INTRODUCTION

During vertebrate embryogenesis neural crest cells delaminate from the dorsal neural tube, migrate throughout the body and differentiate into a remarkably diverse array of cell types (Le Douarin and Kalcheim, 1999; Smith et al., 1994). These neural crest fates can be broadly categorized as ectomesenchymal and nonectomesenchymal. The ectomesenchymal neural crest fates include cranial cartilage and fin mesenchyme (in fish) whereas the nonectomesenchymal fates include neurons and glia of the peripheral nervous system and pigment cells. Defects in neural crest development are a significant cause of human disease and the resulting syndromes are termed neurocristopathies (Le Douarin and Kalcheim, 1999). One such neurocristopathy is Waardenburg’s Syndrome, in which individuals have dominant pigmentation defects. Waardenburg’s Syndrome types IIa and IV are associated with haploinsufficiency for the transcription factor genes MITF and SOX10, respectively (Pingault et al., 1998; Tachibana et al., 1994; Tassabehji et al., 1994).

Zebrafish or mice homozygous for mutations in the sox10 transcription factor gene [previously called colourless (cls) in zebrafish] have severe defects in all the nonectomesenchymal neural crest cell fates (Dutton et al., 2001; Herbarth et al., 1998; Southard-Smith et al., 1998). In cls/sox10–/– zebrafish many neural crest cells undergo apoptotic cell death near the neural tube. They do so after failing to become specified or migrate (Dutton et al., 2001). Apoptotic death of cells on the neural crest migration pathways has also been reported in Sox10–/– mouse embryos (Kapur, 1999). In cls/sox10–/– zebrafish and in Sox10–/– mouse embryos some of the nonectomesenchymal neural crest cell fates such as melanocytes (also called melanophores in zebrafish) and peripheral glia are essentially absent whereas others such as the dorsal root ganglia sensory neurons do form but with fewer and disorganized cells (Britsch et al., 2001; Kelsh and Eisen, 2000; Sonnenberg-Riethmacher et al., 2001; Southard-Smith et al., 1998).

In mammalian systems it has been shown that in the case of the peripheral glia a major requirement of Sox10 is to directly regulate expression of terminal differentiation genes such as P0 and Cx32 (Gjb1 – Mouse Genome Informatics) (Bondurand et al., 2001; Peirano et al., 2000). Sox10 also regulates expression of the neuregulin receptor gene, Erbb3 (Britsch et al., 2001). Signaling through Erbb3 promotes acquisition of the glial fate

SUMMARY

The transcription factor Sox10 is required for the specification, migration and survival of all nonectomesenchymal neural crest derivatives including melanophores. sox10–/– zebrafish lack expression of the transcription factor mitfa, which itself is required for melanophore development. We demonstrate that the zebrafish mitfa promoter has sox10 binding sites necessary for activity in vitro, consistent with studies using mammalian cell cultures that have shown that Sox10 directly regulates Mitf expression. In addition, we demonstrate that these sites are necessary for promoter activity in vivo. We show that reintroduction of mitfa expression in neural crest cells can rescue melanophore development in sox10–/– embryos. This rescue of melanophores in sox10–/– embryos is quantitatively indistinguishable from rescue in mitfa–/– embryos. These findings show that the essential function of sox10 in melanophore development is limited to transcriptional regulation of mitfa. We propose that the dominant melanophore phenotype in Waardenburg syndrome IV individuals with SOX10 mutations is likely to result from failure to activate MITF in the normal number of melanoblasts.

Key words: Zebrafish, Danio rerio, Neural crest, Fate specification, Melanocyte, sox10, colourless, mitf, nacre, Survival, Transcriptional regulation

DEVELOPMENT AND DISEASE

Transcriptional regulation of mitfa accounts for the sox10 requirement in zebrafish melanophore development

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by neural crest cells and is required for peripheral glial cell migration and survival (Paratore et al., 2001). However it is not known whether this Erbb3 regulation by Sox10 is direct.

In the case of melanocytes it is not clear to what extent Sox10 is required for direct transcriptional regulation of terminal differentiation genes. One plausible hypothesis is that in the melanocyte lineage Sox10 is simply required for direct activation of the Mitf transcription factor gene, which then acts as a master regulator of melanocyte cell fate. Evidence for the pivotal role of Mitf in melanocyte development has come from studies with both mammals and zebrafish. In mammalian systems Mitf transactivates expression of melanogenic enzyme genes such as Tyr and Trp1 as well as the receptor tyrosine kinase gene Kit. Kit signaling potentiates Mitf activity in turn and is also required for melanocyte proliferation and survival in both zebrafish and mice (Goding, 2000; Hemesath et al., 1998; Hou et al., 2000; Opdecamp et al., 1997; Parichy et al., 1999; Steel et al., 1992; Yasumoto et al., 1997). In mammalian systems Mitf also directly regulates expression of the antiapoptotic factor gene Bcl2 required for melanocyte survival (McGill et al., 2002). Similarly, ectopic Mitf (previously known as nac) expression in zebrafish embryos causes ectopic expression of the melanogenic enzyme gene dct (Lister et al., 1999). Forced expression of Mitf in cultured mouse fibroblasts can induce some aspects of melanocyte differentiation and ectopic nac/mitfa expression in zebrafish embryos causes ectopic abnormal melanized cells (Lister et al., 1999). Thus loss of mitf expression would be sufficient to account for the melanocyte defect in sox10+/− mutant embryos.

Although regulation of Mitf expression is clearly part of the Sox10 requirement in the melanocyte lineage it is also possible that there are other essential Sox10 functions in this lineage. Unlike zebrafish, mice show a haploinsufficiency phenotype when heterozygous for Sox10 mutations (Britsch et al., 2001). This phenotype includes a mild melanocyte deficiency. Melanocytes from these mice show little reduction in Mitf expression and yet transiently have a severe reduction in expression of the melanogenic enzyme gene Det (Potterf et al., 2001). In addition, Sox10 can transactivate expression from a Det promoter construct in cultured cells (Britsch et al., 2001; Potterf et al., 2001). These findings could suggest a requirement for Sox10 in regulating Det expression that is not mediated via Mitf. A critical question is whether any such non-Mitf-mediated effects of Sox10 have a significant role in melanocyte development.

We show here that the direct regulation of Mitf expression by Sox10 reported in cultured mammalian cells also occurs in developing melanophores in zebrafish embryos. We extend these studies by showing that forced expression of nac/mitfa in the neural crest of cls/sox10+/− mutant zebrafish embryos is sufficient to rescue melanophore development. Furthermore, we show that rescue of melanophores in cls/sox10+/− embryos is quantitatively indistinguishable from rescue in nac/mitfa+/− embryos. Together, these data suggest that regulation of nac/mitfa by cls/sox10 can fully account for the cls/sox10 requirement in the zebrafish melanophore lineage.

**MATERIALS AND METHODS**

**Fish**

Embryos were obtained through natural crosses and staged according to Kimmel et al. (Kimmel et al., 1995). We used three cls alleles (m618, t3 and tw2) which all have equally strong phenotypes (Dutton et al., 2001). We used the nac+ allele (Lister et al., 1999) except where it is stated that we used the nac− allele (Lister et al., 2001).

**PCR genotyping**

Embryos were tested for heterozygosity or homozygosity of the nac mutations by PCR on genomic DNA. The nac− test used PCR primers cattctggtctagatcagcag and gcgaagtcgaagggcagagag followed by digestion with DraI which cleaves the mutant allele (Lister et al., 1999). The nac+ test used PCR primers gcaagtaagagccctggc and acggatcatttgacttgggaattaaag followed by digestion with BsrD1 which cleaves the mutant allele.

**Whole-mount in situ hybridization**

Embryos were processed for whole-mount in situ hybridization with nac/mitfa digoxigenin-labeled riboprobe as in Dutton et al. (Dutton et al., 2001).

**Cell culture and luciferase assays**

Promoter truncations were made from plasmid nac>lac (Dorsky et al., 2000) using the restriction sites indicated in Fig. 3. Mutation to the M1 sequence (see Table 1) was made by replacing the SpeI-AgeI region with the annealed oligonucleotides cttactgcggctgttgcggtttgccgtt between the BamH1 and EcoR1 sites of pGEX-3X (Amersham Pharmacia). Cls/Sox10-GST fusion protein was expressed in E. coli BL21(RIL) (Stratagene) and affinity purified using glutathione agarose following the manufacturer’s instructions.

**Electrophoretic mobility shift assays**

The pCls/Sox10-GST expression plasmid was constructed by cloning a PCR product amplified from hs>sox10 (Dutton et al., 2001) (using primers cgggatcccgatgtcggcggaggagcacag and gcgaattcaggaacccgaacggccggtttgccggtg) between the BamH1 and EcoRI sites of pGEX-3X (Amersham Pharmacia). Cls/Sox10-GST fusion protein was expressed in E. coli BL21(RIL) (Stratagene) and affinity purified using glutathione agarose following the manufacturer’s instructions. Approximate relative concentrations of Cls/Sox10-GST protein were estimated by comparison to a dilution series of bovine serum albumin (BSA) standard using Coomassie-stained polyacrylamide gel electrophoresis (PAGE). The SpaeGene DNA probe was oligonucleotides cttactgcggctgttgcggtttgccgtt between the BamH1 and EcoRI sites of pGEX-3X. Whole-mount in situ hybridization

Embryos were obtained through natural crosses and staged according to Kimmel et al. (Kimmel et al., 1995). We used three cls alleles (m618, t3 and tw2) which all have equally strong phenotypes (Dutton et al., 2001). We used the nac+ allele (Lister et al., 1999) except where it is stated that we used the nac− allele (Lister et al., 2001).
with the embryos anesthetized using 0.003% MS222 (Sigma) and fertilization (hpf) embryos using an Axioplan 2 microscope (Zeiss). GFP fluorescence was scored in 24 hours-post-site.

0.5 mM EDTA, 0.1 mM dithiothreitol, 1 mg/ml BSA and sometimes specific competitor oligonucleotides was incubated on ice for 20 minutes then electrophoresed on a gel (5% (w/v) polyacrylamide (37:1), 0.5% TBE) at 120 V, at 4°C, for 3 hours. Dried gels were exposed to Biomax MS film (Kodak) for autoradiography.

**Embryo injections**

One- or two-cell stage embryos were injected with plasmids and/or RNA using standard methods as in Dutton et al. (Dutton et al., 2001). RNA was produced using the mMESSAGE mACHINE kit (Ambion) from hsp>sox10 or hsp>sox10(L142Q) templates (Dutton et al., 2001) linearized with Asp718.

Plasmids nac>GFP and nac>nac were generated as follows: the SalI-Xba CMV promoter fragment of pCS2+ replaced by 6.8 kb cloning the PCR fragment into the Xba1 site of CS26.8. CS26.8 has N-terminal myc tags from pHS-MT3A.1 (Lister et al., 1999) and constructed by PCR amplifying the corresponding Fspnac>luc constructs (see above). cls>nac was mutated to the M1, M2, M3, M4, M1M3 and M3M4 sequences by MT3A.1) (Lister et al., 1999). Plasmids nac>GFP and nac>nac were rescued if they had wild-type morphology.

**Photography**

Live embryos were anesthetized with 0.003% MS222 (Sigma), mounted in methylcellulose or between bridged coverslips and photographed using a Spot digital camera mounted on an Eclipse E800 microscope (Nikon) or Axioplan 2 microscope (Zeiss) with DIC optics. Embryo whole-mount in situ hybridization specimens were photographed using a Spot digital camera mounted on a MZ12 microscope (Leica) with epi-illumination. The GFP fluorescent gastrula image was captured using a LSM510 confocal microscope (Zeiss) with DIC and confocal fluorescence images superimposed.

**RESULTS**

**nac/mitfa<sup>−/−</sup>;cls<sup>sox10<sup>+/−</sup></sup> double mutant embryos have minute melanophores**

cls<sup>sox10<sup>+</sup></sup> embryos show no nac/mitfa expression detectable by in situ hybridization and nac/mitfa<sup>−/−</sup> embryos have a complete absence of melanophores (Dutton et al., 2001; Lister et al., 1999). Although cls<sup>sox10<sup>+</sup></sup> embryos never have any normal melanophores, they do have a small number of tiny rounded cells expressing melanin (Kelsh et al., 1996; Kelsh et al., 2000). To determine whether these melanized cells result from residual mitfa expression below the sensitivity of in situ hybridization, we examined nac/mitfa<sup>−/−</sup>;cls<sup>sox10<sup>+/−</sup></sup> double mutant embryos.

Intercrossing nac/mitfa<sup>h692</sup>;cls<sup>sox10<sup>+/−</sup></sup> parents gave embryos with three different phenotypes: wild-type (Fig. 1A), embryos with the typical nac/mitfa<sup>−/−</sup> phenotype of complete loss of all melanophores but no reduction in iridophores (Fig. 1B), and embryos with the typical cls<sup>sox10<sup>+</sup></sup> phenotype of a severe reduction in all pigment types including iridophores but a persistence of tiny melanized spots (Fig. 1C,D). All embryos classified as having a cls phenotype were similar, having at least five tiny melanized cells, and importantly we did not observe any embryos with both a complete absence of these tiny melanized cells and loss of iridophores. The numbers of embryos with these specific phenotypes, 168 wild type: 59 nac<sup>−/−</sup> cls<sup>−/−</sup> homozygotes by PCR genotyping. Of the 27 such embryos we tested, four were nac/mitfa<sup>−/−</sup>;cls<sup>sox10<sup>+/−</sup></sup> (Fig. 1D), 14 were nac/mitfa<sup>−/−</sup>;cls<sup>sox10<sup>−/−</sup></sup> and nine were nac/mitfa<sup>−/−</sup>;cls<sup>sox10<sup>−/−</sup></sup>.

To test whether this surprising result was also observed with other nac/mitfa and cls<sup>sox10</sup> alleles we crossed nac/mitfa<sup>h692;h692</sup>;cls<sup>sox10</sup><sup>+/−</sup> and nac/mitfa<sup>h692;h692</sup>;cls<sup>sox10</sup><sup>−/−</sup> parents. This gave 36 wild-type embryos, 39 embryos with the typical nac/mitfa<sup>−/−</sup> phenotype and 18 embryos with a severe reduction in all pigment types. These 18 embryos each had at least five tiny melanophores and PCR genotyping showed that of the 17 such embryos we tested, 12 were nac/mitfa<sup>−/−</sup>;cls<sup>sox10<sup>−/−</sup></sup> and five were nac/mitfa<sup>−/−</sup>;cls<sup>sox10<sup>−/−</sup></sup> double mutant embryos as compared to nac/mitfa<sup>−/−</sup>.
Ectopic cls/sox10 expression in the embryo can induce ectopic nac/mitfa expression

In zebrafish embryos cls/sox10 has been shown to be necessary for nac/mitfa expression (Dutton et al., 2001). In mammalian cells Sox10 has also been reported to directly activate Mitf expression (Bondurand et al., 2000; Lee et al., 2000; Potterf et al., 2000; Verastegui et al., 2000). We used forced ectopic expression of cls/sox10 to test whether cls/sox10 was also sufficient to induce nac/mitfa expression in the zebrafish embryo. Embryos injected with cls/sox10 RNA were probed for nac/mitfa expression by in situ hybridization. cls/sox10 RNA injection induced nac/mitfa transcription at 6 hpf (Fig. 2C), 12 hours before the onset of endogenous nac/mitfa expression (Lister et al., 1999). The induced nac/mitfa expression was unevenly distributed as patches or spots, with the pattern of expression varying greatly from embryo to embryo. Ectopic nac/mitfa expression was not seen when embryos were injected with point mutant cls/sox10L142Q RNA (Fig. 2B), the mutation in the cls/m618 allele (Dutton et al., 2001). These results show that cls/sox10 can induce nac/mitfa expression in embryonic contexts other than the neural crest cells where nac/mitfa is normally expressed.

Fig. 2. Precocious nac/mitfa expression in 6 h.p.f embryos following injection with cls/sox10 RNA. Lateral views of uninjected (A) and cls/sox10 RNA injected embryos (250 pg per embryo; B) and cls/sox10 RNA injected embryos (250 pg per embryo; C) 6 hpf embryos following in situ hybridization with a nac/mitfa probe. Spots and/or patches of nac/mitfa expression were detected in 39% of cls/sox10 RNA injected embryos (n=136) but not in any of the un.injected embryos (n=58) nor in any of the cls/sox10L142Q RNA injected embryos (n=92). (D) Superimposed fluorescent confocal and DIC images of an animal/lateral view of a 6 hpf embryo coinjected with cls/sox10 RNA (250 pg per embryo) and nac>GFP reporter plasmid (150 pg per embryo) show cells with GFP fluorescence. GFP fluorescence was observed in 75% (n=224) of embryos coinjected with cls/sox10 RNA and nac>GFP.

nac/mitfa upstream sequence responds to cls/sox10

To establish whether cls/sox10 acts directly or indirectly on nac/mitfa transcription it was necessary to identify sequence elements in the nac/mitfa promoter mediating cls/sox10 responsiveness. Dorsky et al. (Dorsky et al., 2000) showed that an 836 b.p. nac/mitfa promoter (extending from −753 to +83 b.p. relative to the transcriptional start site) was able to direct expression from a GFP reporter plasmid (nac>GFP) to melanophores. We found that this reporter responded to cls/sox10 RNA coinjection (Fig. 2D), but not cls/sox10L142Q RNA coinjection (n=146 embryos), in gastrula embryos, recapitulating the ectopic expression of nac/mitfa. This indicates that this 836 b.p. region of the nac/mitfa promoter contains sequence elements responsible for the cls/sox10 response in zebrafish embryos. We used a cell line transfection assay to further localize sequence elements in the nac/mitfa promoter responsible for cls/sox10 responsiveness. In transfected NIH3T3 cells a luciferase reporter construct with the point mutant construct pCS2sox10L142Q. Successive 5' truncations of the promoter. Sox10 induction was compared with the baseline value obtained by co-transfection with the point mutant construct pCS2sox10G Q. Successive 5' truncations of the nac/mitfa promoter resulted in incremental decreases in the level of induction in response to cls/sox10 (Fig. 3). Thus elements conferring response to cls/sox10 appeared to be widely distributed throughout the 836 b.p. nac/mitfa promoter. We chose to focus on the most proximal regions that conferred cls/sox10 response. A promoter with a 5' truncation to the Spe1 site (at −173 b.p.) could still respond to cls/sox10, and was significantly different than control transfection (p=0.01), but further truncation to the Age1 site (at −134 b.p.) prevented significant response (indistinguishable from control, P>0.1). These results tentatively localized a sequence element(s) responsible for some of the response to cls/sox10 to this 41 b.p. region of the nac/mitfa promoter.

Cls/Sox10 binds nac/mitfa promoter sequences in vitro

The 41 b.p. critical region of the nac/mitfa promoter between the Spe1 and Age1 sites contains a sequence element (site S1)
was found to reduce the plasmid’s response to with pCS2Sox10, mutation of site S1 (to make FspM1nac>luc) sites in vitro (see Fig. 4; Table 1). In co-transfection assays effect than mutating S1 alone (Fig. 5). S3 and S4 had only a slight effect in this assay (Fig. 5). NIH3T3 cells (Fig. 5). Mutation of sites S2, S3 or S4 or both these binding sites mutated did not compete as effectively (Fig. 4D).

The sequence upstream of the Spe1 site contains additional sequence elements similar to the consensus sox binding site WWCAAWG (Mertin et al., 1999) (Table 1). We used an in vitro DNA binding assay to establish whether Cls/Sox10 could be acting by binding to site S1. An EMSA showed that a Cls/Sox10-GST fusion protein (with Cls/Sox10 residues 1-189) binds to the Spe1Age1 fragment that contains site S1 (Fig. 4B). However, when site S1 is mutated this binding is greatly reduced. Similarly, binding to the Spe1-Age1 fragment is effectively competed by a 19 b.p. double-stranded oligonucleotide with the site S1 sequence but not by an equivalent oligonucleotide with the site S1 mutated (Fig. 4C).

The sequence upstream of the Spe1 site contains additional sequence elements similar to the consensus sox binding site (sites S2, S3 and S4; Table 1). We tested 19 b.p. double-stranded oligonucleotides corresponding to these sequence elements for their ability to compete with the Spe1-Age1 fragment in the EMSA binding assay with Cls/Sox10-GST fusion protein. These oligonucleotides also effectively competed for Cls/Sox10-GST protein binding whereas equivalent oligonucleotides with the Sox consensus binding sites mutated did not compete as effectively (Fig. 4D). However, the short and degenerate nature of the sox binding site consensus sequence means that it occurs frequently, making it difficult to identify functional sox response elements by sequence alone. It was thus important to test what relevance these binding sites had for clsox10 responsiveness in vivo.

| Table 1. Potential Sox binding sites in the nac/mitfa promoter and mutated versions |
|------------------------|-----------------|-----------------|
| Position in promoter   | Oligonucleotides used to test Sox10 binding |
| ~157 b.p.     | S1  | 5’ccatctCTCAAAAGagctg3’ |
| M1 (mutated S1) | 3’tgacgAGGTTCCtccgaac5’ |
| ~247 b.p.     | S2  | 5’gagaacaAACAATGttataa3’ |
| M2 (mutated S2) | 3’ctcttTTGGTTAAaataac5’ |
| ~262 b.p.     | S3  | 5’gcctgtGTTGTTATgctg3’ |
| M3 (mutated S3) | 3’cgatcaCTAACATgcgccct5’ |
| ~284 b.p.     | S4  | 5’tagccACCCGGGctaggg3’ |
| M4 (mutated S4) | 3’atcttgTGGTGCagcatc5’ |

Fig. 4. Electrophoretic mobility shift assays (EMSA) showing binding of Cls/Sox10-GST fusion protein to sites in the nac/mitfa promoter. (A) Schematic diagram of the 836 b.p. nac/mitfa promoter showing the positions of the putative sox binding sites S1, S2, S3 and S4. (B) The Spe1-Age1 fragment of the nac/mitfa promoter (SpeAge probe) shows a band of reduced electrophoretic mobility (black arrow) with ~20 nM, 10 nM, 5 nM and 2.5 nM Cls/Sox10-GST fusion protein (Sox10) which is not seen without the Cls/Sox10-GST protein (no prot). When site S1 is mutated in this DNA fragment (mutated SpeAge probe) binding under these same Cls/Sox10-GST protein concentrations is greatly reduced. (C) Binding of ~10 nM Cls/Sox10-GST protein to the Spe1-Age1 fragment of the nac/mitfa promoter is effectively competed by an oligonucleotide with site S1 (S1 comp) but not by the mutated site oligonucleotide M1 (M1 comp). Shown are binding reactions with a serial five-fold dilution series of this competitor oligonucleotide giving 0.13 to 75 pmol per reaction and also controls with no specific competitor (no comp) and with no Cls/Sox10-GST protein (no prot). (D) Binding of ~10 nM Cls/Sox10-GST protein to the Spe1-Age1 fragment of the nac/mitfa promoter is effectively competed by oligonucleotides with binding sites S2, S3 or S4 but less effectively by the mutated versions M2, M3 or M4. Shown are binding reactions with 75 pmol (75) or 15 pmol (15) of these competitor oligonucleotides and also controls with no specific competitor (no comp) and with no Cls/Sox10-GST protein (no prot).

We used an in vivo melanophore rescue assay to test whether these sox binding sites controlled expression in neural crest cells in the zebrafish embryo. As shown by Dorsky et al. (Dorsky et al., 2000), a plasmid with the nac/mitfa cDNA under control of the 836 b.p. nac/mitfa promoter (nac+nac) can rescue melanophores when injected into nac/mitfa−/− embryos. Mutation of sox binding site S1 in this plasmid’s promoter

A Cls/Sox10 binding site is needed for the clsox10 response of the clsox10 promoter.

In order to test whether sox binding sites S1, S2, S3 or S4 could act as clsox10 response elements, we mutated each of them in a luciferase reporter construct (Fspnac>luc) with a nac/mitfa promoter truncated to the Fsp1 site (~434 b.p.). The mutations used were the same as those used to disrupt binding to these sites in vitro (see Fig. 4; Table 1). In co-transfection assays with pCS2Sox10, mutation of site S1 (to make FspM1nac>luc) was found to reduce the plasmid’s response to clsox10 in NIH3T3 cells (Fig. 5). Mutation of sites S2, S3 or S4 or both S3 and S4 had only a slight effect in this assay (Fig. 5). Similarly, mutation of both S1 and S3 did not have more of an effect than mutating S1 alone (Fig. 5).
(making M1nac>nac) greatly reduced the plasmid’s effectiveness at melanophore rescue (Table 2). Mutation of sox binding site S3 (making M3nac>nac) caused a less dramatic reduction in effectiveness, whereas mutations of sites S2 or S4 (making M2nac>nac and M4nac>nac) had little effect (Table 2). We combined the S1 and S3 mutations (making M1M3nac>nac) which had more effect than mutating S1 alone (P<0.0001 by chi-square analysis). Combining the S3 and S4 mutations (making M3M4nac>nac) had no more effect than mutating S3 alone (P>0.5). These results show that the ability of a binding site to act as a response element in vivo is not accurately reflected by binding affinity in vitro, because sites S2 and S4 compete effectively for Cls/Sox10-GST protein binding in vitro (Fig. 4C) and yet show little evidence of being cls/sox10 response elements in vivo (Table 2). Presumably other characteristics such as the context of the binding site in the promoter are just as important in defining a site as active in vivo.

We used the GFP reporter plasmid nac>GFP to further test the effect of mutating sox binding sites S1 and S3. As shown by Dorsky et al. (Dorsky et al., 2000), the 836 b.p. nac/mitfa promoter in nac>GFP directs expression of GFP to prospective pigment cells in injected embryos at 24 hpf. This assay differs from the nac>nac melanophore rescue assay in that it assesses promoter function in melanoblasts at an earlier developmental stage. Mutation of sox binding site S1 in nac>GFP (making M1nac>GFP) markedly reduced GFP reporter expression (Table 2). Mutation of site S3 (making M3nac>GFP) also reduced GFP reporter expression and combining the two mutations (making M1M3nac>GFP) had more effect than mutating S1 alone. The mutant rescue and GFP expression assays are different and so it is not prudent to compare the magnitude of the effects observed with each. However, both assays show similar trends in which mutating site S1 has a major effect, mutating site S3 has less of an effect, and mutating both sites has more effect than mutating S1 alone.

These results demonstrate that the nac/mitfa promoter contains a Cls/Sox10 protein binding site (site S1) that acts as a cls/sox10 response element and that is necessary for adequate nac/mitfa expression in developing melanophores in the zebrafish embryo. The Cls/Sox10 protein binding site S3 also contributes to activation of nac/mitfa expression but to a lesser extent. These results suggest that in zebrafish neural crest cells in the embryo, Cls/Sox10 activates nac/mitfa expression by directly binding to the nac/mitfa promoter.

**Forced nac/mitfa expression rescues the cls/mitfa allele: melanophore phenotype**

cls/mitfa–/– mutant embryos lack nac/mitfa expression and nac/mitfa–/– mutant embryos lack melanophores (Dutton et al., 2001; Lister et al., 1999). This prompted us to investigate whether activation of nac/mitfa transcription could account for
the required role of cls/sox10 in the melanophore lineage. We tested this by forcing nac/mitfa expression in cls/sox10+/– embryos, thus bypassing the role of cls/sox10 in activating nac/mitfa expression. Because ectopic expression of mitf can confer some melanophore characteristics upon other cell types (Lister et al., 1999; Tachibana et al., 1996), we wanted to express nac/mitfa specifically in neural crest cells. We constructed a plasmid with the nac/mitfa cDNA under control of a cls/sox10 promoter (cls>nac). The cls/sox10 promoter used had previously been shown to target expression of a GFP reporter plasmid to the endogenous sites of cls/sox10 expression such as neural crest and otic vesicle (T.J.C., J. Dutton and R.N.K., unpublished). Injected cls>nac was able to rescue melanophores with normal morphology and migratory ability in cls/sox10–/– mutant embryos and in nac/mitfa–/– mutant embryos (Fig. 6). In both genotypes, and in agreement with previous rescue studies of mitf/nac–/– (Lister et al., 1999), only a few melanophores were rescued in each embryo, presumably because of the highly mosaic distribution of injected DNA typical for zebrafish injection experiments. These results show that reintroduction of nac/mitfa expression rescues the differentiation, migration and survival deficiencies of cls/sox10–/– neural crest cells in the melanophore lineage. We were also able to rescue melanophores by expression of nac/mitfa using a hsp70 promoter construct (Lister et al., 1999) (data not shown).

We tested whether forced expression of nac/mitfa was as effective at rescuing melanophores in cls/sox10+/– embryos as in nac/mitfa–/– embryos. We injected cls>nac into embryos from intercrossed cls/sox10+/–;nac/mitfa+/– double heterozygous parent fish to compare rescue in cls/sox10+/– and nac/mitfa+/– siblings that were laid, injected and raised together. Because both cls/sox10+/– and nac/mitfa+/– embryos have melanophore defects, we used the iridophore phenotype of the cls/sox10+/– embryos to distinguish them from embryos mutant for nac/mitfa alone (see Fig. 1). As mentioned above, double homozygous cls/sox10+/–;nac/mitfa+/– embryos have melanophore and iridophore defects as in cls/sox10+/– embryos and this is reflected in the ratio of phenotypes (Table 4). The cls/sox10+/– embryos were rescued to the same extent as the embryos mutant for nac/mitfa+/– alone, both in terms of the proportion of embryos showing any rescued melanophores and in terms of the number of rescued melanophores per embryo (Table 4, Fig. 7). This result indicates that in the melanophore lineage, cls/sox10 is required only to induce nac/mitfa expression.

**DISCUSSION**

cls/sox10+/–; nac/mitfa+/– embryos have a less severe melanophore phenotype than nac/mitfa–/–

Previous reports indicated that nac/mitfa–/– embryos lack all melanophores whereas cls/sox10+/– embryos still have a few tiny, rounded, melanized cells that fail to migrate (Kelsh and Eisen, 2000; Kelsh et al., 2000; Lister et al., 1999). We report...
here that the presence of these melanized cells cannot be attributed to putative residual nac/mitfa expression in cls/sox10+/− embryos because they are also found in cls/sox10+/−; nac/mitfa+/− embryos. The stronger phenotype of nac/mitfa+/− embryos may, therefore, imply the presence of a cls/sox10-dependent activity that inhibits melanophore development. Obviously, in normal development any such effect must be greatly outweighed by the positive activation of melanophore development mediated by cls/sox10. The source of any such inhibitory activity is completely unknown. However, nac/mitfa+/− embryos have an increased number of iridophores (Lister et al., 1999) and so it is conceivable that there might be some mechanism for mutual repression between pigment cell types. Sox10 is expressed in neural crest lineages other than that giving rise to melanophores, and perhaps the inhibitory activity functions to prevent expression of melanogenic genes in these cell types.

**Role of sox10 in nonectomesenchymal crest fate specification**

Several groups have shown that Sox10 can directly activate Mitf expression in cultured mammalian cells (Bondurand et al., 2000; Lee et al., 2000; Potterf et al., 2000; Verastegui et al., 2000). We found that the zebrafish nac/mitfa promoter is also directly activated by zebrafish Cls/Sox10 and that this direct regulation is necessary for expression from the zebrafish nac/mitfa promoter in neural crest cells in the developing embryo. Most significantly we found that this activation of nac/mitfa expression can account quantitatively for all of the cls/sox10 requirement in the melanophore lineage. Studies in zebrafish and in mice have revealed defects in neural crest cell fate specification, migration, survival and differentiation in sox10 mutants. We have previously proposed that the complex phenotype of cls/sox10 mutants might be explained by a primary defect in specification of nonectomesenchymal crest fates, with defects in migration, survival and differentiation being secondary consequences of this (Dutton et al., 2001; Kelsh and Raible, 2002). Our demonstration here that cls/sox10 directly activates nac/mitfa, a key gene in melanophore fate specification, and that this is vital for melanophore rescue in nac/mitfa mutants, is clearly consistent with our model.

Although not usually interpreted in the same way, the mouse Sox10 mutant phenotype is plainly consistent with the model proposed. For example, the recent demonstration that Mitf regulates the antiapoptotic gene Bcl2 provides a molecular explanation for the apoptosis of melanoblast progenitors in Sox10 mutants (McGill et al., 2002). Furthermore, in mice the regulation of Erbb3 (directly or indirectly) by Sox10 (Britsch et al., 2001) provides evidence that Sox10 regulates glial fate specification, because neuregulin signaling has been shown to direct neural crest stem cells to a glial fate (Shah and Anderson, 1997; Shah et al., 1994).

At first glance, our findings with the melanophore lineage contrast with the body of work establishing that Sox10 directly activates a variety of differentiation genes in developing glia. However, these findings are consistent with the observation that cls/sox10 expression is downregulated in melanoblasts but retained in developing peripheral glia (Dutton et al., 2001), and suggests that in addition to its roles in nonectomesenchymal fate specification, sox10 is also required for glial cell differentiation.

Only a subset of sox10-expressing neural crest cells express mitfa and become melanophores. Dorsky et al. (Dorsky et al., 2000) showed that wnt signaling also directly activated nac/mitfa expression. These findings are consistent with a model for cls/sox10 function in the melanophore lineage in which sox10 is required in conjunction with Wnt signaling to activate nac/mitfa expression in neural crest cells (Kelsh and Raible, 2002). nac/mitfa then in turn specifies the melanophore fate by activating expression of differentiation genes such as dct and genes such as spa/kit required for survival and migration. The NIH3T3 cell transfection work described here was conducted in the absence of any known Wnt signaling. Furthermore, eliminating the Tcf/Lef binding sites as described by Dorsky et al. (Dorsky et al., 2000) from the nac/mitfa promoter reporter construct did not prevent the observed cls/sox10 response in NIH3T3 cells (data not shown). Recently, Saito et al. (Saito et al., 2002) have shown that LEF-1 activates transcription from the MITF promoter in Hela cells much more effectively when bound together as a complex with the MITF-M protein itself. Future studies using coexpression of sox10, mitfa and Wnt signaling components could help to reveal how Wnt signaling and sox10 interact to establish mitfa expression. Work by others using mammalian systems has also shown that the transcription factors Pax3, OC-2 and CREB transactivate Mitf transcription (Bertolotto et al., 1998; Jacquemin et al., 2001; Potterf et al., 2000; Watanabe et al., 1998).

**SOX10, MITF and human disease**

Our demonstration that sox10 function in melanophores may be limited to regulation of mitfa helps to explain the similar pigmentation defects of the Waardenburg Syndromes IIa and IV. Waardenburg Syndromes IIa and IV are associated with human haploinsufficiency for MITF and SOX10, respectively (Pingault et al., 1998; Tachibana et al., 1994; Tassabehji et al., 1994). Although zebrafish cls/sox10 mutants have no dominant phenotype, our results suggest a model for the aetiology of Waardenburg Syndrome IV. We propose that in heterozygous SOX10 mutant humans, activation of MITF by SOX10 is less efficient, resulting in specification of fewer melanoblasts. Consistent with this, in heterozygous Sox10 mutant mice, which share the dominant pigment defects of human individuals, Kit-positive melanoblasts are reduced in number (Potterf et al., 2001); although not reported in these studies, we predict that the number of Mitf-expressing cells would be reduced in these mice compared to wild-types.

That we can, in zebrafish, account quantitatively for the role of sox10 in the melanophore lineage by its activation of mitfa is perhaps surprising in view of the reports that the mouse Dct promoter can be directly regulated by Sox10 (Britsch et al., 2001; Potterf et al., 2001). However, these studies used co-transfection assays in cultured cells and thus leave open the question of whether Dct is regulated directly by Sox10 in the developing neural crest. Our findings strongly suggest that even if Sox10 does directly regulate dct expression in vivo, this requirement may be dispensable for melanophore development. Such an interpretation is consistent with the phenotype in heterozygous Sox10 mutant mice. Thus, a transient reduction in Dct expression seen in developing melanoblasts was attributed to an effect of the reduced levels of Sox10 (Potterf et al., 2001), although an alternative explanation that sub-wild-type levels of Mitf expression result...
in lowered Dct expression cannot be ruled out; indeed, more recent studies in culture show that MITF interacts with LEF-1 to directly coactivate the DCT promoter (Yasumoto et al., 2002). However, regardless of the mechanism mediating this reduction in detectable Dct expression, the Dct phenotype rapidly recovers, suggesting that in melanophores in which Mitf expression is above a threshold level, the requirement for Sox10 is only transient and non-essential. The alternative explanation, that the precise contributions of Sox10 and Mitf in melanocyte development may not be fully conserved between zebrafish and mice, is less attractive because of the striking similarities in the genetic control of melanocyte development already demonstrated between mouse and zebrafish (Rawls et al., 2001).

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REFERENCES


