Translation of SOX10 3′ untranslated region causes a complex severe neurocristopathy by generation of a deleterious functional domain

Ken Inoue1,2,*, Tomoko Ohyama2, Yosuke Sakuragi1, Ryoko Yamamoto1, Naoko A. Inoue1, Yu Li-Hua1, Yu-ichi Goto1, Michael Wegner3 and James R. Lupski2,4,5

1Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo 187-8502, Japan, 2Department of Molecular and Human Genetics, 3Institut für Biochemie, Emil-Fischer-Zentrum, Universität Erlangen, Erlangen D-91054, Germany, 4Department of Pediatrics, Baylor College of Medicine and 5Texas Children’s Hospital, Houston 77030, TX

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Peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome and Hirschsprung disease (PCWH) is a complex neurocristopathy caused by SOX10 mutations. Most PCWH-associated SOX10 mutations result in premature termination codons (PTCs), for which the molecular mechanism has recently been delineated. However, the first mutation reported to cause PCWH was a disruption of the native stop codon that by conceptual translation extends the protein into the 3′ untranslated region (3′-UTR) for an additional 82 residues. In this study, we sought to determine the currently unknown molecular pathology for the SOX10 extension mutation using in vitro functional assays. Despite the wild-type SOX10 coding sequence remaining intact, the extension mutation led to severely diminished transcription and DNA-binding activities. Nevertheless, it showed no dominant-negative interference with wild-type SOX10 in vitro. Within the 82-amino acid tail, an 11-amino acid region (termed the WR domain) was responsible primarily for the deleterious properties of the extension. The WR domain, presumably forming an α-helix structure, inhibited SOX10 transcription activities if inserted in the carboxyl-terminal half of the protein. The WR domain can also affect other transcription factors with a graded effect when fused to the carboxyl termini, suggesting that it probably elicits a toxic functional activity. Together, molecular pathology for the SOX10 extension mutation is distinct from that of more common PTC mutations. Failure to properly terminate SOX10 translation causes the generation of a deleterious functional domain that occurs because of translation of the normal 3′-UTR; the mutant fusion protein causes a severe neurological disease.

INTRODUCTION

Mutations in the SOX10 gene, encoding a transcription factor, that is essential for neural crest development and myelin formation both in the central and peripheral nervous systems (CNS and PNS) (1), are associated with two distinct ‘neurocristopathies’. A milder more restricted spectrum trait, Waardenburg–Hirschsprung disease (WS4, OMIM: 277580) combines Waardenburg syndrome and Hirschsprung disease (2), whereas a more severe and complex neurological trait, peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome and Hirschsprung disease (PCWH, OMIM: 609136) reveals additional de-/dysmyelinating phenotypes in the PNS and CNS (3–7). The vast majority of disease-associated SOX10 mutations result in premature termination codons (PTCs), causing either WS4 or PCWH depending on the position of the mutations. The exon position of the PTC is directly associated with a sensitivity or resistance to mRNA degradation by nonsense-mediated mRNA decay (NMD) (3). Functional analyses

*To whom correspondence should be addressed at: Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi-cho, Kodaira, Tokyo 187-8502, Japan. Tel: +81-42-346-1713; Fax: +81-42-346-1743; Email: kinoue@ncnp.go.jp

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indicated that WS4 resulted from SOX10 haploinsufficiency because the WS4-associated mutant SOX10 mRNAs contain PTCs in the upstream exons that are subject to rapid degradation by activating the NMD surveillance pathway. In contrast, PCWH is caused by dominant-negative SOX10 alleles because NMD is unable to detect PCWH-associated mutant mRNAs typically carrying PTCs in the last exon, thereby allowing stable translation of truncated proteins that can interfere with the wild-type SOX10 protein.

However, the first SOX10 mutation found in a PCWH patient did not result in a PTC. It was a 12 bp deletion (designated as 1400del12) that only disrupted the normal stop codon, presumably leading to an extended in-frame translation into the 3’ untranslated region (3’-UTR) (5). As a result, an additional 82 amino acids are attached to the carboxyl terminus of the normal SOX10 protein creating a mutant fusion protein. Because of the severe neuromuscular phenotype (i.e. PCWH) associated with this extension mutation, it is unlikely that the extension simply causes a loss-of-function allele. However, the exact molecular pathobiology for this extension mutation remains unknown. Here, we examined the functional properties of the 1400del12 extension mutant by in vitro functional assays. We thus hypothesized that the extension may convey the severe neurological phenotype by a pathologic function, that is, distinct from PTC mutations, possibly mediated by a gain-of-function effect.

RESULTS

The extension mutant affects SOX10 transcription activities

We previously hypothesized that the proline-rich region within the 82-amino acid extension tail may confer an additional function to the adjacent transactivation domain (5). We surmised that proline may change the native protein structural conformation and the proline-rich region of the SOX10 extension has a moderate homology to one of the proline-rich domains of Wilms tumor 1 transcription factor, a potent transcriptional repression domain (5). We thus tested this hypothesis using SOX10 expression plasmids carrying the 82-amino acids extension (S mutant, pCMV.SOX10.S) and an extension lacking 19 residues of proline-rich region (D mutant, pCMV.SOX10.D) (Figures 1B and 6).

First, we determined the subcellular localization of these extension mutant proteins by immunocytochemistry using Cos7 cells transiently transfected with SOX10 expression plasmids (Fig. 1A). Similar to the wild-type and truncated Q250X SOX10 proteins, both of which predominantly localized to the nucleus, SOX10 protein with either the S or D extension is present in the nucleus. In the SOX10 protein, two nuclear localization signals (NLSs) are present flanking the high mobility group (HMG) domain (8). SOX10 also contains one nuclear export signal (NES) within the HMG domain (8). Although SOX10 normally shuttles between the nucleus and the cytoplasm, it is predominantly observed in the nucleus because the rate of import probably exceeds the rate of export (8). In agreement with the fact that the NLS and NES are maintained in all mutants examined in this study, the extension mutants localized to the nucleus, suggesting that the extensions did not affect the subcellular localization.

Next, we determined whether the extension tail can affect the SOX10 transcription activity by luciferase reporter assays. Wild-type and mutant SOX10 expression plasmids were transiently transfected into U138 human glioblastoma cells with a luciferase reporter plasmid containing either a synthetic SOX-responsive minimal promoter (3xSXluc) (9) or the human GJB1 promoter (pGL3-GJB1) (10), that is, directly regulated by SOX10. In comparison to the wild type, transcriptional activity of the S extension mutant was dramatically diminished regardless of the promoter utilized to drive expression of the reporter (Fig. 1C showing results for GJB1 promoter; Data for minimal promoter were not shown), suggesting that the extension completely inactivated the SOX10 transcriptional activity. Interestingly, removal of the proline-rich region from the extension tail did not restore the transcriptional activity, indicating that, contrary to our initial prediction, this proline chain is dispensable for the pathologic nature of the extension.

We then examined whether the extension affects the DNA-binding capability of SOX10 protein by electrophoretic mobility shift assay (EMSA). Surprisingly, both S and D extension clones dramatically diminished the DNA-binding ability, despite the fact that the DNA-binding domain is located physically far from the extension in the primary sequence (Fig. 1D). These findings suggest that the extension may involve complex pathologic mechanisms including a major change in structural conformation.

SOX10 harbors a transactivation domain at the carboxyl terminus, that is essential for its transcriptional activity (11). Because the extension tail is located adjacent to the transactivation domain, we examined if the extension can affect the transactivation capability directly and independently. We isolated the carboxyl-terminal region of SOX10 with extension from the HMG DNA-binding domain and amino-terminal side of the SOX10 protein, and fused it to a POU DNA-binding motif of mouse POU3F1, that is essential for DNA binding and nuclear localization, but not for transcriptional activation (12). In contrast to the prominent activation of over 15-fold by the wild-type SOX10/POU chimera (POU.C354), no activation was observed for fusion constructs with the extension tail (POU.S and POU.D), regardless of the presence or absence of the proline-rich chain (Fig. 1E). These findings suggest that the extension tail also diminishes the transactivation ability of SOX10. These results reveal that the extension mutant dramatically affects SOX10 transcriptional activity, DNA-binding ability and transactivation activity.

Identification of the critical region for the toxicity of the extension

We sought to determine if the pathogenic function of the extension mutant is conveyed by a specific protein sequence motif with functional properties, or is non-specifically determined...
by the physical length of extension and not dependent on particular protein sequences. We generated a series of carboxyl-terminal deletions based on S and D clones, as shown in Fig. 1B, and examined their transcriptional activities by luciferase reporter assays. Among these six deletion clones, only S-ext1, which carries a 23 amino acid extension including the proline-rich region, revealed full transcriptional activity, again showing that the proline-rich region is not responsible for pathogenicity (Fig. 1C). Meanwhile, the other five clones similarly resulted in a large reduction of activity. Although there was a slight tendency for transcriptional activities to inversely increase with a decrease in length of the extension from S to D-ext4 clones, the major change in the activity (＞6-fold) from D-ext4 (15 amino acid extension) to S-ext1 (23 amino acid extension) was not likely associated with the difference in the length of the extension.
Comparison of the contents of each clone revealed that the presence of an 11 amino acid region is likely sufficient to diminish the transcriptional activity. The 11 amino acid region consists of WWWQWRRLRRL, enriched by tryptophan in the first half and by arginine in the latter half, thus we designated it as a ‘WR domain’ (Fig. 1B). EMSA using five of these clones showed that extension mutants with diminished transcriptional activity had defective DNA-binding ability, whereas the S-ext1 clone retained adequate DNA-binding affinity (Fig. 2A), suggesting that the WR domain is sufficient to diminish the DNA binding of the extension mutants.

To further characterize the WR domain, we determined if this 11 amino acid domain can be minimized to a smaller functional unit and yet retain pathogenicity. We removed each half of this domain from the D-ext1 clone, resulting in D-ext5 (carrying WWWQW) and D-ext6 (carrying RRLRRL), respectively (Fig. 2B). Both constructs resulted in a transcriptional up-regulation of 3-fold compared with D-ext4, leading to a restoration of transcriptional activity to half the wild type. These findings suggested that the 11 amino acids probably forms a minimal functional unit to effectively elicit the transcriptional toxicity. Furthermore, elimination of the WR domain from the S extension (designated as S-WR, Fig. 2C) up-regulated the activity by 10-fold, effectively restoring the transcriptional activity to half the wild type. These findings indicated that the WR domain is critical to the toxic function of the extension mutant, although the rest of the extension excluding the WR domain can also suppress the SOX10 activity to some extent.

**Figure 2.** Functional determination of the WR domain. (A) DNA-binding assay using SOX10 proteins with different length of extension. SOX10 proteins in nuclear extracts from HeLa cells transfected with wild type (wt) or mutant SOX10 expression plasmids (listed in Fig 1B), shown in the western blot (top), were utilized for EMSA (bottom). As a negative control (NC), HeLa cells transfected with an empty vector (pCMV5) was used. High-affinity SOX10 monomer-binding probe from MPZ was used. A molecular weight marker 89 kDa is shown on left. An arrowhead indicates specific binding to the target DNA. (B and C) Transcription activities determined by luciferase reporter assays using SOX10 extension plasmids, listed on left, and pGL3-GJB1 reporter plasmid. Filled bars at the C terminus indicate various lengths of extensions. Black rectangle below and light shade above each clone indicate the position of the WR domain. HMG, high mobility group DNA-binding domain; TA, transactivation domain. The x-axis shows the luciferase activities as the relative induction above the mean activity from transfections with luciferase reporter and empty expression plasmid, which was arbitrarily set as 1. In (B) and (C), bars indicate mean + s.d.

**Inhibitory effect of the inserted WR domain within the SOX10 protein**

To determine if the toxicity of the WR domain is position-specific, we inserted this WR domain into various positions of the SOX10 protein (Fig. 3A). These positions were selected so as not to disrupt either the HMG or transactivation domains of SOX10. As controls, we inserted a 10 amino acid myc tag (EQKLISEEDL) at the same positions. Luciferase reporter assays using the GJB1 reporter revealed that the WR domain, but not the myc tag, diminished the SOX10 transcriptional activity when it was placed downstream of the HMG domain (Fig. 3B). Data obtained using the minimal promoter (3xSXluc) conveyed similar results (data not shown). The inhibitory effect was strong at the NaeI and DraIII sites and modest at the PstI site. Because, insertion of the myc tag at the same positions did not reduce the transcriptional activity, the inhibitory effect of the WR domain insertion is likely specific to its sequence content. In contrast, both the WR and myc insertions at the NarI site upstream of the HMG domain conveyed an inhibitory effect, wherein the WR insertion showed a milder inhibition. This inhibition by myc insertion at the NarI site was not observed when the minimal promoter was used (data not shown), suggesting that the effect of the insertions at this position may be different among different promoters.

Accordingly, EMSA showed that the WR domain insertions, but not the myc insertions, in the carboxy-terminal half of the protein resulted in a diminished DNA-binding ability (Fig. 3C), suggesting that the WR domain specifically
affected the binding to the target. Insertions upstream of the HMG domain also resulted in reduced DNA-binding affinity, however, these may not be sequence specific. Because the NarI insertion is located within the amino acid 61–100 region that immediately precedes the HMG domain and modulates DNA binding of SOX10 in a site-specific manner (13), the insertion may have changed the affinity to the SOX10-binding sequences present in the different probe and promoters used in the EMSA and reporter assays. Nevertheless, these findings demonstrated that the WR domain can also affect the SOX10-binding and transcriptional activities from different positions within the protein.

No dominant-negative interference with the wild-type SOX10 protein

To examine whether the SOX10 mutant proteins with extension can possibly interfere with the wild-type SOX10 protein, we performed competition assays. None of the extension mutants that harbor diminished transcription activity by itself inhibited the activity of the co-existing wild-type SOX10, an observation in sharp contrast to the Q250X truncated SOX10 that elicited dominant-negative interference (Fig. 4). As predicted, S-ext1 showed an additive effect, because it retains transcription activity similar to the wild-type SOX10. These findings suggest that, at least in this experimental setting in vitro, extension mutants do not function as dominant-negative alleles by competing with the wild-type SOX10.

WR polypeptide inhibited SOX11, SOX9 and POU3F1 transcription activities with a graded effect

If the WR domain has a specific toxic property, it may also be able to affect other transcription factors. We thus examined the effects of the WR domain when fused to two other SOX family member proteins, SOX9 and SOX11, and to a POU domain transcription factor, POU3F1. Again, the myc tag was used as a control. Addition of the WR domain in SOX9, that belongs to the same group as SOX10 (group E), resulted in a ~70% reduction of activity on the SOX-responsive minimal reporter 3xSXluc, but only 30% reduction on the GJB1 native promoter pGL3-GJB1 (Fig. 5A). Meanwhile, the WR domain dramatically affected SOX11, which belongs to a different subclass (group C) (1), on both minimal and GJB1 reporters (Fig. 5B). In contrast, both myc and WR domain diminished the POU3F1 activity to one-third, thus the WR domain did not confer specific effects on POU3F1 in comparison with the addition of the myc tag (Fig. 5C). EMSA showed that, unlike SOX10, the WR domain did not confer major changes in the DNA-binding ability of SOX9, SOX11 or POU3F1. Together, the WR domain can also affect transcription factors other than SOX10 with a graded effect, being strong for SOX10 and SOX11, intermediate for SOX9 and similar to the myc tag for POU3F1. These effects may be conveyed by a toxic function of the WR domain. One should note that these findings are not readily applicable to the disease-causing mutations in SOX9, SOX11 or POU3F1, because no naturally occurring mutations in these genes may result in the WR domain due to the sequence divergence in their 3′-UTRs.


DISCUSSION

In this study, we delineated the molecular pathology of a unique SOX10 extension mutation that was found in the first PCWH patient (5) by in vitro functional analyses. The mutation, a 12 bp deletion starting from the second nucleotide of the native stop codon, presumably leads to a failure to properly terminate translation. As a result, by conceptual translation a putative 82-amino acid tail translated from the 3'-UTR was attached to the native carboxyl terminus of intact SOX10 protein sequence creating a mutant fusion protein (5). There is one other SOX10 mutation with a similar extension with 86 amino acids resulting from substitution of the stop codon to lysine (X467K) (16). Although little information about the neurological examination was available for this case, the baby with this mutation showed mental and global developmental delay in addition to Waardenburg syndrome and Hirschsprung disease, suggesting that the patient probably had PCWH rather than WS4. Thus, the in-frame SOX10 extensions are likely associated with PCWH.

Our luciferase reporter assays and EMSA demonstrated that, despite the wild-type SOX10 protein sequence remaining intact, the additive extension completely diminished the transcriptional activity both on the minimal and GJB1 promoters, suggesting that extension may elicit deleterious effects. Because loss-of-function of one allele, or haploinsufficiency, of SOX10 is associated with a WS4 phenotype, whereas the extension mutations cause more severe PCWH phenotype, it is unlikely that the extension mutant simply acts as a loss-of-function allele. Our competition assays demonstrated that the extension mutants do not interfere with co-present wild-type SOX10 activity, indicating that dominant-negative action is less likely, at least in our in vitro experimental setting. Nevertheless, these findings are not readily applicable in vivo without further experimental validation. Interestingly, one study reported an enhanced transcription activity of the extension mutant (1400del12) (17). We were unable to clarify the reason for this discrepancy. This study also reported reduced transcription activity for the PCWH-causing X467K extension mutant (shaded bars). Bars indicate mean ± s.d.

Computational analyses suggest that the WR domain may form an α helix

Our findings strongly suggest that the WR domain may have functional properties that are essential for the pathologic mechanism of the extension mutant. To further delineate such properties and potentially relate them to structural features, we performed computational predictions for the extension region using various protein prediction programs, including SAM-T02 (14), through the ExPASy proteomics server (http://www.expasy.org/) (15).

Multiple secondary structural prediction analyses indicated that the WR domain may form a strong α helix structure (Fig. 6). No other region in the extension tail is predicted to form an apparent secondary structure. Examination and computational analyses of the entire extension showed two other findings: a moderate similarity to type II small proline-rich proteins identified by BLASTP and potential phosphorylation sites predicted by NetPhos (data not shown). However, the WR domain was not directly involved in these features.

Figure 4. Competition assays. Increasing amounts of mutant SOX10 expression plasmid were mixed with a fixed amount of wild-type SOX10 expression plasmid and co-transfected with the luciferase reporter plasmid 3xSxN11c. The x-axis shows the relative amount of mutant SOX10 plasmid to the fixed wild-type SOX10 plasmid. The total amount of DNA per well was kept constant by adding empty expression plasmid. The mean activity from transfections with the luciferase reporter and wild-type SOX10 plasmid was set as 1, and other data are shown relative to this activity. SOX10.S-ext1 (empty bars) retains transcription activity alone, whereas six SOX10 mutants with filled bars show diminished transcription activity, as shown in Fig 1B. Nevertheless, none of the extension mutants showed dominant-negative interference as demonstrated in SOX10.Q250X truncated mutant (shaded bars). Bars indicate mean ± s.d.
ecular mechanism for the potential interaction between the WR domain and HMG domain will help clarify the pathologic basis for the toxic effect of the WR domain.

Disruption of the native stop codon without changing wild-type protein coding sequence is an uncommon cause for human genetic diseases. More frequent frame-shift mutations in the coding sequences can give rise to similar ‘out-of-frame’ translations, but the impact of such ‘out-of-frame’ tails in the disease pathoetiology probably differs case by case, depending on location of the frame-shift alteration, reading frame (i.e. one base plus or two bases plus), or actual DNA sequences comprising the coding and non-coding region of a gene, as we previously demonstrated in the myelin protein zero; MPZ, gene (18). In fact, at least four disease-causing frame-shift mutations have been reported in SOX10, namely 778delG, 795delG, 847insT and 1076delGA, resulting in 25, 19, 10 and 41 amino acid extension tails, respectively (2,3,6,19,20). Clinical severity conveyed by these mutations appears to be associated with the location of PTCs (i.e. the more proximal the truncation is located, the more severe the phenotype), but not with the length, position or protein sequence of the ‘out-of-frame’ tails. Of note, 1076delGA mutation, which produces the longest tail, was associated with WS4 in a family; no PCWH-associated neurological symptoms were apparent and consequently no dominant-negative interference was observed by functional assays (3). Theses findings further support the unique toxic property of the WR domain that is only conveyed by in-frame translation of the SOX10 3’-UTR.

Collectively, our in vitro functional analyses showed that molecular pathogenesis for PCWH caused by extension mutations is distinct from that caused by PTC mutations. We determined that the WR domain within the extension probably elicited a functional property that is responsible for the toxicity of the extension, thus causing the PCWH phenotype. Although the exact nature of this toxicity is not fully delineated yet, it probably acts through a gain-of-function mechanism.

With these findings, now the genotype–phenotype correlation of SOX10 mutations has been consolidated. Loss-of-function alleles are associated with a milder and more restricted spectrum trait, WS4, in which no de-/dysmyelinating phenotypes in the PNS and CNS are involved. In contrast, dominant-negative alleles, mainly resulting from common

Figure 5. Inhibitory effects of the WR domain on other transcription factors. Transcription activities were determined by luciferase reporter assays using SOX9 (A), SOX11 (B) and POU3F1 (C) plasmids, as listed on left. Black rectangles indicate the WR domain insertions whereas grey rectangles indicate the myc tag insertions. Each expression plasmid was co-transfected with either 3xSxluc (middle) or pGL3-GJB1 (right) reporter plasmid for SOX protein and HsvOCT reporter plasmid for POU3F1 protein. The x-axis shows the luciferase activities as the relative induction above the mean activity from transfections with luciferase reporter and empty expression plasmid, which was arbitrarily set as 1. Bars indicate mean ± s.d. DNA- binding assays and western blottings using each protein listed on left were shown on right. Protein extracts from Cos7 cells transfected with wild type (wt), WR, Myc or empty vector (negative control, nc) plasmid, shown in the western blot (top), were utilized for EMSA (bottom). SOX-binding probe was used for SOX9 and SOX11, whereas POU-binding probe was utilized for POU3F1. Molecular weight markers 75 or 50 kDa are shown on right. Arrowheads indicate specific binding to the target DNA.
PTC mutations, and gain-of-function alleles, conveyed by rare extension mutations as demonstrated in this study, give rise to more severe and complex trait, PCWH.

MATERIALS AND METHODS

Construction of expression plasmids

Human SOX10 cDNA was inserted into a mammalian expression plasmid pCMV5 under the control of a cytomegalovirus promoter, as described (3,9). Various mutations were incorporated by PCR or PCR-based site-directed mutagenesis. Resultant mutants were: pCMV.SOX10.S that carries the 1400del12 mutation, pCMV.SOX10.D that lacks the proline-rich 19 amino acid region (HHPPPSPCPQPVCPVPC; see Fig. 6 for the actual location), and a 3' end deletion series as shown in Figures 1B and 2B, designated as pCMV.SOX10.S-ext1 to pCMV.SOX10.D-ext6. The carboxyl-terminal half of SOX10 that contains the transactivation domain was fused to a POU cassette of rat POU3F1, that contains a DNA-binding domain and nuclear localization signal, to generate pCMV.POU.C354(11), pCMV.POU.S and pCMV.POU.D (Fig. 1E). An 11 amino acid sequence (WWWQWRRLRRL; designated as the WR domain) was inserted at various positions within the HOX10 protein (Figures 3A and 6). The WR domain was removed from pCMV.SOX10.S to generate pCMV.SOX10.S-WR (Fig. 2C). The WR domain or myc tag was inserted in frame before the stop codon of rat SOX11, mouse SOX9 and mouse POU3F1 cDNA in the pCMV5 expression plasmid to generate WR or myc fusion constructs (Fig. 5).

Immunocytochemistry

Cos7 cells were grown on chamber slides in DMEM medium supplemented with 10% fetal bovine serum (FBS) and transiently transfected with SOX10 expression plasmids using PolyFect transfection reagent (Qiagen, Germany). Cells were fixed

Figure 6. Predicted secondary structure of SOX10 protein with the 82-amino acid extension. Data obtained by SAM-T02 protein structure prediction program (14) are summarized. Predicted secondary structure is shown above the protein sequence for the SOX10 extension mutant. H, α helix; E, β bridges and strands; C, others including coils. The height of the letter represents the likelihood of the prediction. Shaded sequences indicate HMG domain that consists of three blocks of α helix, and the sequences written in white show the 82-amino acids extension. A rectangle indicates the WR domain, showing an α helix structure. A horizontal line indicates the proline-rich 19-amino acids region deleted in SOX10.D extension mutant. Arrowheads show the position of either WR domain or myc tag insertion indicated in Fig 3A.
in 4% paraformaldehyde in PBS for 5 min, then washed and permeabilized with 0.1% Tween 20 in PBS for 5 min. After twice rinsing with PBS, cells were blocked with 5% normal goat serum at room temperature for 1 h and incubated with rabbit anti-SOX10 antibody (1:500 dilution; Chemicon, CA, USA) at 4°C overnight, followed by two washes with PBS and incubation with AlexaFluor488 goat anti-rabbit antibody (1:5000: Invitrogen, CA, USA) for 1 h at room temperature before visualization by standard fluorescent microscopy. For nuclei staining, an AntiFade Kit with 4',6'-diamidino-2-phenylindole (Invitrogen) was used.

Transfection assays using luciferase reporter system

Two SOX10-responsive luciferase reporter plasmids, 3xSXIuc and pGL3-GJB1 [term pGL3-Cx32 in our previous study (3)], and one POU-responsive reporter plasmid, HsvOCTluc, were prepared as described previously (3,9,10). U138 human glioblastoma cells were grown in DMEM medium supplemented with 10% FBS and were transiently transfected using PolyFect or Lipofectamine2000 (Invitrogen) transfection reagents. We selected U138 for these studies specifically because it does not express endogenous SOX10 protein that might interfere with transfection assays (9). A total of 0.5–0.8 μg of plasmid DNA per well was used. Typically, we used 0.2 μg reporter plasmid, 0.2 μg test plasmid and 0.1 μg inner control plasmid per well. We collected cells from 24-well trays after 48 h of transfection and assayed luciferase activity using a monolight luminometer (Pharmingen, CA, USA) or TD-20/20 luminometer (Promega, WI, USA). A β-galactosidase expression plasmid, pCMVβ (Clontech, CA, USA), or Renilla luciferase expression plasmid, pRT-TK (Promega), were used as a reference for transfection. Each experiment was repeated at least three times with at least four independent samples per experiment. Representative results from one experiment were shown in figures.

Western blotting and EMSA

HeLa cells were grown in DMEM medium supplemented with 10% FBS. Nuclear extract was obtained from HeLa cells after 48 h of transfection (21), which was used for western blotting and EMSA. Rabbit antibody to SOX10 (1:3000 dilution) was used for western blots. Other antibodies utilized were: goat anti-SOX9 antibody (Santa Cruz P-20, 1:500 dilution), guinea pig anti-SOX11 antibody (1:3000 dilution) (22) and goat anti-POU3F1 antibody (Santa Cruz H-13, 1:1000 dilution). We determined structural and functional characteristics of the SOX10 mutant proteins by a series of web-based prediction programs, primarily available through the ExPASy proteomics web server (http://www.expasy.org/).

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Conflict of Interest statement. None declared.

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