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Analysis of *SOX10* Mutations Identified in Waardenburg-Hirschsprung Patients: Differential Effects on Target Gene Regulation

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Abstract *SOX10* is a member of the *SOX* gene family related by homology to the high-mobility group (HMG) box region of the testis-determining gene *SRY*. Mutations of the transcription factor gene *SOX10* lead to Waardenburg-Hirschsprung syndrome (Waardenburg-Shah syndrome, WS4) in humans. A number of *SOX10* mutations have been identified in WS4 patients who suffer from different extents of intestinal aganglionosis, pigmentation, and hearing abnormalities. Some patients also exhibit signs of myelination deficiency in the central and peripheral nervous systems. Although the molecular bases for the wide range of symptoms displayed by the patients are still not clearly understood, a few target genes for *SOX10* have been identified. We have analyzed the impact of six different *SOX10* mutations on the activation of *SOX10* target genes by yeast one-hybrid and mammalian cell transfection assays. To investigate the transactivation activities of the mutant proteins, three different SOX target binding sites were introduced into luciferase reporter gene constructs and examined in our series of transfection assays: consensus HMG domain protein binding sites; SOX10 binding sites identified in the *RET* promoter; and Sox10 binding sites identified in the *P0* promoter. We found that the same mutation could have different transactivation activities when tested with different target binding sites and in different cell lines. The differential transactivation activities of the *SOX10* mutants appeared to correlate with the intestinal and/or neurological symptoms presented in the patients. Among the six mutant SOX10 proteins tested, much reduced transactivation activities were observed when tested on the SOX10 binding sites from the *RET* promoter. Of the two similar mutations X467K and 1400del12, only the 1400del12 mutant protein exhibited an increase of transactivation through the *P0* promoter. While the lack of normal SOX10 mediated activation of *RET* transcription may lead to intestinal aganglionosis, overexpression of genes coding for structural myelin proteins such as P0 due to mutant SOX10 may explain the dysmyelination phenotype observed in the patients with an additional neurological disorder. J. Cell. Biochem. 90: 573–585, 2003.

Key words: *SOX10*; Waardenburg-Hirschsprung syndrome; HMG domain; intestinal aganglionosis; neural crest; *RET*; *P0*

SOX genes encode HMG domain-containing transcription factors that share at least 50% amino acid sequence identity with the HMG DNA-binding domain of the sex-determining gene *SRY* [Pevny and Lovell-Badge, 1997; Kamachi et al., 2000]. Based on sequence homology within and outside the HMG domain, *SOX* genes are divided into seven different subgroups [Wegner, 1999; Bowles et al., 2000]. *SOX10* is a member of the group E *SOX* genes, it was identified as a transcriptional activator preferentially expressed in neural crest cells during early development and later in glial cells of both the peripheral and central nervous systems [Kuhlbrodt et al., 1998; Paratore et al., 2001; Potterf et al., 2001]. The neural crest gives rise to a number of different cell types and

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tissues, which include cells of the peripheral and the enteric nervous system as well as melanocytes. Mutation of the *Sox10* gene in the *Dominant megacolon* (*Dom*) mouse mutant causes aganglionosis of the colon, pigmentation defects, as well as a substantial loss of neurons and glia in the PNS [Herbarth et al., 1998; Southard-Smith et al., 1998, 1999]. In a knockout mutant mouse study, *Sox10* was demonstrated to be an important regulator of peripheral glia development [Britsch et al., 2001]. Other cellular studies showed that *Sox10* was required for the survival and glial fate acquisition of neural crest cells, as well as terminal differentiation of myelin-forming oligodendrocytes [Paratore et al., 2001, 2002; Stolt et al., 2002].

Waardenburg-Hirschsprung syndrome (OMIM #277580), also called Waardenburg-Shah (WS4) syndrome, combines features of Hirschsprung's disease (HSCR) (OMIM #142623) and Waardenburg syndrome (WS) (OMIM #193500). Patients with Waardenburg-Hirschsprung syndrome have pigmentary defects (white forelock, eyebrow, and eyelashes) and deafness, they also suffer from colon aganglionosis, which leads to intestinal obstruction in neonates and severe constipation in adults [Shah et al., 1981; Ambani, 1983; Badner and Chakravarti, 1990]. Three genes have been shown to lead to WS4 when mutated, these include the endothelin-B receptor gene (*EDNRB*), the gene for the ligand endothelin-3 (*EDN3*), and the *SOX10* gene [Amiel and Lyonnet, 2001]. The presence of *SOX10* mutations in the Waardenburg-Shah patients suggest that *SOX10*, together with *EDN3* and *EDNRB* genes, are involved in regulatory and signaling pathways for the normal development of the neural crest cell lineages which differentiate into melanocytes and enteric ganglia. So far 15 different *SOX10* mutations have been reported in patients presented with clinical features of WS4 [Pingault et al., 1998, 2002; Inoue et al., 1999; Southard-Smith et al., 1999; Touraine et al., 2000; Sham et al., 2001a]. Analysis of these 15 *SOX10* mutations and their associated intestinal phenotype shows that generally, there seems to be a correlation between the specific location of the mutation in the *SOX10* sequence and the severity of intestinal aganglionosis. Mutations around the HMG domain would lead to hypoganglionosis or short segment HSCR; mutations that affect the Group E conserved region and the C-terminal transactivation domain would lead to total aganglionosis or long segment HSCR [Sham et al., 2001a] (Fig. 1). A number of the patients with total or long segment HSCR also exhibited additional signs of deficiency in myelinating Schwann cells of the peripheral nervous system and oligodendrocytes of the central nervous system [Inoue et al., 1999; Pingault et al., 2000, 2001; Touraine et al., 2000]. There are other *SOX10* mutations identified in patients without full clinical features of WS4. A *SOX10* mis-sense mutation (S135T) has been reported in a case of Yemenite deaf-blind hypopigmentation syndrome [Bondurand et al., 1999], the patient described had characteristics of hypopigmentation, but not all the features of Waardenburg syndrome nor intestinal aganglionosis. In another case, a single nucleotide deletion (795delG) in the *SOX10* gene has been reported in a patient with peripheral neuropathy, hypopigmentation, deafness, and chronic intestinal pseudo-obstruction, which are not clinical features of WS4 [Pingault et al., 2000].

The wide range of phenotypic abnormalities caused by SOX10 dysfunction may be explained by its function in cooperation with other transcription factors in regulating the expression of different target genes during the development of several neural crest-derived cell lineages. In the development of melanocytes, SOX10 interact with PAX3 to activate transcription through the *MITF* promoter [Lee et al., 2000, Potterf et al., 2000, Verastegui et al., 2000]. In the development of myelinating glia, SOX10 interact with EGR2 to activate transcription through the *Connexin32* (*Cx32*) promoter [Bondurand et al., 2001]. On the other hand, Sox10 can directly regulate the expression of myelin protein zero (P0) without cofactors through binding with a 1.1 kb promoter fragment of the *P0* gene [Peirano et al., 2000]. In the development of the enteric nervous system, SOX10 was also shown to cooperate with PAX3 to activate transcription of RET [Taraviras et al., 1999; Bondurand et al., 2000]. A 45-bp element has been identified in the *c-RET* enhancer, which contains adjacent Pax3 and Sox10 binding sites required for synergistic Pax3 and Sox10 activation [Lang et al., 2000].

Among the *SOX10* mutations identified in WS4 patients (Fig. 1), only some of them were functionally characterized. Most of these studies indicated the loss of transactivation

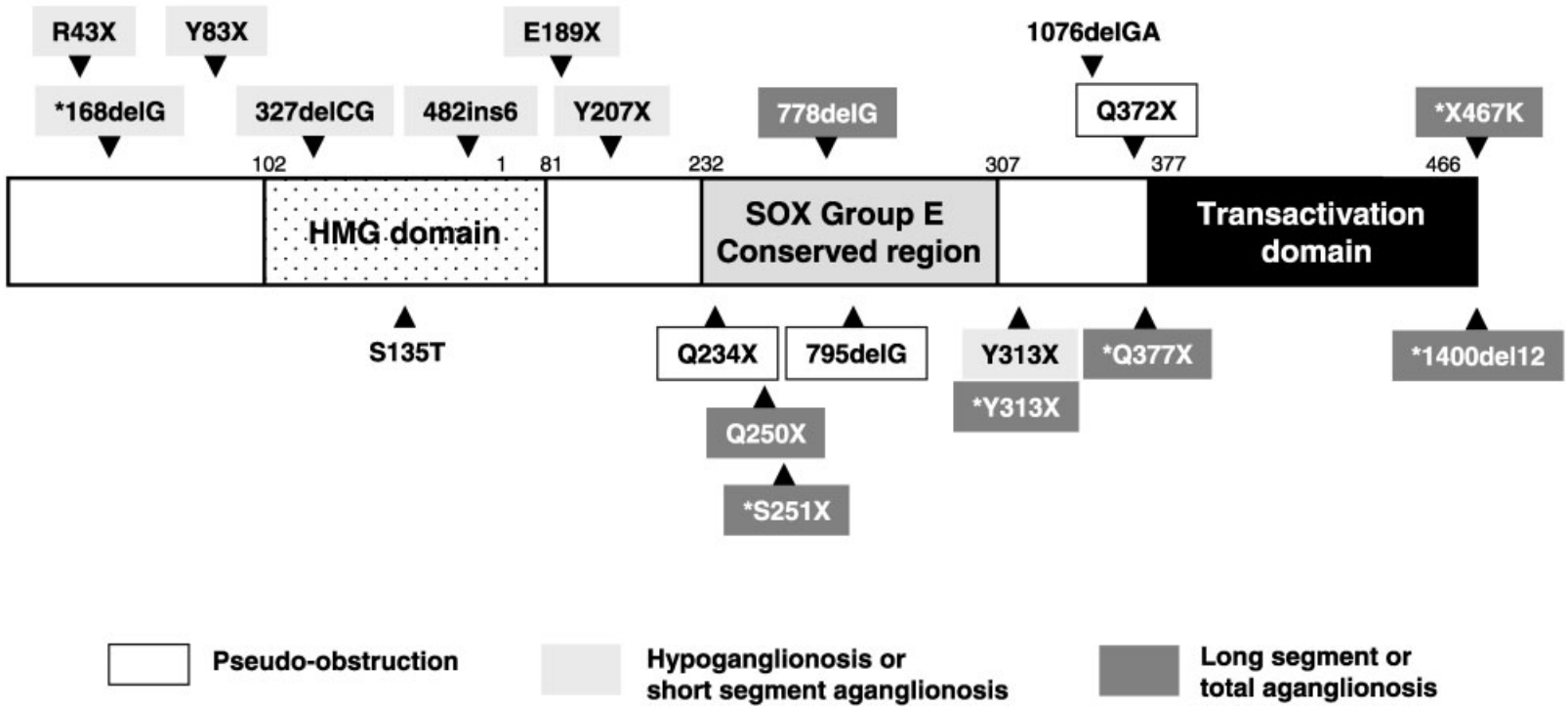

Fig. 1. A schematic diagram of the functional domains of the SOX10 protein and a summary of all *SOX10* mutations reported so far. Analysis of the intestinal phenotypes of the patients with *SOX10* mutations shows that mutations around the HMG domain all led to hypoganglionosis or short segment aganglionosis as indicated in light gray shading, mutations in the Group E conserved domain and the transactivation domain led to long segment or total aganglionosis as indicated in dark gray shading, mutations led to intestinal pseudo-obstruction are shown in clear boxes. The other mutations shown are either with unknown or no abnormal intestinal phenotype. Mutations associated with neurological disorders are shown below the schematic diagram of the SOX10 protein, those without associated neurological phenotype are shown above. The six *SOX10* mutations we examined in this study are highlighted with an asterisk (*).

activities due to the lack of the HMG domain or the structural truncation of the C-terminal transactivation domain in the mutant. It has been shown that SOX10-dependent activation of *MITF* promoter as well as the synergistic activation of the *MITF* promoter with PAX3 were lost almost completely for several mutants tested in HeLa and mouse melanoma cell lines [Bondurand et al., 2000; Verastegui et al., 2000]. Transactivation of the promoter of another target gene *Cx32* in HeLa cells by several *SOX10* mutants tested was markedly reduced, the synergistic effects of SOX10 and EGR2 on the activation of the *Cx32* promoter were also greatly reduced [Bondurand et al., 2001]. Two C-terminal truncated *SOX10* mutants were also tested in neuroblastoma cells, which showed severely reduced activation of the *P0* promoter [Peirano and Wegner, 2000; Peirano et al., 2000]. Despite the functional analyses performed on several *SOX10* mutants, the correlations between the abnormal regulation of SOX10 target genes and the phenotypic abnormalities observed in the WS4 patients had not been addressed in these studies.

We have performed yeast one-hybrid assays and transfection assays in different neural and glial cell lines in the study of the two *SOX10* mutations 168delG and X467K we identified [Sham et al., 2001a], and four other mutations reported by others [Inoue et al., 1999; Southard-Smith et al., 1999; Touraine et al., 2000] (Fig. 1) (Table I). In this study, we have analyzed the impact of these *SOX10* mutations on the activation of SOX10 target genes in different cell types in order to further understand the genotype-phenotype relationship between the different mutations and the manifestation of symptoms, and correlate these mutations with the abnormal intestinal and neurological phenotype observed in the WS4 patients.

MATERIALS AND METHODS

DNA Constructs

Wild-type full-length human *SOX10* cDNA was isolated by reverse transcription-polymerase chain reaction (RT-PCR) as previously described [Sham et al., 2001b]. The sequences of the forward and reverse primers were 5′-ATG GCG GAG GAG CAG GAT CTA-3′and 5′-TTA GGG CCG GGA CAG TGT CGT-3′, respectively. Six *SOX10* mutations previously identified in Waardenburg-Hirschsprung patients, 168delG, S251X, Y313X, Q377X, 1400del12, and X467K [Inoue et al., 1999; Southard-Smith et al., 1999; Touraine et al., 2000; Sham et al., 2001a] were introduced into the wild-type full-length sequence by PCR based site-directed mutagenesis to produce mutant *SOX10* cDNAs. The nucleotide sequences of all the cDNA fragments were verified by sequencing reactions.

For the yeast one-hybrid assay, wild-type and mutant *SOX10* cDNAs were cloned into the yeast expression vector pLexA (Clontech), which contains the GAL4 DNA binding domain (GAL4-DBD) to produce GAL4-DBD-SOX10 wild-type and mutant fusion proteins.

For mammalian cell transfection assays, wild-type and mutant *SOX10* cDNAs were cloned in-frame into the mammalian expression vector pcDNA3 (Invitrogen) which contained the CMV promoter. Three different luciferase reporter constructs 5X-SOX, 2X-RET, and 3X-P0, named after the sequence origin of SOX10 binding sites, were generated by inserting PCR

TABLE I. Summary of the *SOX10* Mutations and the Associated Phenotypes of the Affected Patients Included in This Study

Sox10 mutation	Effect on protein sequence	Affected colon phenotype	Additional neurological phenotypes	Reference
168delG	Frameshift, truncation before HMG	Short segment aganglionosis	None	Sham et al. [2001a]
S251X	Truncation at the Group E conserved region	Total colonic aganglionosis	Impairment of CNS and ANS	Touraine et al. [2000]
Y313X	Truncation after HMG domain	Total colonic aganglionosis	Impairment of CNS and ANS	Touraine et al. [2000]
Q377X	Truncation of C-terminal domain	Variable diagnosis, ranging from hypoganglionosis to long segment aganglionosis	Ataxic cerebral palsy	Southard-Smith et al., [1999]
1400del12	82aa added at stop codon	Long segment aganglionosis	Demyelinating neuropathy of the CNS and PNS severe leukodystrophy	Inoue et al. [1999]
X467K	86aa added at stop codon	Total colonic aganglionosis	None	Sham et al. [2001a]

fragments or oligonucleotides upstream of the SV40 promoter of the pGL3-promoter vector (Promega). For the 5X-SOX reporter construct, oligonucleotides containing five tandem copies of the consensus SOX-HMG binding sites (-CTTTGT-) [Kuhlbrodt et al., 1998] were inserted. For the 2X-RET reporter construct, two tandem copies of oligonucleotides (5′-GCC TCA GAG TCT GTG CTT CCA ACC ACC ATG TCA CAC TGC CCA TGG-3′) corresponding to part of the *RET* enhancer with a SOX10 binding site [Lang et al., 2000] were inserted into the pGL3-promoter vector. For the 3X-P0 reporter construct, a DNA fragment corresponding to positions −229 to −116 of the rat *P0* promoter, which contains three Sox10 binding sites [Peirano and Wegner, 2000] was amplified by PCR using primers 5′-TAG GGC GGT ACC CCT AGG GTA GGG GGA GGT CAG TAT A-3′and 5′-TAG GGC CTC GAG TGG CAG GGG CAT GGA GCA GAA GG-3′ and inserted into the pGL3-promoter vector.

Yeast Transformation and One-Hybrid Transactivation

Yeast reporter host strain EGY48 (Clontech), which had a wild-type *LEU2* gene under the control of LexA operators, was grown on yeast-peptone-dextrose (YPD) plates, YPD medium, or in supplemented Synthetic Dropout (SD) medium. The pLexA-hSOX10 or pLexA-hSOX10m constructs were co-transformed with the p8op-lacZ reporter construct into yeast strain EGY48 by the lithium acetate method. All procedures were carried out according to the instructions of the manufacturer (Clontech). The transformants were selected in SD agar plates lacking His and Ura. Trp^+ Ura^+ transformants were then tested for the expression of *lacZ* reporter gene by blue/white screening of the colony lift β-galactosidase filter assay as previously described [Chan et al., 2000; Ng et al., 2002]. Quantitative liquid culture assay with ONPG as substrate of β-galactosidase activity was performed as described by manufacturer's protocol (Clontech). The OD_{600} of the yeast culture and the total time of the reaction were recorded. Upon terminating the reaction, the OD_{420} was determined by spectrophotometry. The β-galactosidase units for each transformed yeast culture, an indirect measure of transactivation potential, were calculated by applying the formula: $(OD_{420} \times 1000)/(t \times V \times OD_{600}) = \beta$-galactosidase units, where t is time of reaction in minutes and V is the volume of the cells used in milliliter. Each culture was assayed in triplicate and each GAL4-DBD SOX10 wild-type and mutant fusion protein was assessed in a minimum of two separate experiments. Control transformations were performed and assessed in parallel.

Cell Culture and Transfection

NIH/3T3 mouse fibroblast, Neuro2A mouse neuroblastoma, C6 rat glioma, and U-138 human glioblastoma cells were obtained from American Type Culture Collection (ATCC) and were maintained in DMEM medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS). NIH/3T3, Neuro2A, C6, and U-138 cells were seeded in 24-well culture plates and co-transfected with various plasmid constructs on the following day. Transfections were performed in quadruplicate using LipofectAMINE PLUS^{TM} reagent (Life Technologies, Inc.). A total of 1 μg of plasmid DNA (containing 0.45 μg of 5X-SOX, 2X-RET, or 3X-P0 luciferase reporter plasmid, 0.5 μg of pcDNA3 expression vector with either mutant or wild-type *SOX10* cDNAs, 0.05 μg of pRc/RSV-lacZ) and 1 μg of lipofectamine reagent were added to the cells and incubated for 3 h according to the manufacturer's instructions. After 3 h, the cells were rinsed in PBS and cultured for 24 h in fresh medium supplemented with 10% FBS. Transfection with control pcDNA3 plasmids without *SOX10* insert was performed in parallel. All transfection experiments were performed at least twice using different batches of cells with different preparations of plasmid DNAs and similar results were obtained.

Luciferase Activity Assay and β-Galactosidase Assay

Cells were harvested after transfection and assayed for luciferase activity using a commercially available Luciferase Assay System (Promega). The cells were washed three times with PBS buffer and lysed with cell culture lysis reagent (CCLR). Cell lysate (20 μl) was mixed with 100 μl of Luciferase Assay Reagent in a microplate and the luciferase activities were measured by a luminometer (EG&G Berthold). As internal controls for normalization of the differences in transfection efficiencies between samples, the activities of β-galactosidase were measured using aliquots of cell lysates by liquid assays. The background luciferase activity of

the negative control (pcDNA3 vector alone) was subtracted from the luciferase activities of those tested *SOX10* cDNAs. The normalized luciferase activities of wild-type *SOX10* was arbitrarily set as 1, the activities of the mutant *SOX10* cDNAs were expressed as fold induction relative to the wild-type cDNA.

RNA Extraction and RT-PCR

RNA samples were extracted from transfected C6 cells using TRIZOL reagent (Life Technologies, Inc.) and reverse transcribed into cDNA using Superscript reverse transcriptase (Life Technologies, Inc.) according to the manufacturer's instructions. The cDNAs were then used for PCR analyses with primer pairs specific for the expressed mutant *SOX10*, wild-type *SOX10*, and endogenous *Sox10*. The sequences of the primers for X467K, 1400del12, and wtSOX10 were 5′-ACA CAC TGG GAG CAG CCA GT-3′, and 5′-ACA GTG GGA GTG GCA CCT TC-3′, and the sequences of the primers for 168delG were 5′-TAC GAC TGG ACG CTG GTG CCC A-3′ and 5′-ACA GTG GGA GTG GCA CCT TC-3′. For amplification of endogenous *Sox10*, specific primers for the 3′-untranslated region were used and their sequences were 5′-ACA CAC TGG GAG CAG CCA GT-3′ and 5′-GGT CCT GGG ATA GAG GGT TA-3′. The expressed *SOX10* and endogenous *Sox10* cDNAs were amplified in separate PCR reactions. Samples that lacked RT were also amplified to control for the presence of any contaminating genomic DNA.

RESULTS

We have examined the transactivation activity of two *SOX10* mutations we identified, 168delG and X467K [Sham et al., 2001a], and of four other *SOX10* mutations (S251X, Y313X, Q377X, and 1400del12) previously described [Inoue et al., 1999; Southard-Smith et al., 1999; Touraine et al., 2000]. The positions of these mutations along the SOX10 protein sequence are schematically summarized in Figure 1. In the 168delG mutant, the deletion of a single nucleotide (G) at position 168 from the start codon resulted in a frame-shift leading to the truncation of the SOX10 polypeptide to only 107 amino acids. The predicted mutant protein has a novel C-terminal sequence, but lacks the HMG DNA-binding domain and the normal C-terminal transactivation domain. In the X467K mutant, transversion of T to A at the first base of the stop codon changed it to a codon for lysine. The mutant SOX10 open reading frame encodes an extra 86 amino acid long, proline-rich peptide at the C-terminus [Sham et al., 2001a]. Four other *SOX10* mutations, which could lead to different truncations of SOX10 protein, including truncation at the Group E conserved region (S251X) or truncation of the C-terminal domain (Y313X, Q377X) [Southard-Smith et al., 1999; Touraine et al., 2000], were examined in order to test the functional importance of different protein domains in target gene activation. Mutant 1400del12 [Inoue et al., 1999], which has a deletion of 12 nucleotides at the stop codon, and has an additional 82 amino acid C-terminal tail similar to that in X467K, was also included in this study.

Yeast One-Hybrid Transactivation Assay

To determine the transactivation potential for six of the SOX10 mutant proteins, yeast one-hybrid assays were performed. *SOX10* cDNAs, either wild-type or mutant forms, were fused to the GAL4-DBD in the yeast expression vector pLexA (Fig. 2A) and co-transformed with a *lacZ* reporter construct containing upstream GAL4-DNA-binding sites. The transactivation activities of the mutant *SOX10* cDNAs were determined by colony lift β-galactosidase filter assay, the relative level of transactivation was measured quantitatively by liquid culture β-galactosidase assay.

In the colony lift β-galactosidase filter assay, among the seven different human *SOX10* cDNAs tested, only the wild-type and 1400del12 mutant cDNAs were able to transactivate the reporter in yeast cells as indicated by the blue colour on the filter (Fig. 2B). All the other mutants examined (168delG, S251X, Y313X, Q377X, X467K) did not show any transactivation activity. When the levels of transactivation activity were measured by liquid culture β-galactosidase assay, similar results were obtained (Fig. 2C). Only wild-type *SOX10* and the 1400del12 mutant were shown to have transactivation activity. Wild-type SOX10 fusion protein had the highest transactivation activity, while the activity of the 1400del12 mutant was about 20% of that of the wild-type (Fig. 2C). Interestingly, the X467K mutant, which only differs from the 1400del12 mutant by having 4 additional amino acids KRGP at codon 467, did not demonstrate any transactivation activity

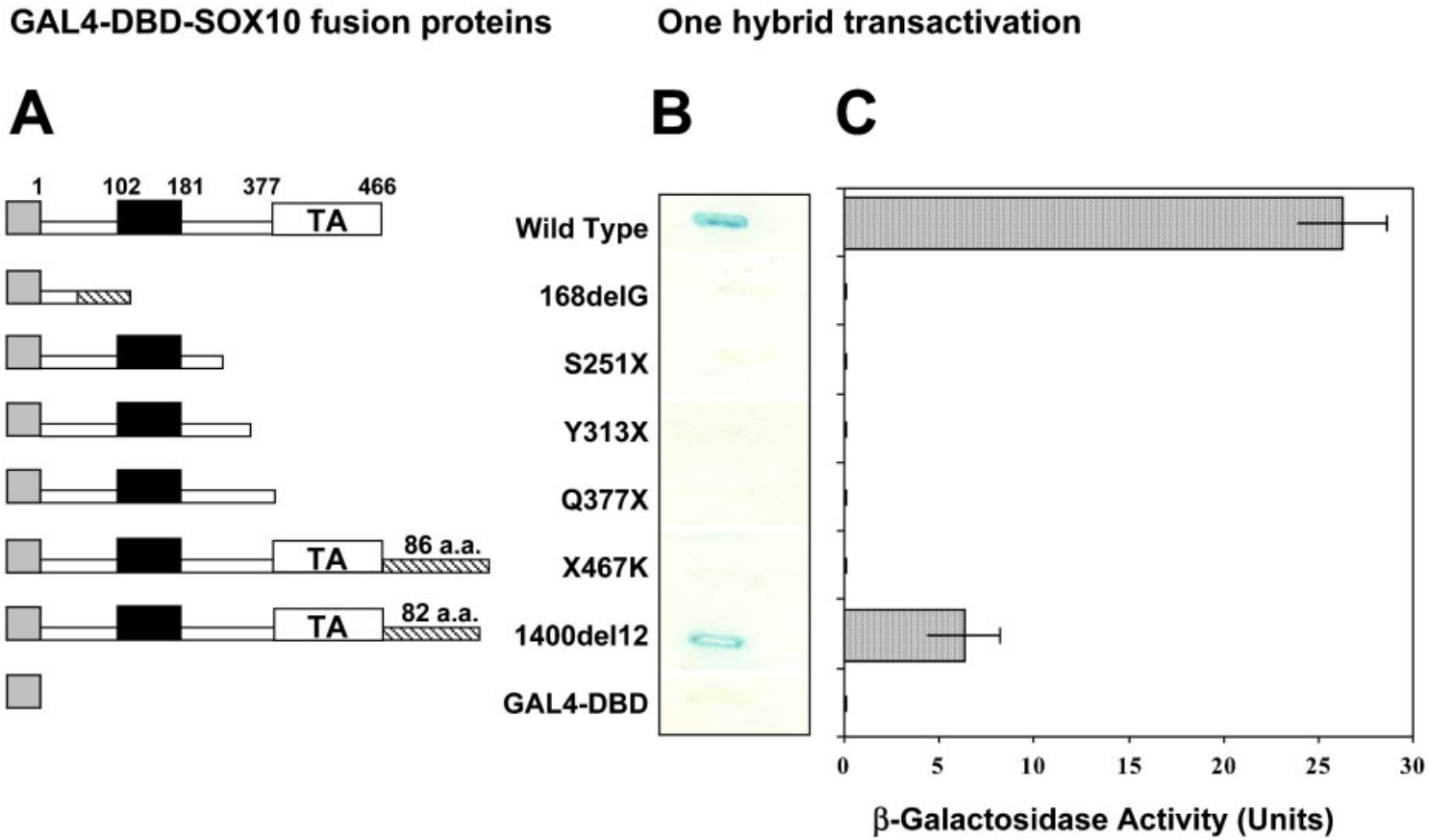

Fig. 2. Transactivation activity of wild-type and mutant SOX10 proteins determined by yeast one-hybrid transactivation assay. **A**: Schematic diagram of the wild-type and mutant GAL4-DBD-SOX10 expression constructs. Gray boxes represent the GAL4-DBD, black boxes represent the HMG domain, clear boxes represent the transactivation domain (TA), shaded bars represent the unrelated C-terminal tails, clear bars represent coding region of wild-type and various mutant SOX10 proteins. **B**: Colony lift β-galactosidase assay of the yeast one-hybrid transactivation experiment. Wild-type and mutant 1400del12 were able to transactivate the *lacZ* reporter gene, as shown by the blue colored product. GAL4-DBD is included to indicate the level of background transactivation in the assay. **C**: Quantitative analysis of yeast one-hybrid experiments determined by liquid culture assay. The average β-galactosidase units ± SD ($n = 6$) is shown. Only the wild-type and mutant 1400del12 were shown to have transactivation activities.

in the yeast one-hybrid assay system. Therefore, the 1400del12 mutant is different from all the other mutants tested, in retaining some transactivating function. However, previous cell transfection studies have shown that this 1400del12 mutant did not have any transactivating activity, implying that all *SOX10* mutations would abolish the transactivating function [Bondurand et al., 2000]. In order to examine the transactivating potential of 1400del12 and other mutations on specific target genes in mammalian cells, we analyzed the activities of *SOX10* mutants in different neural and glial cell lines using reporters with three different SOX10 binding sites.

Effects of *SOX10* Mutations on Transcriptional Activation

To assess the effects of the mutations on the ability of the mutant SOX10 proteins in activating the transcription of their target genes in mammalian cells, we performed a series of cell transfection experiments using luciferase reporter constructs. The transactivating effects were examined in four different cell lines including NIH/3T3 mouse fibroblast, N2A mouse neuroblastoma, and two glial cell lines C6 rat glioma and U-138 human glioblastoma. Apart from the C6 cell line, the other three cell lines used in this study do not have any endogenous *Sox10* expression [Kuhlbrodt et al., 1998]. By RT-PCR assay, we demonstrated that the C6 glioma expressed endogenous *Sox10*. As shown in Figure 3, DNA bands of 445 bp corresponding to the expected mutant *SOX10* fragments for X467K and 1400del12 were detected in the transfected cells. Fragments of about 300 bp were amplified from the *SOX10* mutant 168delG and wild-type *SOX10* in the transfected cells. Absence of human *SOX10* expression in the pcDNA3 expression vector transfected cells and in mock transfection were also confirmed (Fig. 3). The control endogenous *Sox10* band of about 300 bp was amplified from all cDNA samples. No DNA fragment was amplified from samples that had not undergone reverse transcription, indicating that the amplified bands were derived from RNA without any contaminating genomic DNA.

SOX10 cDNA clones (mutants and wild-type control) were co-transfected with a luciferase reporter construct containing five tandem copies of SOX-HMG consensus binding sites fused to the promoter. In the co-transfection assays using four different cell lines, all the SOX10 mutants tested have diminished or reduced transactivation activity when compared with the wild-type (Fig. 4A,B) except the 1400del12 mutant. In the U-138 human glioblastoma cell line, the 1400del12 mutant cDNA had a level of transactivation activity comparable to wild-type *SOX10* cDNA (Fig. 4B). These results were consistent with previous reports which showed that the transactivation activities of *SOX10* mutants were greatly reduced when tested in other cell lines. However, the 1400del12 mutant did have a high level of transactivation activity in the U-138 human glioblastoma cells.

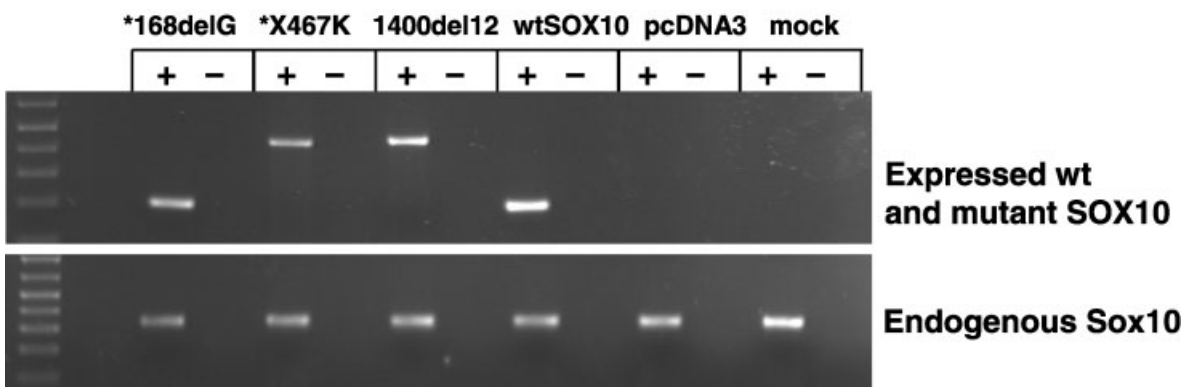

Fig. 3. Expressions of wild-type (wtSOX10) and SOX10 mutant 168delG, X467K, 1400del12 transcripts in the C6 glioma cell line examined by reverse transcription-polymerase chain reaction (RT-PCR) in the presence (+) and absence (−) of reverse transcriptase. (*) indicates the two *SOX10* mutations we recently identified in WS4 patients. Mock transfection and transfection with pcDNA3 plamid alone were included as controls. In all the samples, endogenous Sox10 bands of about 300 bp were amplified; wild-type and mutant SOX10 bands of expected sizes were also amplified from the transfected C6 glioma cells.

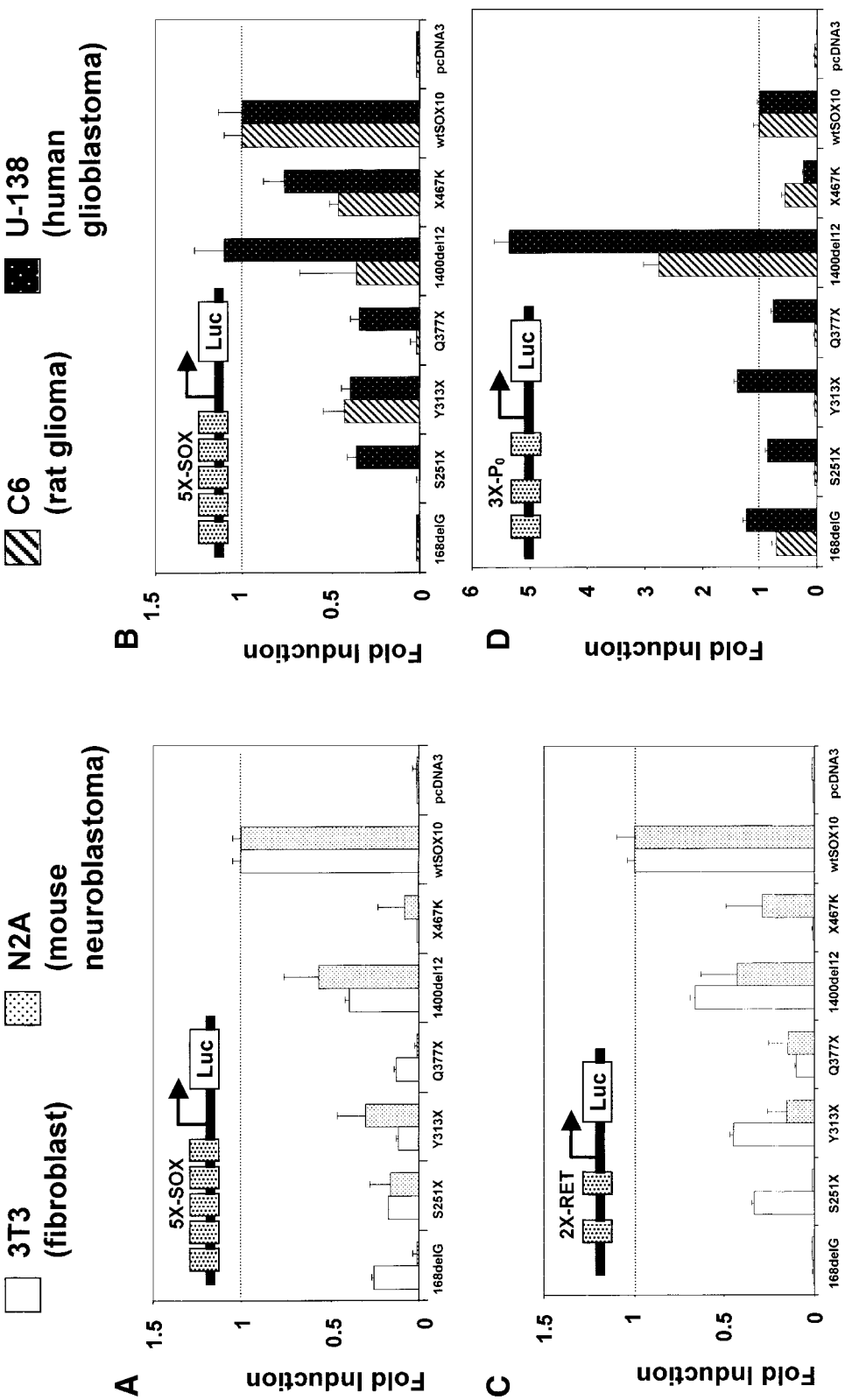

Fig. 4. Transactivation activity of wild-type and mutant SOX10 proteins determined by transfection assays in different neuronal and glial cell lines. The luciferase reporter constructs 5X-SOX (**A**,**B**), 2X-RET (**C**), and 3X-P0 (**D**) were co-transfected into NIH/3T3 mouse fibroblast, Neuro2A neuroblastoma (mouse), C6 glioma (rat), and U-138 glioblastoma (human) cells together with expression constructs for wild-type or mutant SOX10 cDNA, or control pcDNA3 expression plasmid as indicated in the *X*-axis of each of the panels. The normalized luciferase activities of the wild-type SOX10 was arbitrarily set as 1, the activities of the mutant SOX10 cDNAs were expressed as fold induction relative to the wild-type. Details of the calculation are described in the Materials and Methods section. Data are illustrated as mean ± SD of a minimum of two experiments, each performed in quadruplicate.

Effects of *SOX10* Mutations on *RET* Promoter Transactivation

To examine the effects of *SOX10* mutations on target genes involved in intestinal aganglionosis, we studied the effects of mutations on the transactivation of reporter gene expression mediated by SOX10 binding site from the human *RET* promoter [Lang et al., 2000]. As the *RET* gene is normally expressed in enteric neuroblasts, and as the other co-factors required for *SOX10* mediated *RET* activation would probably be present in neuronal cells, we used N2A neuroblastoma cells for the transactivation assay, and 3T3 fibroblast cells for comparison. In both 3T3 and N2A cell lines, all the mutants tested had reduced transactivation activity when compared with wild-type *SOX10* (Fig. 4C). Therefore, none of the SOX10 mutants tested were as efficient as the the wild-type protein in interacting with the target binding sites in the *RET* promoter.

Effects of *SOX10* Mutations on *P0* Promoter Transactivation

In order to correlate the transactivation activity of SOX10 with the neurological abnormality of myelin deficiency observed in some of the patients, we examined the effect of *SOX10* mutations on the *P0* promoter-mediated expression [Peirano and Wegner, 2000] using two glial cell lines C6 glioma and U-138 glioblastoma. In transfection experiments using the C6 cell line, five of the *SOX10* mutants tested had much reduced transactivation activity, only the 1400del12 mutant had a 2.6-fold higher activity when compared with the wild-type. Similarly, when tested in the U-138 cell line, the 1400del12 mutant had a 5.5-fold higher transactivation activity, significantly higher than that of wild-type. In the U-138 cell line, four of the mutants tested (168delG, S251X, Y313X, and Q377X) had transactivation activity comparable to the wild-type, but the X467X mutant had much reduced activity. The high transactivation activity of the 1400del12 mutant in both of the glial cell lines tested appeared to correlate with the severe dysmyelination phenotype observed in the patient.

DISCUSSION

By transcriptional activation experiments using wild-type and mutated human *SOX10*, we have demonstrated differential effects of *SOX10* mutations on target gene regulation, which could explain the differences in clinical symptoms. We examined six *SOX10* mutations, including 168delG, S251X, Y313X, Q377X, 1400del12, and X467K; and we found that for the same mutation it could have different transactivation activities when tested with different target binding sites and in different cell lines. The differential transactivation activities of the *SOX10* mutants appeared to correlate with the intestinal and/or neurological symptoms presented in the patients. Our results suggest that specific molecular mechanisms could be involved in the pathogenesis of different disease phenotypes observed in Waardenburg-Hirschsprung (WS4) syndrome patients.

The range of abnormalities associated with *SOX10* mutations can be explained by the function of SOX10 in activating the expression of different target genes. In the development of the enteric nervous system, SOX10 has been shown to cooperate with PAX3 to activate the transcription of *RET* [Taraviras and Pachnis, 1999; Taraviras et al., 1999; Lang et al., 2000]. Our functional study by transfection assays in neuronal cell lines showed that none of the six *SOX10* mutants we tested could efficiently transactivate reporter gene expression through the SOX10 binding sites in the *RET* promoter (Fig. 4C). It is anticipated that even in the presence of PAX3, the induction of reporter gene expression due to the *SOX10* mutants would still be lower than that for the wild-type, as had been demonstrated in several other studies [Bondurand et al., 2000, Lang and Epstein, 2003]. Our study on transactivation of *RET* promoter shows that mutations in *SOX10* have a loss-of-function effect, leading to reduced *RET* transcription. Impaired upregulation of the *RET* gene would contribute to intestinal aganglionosis, therefore our results on *SOX10* mutants would explain the phenotype observed in the patients, all of whom had intestinal aganglionosis of various degrees.

It has been shown that SOX10 directly regulates the expression of myelin protein zero (*P0*) without cofactors through binding with its promoter [Peirano et al., 2000]. In this study, we have also demonstrated the ability of the SOX10 to transactivate target gene expression without added cofactors in glial cell types. This shows the intrinsic ability of SOX10 to activate the *P0* promoter either alone or by recruiting necessary endogenous cofactors already present in

the glial cells. Moreover, despite the fact that most of the *SOX10* mutants could not transactivate the *P0* promoter as readily as wild-type SOX10, the 1400del12 mutant actually stimulated reporter gene activity far better than the wild-type SOX10 protein (Fig. 4D). Therefore, in glial cells, certain *SOX10* mutant could have a gain-of-function effect and stimulate target gene expression to a greater extent. Overexpression of myelinating proteins have been demonstrated to cause dysmyelination, as shown in the overexpression of PMP22 in Charcot-Marie-Tooth disease [Lupski, 1992; Magyar et al., 1996; Robaglia-Schlupp et al., 2002], and in the dysmyelinating neuropathy of transgenic mice with overexpression of the P0 glycoprotein [Wrabetz et al., 2000]. It has been suggested that overproduction of myelinating proteins would cause the disorganization of the myelin structure and as a result lead to the dysmyelination of axons. Based on this mechanism, our results of the greatly enhanced transactivation activity of the 1400del12 mutant on the *P0* promoter in glial cells can explain the severe neurological phenotype of dysmyelination observed in the patient with this *SOX10* mutation [Inoue et al., 1999] (Table I).

Apart from the 1400del12 mutation, the other five *SOX10* mutations when examined using binding sites derived from the *RET* promoter or from the HMG domain protein consensus binding sequence, the transactivation activities as shown by the reporter expression assays were reduced regardless of the cell types used (Fig. 4A–C). There did not appear to be any correlations between the presence of certain functional protein domains in the SOX10 mutants and target gene activation. In glial cells where *P0* is a downstream target of SOX10, the transactivation activities of the mutant SOX10 proteins were either diminished or comparable with the wild-type. Three of these *SOX10* mutations (S251X, Y313X, and Q377X) were apparently associated with myelination and neurological abnormalities in the patients (summarized in Fig. 1 and Table I). However, unlike the 1400del12 mutant, in our transfection assays in human glioblastoma cells U-138 (Fig. 4D), the transactivation activities of these three mutants were only comparable to the wild-type and no significant upregulation of *P0* promoter could be observed. We think that the level of *P0* upregulation may contribute to the severity of neurological abnormalities displayed in these selected WS4 patients, alternatively other *SOX10* target genes might be affected by these mutations which contributed to the complex phenotypes.

Among the six *SOX10* mutations we examined, the 1400del12 mutation displayed different activities from the others. The 1400del12 mutation is a deletion of 12 nucleotides at the stop codon of the *SOX10* gene, as a result the open reading frame is extended further downstream and an additional 82-amino acid, proline-rich C-terminal tail will be appended to the SOX10 protein upon translation [Inoue et al., 1999]. In yeast one-hybrid assays, 1400del12 was the only *SOX10* mutant, which retained some transactivation activity. Although the transactivation activity of the 1400del12 mutant was only 20% of that of the wild-type in the yeast assay system (Fig. 2), our data suggests that the 1400del12 mutation could produce functionally active mutant protein. Despite the fact that mutants 1400del12 and X467K differ from one another by only 4 amino acid residues, this small difference had a critical effect on their transactivation of target gene involved in myelination of glial cells. In this study, 1400del12 was shown to have a significant increase in transactivation activity through *P0* promoter in the glial cell lines, whereas X467K had little effect. The structure-function relationships for these two mutant proteins deserve further investigations, but the different transactivation activities demonstrated by these two similar mutants may explain their respective phenotype observed in the patients: the patient with 1400del12 mutation suffered from severe neurological abnormalities [Inoue et al., 1999] but the patient with the X467K mutation did not have any neurological disorder [Sham et al., 2001a]. Previous studies of the 1400del12 mutant, which showed that this *SOX10* mutant failed to activate gene transcription through *MITF* or *Cx32* promoters with or without added cofactors, were based on analyses in HeLa cell lines [Bondurand et al., 2000, 2001; Verastegui et al., 2000]. In this study, we have for the first time investigated a series of SOX10 mutants together with two recently identified mutations 168delG and X467K in neuronal and glial cell lines through analysis of different target genes. While the lack of normal SOX10 mediated activation of *RET* transcription may lead to intestinal aganglionosis, overexpression of genes coding for structural

myelin proteins such as P0 due to mutant SOX10 may explain the dysmyelination phenotype observed in the patients with additional neurological disorder.

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