Human Molecular Genetics, 1999, Vol. 8, No. 9 ***1785–1789***

A molecular analysis of the Yemenite deaf-blind hypopigmentation syndrome: SOX10 dysfunction causes different neurocristopathies

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Received April 28, 1999; Revised and Accepted June 30, 1999

The Yemenite deaf-blind hypopigmentation syndrome was first observed in a Yemenite sister and brother showing cutaneous hypopigmented and hyperpigmented spots and patches, microcornea, coloboma and severe hearing loss. A second case, observed in a girl with similar skin symptoms and hearing loss but without microcornea or coloboma, was reported as a mild form of this syndrome. Here we show that a SOX10 missense mutation is responsible for the mild form, resulting in a loss of DNA binding of this transcription factor. In contrast, no SOX10 alteration could be found in the other, severe case of the Yemenite deaf-blind hypopigmentation syndrome. Based on genetic, clinical, molecular and functional data, we suggest that these two cases represent two different syndromes. Moreover, as mutations of the SOX10 transcription factor were previously described in Waardenburg–Hirschsprung disease, these results show that SOX10 mutations cause various types of neurocristopathy.

INTRODUCTION

The association of hearing loss and pigmentary abnormalities has long been known in a variety of mammals (1). In humans, Waardenburg syndrome (WS) is a rare (1/40 000) autosomal dominant disorder characterized by pigmentation defects and sensorineural deafness (for a review see ref. 2) caused by the absence of melanocytes in the skin and the stria vascularis of the inner ear. The syndrome has been clinically classified into four types. Type I WS, characterized by dystopia canthorum, is caused by loss of function mutations in the *PAX3* gene. In the genetically heterogenous type II WS, dystopia canthorum is absent. In some but not all families, the disease is caused by mutations in the *MITF* (microphthalmia-associated transcription factor) gene (3). Type III WS, also called Klein–Waardenburg syndrome, is a rare and severe presentation of type I that includes limb abnormalities. Like type I WS, type III WS is caused by *PAX3* mutations. Finally, type IV WS (WS4, also called Shah–Waardenburg syndrome or Waardenburg–Hirschsprung disease) is characterized by an association with Hirschsprung disease, a defect of enteric innervation that results in intestinal aganglionosis. A few cases of homozygous mutations in the genes encoding the endothelin B receptor (*EDNRB*) (4,5) or its ligand endothelin 3 (*EDN3*) (6–8) have been described in this syndrome. More recently, heterozygous mutations in the gene coding for the transcription factor SOX10 (for SRY box 10) have been detected in WS4 patients (9,10). However, these three genes still seem unable to account for all the cases of Waardenburg–Hirschsprung disease.

Melanocytes, as well as enteric neurons, are derived from the neural crest. The broad and early expression of *SOX10* in the neural crest (11) argues for its possible involvement in other syndromes involving neural crest derivatives (i.e. neurocristopathies). We therefore searched for mutations affecting the *SOX10* gene in such disorders, including the Yemenite syndrome.

The Yemenite deaf-blind hypopigmentation syndrome was first described by Warburg in 1990. It was observed in a Yemenite sister and brother, born from unaffected parents, possibly consanguineous. The two patients showed cutaneous hypopigmented and hyperpigmented spots and patches on the trunk and extremities, grey hairs, white brows and lashes, microcornea, coloboma and abnormalities of the anterior chamber of the eye, severe sensorineural hearing loss, dental abnormalities and no mental retardation (12). This case is here referred to as

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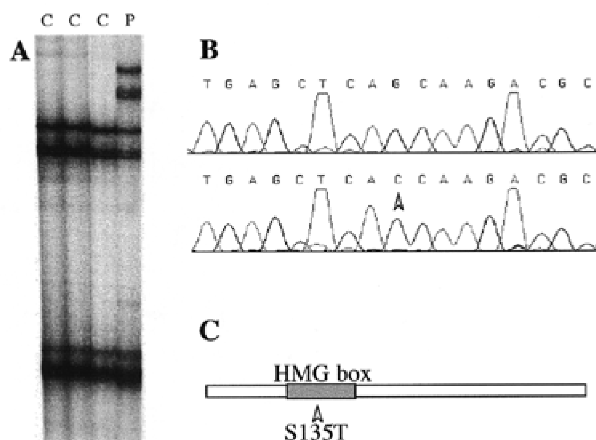

Figure 1. The S135T mutation. (**A**) SSCP analysis shows a band shift in the distal part of exon 3 (C, control DNAs; P, patient DNA). (**B**) After cloning of the PCR product, DNA sequencing revealed a G (normal allele, upper sequence) to C (mutated allele, lower sequence) transversion. (**C**) This mutation results in a Ser→Thr missense mutation localized in the central part of the HMG box, which is the DNA-binding domain of the SOX factors.

the severe form. A second case was observed later in a girl born from non-consanguineous parents. She had similar skin symptoms and hearing loss but without microcornea or coloboma and was reported as a mild form of this syndrome (13).

We examined the *SOX10* gene in the two cases and identified a *SOX10* mutation in the mild form, whereas no alteration could be found in the other. Based on genetic, clinical, molecular and functional data, we now suggest that these two cases represent two different syndromes.

RESULTS

We searched for variations in the *SOX10* coding region in patients affected by the Yemenite syndrome by using single strand conformation polymorphism (SSCP) analysis screening as previously described (9). A band shift was observed in the girl with the mild form. Cloning and sequencing of the variant PCR product revealed a G→C transversion which results in a S135T missense mutation localized in the central region of the high mobility group (HMG) box, the DNA binding domain of SOX proteins (Fig. 1). This variation was not found in 50 control DNAs (100 chromosomes).

The heterozygous status of this transversion is consistent with the previously described dominant inheritance of *SOX10* mutations (9). As the parents are phenotypically unaffected, it is likely to be a *de novo* mutation. Unfortunately, we were unable to analyse their genotypes as they were unavailable for testing.

The three-dimensional structure of SOX10 is unknown, but the structure of the HMG domain of the related factor SRY (sex determining factor) has been studied in its DNA-bound state by multidimensional NMR spectroscopy (14). This analysis showed that the mutated serine (S36 of the SRY HMG box) is one of the seven residues in direct contact with the DNA double strand (Fig. 2B). Moreover, it is involved in DNA binding through a hydrogen bond between its hydroxyl group and a thymine. Although S135T is a conservative mutation, addition of a methyl residue in this region of close contact between the HMG domain and the DNA can result in steric hindrance (Fig. 2C). As a result, this mutation is likely to impair DNA binding of the SOX10 factor.

In order to test this hypothesis, an *in vitro* functional study of the mutation was performed as previously described for Waardenburg–Hirschsprung *SOX10* mutations (15). When transiently transfected into tissue culture cells, the SOX10^{S135T} factor was localized to the nuclei, as indicated by its presence in nuclear extracts (Fig. 3A). Using a double-stranded oligonucleotide probe with adjacent binding sites for SOX and POU domain proteins, we next tested SOX10^{S135T} in gel shift assays for its ability to bind to DNA. As shown in Figure 3A, wild-type SOX10 efficiently recognized its site in the probe, as did the ubiquitous POU domain OCT1, which was also present in the nuclear extract. Adjacent binding of both proteins to the probe, furthermore, led to formation of a ternary complex with low mobility. When the same experiment was performed with nuclear extracts containing the SOX10^{S135T} factor, no binding to the probe was observed either alone or in combination with OCT1, which served as an internal control (Fig. 3A). Similar results were obtained with purified fusion proteins between glutathione *S*-transferase and the HMG domain of SOX10^{S135T}. Only at very high amounts did we obtain residual DNA binding of the fusion protein (data not shown). Consistent with these results, the SOX10^{S135T} factor completely lost its activity as an autonomous transcription factor, as is evident from its inability to stimulate a luciferase reporter construct with multiple SOX binding sites in the promoter in transiently transfected U138 glioblastoma cells (Fig. 3B). SOX10^{S135T} was likewise unable to synergistically enhance the activity of the POU domain protein Tst-1/Oct6/SCIP on a promoter that is efficiently activated by Tst-1/Oct6/SCIP and wild-type SOX10 (Fig. 3B; see also refs 15,16).

This demonstration of the deleterious effect of the S135T mutation prompted us to search more carefully for a *SOX10* mutation in the severe case of Yemenite deaf-blind hypopigmentation syndrome. Sequencing of the coding sequence revealed no variation in the affected boy. Moreover, a Southern blotting experiment showed no rearrangement of the gene in this family (data not shown). A large deletion of a DNA region containing *SOX10* (which would not have been detected by Southern blot analysis) could be excluded by the results of microsatellite analysis. Indeed, the parents and the two children are heterozygous for a microsatellite marker localized very close to the *SOX10* gene (~1.3 kb upstream of exon 2).

DISCUSSION

The SOX factors constitute a family of transcription factors that are characterized by a DNA-binding domain similar to the HMG domain of the sex determining factor SRY and are therefore able to bind and bend DNA (17). They play important roles during embryogenesis and show diverse and changing patterns of expression throughout development. Following SRY in XY sex reversal (18,19) and SOX9 in campomelic dysplasia (20,21), SOX10 is the third factor of this family known to be involved in a human disease. To date, six SOX10 mutations have been described in Waardenburg–Hirschsprung disease (9,10), a syndrome characterized by the association of

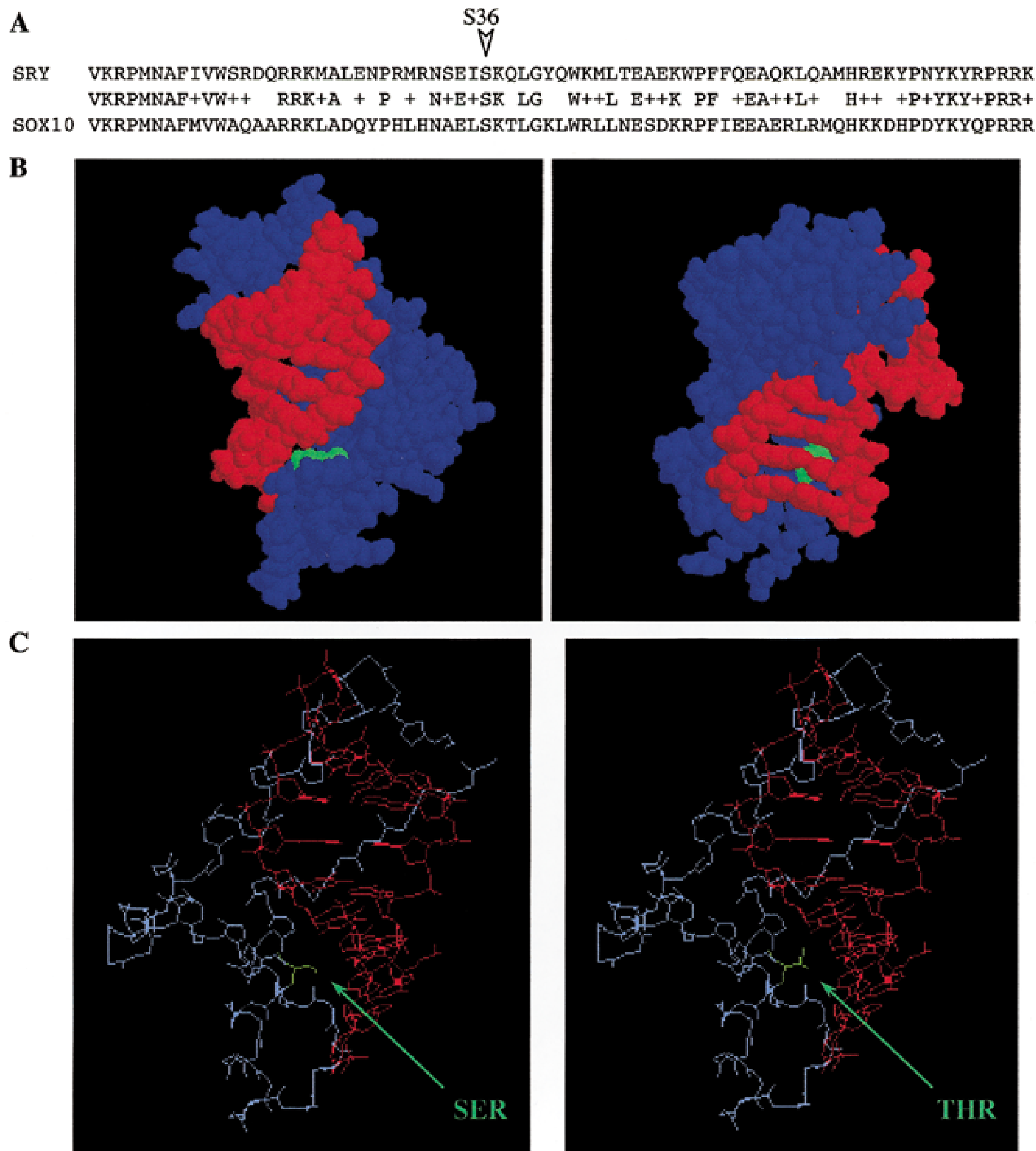

Figure 2. Structure of the HMG domain of SRY and the SOX10^{S135T} mutation. (**A**) Homology between the SRY and the SOX10 HMG domains. (**B**) Three-dimensional representation of the interaction between the HMG domain of SRY and the target DNA sequence. The two views illustrate the close interaction between the DNA helix (red) and the Ser (green) at position 36 of the SRY HMG domain (blue). (**C**) The Ser→Thr (green) mutation is placed in the SRY HMG domain (blue) to illustrate its possible effect on the interaction with DNA (red). Except for the Ser/Thr, only the backbone of the HMG domain is represented.

aganglionic megacolon of Hirschsprung disease with pigmentary-auditory defects of Waardenburg syndrome. This phenotype can be explained by the absence of a functional SOX10 factor in neural crest cells and derivatives, where it is consistently expressed (11,16,22,23).

However, the wide range of *SOX10* expression in the central and peripheral nervous systems, as well as in some mesectodermic derivatives of neural crest cells, suggested that it could be involved in other neurocristopathies (11). Indeed, we identified and characterized an S135T mutation in the previously described mild form of the Yemenite syndrome. Although the mutation is conservative, we provide evidence that it is deleterious.

First, a Ser at the same position is found in all SOX factors. It is worth noting that a missense mutation of this Ser in SRY factor reduces DNA binding and leads to sex reversal (24).

Second, a comparison with the three-dimensional structure of the DNA-bound HMG domain of SRY (14) shows that this residue plays an essential role in DNA binding. The twisted L shape of the SRY HMG domain presents a concave surface made up of three α-helices and accepts the double-strand DNA helix in a sequence-specific manner. The Ser is localized in the second α-helix of the HMG domain and is involved in DNA binding by direct interaction with a DNA base. The replacement of this Ser by a Thr is likely to result in steric hindrance that prevents binding.

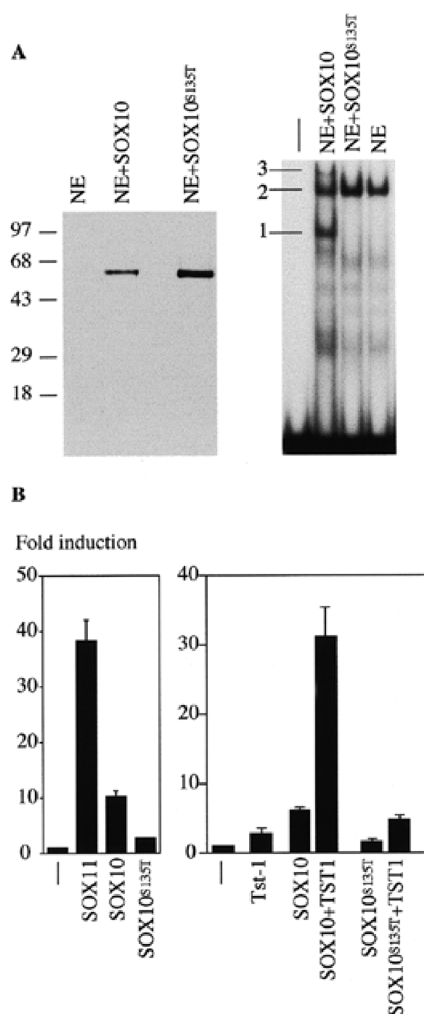

Figure 3. *In vitro* studies of the S135T mutation. (**A**) Western blot analysis of 8 µg of nuclear extract (NE) containing wild-type SOX10 or SOX10^{S135T} factor (size markers are shown on the left in kDa), and gel shift assay with 0.8 µg of the same extracts and the FXO oligonucleotide probe (16) showing the absence of DNA binding by the mutated factor. Complex 1, SOX10 + DNA; complex 2, OCT1 + DNA; complex 3, SOX10 + OCT1 + DNA. (**B**) Transient transfection experiments in U138 glioblastoma cells confirm the absence of autonomous transcriptional activity (3×SX luc reporter) of the SOX10^{S135T} factor in comparison with the wild-type SOX10 factor and the inability to synergistically stimulate the activity of the POU domain protein Tst-1/Oct6/SCIP (3×FXO luc reporter). Luciferase activities were determined in three independent experiments each performed in duplicate. Data are presented as fold induction above the level obtained for the luciferase reporter without co-transfected transcription factor.

Finally, functional *in vitro* assays show that the resulting SOX10 factor has a strongly reduced ability to bind DNA and is therefore unable to exert its activity as a transcriptional activator and as a stimulatory cofactor for other transcription factors. The results obtained with the S135T mutation are very similar to those obtained with another SOX10 mutation characterized in one case of Waardenburg–Hirschsprung disease, 482ins6, which results in the insertion of two amino acids in the third α-helix of the SOX10 HMG domain (9).

The girl who carries the S135T mutation was initially diagnosed as having Waardenburg syndrome, but because of doubts regarding the diagnosis she was re-evaluated at the age of 12. The diagnosis of Yemenite deaf-blind hypopigmentation syndrome was based on the presence of congenital sensorineural hearing loss, nystagmus, hypopigmentations, multiple freckles, patchy white hair and similar dental findings to those from the original two patients with the severe form (13). The main difference from the two original patients is the absence of anomalies of the anterior chamber of the eye and of choroidea coloboma in the patient with the milder syndrome. The girl has no constipation, dystopia canthorum or abnormal shape of the nose.

Our results established that the milder syndrome presented by this girl is due to a heterozygous mutation in the *SOX10* gene that results in a loss of function of this transcription factor. This observation indicates that the *SOX10* gene is involved in different types of neurocristopathy. The mutation in this patient without intestinal dysfunction, like those we described previously in Waardenburg–Hirschsprung disease, appears to be a loss-of-function allele. However, it is worth pointing out that up to now, the search for SOX10 mutations has been largely confined to patients with Hirschsprung disease, so the cases described are probably not an unbiased sample of phenotypes.

The fact that the parents' DNAs were not available precluded testing for a possible *de novo* dominant mutation in the mild form. On the other hand, the severe case seems to be transmitted in an autosomal recessive manner, as the two affected siblings were born from unaffected parents (12) and no SOX10 mutation or rearrangement can be detected. These results strongly suggest that these two syndromes are in fact different entities, the mild one being allelic to Waardenburg–Hirschsprung disease.

MATERIALS AND METHODS

Mutation detection and sequencing

The whole coding region of the human *SOX10* gene was screened using SSCP as previously described (9). The PCR product showing an aberrant mobility was cloned using the Topo Cloning kit (Invitrogen) and sequenced using the ABI PRISM Dye Terminator Cycle DNA Sequencing kit.

For the severe case, the absence of mutation in the *SOX10* gene was verified by direct sequencing of the purified PCR products. The absence of a partial deletion or rearrangement was tested by Southern blot. The parents' and children's DNAs were digested with *Bam*HI and the human *SOX10* cDNA was used as a probe (GenBank accession no. AJ001183). The primer sequences for the microsatellite analysis were: forward, 5'-GAGATTGTCCAAGGCCAGCA-3'; reverse, 5'-GTCACACGAGGCAGAGGCAT-3'. Tested on 50 control DNAs, the heterozygosity was 0.58.

Three-dimensional structure of DNA-bound SRY HMG

The NMR spectroscopy data for the complex between the HMG domain of SRY and its target DNA (14) are available at the Protein Data Bank (PDB accession code 1HRY). The three-dimensional structure is represented using RasMol (Fig. 2B) or Swiss-PdbViewer (Fig. 2C) software.

In vitro functional study of the mutation

Tissue culture cells were transfected by the calcium phosphate technique. For transcription assays, U138 cells were transfected with 2 µg of luciferase reporter (3×SX luc or 3×FXOluc) and 0.2 µg of CMV expression plasmid for Sox10, its S135T mutant or Tst-1/Oct6/SCIP per 60 mm plate. Cells were harvested 48 h after transfection and extracts were assayed for luciferase activity (16).

For extract preparation, COS cells were transfected with 10 µg CMV expression plasmid per 100 mm plate. Nuclear extracts were prepared and used for western blot analysis as described (16). A rabbit antiserum against rat Sox10 served as primary antibody at a dilution of 1:3000. Horseradish peroxidase-coupled protein A and the enhanced chemiluminescence system (Amersham) were used for detection.

Nuclear extracts were also analysed in gel shift assays in the presence of 2 µg poly(dG·dC) competitor DNA using ^{32}P-labeled FXO oligonucleotide as probe, as reported elsewhere in detail (16).

ACKNOWLEDGEMENTS

This work was supported by Biomed (contract BMH4-CT97-2107), the Institut National de la Science et de la Recherche Médicale (INSERM), the Deutsche Forschungsgemeinschaft (grant We1326/7–1), the Danish Medical Research Councils (9700832) and the Aage Bangs Foundation.

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