes are generally apparent by 6–10 days in neural tube cultures. In 14-day cultures, in explants derived from *Sox10*^{Dom}/*Sox10*^{Dom} embryos (Table 1), pigmentation was absent and no MITF or DCT expression was observed. In fact, *Sox10*^{Dom}/*Sox10*^{Dom} cultures grew to smaller diameters than wild-type and heterozygous cultures, suggesting extensive defects in cell growth. Since all cultures include EDN3, this experiment also showed that EDN3 could not complement the absence of functional SOX10 *in vitro*. Even in early (2-day) *Sox10*^{Dom}/*Sox10*^{Dom} explants, MITF expression was absent. However in 2-day wild-type and heterozygous cultures, NC cells expanded from the neural tube and NC-derived melanoblasts expressed MITF (Table 1). This is consistent with our previous observation that *in vivo Sox10*^{Dom}/*Sox10*^{Dom} embryos ini-

tially form NC cells expressing *Sox10*, but fail to develop further due to extensive apoptosis (Southard-Smith *et al.*, 1998). These data also support our hypothesis that mutations in *Sox10* disrupt *Mitf* expression, resulting in a failure of melanocytes to develop (Lee *et al.*, 2000; Potterf *et al.*, 2000). Together, these results are consistent with an essential role for SOX10 early in NC-derived melanocyte development.

Examination of melanocyte development in $Sox10^{Dom}$/+ neural tube cultures yielded an unexpected finding. While MITF+, DCT+, and pigmented cells were present in 14-day $Sox10^{Dom}$/+ neural tube cultures, DCT was not detected in 2-day $Sox10^{Dom}$/+ neural tube cultures even though MITF was present (Table 1). This result suggested that SOX10 might have a selective role in *Dct* expression and that the presence of MITF was not sufficient to induce *Dct* production.

Transient Absence of DCT + Melanoblasts in $Sox10^{Dom}$/+ Embryos in Vivo

We had previously shown that *Dct* expression is absent in NC-derived cells in E11.5 $Sox10^{Dom}/Sox10^{Dom}$ embryos *in vivo* (Southard-Smith *et al.*, 1998) although it is present in the retinal pigmented epithelium (RPE) and the telencephalon. To further investigate our current *in vitro* observation that DCT is also absent in 2-day $Sox10^{Dom}$/+ cultures, we extended the analysis to *in vivo* studies. *In situ* hybridization in E11.5 and E12.5 wild-type embryos revealed NC-derived DCT+ cells in a characteristic distribution (Steel *et al.*, 1992) in the head and trunk regions, as well as pigmented neuroepithelium-derived cells in the eye (Figs. 1A, 1C, 1E, 1G). Interestingly, and consistent with our neural tube culture studies, DCT+ melanoblasts were almost entirely lacking in NC-derived cells of the head and trunk regions in E11.5 and E12.5 $Sox10^{Dom}$/+ mutant embryos (Figs. 1B, 1D, 1F, 1H). Only a very few *Dct*-expressing cells were present in the most caudal tail region of these two stages. Neuroepithelial *Dct* expression in the eye, however, was not affected. An absence of DCT+ melanoblasts was unexpected given that $Sox10^{Dom}$/+ mice are mostly pigmented with only ventral and occasional head spotting (Southard-Smith *et al.*, 1999). In the developing eye, *Dct* expression and pigmentation were not disrupted in $Sox10^{Dom}$/+ mutants and an occasional DCT+ cell could be seen around the otocyst or in the head of the embryo. *Dct* expression in the eye is expected since *Sox10* is not expressed in the RPE (Southard-Smith *et al.*, 1999; Herbarth *et al.*, 1998) and no apparent abnormalities are seen in the eye of *Dominant megacolon* mice. As we observed in neural tube cultures, the absence of *Dct* expression in $Sox10^{Dom}$/+ mutant embryos was transient, with a large increase in *Dct*-positive melanoblast numbers appearing at E13.5 (Figs. 1J and 1L). The melanoblasts populate the $Sox10^{Dom}$/+ embryos in a correct spatial manner, but are still significantly reduced in number compared to wild type (Figs. 1I–1L). The transient absence of DCT+ melanoblasts in restricted portions of E11.5–E12.5 $Sox10^{Dom}$/+ embryos could be due either to a lack of melanoblasts at early stages or to the absence of *Dct* expression in otherwise correctly specified melanoblasts. Our results indicate the latter (*vide infra*). Furthermore, the latter possibility is supported by the observation that $Sox10^{Dom}$/+ adult mice are pigmented, indicating that functional melanocytes are present at birth.

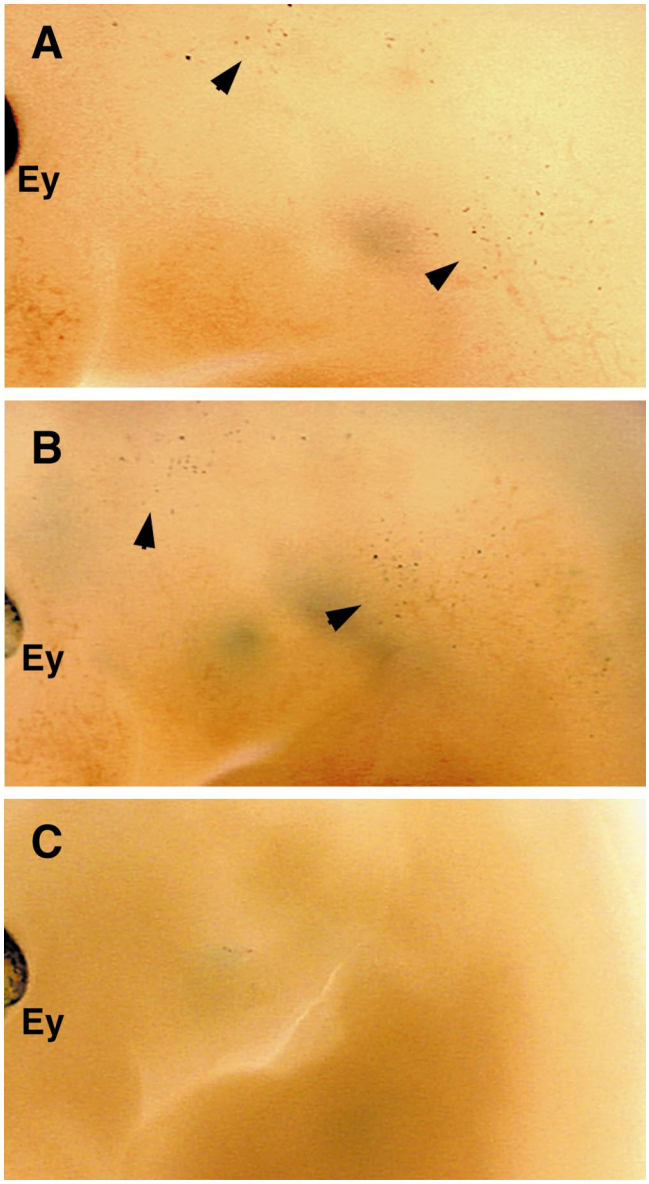

FIG. 5. *Dct* transgene expression in E11.5 embryos heterozygous for mutations in early melanoblast transcription factors. *Dct* transgene expression in head and ear regions in $Pax3^{Sp}$/+ (A), $Mitf^{mi}$/+ (B), and $Sox10^{Dom}$/+ (C) E11.5 mouse embryos. All three NC mutants were mated to *Dct-LacZI* transgenic mice. Ey, eye. Arrowheads point to clusters of DCT+ melanoblasts.

Transient Lack of Dct-LacZ Transgene Expression in Sox10^{Dom}/+ Melanocyte Development

To further test whether there is a reduction of DCT expression in *Sox10^{Dom}*/+ melanoblasts, we engineered a transgenic line that expresses β-galactosidase from a 3.5-kb minimal *Dct* promoter (see Materials and Methods). Xgal staining in E10.5–E11.5 transgenic embryos exhibited a pattern similar to that of the endogenous *Dct* mRNA *in situ* expression, indicating that the DCT transgenic line represents an accurate measure of DCT production at these ages (Figs. 2A–2J). At E10.5, a typical melanoblast distribution pattern in wild-type embryos was found, but in age-matched *Sox10^{Dom}*/+ embryos, DCT expression pattern was mostly absent in NC-derived melanoblasts and was retained only in the optic cup (Figs. 2A and 2B). The lack of β-galactosidase expression was also observed in rostral regions of E11.0 and E11.5 *Sox10^{Dom}*/+ embryos (Figs. 2D, 2F, 2H, 2J), with only few lightly staining cells present in the rostral tail region and an occasional cell in the otocyst or head of the embryo. Similar results were observed in a second line of *Dct-LacZ* mice (Hornyak *et al.*, 2001) (data not shown). These data are consistent with the *in situ* hybridizations (Figs. 1E and 1F), indicating that there is an almost complete lack of DCT+ melanoblasts in restricted portions of E10.5–E11.5 *Sox10^{Dom}*/+ embryos. These data also indicate that the information required to mediate the induction of *Dct* expression in the usual temporal pattern observed in embryos is contained within the minimal promoter segment used to make the *Dct-LacZI* transgenic mice (see transfection studies below).

Examination of β-galactosidase production at later ages also confirmed that the lack of DCT+ cells was transient. Late E12.5 *Sox10^{Dom}*/+; *Dct-LacZI* transgenic embryos showed a few DCT-positive cells around the developing ear and in the face, with greater numbers appearing in the trunk region adjacent to the hindlimb (data not shown). These cells were still very reduced in number and signal intensity compared to what we observe in E12.5 *Dct-LacZI* transgenic embryos. However, E13.5 *Sox10^{Dom}*/+; *Dct-LacZI* mutant embryos showed a large number of DCT-producing melanoblasts that are in a correct spatial and temporal distribution pattern (Figs. 3B and 3D), with β-galactosidase-expression patterns overlapping that of wild-type transgene. The recovery of DCT-producing cells continued at E14.5 (Figs. 3E–3H). At this age, nearly comparable numbers of melanoblasts are detected in the caudal trunk region (not shown), while only 65% recovery of DCT-producing cells is observed in the eye region and in the rostral posterolateral trunk (not shown). The most marked reduction of DCT-expressing melanoblasts occurs in the ventral region (Figs. 3J and 3L), as few cells were observed to populate the ventrum in mutant embryos. Melanoblast entry to the invaginating hair follicle begins at this developmental stage and a lack of melanoblasts in the *Sox10^{Dom}*/+ ventrum may explain the depigmented patch in this region in adult *Sox10^{Dom}*/+ mice. Alternatively, melanoblasts may be present on the ventrum of E14.5 *Sox10^{Dom}*/+ mice but are representative of the endpoint of delayed *Dct* expression. The delayed appearance of DCT+ melanoblasts suggests that either melanoblasts newly recruited into the melanoblast pathway have very rapidly repopulated the entire mutant embryo or, more likely, SOX10 transiently controls *Dct* expression in melanoblasts.

Consistent with a transient effect of SOX10 on *Dct* expression, mice doubly heterozygous for *Sox10^{Dom}* and *Dct$^{slt\text{-}2J}$* (*slaty-2J,* dark gray coat color) do not exhibit an altered coat color (data not shown) that might be expected if the *Sox10^{Dom}* mutation decreased the DCT level below the threshold needed for normal pigmentation or melanocyte survival. Also, aging *Sox10^{Dom}*/+ mice do not appear to lose melanocytes during successive hair cycles, suggesting that melanoblast stem cell to melanocyte transitions are not sensitive to reduced SOX10 function.

The Lack of Dct Expression Is Not Attributed to a Lack of Melanoblasts

To address whether melanoblasts are present in E10.5–E11.5 *Sox10* mutant embryos, we followed the expression of another early melanoblast marker, KIT, in embryos derived from *Sox10^{Dom}*/+; *Kit$^{W\text{-}lacZ}$*/+ matings. Analysis of embryos from this mating utilized the endogenous *Kit* promoter expression of *LacZ* and allowed us to determine whether *Kit*-expressing melanoblasts are present on a *Sox10^{Dom}* background. Greatly reduced numbers of KIT+ cells were detected in E10.5 *Sox10^{Dom}*/*Sox10^{Dom}* embryos compared to wild type (Figs. 4A and 4B). Consistent with our hypothesis that there is total absence of melanoblasts in early *Sox10^{Dom}*/*Sox10^{Dom}* embryos, no KIT+ melanoblasts were observed in mutant E11.5 embryos (Fig. 4D). KIT was detected in the trigeminal ganglion in wild-type and mutant E10.5 embryos (Figs. 4A and 4B); however, soon after in early E11.5 trigeminal ganglion, expression of KIT was not detectable in *Sox10^{Dom}*/Sox10^{Dom} embryos (Fig. 4D), consistent with a loss of peripheral nervous system derivatives as a result of deficient NC development. In contrast, in E11.5 *Sox10^{Dom}*/+ embryos, KIT+ melanoblasts could be observed in a characteristic melanoblast distribution (Figs. 4E–4H). Although the number of KIT+ melanoblasts was reduced to 27% of wild type in the mid-trunk region, these embryos have comparable numbers of cells in eye region of the head, and KIT+ cells are only reduced to 90% of wild type in the caudal trunk area near the hindlimb (Figs. 4G and 4H). Contrary to our *Dct* expression observations in *Sox10^{Dom}*/+ embryos, many KIT+ melanoblasts were still present in a proper spatiotemporal patterning, indicative of melanoblast development at this time point. Therefore, large numbers of KIT+ melanoblasts were still present on a *Sox10^{Dom}*/+ background at E11.5. While we cannot rule out that the restricted absence of DCT expression in melanoblasts of *Sox10^{Dom}*/+ embryos is due to a developmental delay, taken together with our observation that MITF+ melanoblasts are present in 2-day *Sox10^{Dom}*/+ neural tube cultures (Table 1)

and KIT+ cells are present at 10.5 and 11.5 days *in vivo*, our data strongly suggest that the lack of DCT is due to diminished induction of *Dct* within melanoblasts.

Embryonic DCT Production in Pax3, Mitf, and Sox10 Heterozygous Spotting Mutants

We sought to determine whether the transient absence of DCT+ cells is a phenomenon that is common in heterozygous spotting mutants harboring mutations in transcription factors required for early melanoblast development. The transcription factors SOX10, PAX3, and MITF have been proposed to have a hierarchical relationship in melanoblast development (Bondurand *et al.*, 2000; Potterf *et al.*, 2000), and mutations in these transcription factors result in disruption of NC-derived melanocyte development. Mice heterozygous for these mutations are almost totally pigmented, with variable white ventral spots (not shown). If the absence of DCT+ cells was a common phenomenon in mice heterozygous for spotting mutant genes, then *Pax3* and *Mitf* mutants may show a similar absence of DCT between E10.5 and E12.5. However, in E11.5 mutant embryos, both $Pax3^{Sp}$/+ and $Mitf^{mi}$/+ embryos possess DCT+ melanoblasts around the eye and otocyst regions (Figs. 5A and 5B), as well as in the trunk (not shown). In contrast, DCT production at this age is almost completely absent in $Sox10^{Dom}$/+ embryos (Fig. 5C), suggesting that the lack of DCT may be unique to $Sox10^{Dom}$ mutants and strongly supporting a critical role for SOX10 in transiently regulating *Dct* expression.

SOX10 and DCT Coexpression in Melanoblasts

To further investigate whether SOX10 function in melanocyte development is intrinsic to melanoblasts, colocalization of SOX10 and DCT was determined in order to establish that these factors are expressed in the same cells during the same stage of development. Neural tube explants were obtained from $Sox10^{Dom}$/+ X *Dct-LacZI/Dct-LacZI* matings and transgene expression was tested for comparability to *Dct* transgene expression *in vivo*. Indeed, 2-day wild-type cultures corresponding to E11.5 embryos expressed β-galactosidase while $Sox10^{Dom}$/+ cultures did not (Figs. 6A and 6B), recapitulating the phenotypes observed *in vivo* (Figs. 2G and 2H). Wild type 2-day neural tube cultures have a large number of cells that express SOX10 in the nucleus, with a small number of DCT-LacZ cells that show SOX10 and LacZ colocalization (Fig. 6C), indicating that SOX10 and DCT-LacZ are expressed together early in wild-type melanoblasts. SOX10 and DCT-LacZ were also coexpressed in both wild-type and $Sox10^{Dom}$/+ 5-day neural tube cultures (Figs. 6D and 6E). The restoration of DCT-LacZ expression in 5-day mutant neural tube cultures temporally correlates with E14.5 DCT expression patterns in $Sox10^{Dom}$/+ embryos (Figs. 3G and 3H). Because SOX10 and DCT-LacZ colocalize in $Sox10^{Dom}$/+ melanoblasts at this time (Fig. 6E), the restoration of DCT-LacZ production in mutant embryos does not completely depend on reduced SOX10 expression. Taken together, the results show that SOX10 and DCT are expressed together early in melanocyte development, presenting a framework for SOX10 regulation of *Dct* in melanoblasts, and suggest that melanocyte developmental defects in *Sox10* mutants are due to effects intrinsic to melanoblasts.

In Vitro SOX10 Induction of Dct Expression

The ability of SOX10 to induce *Dct* expression was determined by using *in vitro* transient transfection assays. The minimal promoter fragment is capable of recapitulating the endogenous *Dct* expression (Fig. 1), both *in vivo* (Fig. 2) and *in vitro* (Figs. 6A and 6B). Using this fragment for *in vitro* cotransfection studies, we found that SOX10 was able to induce nearly 7-fold increases in *Dct* reporter activity in murine melanocytes (Fig. 7A). Somewhat lesser induction levels (5-fold) were observed by using a human *Dct* promoter fragment (Fig. 7A). The murine *Dct* promoter was also effectively activated by SOX10 in nonmelanocyte cell lines HeLa and NIH3T3 (Fig. 7A). To examine the effects of truncating mutations on SOX10 function, we used a SOX10 deletion construct (Potterf *et al.*, 2000) that models human and murine mutations that truncate the C-terminal transactivation domain (Pingault *et al.*, 1998; Southard-Smith *et al.*, 1999). The SOX10 deletion construct completely failed to activate *Dct* expression (Fig. 7A). In murine melanocytes, the SOX10 deletion mutant acted in a dominant-negative manner to completely inhibit the wild-type SOX10 induction of the *Dct* promoter (Fig. 7B). These data may explain the mode of action by which the $Sox10^{Dom}$ mutation exerts its effect on *Dct* expression and strongly support a role for SOX10 in initiating control of *Dct* expression in early melanoblast development.

DISCUSSION

SOX10 Is Essential for Neural Crest-Derived Melanocyte Development

Genetic studies in mouse and human have implicated SOX10 as possessing a critical function for mammalian NC development. Using both *in vivo* and *in vitro* analyses of melanoblast marker expression in the *Dominant megacolon* mouse model for human Waardenburg syndrome IV, we have determined that SOX10 is essential for NC-derived melanocyte development. We speculate that SOX10 functions early in melanocyte development, being necessary but not sufficient for promoting the initial determination of NC cells to melanoblasts. This early action of SOX10 is supported by several results. First, in mice that lack functional SOX10 (as seen in $Sox10^{Dom}$/$Sox10^{Dom}$ embryos), there is no evidence of pigmentation or melanoblast marker expression *in vitro* (DCT, MITF), or *in vivo* (DCT, KIT) by E11.5. The absence of MITF+ cells in these mutants supports our hypothesis that SOX10 directly regulates *Mitf* expression in

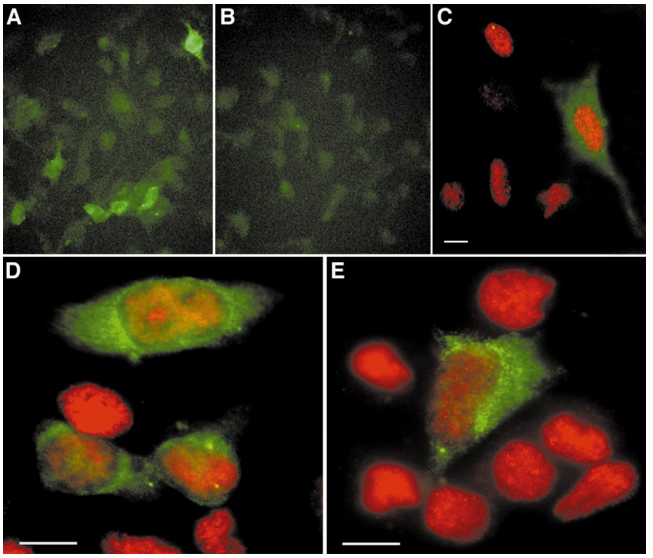

FIG. 6. *Dct* transgenic expression in 2-day neural tube explants. Immunohistochemical colocalization of SOX10 and *Dct* transgene in 2-day and 5-day neural tube explants. *Dct* transgene expression in wild-type (A) or $Sox10^{Dom}$/+ (B) 2-day NC cultures. SOX10 (nuclear, red) colocalizes with DCT transgene (cytoplasmic, green) in 2-day wild-type NC cultures (40× objective) (C). SOX10 (nuclear, red) colocalizes with DCT transgene (cytoplasmic, green) in both 5-day wild-type (D) and $Sox10^{Dom}$/+ (E) NC cultures (100× objective). Scale bar, 10 μm.

melanoblasts, and the resultant lack of MITF results in failure of melanoblasts to develop (Potterf *et al.*, 2000). Second, in $Sox10^{Dom}$/+ mice, the absence of most of the DCT+ cells is transient, suggesting that the $Sox10^{Dom}$ mutation exerts its effect on melanoblast development until late E12.5–E13.5, with any differences in melanoblast number after this stage attributable to earlier effects prior to E12.5. Our results showing early involvement of SOX10 in melanocyte development is similar to what has been proposed for *Ednrb* (Shin *et al.*, 1999). Using different methods, these authors showed that *Ednrb* is also required early in melanocyte development, but not at later stages (Shin *et al.*, 1999). More studies are needed to delineate further the temporal requirements for SOX10 in proper melanocyte development and function.

Transient Absence of DCT+ Cells in $Sox10^{Dom}$/+ Embryos

Analysis of *Dct* expression in $Sox10^{Dom}$/+ age-matched embryos revealed that DCT+ melanoblasts were almost completely absent from E10.5 to E12.5. This was surprising since adult $Sox10^{Dom}$/+ mice are almost completely pigmented. The early absence of DCT+ melanoblasts may be unique to mice with mutations in *Sox10* as we have shown that Pax^{Sp}/+ and $Mitf^{mi}$/+ heterozygous E11.5 embryos clearly retain DCT+ melanoblasts in predicted regions.

How do we account for the appearance of melanoblasts in regions previously lacking DCT+ cells? One possibility is that DCT+ cells arise at 13.5 from proliferation of existing DCT+ cells. Populations of late migrating melanoblasts have been described in avian and zebrafish developmental systems (Johnson *et al.*, 1995; Milos *et al.*, 1983; Sharma *et al.*, 1995). Consistent with these observations, recent findings suggest the possibility of a second wave of late migrating melanoblasts in the mouse beginning at E15.5–E16.5

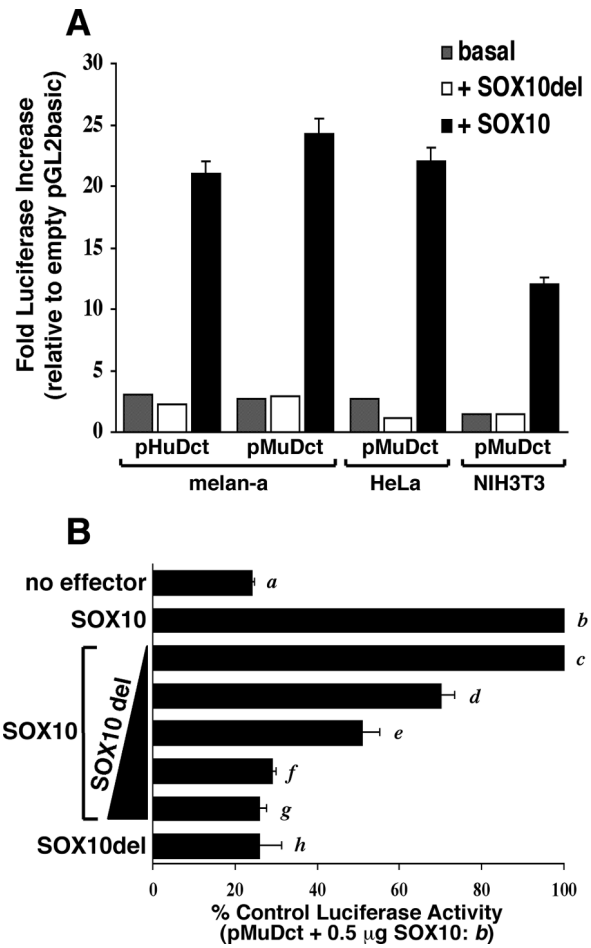

FIG. 7. Transient transfections of murine melanocytes with SOX10 effector and *Dct* promoter/reporter constructs. Cotransfection with CMV-expressing SOX10 (SOX10) or CMV-expressing SOX10 deletion (SOX10del) with *Dct* reporter (pMuDct or pHuDct) (A). SOX10 or SOX10del was cotransfected with an equal amount of pMuDct or pHuDct. Cotransfection with SOX10 resulted in 5- to 12-fold increases in luciferase reporter activity in three different cell lines. Cotransfection with SOX10del failed to activate the *Dct* promoter in any cell line. Dominant negative effect of C-terminal SOX10 mutant (B). Increasing amounts of SOX10del added to a constant amount of SOX10 abolished the ability of wild-type SOX10 to activate the *Dct* promoter. All samples contain 0.5 μg pMuDct. a, no effector; b, 0.5 μg SOX10; c–g, 0.1–0.5 μg SOX10del, plus 0.5 μg SOX10 for all; h, 0.5 μg SOX10del, no SOX10.

(Jordan and Jackson, 2000). In *patch* and *rump-white* (*Rw*) mice, a repopulation of melanoblasts occurred from a small number of DCT+ cells located at the dorsal most portions of the trunk (as in *patch*) or as a result of rostrocaudal migration from regions of high density of melanoblasts to low (*Rw*). While we cannot rule out a second wave of migration accounting for the expansion of DCT+ cell numbers, the melanoblast expansion reported in our study differs from the Jordan and Jackson model in several ways. We observed in $Sox10^{Dom}$/+ mutant embryos that both the KIT+ melanoblasts at E10.5–E11.5 and the rapidly appearing DCT+ melanoblasts at E13.5 are present in their proper positions in the head, ear, and trunk and did not appear to spread out or fill in from a very small population of survivor clones along the dorsal midline. Although initially reduced in number, melanoblasts populated the majority of the embryo in a correct spatial configuration by E14.5, much earlier than proposed for the late wave of melanoblast migration.

A second possibility to account for the appearance of melanoblasts in regions lacking DCT+ cells is that melanoblasts are completely absent early on and then develop from a nonmelanoblast population of cells (for example, Schwann cells or their precursors). The reduction of KIT+ cells observed in $Sox10^{Dom}$/+; $Kit^{W\text{-}lacZ}$/+ embryos could represent the specific lack of a distinct KIT+DCT+ population of melanoblast cells. The second population, consisting of KIT+DCT− cells (melanoblasts?), may not be affected by the $Sox10^{Dom}$ mutation. This population would then become KIT+DCT+ around E13.5, and though reduced in number, these cells would proceed to develop into melanocytes. This interpretation is less likely given the comparison of KIT+ and DCT+ melanoblast numbers in E11.5 embryos. Comparable numbers of KIT+ and DCT+ melanoblasts are found in the head and tail regions of $Kit^{W\text{-}lacZ}$/+ and *Dct-LacZ* E11.5 embryos (both wild type at the *Sox10* locus). However, in $Sox10^{Dom}$/+; $Kit^{W\text{-}lacZ}$/+ embryos, there is only a 10% reduction in KIT+ melanoblasts in the hindlimb and head regions whereas there is a >98% reduction in DCT+ cells in these regions in $Sox10^{Dom}$/+ embryos (Figs. 1E and 1F). If a KIT+DCT+ melanoblast lineage were selectively lost in $Sox10^{Dom}$/+ embryos, there would be a far greater lack in KIT+ cells than was observed.

These data argue that, in $Sox10^{Dom}$/+ embryos, melanoblasts are present, but *Dct* is transiently reduced to undetectable levels in most of these cells. *In vitro*, MITF+ melanoblasts were present in 2-day $Sox10^{Dom}$/+ neural tube cultures, and *in vivo* KIT+ melanoblasts were present in the normal locations of E11.5 $Sox10^{Dom}$/+; $Kit^{W\text{-}lacZ}$/+ embryos. While the melanoblast numbers were reduced compared to those of $Kit^{W\text{-}lacZ}$/+ embryos, the KIT+ melanoblasts were in the correct spatiotemporal arrangement in these embryos, suggesting that migration was not severely delayed. The normal timing of expression of the MITF and pigmentation melanocyte markers *in vitro* and KIT expression *in vivo* suggests that the lack of DCT is not the result of an overall developmental delay in melanocyte development.

SOX10 Is a Transcriptional Regulator of Dct

Consistent with the role of SOX10 as a transcriptional regulator in NC derivatives (Kuhlbrodt *et al.*, 1998a; Peirano *et al.*, 2000; Potterf *et al.*, 2000), we have shown that DCT-LacZ was colocalized with SOX10 in NC cultures (Fig. 6C) and that SOX10 could induce *Dct in vitro*, utilizing a *Dct* promoter fragment (Fig. 2) that exhibited expression patterns nearly identical to endogenous *Dct* (Fig. 1). Wild-type SOX10 had the ability to significantly induce both the murine and human *Dct* promoter whereas a C-terminal deletion mutant completely failed to activate them. Two recent reports support our findings of transcriptional regulation of *Dct* by *SOX10*. One report described a consensus HMG-box binding sequence within the human *Dct* 5′ flanking region that is needed for appropriate *Dct* expression in melanoma cells (Amae *et al.*, 2000). Further analysis is needed to determine whether SOX10 can bind to this region directly and if this region is needed for expression *in vivo*. A second report examined melanocyte markers in the hindlimb of E12.5 embryos carrying a null allele of *Sox10* (Britsch *et al.*, 2001). Homozygous mutants demonstrated a reduction of KIT+ cells and absence of MITF and DCT cells in this region. In heterozygotes, they noted reduced number of DCT+ cells in E12.5 embryo and demonstrated an *in vitro* induction from the murine Dct promoter. This study suggested that haploinsufficiency can account for some of the traits observed in *Sox10* mutations.

MITF has been proposed as a possible transcriptional regulator of *Dct*, suggesting that SOX10 influence over *Dct* may be indirect. However, very little interaction between MITF and the *Dct* promoter has been found, even though the *Dct* promoter has *cis* elements similar to those contained within the melanocyte-specific promoters for tyrosinase and TYRP-1 (Goding, 2000). These data, taken together with our demonstrations that $Mitf^{mi}$/+ E11.5 embryos produce DCT and that age-related $Sox10^{Dom}$/+ neural tube cultures express MITF but no DCT, strongly suggest that early transcriptional regulatory control of *Dct* lies primarily with SOX10.

SOX10 alone cannot account for all *Dct* expression. *Sox10* and *Mitf* mutants still exhibit *Dct* expression in the non-NC-derived RPE, suggesting another transcriptional regulator(s) of *Dct* in the RPE (Southard-Smith *et al.*, 1998; Nakayama *et al.*, 1998; Opdecamp *et al.*, 1997). Furthermore, the SOX10 control of *Dct* is transient since $Sox10^{Dom}$/+ melanoblasts can express *Dct* after E13.5. SOX10 is also not sufficient for *Dct* or *Mitf* expression since other neural crest cells express SOX10 but do not form melanocytes. Additional studies are needed to determine all necessary factors for initial expression of MITF and for the maintenance of *Dct* expression during later stages of melanocyte development.

ACKNOWLEDGMENTS

We thank Drs. Jean-Jacques Panthier for the $Kit^{W\text{-}lacZ}$/+ mice, Vincent Hearing for the anti-DCT antibody, Dorothy Bennett for the melanocyte cell lines, and Minao Furumura for the human *Dct*

reporter construct. We also thank Drs. Karen J. Dunn for insightful comments and discussion, Arturo Incao for genotyping of the mice, and the National Human Genome Research Institute Embryonic Stem Cell core for the *Dct-LacZ* transgenic construction.

REFERENCES

Amae, S., Yasumoto, K., Takeda, K., Udono, T., Takahashi, K., and Shibahara, S. (2000). Identification of a composite enhancer of the human tyrosinase-related protein 2/DOPAchrome tautomerase gene. *Biochim. Biophys. Acta* **1492,** 505–508.

Bernex, F., De Sepulveda, P., Kress, C., Elbaz, C., Delouis, C., and Panthier, J. J. (1996). Spatial and temporal patterns of c-kit-expressing cells in WlacZ/+ and WlacZ/WlacZ mouse embryos. *Development* **122,** 3023–3033.

Bondurand, N., Pingault, V., Goerich, D. E., Lemort, N., Sock, E., Caignec, C. L., Wegner, M., and Goossens, M. (2000). Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome. *Hum. Mol. Genet.* **9,** 1907–1917.

Britsch, S., Goerich, D. E., Riethmacher, D., Peirano, R. I., Rossner, M., Nave, K. A., Birchmeier, C., and Wegner, M. (2001). The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev.* **15,** 66–78.

Budd, P. S., and Jackson, I. J. (1995). Structure of the mouse tyrosinase-related protein-2/dopachrome tautomerase (Tyrp2/Dct) gene and sequence of two novel slaty alleles. *Genomics* **29,** 35–43.

Dunn, K. J., Williams, B. O., Li, Y., and Pavan, W. J. (2000). Neural crest-directed gene transfer demonstrates Wnt1 role in melanocyte expansion and differentiation during mouse development. *Proc. Natl. Acad. Sci. USA* **97,** 10050–10055.

Goding, C. R. (2000). Mitf from neural crest to melanoma: Signal transduction and transcription in the melanocyte lineage. *Genes Dev.* **14,** 1712–1728.

Hearing, V. J. (1993). Unraveling the melanocyte. *Am. J. Hum. Genet.* **52,** 1–7.

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.

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., Meijers, C., and Buys, C. H. (1996). A homozygous mutation in the endothelin-3 gene associated with a combined Waardenburg type 2 and Hirschsprung phenotype (Shah-Waardenburg syndrome). *Nat. Genet.* **12,** 445–447.

Hornyak, T. J., Hayes, D. J., and Ziff, E. B. (2000). Cell-density-dependent regulation of expression and glycosylation of dopachrome tautomerase/tyrosinase-related protein-2. *J. Invest. Dermatol.* **115,** 106–112.

Hornyak, T. J., Hayes, D. J., Chiu, L-Y., and Ziff, E. B. (2001). Transcription factors in melanocyte development: Distinct roles for Pax-3 and Mitf. *Mech. Dev.* **101,** 47–59.

Hou, L., Panthier, J. J., and Arnheiter, H. (2000). Signaling and transcriptional regulation in the neural crest-derived melanocyte lineage: Interactions between KIT and MITF. *Development* **127,** 5379–5389.

Jackson, I. J. (1988). A cDNA encoding tyrosinase-related protein maps to the brown locus in mouse [published erratum appears in *Proc. Natl. Acad. Sci. USA* (1989). **86,** 997]. *Proc. Natl. Acad. Sci. USA* **85,** 4392–4396.

Jackson, I. J., Chambers, D. M., Tsukamoto, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Hearing, V. (1992). A second tyrosinase-related protein, TRP-2, maps to and is mutated at the mouse slaty locus. *EMBO J.* **11,** 527–535.

Johnson, S. L., Africa, D., Walker, C., and Weston, J. A. (1995). Genetic control of adult pigment stripe development in zebrafish. *Dev. Biol.* **167,** 27–33.

Jordan, S. A., and Jackson, I. J. (2000). A late wave of melanoblast differentiation and rostrocaudal migration revealed in patch and rump-white embryos. *Mech. Dev.* **92,** 135–143.

Kapur, R. P. (1999). Early death of neural crest cells is responsible for total enteric aganglionosis in Sox10(Dom)/Sox10(Dom) mouse embryos. *Pediatr. Dev. Pathol.* **2,** 559–569.

Kos, L., Aronzon, A., Takayama, H., Maina, F., Ponzetto, C., Merlino, G., and Pavan, W. (1999). Hepatocyte growth factor/scatter factor-MET signaling in neural crest-derived melanocyte development. *Pigment Cell Res.* **12,** 13–21.

Kuhlbrodt, K., Herbarth, B., Sock, E., Hermans-Borgmeyer, I., and Wegner, M. (1998a). Sox10, a novel transcriptional modulator in glial cells. *J. Neurosci.* **18,** 237–250.

Kuhlbrodt, K., Schmidt, C., Sock, E., Pingault, V., Bondurand, N., Goossens, M., and Wegner, M. (1998b). Functional analysis of Sox10 mutations found in human Waardenburg–Hirschsprung patients. *J. Biol. Chem.* **273,** 23033–23038.

Lee, M., Goodall, J., Verastegui, C., Ballotti, R., and Goding, C. R. (2000). Direct regulation of the microphthalmia promoter by sox10 links waardenburg-shah syndrome (WS4)-associated hypopigmentation and deafness to WS2. *J. Biol. Chem.* **275,** 37978–37983.

Loftus, S. K., and Pavan, W. J. (2000). The use of expression profiling to study pigment cell biology and dysfunction. *Pigment Cell Res.* **13,** 141–146.

Mackenzie, M. A., Jordan, S. A., Budd, P. S., and Jackson, I. J. (1997). Activation of the receptor tyrosine kinase Kit is required for the proliferation of melanoblasts in the mouse embryo. *Dev. Biol.* **192,** 99–107.

Milos, N., Dingle, A. D., and Milos, J. P. (1983). Dynamics of pigment pattern formation in the zebrafish, Brachydanio rerio. III. Effect of anteroposterior location of three-day lateral line melanophores on colonization by the second wave of melanophores. *J. Exp. Zool.* **227,** 81–92.

Nakayama, A., Nguyen, M. T., Chen, C. C., Opdecamp, K., Hodgkinson, C. A., and Arnheiter, H. (1998). Mutations in microphthalmia, the mouse homolog of the human deafness gene MITF, affect neuroepithelial and neural crest-derived melanocytes differently. *Mech. Dev.* **70,** 155–166.

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.

Pavan, W. J., and Tilghman, S. M. (1994). Piebald lethal (sl) acts early to disrupt the development of neural crest-derived melanocytes. *Proc. Natl. Acad. Sci. USA* **91,** 7159–7163.

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.

Pingault, V., Bondurand, N., Kuhlbrodt, K., Goerich, D. E., Prehu, M. O., Puliti, A., Herbarth, B., Hermans-Borgmeyer, I., Legius, E., Matthijs, G., Amiel, J., Lyonnet, S., Ceccherini, I., Romeo, G., Smith, J. C., Read, A. P., Wegner, M., and Goossens, M. (1998). SOX10 mutations in patients with Waardenburg–Hirschsprung disease. *Nat. Genet.* **18,** 171–173.

Potterf, S. B., Furumura, M., Dunn, K. J., Arnheiter, H., and Pavan, W. J. (2000). Transcription factor hierarchy in Waardenburg syndrome: Regulation of MITF expression by SOX10 and PAX3. *Hum. Genet.* **107,** 1–6.

Puffenberger, E. G., Hosoda, K., Washington, S. S., Nakao, K., deWit, D., Yanagisawa, M., and Chakravart, A. (1994). A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. *Cell* **79,** 1257–1266.

Read, A. P., and Newton, V. E. (1997). Waardenburg syndrome. *J. Med. Genet.* **34,** 656–665.

Sharma, K., Korade, Z., and Frank, E. (1995). Late-migrating neuroepithelial cells from the spinal cord differentiate into sensory ganglion cells and melanocytes. *Neuron* **14,** 143–152.

Shibahara, S., Tomita, Y., Sakakura, T., Nager, C., Chaudhuri, B., and Muller, R. (1986). Cloning and expression of cDNA encoding mouse tyrosinase. *Nucleic Acids Res.* **14,** 2413–2427.

Shin, M. K., Levorse, J. M., Ingram, R. S., and Tilghman, S. M. (1999). The temporal requirement for endothelin receptor-B signalling during neural crest development. *Nature* **402,** 496–501.

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.

Southard-Smith, E. M., Kos, L., and Pavan, W. J. (1998). Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. *Nat. Genet.* **18,** 60–64.

Steel, K. P., Davidson, D. R., and Jackson, I. J. (1992). TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit ligand) is a survival factor. *Development* **115,** 1111–1119.

Tachibana, M. (1999). A cascade of genes related to Waardenburg syndrome. *J. Invest. Dermatol. Symp. Proc.* **4,** 126–129.

Tanaka, S., Yamamoto, H., Takeuchi, S., and Takeuchi, T. (1990). Melanization in albino mice transformed by introducing cloned mouse tyrosinase gene. *Development* **108,** 223–227.

Tsukamoto, K., Jackson, I. J., Urabe, K., Montague, P. M., and Hearing, V. J. (1992a). A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. *EMBO J.* **11,** 519–526.

Tsukamoto, K., Palumbo, A., D'Ischia, M., Hearing, V. J., and Prota, G. (1992b). 5,6-Dihydroxyindole-2-carboxylic acid is incorporated in mammalian melanin. *Biochem. J.* **286,** 491–495.

Verastegui, C., Bille, K., Ortonne, J. P., and Ballotti, R. (2000). Regulation of the microphthalmia-associated transcription factor gene by the waardenburg syndrome type 4 gene, SOX10 [In Process Citation]. *J. Biol. Chem.* **275,** 30757–30760.

Wegner, M. (1999). From head to toes: The multiple facets of Sox proteins. *Nucleic Acids Res.* **27,** 1409–1420.

Wehrle-Haller, B., and Weston, J. A. (1995). Soluble and cell-bound forms of steel factor activity play distinct roles in melanocyte precursor dispersal and survival on the lateral neural crest migration pathway. *Development* **121,** 731–742.

Received for publication January 29, 2001
Revised May 23, 2001
Accepted May 30, 2001
Published online August 9, 2001