Compound Effects of Point Mutations Causing Campomelic Dysplasia/Autosomal Sex Reversal upon SOX9 Structure, Nuclear Transport, DNA Binding, and Transcriptional Activation*

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Human mutations in the transcription factor SOX9 cause campomelic dysplasia/autosomal sex reversal. Here we identify and characterize two novel heterozygous mutations, F154L and A158T, that substitute conserved "hydrophobic core" amino acids of the high mobility group domain at positions thought to stabilize SOX9 conformation. Circular dichroism studies indicated that both mutations disrupt α-helicity within their high mobility group domain, whereas tertiary structure is essentially maintained as judged by fluorescence spectroscopy. In cultured cells, strictly nuclear localization was observed for wild type SOX9 and the F154L mutant; however, the A158T mutant showed a 2-fold reduction in nuclear import efficiency. Importin-β was demonstrated to be the nuclear transport receptor recognized by SOX9, with both mutant proteins binding importin-β with wild type affinity. Whereas DNA binding was unaffected, DNA binding was drastically reduced in both mutants (to 5% of wild type activity in F154L, 17% in A158T). Despite this large effect, transcriptional activation in cultured cells was only reduced to 26% in F154L and 62% in A158T of wild type activity, suggesting that a small loss of SOX9 transactivation activity could be sufficient to disrupt proper regulation of target genes during bone and testis formation. Thus, clinically relevant mutations of SOX9 affect protein structure leading to compound effects of reduced nuclear import and reduced DNA binding, the net effect being loss of transcriptional activation.

Campomelic dysplasia/autosomal sex reversal (CD/SRA1) is

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The abbreviations used are: CD/SRA1, campomelic dysplasia/autosomal sex reversal; CAT, chloramphenicol acetyltransferase; HMG, high mobility group; SRY, sex determining region of the Y chromosome; SSCP, single strand conformation polymorphism; WT, wild type; ELISA, enzyme-immunoassay test; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; NLS, nuclear localization signal; HA, hemagglutinin.

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chondrocyte cell lines and ectopic expression of SOX9 in transgenic mice reveal that SOX9 activates Col2a1 gene, Col11a2, and possibly aggrecan (16–22).

Clinical mutations in SOX9 resulting in CD/SRA1 include splice acceptor/donor changes, missense, nonsense, translocation, and frame-shift mutations (4, 23–27). There appears to be no correlation between mutation type or position and severity of the disease or associated sex reversal, with the same mutation causing varying degrees of gonadal dysgenesis (23). Together with the lack of polymorphisms in the SOX9 gene, it appears that SOX9 is intolerant to variation, perhaps acting at a high biochemical threshold which if not reached, CD is manifest. Another possibility is that the genetic background in which the mutant allele resides influences SOX9 activity. The two major classes of mutations causing CD/SRA1 are amino acid substitutions in the HMG domain and truncations or frame-shifts that alter the C-terminal domain of SOX9. Two highly conserved domains located at the C terminus, the PQS domain and PQA domain, are required for maximal transcriptional activation, and C-terminal deletions of SOX9 from CD patients show reduced transactivation activity (27). All known point missense in SOX9 occurs in the HMG domain (Fig. 1), the majority of which have been examined biochemically and show altered DNA binding compared with wild type, although there are a few exceptions (23). For example, P170R in the HMG domain had near wild type DNA binding and bending but altered DNA binding specificity (27). A second SOX9 mutant, A119V, showed near wild type DNA binding and bending (27) suggesting that other essential but unknown biochemical activities of the SOX9 HMG domain may exist. These results support a model where reduced HMG domain function will lead to reduced transcriptional activation, although this correlation had not been investigated prior to this study, because previous studies on SOX9 missense mutations in CD/SRA1 have only investigated DNA binding and bending.

The HMG domain consists of three o-helices that come together in an L-shape in which the short arm is formed by helices 1 and 2 and the long arm by helix 3 and the N-terminal strand. The concave surface of the “L” contacts the minor groove of the DNA. A number of hydrophobic amino acids from each helix pack together to form a “hydrophobic core” that is thought to stabilize the helices. We previously constructed a model of the SOX9 HMG domain (27), based on the solution structure of the SRY HMG domain, and we observed that most clinical mutations of SOX9 and SRY lie at or near the DNA-bending surface. Here we describe two novel mutations, A154L and A158T, that substitute amino acids within the hydrophobic core and raise the possibility that these residues contribute to the conformation of the HMG domain. Based on our model, these amino acids on helix 3 do not directly bind to DNA, rather they may destabilize the secondary and/or tertiary structure of the HMG domain. This in turn may affect DNA binding and/or DNA bending function of the HMG domain. Also, a signal for nuclear import of SOX9 is located in helix 3 raising the possibility that nuclear import could be affected (28). This nuclear localization signal (NLS) is likely recognized by one of the importins that dock NLS-containing proteins at the nuclear pore complex. We have investigated which components of the nuclear transport machinery normally recognize SOX9 and show that importin-β strongly interacts with SOX9.

In this study, we examine the structure of the mutant SOX9 domains by fluorescence spectroscopy and circular dichroism and their ability to bind and bend DNA and to be recognized by importins as part of the nuclear import process. We also examined the effect of these mutations in cultured cells where we measured the ability of SOX9 to transport to the nucleus and to activate transcription. We have demonstrated that both mutants affect protein structure leading to compound effects of reduced nuclear import and reduced DNA binding with consequent reduction of transcriptional activation. Thus, CD/SRA1 arises in these patients from a failure to properly activate transcription of target genes during bone and testis formation.

EXPERIMENTAL PROCEDURES

Patients Reports—Patient 51 was a female infant weighing 3080 g with classic CD. She died 1 day postnatal. The head showed micrognathia, with a small mouth and elongated dome-shaped tongue, flat nasal bridge, mild hypertelorism, and slightly low set ears. There was disproportionate shortness of the limbs most obvious in the lower limbs, which also showed symmetrical anterior bowing of the tibiae, pretilial dimples, and talipes equinovarus. There was a cleft palate involving the soft and posterior part of the hard palate. The heart was not enlarged and showed atrial situs solitus with concordant atrioventricular and ventricular-arterial connections. There was bowing of both femora and tibiae convexed laterally. Epiphyseal centers for the lower end of the femur and upper end of the tibiae were absent. The tibia showed anterior bowing at the junction of the middle and lower third of the shaft, and there were bilaterally dislocated hips and abnormal ischial bones. There was a delay in ossification of the epiphyseal centers, diagnostic of campomelic dwarfism. The presence of ovaries was confirmed, and numerous germ cells were seen. The long bones showed abnormal ossification with some irregularity of endochondral ossification. The cortical bone on the posterior aspect of the shaft was thickened.

Patient 61 is a 46, XX female with a milder form of CD. At present, she is 19 years of age and is assisted with mechanical ventilation. This patient was born to healthy non-consanguineous parents at 39 weeks of gestation. Physical examination showed flat face with prominent forehead, cleft palate, and clubfoot. Bone survey revealed bowing of the femurs and tibiae, as well as slim, poorly developed bones and ovoid immaturity. External genitalia were feminized with mild clitoromegaly. At 2 years of age, a human chorionic gonadotropin test yielded no testosterone response, and a human menopause gonadotropin test gave no estradiol response. A gonadotropin-releasing hormone test showed hypersresponses of luteinizing hormone (7.2 → 66.5 IU/liter) and follicle-stimulating hormone (11.3 → 80.2 IU/liter).

Polymerase Chain Reaction Amplification, SSCP, and DNA Sequencing—To screen for the mutation, the entire SOX9 open reading frame was amplified from genomic DNA from blood lymphocytes by polymerase chain reaction and analyzed by SSCP as described previously (24).

Molecular Modeling—Homology modeling was used to generate model structures of SOX9 using the NMR structure of SRY as described previously (27). Molecules were rendered using the Molecular Simulations Inc. software WebLabViewer.

Mutagenesis—The CD mutations were introduced into SOX9 using the pAlter Mutagenesis kit (Promega) according to the manufacturer’s instructions using the following oligonucleotides: P154L, 5'-AGCGGAGGGCGGAGGCTGCGCGTGCAG-3'; A158T, 5'-GCCCCC TCTCGTGGAGGAGACGGAGCGGCTGCGCGTGCAG-3'. pAlter-SOX9 was subsequently digested with EcoRI and XhoI, and the mutant cDNAs were subcloned between the EcoRI and XhoI sites in pcDNA3 for transient transfection in cultured cells and in vitro production of protein. pAlter-SOX9 mutants were also digested with BstHII and SacII, and the 125-base pair fragment was inserted into the BstHII and SacII sites in the pT7SOX9HMGbox expression plasmid (27).

Production of Mutant and Wild Type SOX9 HMG Domains—Recombinant SOX9 HMG protein (amino acids 101–184) was expressed and purified from Escherichia coli as described (29) with a modification to the protocol whereby SOX9 HMG protein was extracted by sonication of PBS-washed cell pellets in 100mL of HEDa buffer (50mM HEPES, pH 7.9, 1mM diithiothreitol, 1mM EDTA, 50mM NaCl, 0.4M b-mercaptoethanol). The sample was then brought to a final concentration of 450mM NaCl and mixed with a 0.1 volume of 50% DEAE-Sephadex equilibrated in HEDA buffer, 450mM NaCl for 10 min at 4°C. Following centrifugation (30,000g, 50mL, 30 min, 4°C), the salt concentration of the supernatant was reduced to about 200mM by diluting the sample (2.5fold with HEDA buffer, the supernatant was filtered, under pressure, through a 0.45-μm membrane. The sample was injected onto a 100-μL SP-Sepharose column pre-equilibrated in HEDA buffer containing 200mM NaCl. Bound proteins were eluted over 40min with a salt gradient of HEDA buffer containing 200–1000mM NaCl, and 5-ml fractions were collected. SOX9 HMG elutes at about 0.7mM NaCl containing fractions 90% SOX9 HMG
protein. SOX9 HMG protein was desalted and concentrated using Ami-cron Centriprep 3 cartridges. Protein concentrations were determined against Bradford assay reagent kit (Bio-Rad) with bovine serum albumin as a standard.

**Trypophan Fluorescence Spectroscopy**—Fluorescence spectra were recorded on a SPEX Fluorolog-2 frequency domain spectrofluorometer. Excitation of tryptophan was accomplished using vertically polarized light at 295 nm from a 450-watt xenon lamp. The emission was observed through a polarizer oriented at the magic angle (54.7°), and spectra were measured in the wavelength range of 300–500 nm. The spectral band pass of excitation and emission was 5 nm. 500-μl cuvettes were used, and the protein concentration is given below. Spectrofluorometer was corrected for the wavelength response of the detection system. The cell block was maintained at 20 °C with a circulating water bath (30, 31).

Acrylamide quenching was performed using an excitation wave- length of 295 nm to avoid tyrosine excitation and distortion by acryl- amide. 10-μl aliquots of 4 M acrylamide were added successively to 400 μl of a 2 μg protein sample, and the fluorescence intensity was recorded at 350 nm with a PerkinElmer Life Science LS-5 spectrofluorometer. The fluorescence was corrected for dilution and inner-filtering by acryl- amide (30, 32).

**Circular Dichroism Spectroscopy**—The circular dichroism of SOX-9 and its mutants were recorded at 222 nm using an AVIV model 62DS spectrometer with a spectral bandwidth of 2 nm. The sample temper- ature was controlled electronically with a Peltier device.

**Production of Mutant and Wild Type Full-length SOX9**—Full-length SOX9 protein was produced in vitro by coupled transcription and translation of SOX9 (wild type and mutants) in pcDNA3, using a TNT kit (Promega), with incorporation of [α-35S]methionine.

**Cell Types and Culture and Transient Transfections**—COS-7 cells were cultured as a monolayer in RPMI 1640, supplemented with 1% (v/v) penicillin/streptomycin, 1% L-glutamine, and 10% (v/v) fetal calf serum, at 37 °C under 5% CO2.

COS-7 cells were transfected by DEAE-dextran-assisted electropora- tion (33). Transactivation by SOX9 was measured in transfection assays, using the reporter plasmid, p53-pE1CAT, in which the CAT gene is under control of the E1b promoter, downstream of 10 SOX core-binding sites (AACAAT). Cells (106) in log growth phase were transfected with 1 μg of pcSOX9 CAT reporter DNA and 28 ng of pcDNA3 or pDNA3-SOX9 (wild type or mutant), and 20 ng of pCMV-lac, in a volume of 600 ml of RPMI 1640 containing 10 ml/ml DEAE-dextran. Pulse conditions were 960 microfarads and 250 mV using a Gene Pulser apparatus (Bio-Rad). Cells from each transfection were seeded into two flasks after addition of 6 ml of RPMI and were grown for 48 h before being harvested. Protein concentrations, in cell lysates, were deter- mined by Bradford assay. CAT expression was determined by enzyme-linked immunosorbent assay, using a CAT enzyme-linked immunosor- bent assay kit (Roche Molecular Biochemicals). To correct for varying transfection efficiencies, β-galactosidase levels were assayed, and CAT levels were normalized for β-galactosidase expression. β-Galactosidase expression was assayed using the β-galactosidase enzyme assay system (Promega).

**Immunohistochemistry**—COS-7 cells were transiently transfected with pcDNA3-SOX9 or mutant using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. For SOX9 staining, COS-7 cells grown in 8-well Chamber slides (Nunc) were fixed in 5% paraformaldehyde/PBS for 30 min at room temperature and perme- abilized with 0.5% Triton X-100 (ICN) in PBS for 5 min at room temperature. Cells were then incubated for 30 min with goat or horse serum in a humidified atmosphere. Cells were then incubated with specific polyclonal antibody anti-SOX9 (9) or anti-hemagglutinin (HA) antibody. Slides were washed three times (5 min each) in PBS and incubated for 90 min at room temperature with fluorescein-conjugated anti-rabbit antibody (dilution 1:500, anti-SOX9 from Sigma) or fluores- cein-conjugated anti-mouse antibody, dilution 1:500 (anti-HA from Sig- ma). After three washes in PBS, coverslips were mounted in glass slides with buffered glycerol (containing 1:2000 dilution of TO-PRO3 (Molec- ular Probes) to detect nuclei of cells) and viewed under the Leica fluorescence microscope using a fluorescein isothiocyanate filter, rho- damine filter, or both. Cells were photographed using a Leica DMR (Ernst- man Kodak Co.) in a camera (Leica) at 10 × 10 and 40 magnification. Fluorescence and subcellular immunogold labelling was performed using a Jeol 100CX (JEOL) transmission electron microscope (MRC-500, Bio-Rad) (34).

**Detection of Novel SOX9 Mutations in Two CD Patients**—Analysis of the SOX9 open reading frame from two CD/SRA1 patients using SSCP and subsequent DNA sequencing identi- fied two novel missense mutations (F154L and A158T) in the SOX9 HMG domain from two patients (Fig. 1A; see Table I for details). Both patients carried the mutation on one allele only, the other showing wild type DNA sequence, indicating that the mutations were heterozygous.

**Fluorescence Properties**—Changes in tryptophan fluorescence from proteins are sensitive to the environment about the indole group. Spectrophotometric results can give information concerning changes in protein structure and dynamics. We have shown previously that the fluorescence from the HMG domain of SRY is sensitive to changes in local environment about the trypto- phan residues and undergoes shifts to distinct wavelength and emission maximum upon DNA binding or calmodulin binding when compared with the free HMG domain (31). Therefore, we

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measured the tryptophan fluorescence of wild type and mutant SOX9 HMG domains. SOX9 HMG domains were expressed in *E. coli* bacteria and purified by ion exchange chromatography to approximately 90% purity by SDS-PAGE analysis (Fig. 2A). Table II shows a comparison of three fluorescence parameters that report on the tryptophan micropolarity (wavelength maximum), the tryptophan dynamics (anisotropy), and the accessibility to solvent (acrylamide accessibility). The physical extremes of these parameters correspond to tryptophan environments that range from being non-polar to polar (320–330 to 355 nm), mobile to immobile (0.05 to 0.24), and accessible to shielded (25 to <1 m<sup>-1</sup>), respectively. Table II shows that the SOX9 HMG tryptophan fluorescence is characterized by a relatively non-polar environment (340 nm), a moderate accessibility to acrylamide (10 m<sup>-1</sup>), and is restricted in its mobility (0.125). The A158T mutation produces no detectable alteration in the micropolarity or mobility of the tryptophan residues, and a 10% decrease in accessibility to acrylamide compared with wild type (a 4% change compared with fully accessible/shielded). The F154L mutation changes all three tryptophan fluorescence parameters. Compared with the wild type protein, the tryptophan micropolarity (339 nm) and mobility (0.131) undergo a slight decrease, whereas the accessibility to acrylamide increases (11 m<sup>-1</sup>). As for the A158T mutation, the change in these parameters represents a fractional change of less than 5% compared with the full range of parameter space.
folded protein is plotted against temperature. Symbols as for Fig. 2 denaturation curves of SOX9 and mutants. The apparent fraction unfolded scale, are superimposable which implies their folded domains have similar stabilities to heat denaturation over the temperatures studied (Fig. 2B).

Circular Dicroism—The circular dichroism ellipticity at 222 nm is often used as an indicator of helicity in proteins and, when combined with measurements over a range of temperatures, can yield insight into protein conformational and thermal stability. Table III summarizes the relative CD at 222 nm of both A158T and F154L, compared with the wild type protein. Second, the CD melt curves of all three proteins, when normalized to an apparent fraction unfolded scale, are superimposable which implies their folded domains have similar stabilities to heat denaturation over the temperatures studied (Fig. 2B).

Nuclear Import and Recognition of SOX9 by Importins—To investigate the properties of mutant SOX9 in cultured cells, mammalian expression plasmids encoding wild type and mutant full-length SOX9 were constructed. SDS-PAGE analysis shows that the full-length mutant SOX9 proteins were translated efficiently from pcDNA3 plasmids in rabbit reticulocyte lysate system (Fig. 3A). SOX9 carries signals for nuclear import at each end of its HMG domain (28). NLSs tend to be amphipathic helices, and so the function of the SOX9 NLSs could be perturbed by structural changes (loss of helicity) in the HMG domain. To assess the effect of SOX9 mutations upon nuclear import, full-length wild type and mutant SOX9 plasmids were transiently transfected into COS-7 cells, and the subcellular localization of SOX9 was determined 24 h after transfection using indirect immunofluorescence and confocal laser scanning microscopy and quantitated using image analysis (Fig. 3B). Wild type SOX9 efficiently accumulated in the nucleus (ratio of accumulation of SOX9 fluorescence in the nucleus relative to the cytoplasm – Fn/c of 30) as did the F154L mutant, but the F158T mutant showed a significant (p = 0.0316) reduction of nuclear accumulation of approximately 2-fold (Fn/c of 13).

NLSs are conventionally recognized by the nuclear import receptor, a heterodimer of importin (karyopherin)-α/β subunits (see Ref. 38 for review). The HMG domain of SRY is recognized directly by importin-β, independently of importin-α, via the NLS at the C-terminal end of helix 3. This NLS sequence is highly conserved in all SOX proteins, so it seemed likely that importin-β would interact with SOX9. To test this possibility, we investigated, by ELISA, the interaction of the SOX9 HMG box with importin-α, importin-β, and the heterodimer importin-α/β. SOX9 HMG domain is recognized more strongly by importin-β (Kd of 1.7) compared with importin-α (55 nM) (Fig. 3C; Table IV) consistent with the results obtained for SRY. 2 No significant differences in importin-β binding were detected for secondary structure at all temperatures investigated, as indicated by the increased ellipticity at 222 nm of both A158T and F154L, compared with the wild type protein.

**Figure 2.** Circular dichroism analysis of normal and mutant HMG domain structure. A, SDS-PAGE analysis of E. coli recombinant wild type (WT) and mutant SOX9 HMG domains produced in E. coli. Cell lysates before (--) and after (+) ion exchange chromatography were resolved on a 10–20% polyacrylamide gradient gel at 20 V/cm for 180 min and stained with Coomassie Brilliant Blue. The position of the SOX9 HMG domain and molecular size markers in kilodaltons are indicated on the left. B, the temperature dependence of the relative ellipticity at 222 nm of SOX9 HMG domains (filled circles) and SOX9 mutants, A158T (unfilled squares), F154L (filled squares). C, thermal denaturation curves of SOX9 and mutants. The apparent fraction unfolded protein is plotted against temperature. Symbols as for Fig. 2B.

**Table II**

| Protein | Wavelength maximum (±0.5 nm) | Anisotropy (±0.002) | Accessibility (±0.5 M⁻¹) |
|---------|------------------------------|---------------------|--------------------------|
| WT      | 340                          | 0.125               | 10                       |
| F154L   | 339                          | 0.131               | 11                       |
| A158T   | 340                          | 0.125               | 9                        |

* Defined as the wavelength at which the first derivative of intensity with respect to wavelength is zero.
* Accessibility determined from quenching of fluorescence by acrylamide.

**Table III**

| Protein | Relative ellipticity at 222 nm |
|---------|-------------------------------|
|         | 4 °C                          |
| WT      | −22.0                         |
| F154L   | −14.1                         |
| A158T   | −14.4                         |
|         | 37 °C                         |
| WT      | −18.5                         |
| F154L   | −12.9                         |
| A158T   | −12.8                         |
|         | 70 °C                         |
| WT      | −10.5                         |
| F154L   | −8.4                          |
| A158T   | −9.0                          |

* Defined as the ellipticity measured at 222 nm divided by the absorbance measured at 280 nm at 20 °C.

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the SOX9 mutants compared with wild type SOX9 HMG box suggesting that the decrease in nuclear accumulation of mutant A158T is not due to defects in importin-β binding (Fig. 3C).

DNA Binding and DNA Bending—SOX9 encoded by some CD patients showed reduced DNA binding activity consistent with the DNA binding ability of SOX9 being essential for its function as a transcription factor. The ability of the mutants to bind DNA, relative to wild type, was measured. DNA concentrations were varied, and SOX9-bound versus free DNA was quantitated from electrophoretic mobility shift gels (Fig. 4A) (29). DNA probe SOX9CON bears the high affinity SOX9-binding site selected in vitro (AGAACATGG) which consists of SOX consensus motif (ATT)CAT(A/T) flanked on either side by the two bases specifically preferred by SOX9 (29). The dissociation constant (Kd) was calculated for wild type and mutant SOX9-DNA complexes, and Scatchard plots were drawn (Fig. 4B; Table V). Wild type SOX9 HMG gave a Kd of 12.6 nM, consistent with the published value of 12.4 nM (29). F154L by comparison gave Kd of 244 nM, a reduction to 5% of wild type SOX9 DNA binding affinity. A158T is less affected with a Kd of 76 nM, a reduction to 16.6% of wild type SOX9 DNA binding affinity.

Certain point mutations in the HMG domain of SRY in patients with XY gonadal dysgenesis alter the DNA bending properties of the protein (8). Since DNA bending is also likely to be important for SOX9 function, we set out to determine the bend angles induced upon binding of the wild type and mutant HMG domains to SOX9CON DNA probe. A circular permutation assay was used to determine the bend angle induced by SOX9 HMG (Fig. 5). Wild type deflected the linear DNA probe SOX9CON by 59 ± 0.5°. Differences between our results and those published (39–41) could be due to slight differences in assay conditions, as have been observed for SRY. Both the mutants and wild type SOX9 show no significant differences in their ability to bend DNA with both mutants also bending DNA 59 ± 0.5° from linearity. Thus, the F154L and A158T mutations did not appear to alter the DNA bending properties of SOX9.

DISCUSSION

In this study, we report the identification of the novel amino acid substitution mutations F154L and A158T in the SOX9 HMG domain of two patients with campomelic dysplasia, the former an XX female and the latter a sex-reversed XY female. Fig. 1B shows the positions of these substitutions on the SOX9 open reading frame together with those of previously reported mutations. Apart from the fact that all known missense mutations occur in the HMG domain, no correlation seems to be emerging between the position of the mutations and their clinical severity and associated sex reversal.

Based upon our molecular model of SOX9 (27), we postulated that Phe154 and Ala158 form part of a hydrophobic core region and would play a role in stabilizing the three-dimensional alignment of the three helices of the HMG domain. The substitution of a phenylalanine by a leucine in mutant F154L may have several consequences including an alteration of hydrophobic packing due to the replacement of a bulkier amino acid by a smaller one (39), a loss of aromatic-aromatic interactions (40–43), or a decrease in helicity due to alterations of phenylalanine-side chain interactions between amino acids proximal to helix 3 (44). The replacement of an alanine by a threonine in mutant A158T substitutes a small non-polar amino acid for a bigger relative non-polar amino acid and thus would be expected to affect hydrophobic interactions in this region of the protein. However, the fluorescence studies did not demonstrate significant changes in tertiary structure. The mutations would appear not to perturb the environment or tertiary structure (relative orientation of the helices) as probed by the tryptophan.
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**TABLE IV**
NLS binding parameters of SOX9 derivatives as measured using an ELISA-based binding assay

Importin binding parameters were determined as described under “Experimental Procedures” (34) from experimental data fitted as shown in Fig. 3C. Results for the apparent dissociation constant (Kd) and maximal level of binding (expressed as a percentage relative to the binding of mlmp αβ) to SOX9 wt represent the mean ± S.E.M. (n in parentheses).

| SOX9          | mlmp α Kd | Bmax | mlmp αβ Kd | Bmax | mlmp ββ Kd | Bmax |
|---------------|-----------|------|------------|------|------------|------|
|               | nM        | %    | nM         | %    | nM         | %    |
| WT            | 50 ± 12 (3) | 88 ± 9 | .8 ± 0.0 (2) | 110 ± 10 | 1.7 ± 0.1 (2) | 97 ± 0 |
| F154L         | 38 ± 6 (2) | 83 ± 2 | 1.7 ± 0.3 (2) | 104 ± 4 | 1.6 ± 0.3 (2) | 96 ± 1 |
| A158T         | 41 ± 12 (2) | 75 ± 12 | 1.8 ± 0.2 (2) | 105 ± 5 | 1.5 ± 0.3 (2) | 94 ± 1 |

**FIG. 4.** Scatchard analysis of the equilibrium DNA binding of wild type and mutant SOX9 HMG domains. A, binding reactions containing a fixed amount of SOX9 HMG domain (0.25 nM) and increasing concentrations of DNA probe (10–150 nM, right to left) were resolved by non-denaturing gel electrophoresis and bound and free DNA probe quantitated from three experiments performed in duplicate. An electrophoretic mobility shift assay is representative of one experiment shown. B, Scatchard analysis of the equilibrium binding of SOX9 HMG domain to DNA probe SOX9CON. The points plotted are the mean of duplicate data points from one experiment, see Table V for pooled data.

**TABLE V**
DNA binding activity of wild type and mutant SOX9

| SOX9 HMG       | Kd       | r       | WT binding |
|---------------|----------|---------|------------|
|               | nM       | %       |            |
| WT (n = 4)    | 12.6 ± 2.5 | 0.98    | 50 ± 9     |
| F154L (n = 5) | 244 ± 75 | 0.96    | 5.0        |
| A158T (n = 5) | 76 ± 6   | 0.94    | 16.6       |

a The Kd values given represent the mean from n experiments (±S.E.).

b Defined as (Kd wt/Kd mutant) × 100.

residues of the hydrophobic core, nor does it affect the stability of the (residual) folded structure of the mutants as compared with the wild type protein. This could suggest functional redundancy in amino acids that form the hydrophobic core.

In contrast, our circular dichroism results suggest that the main effect of mutation at Phe154 and Ala158 is upon loss of secondary structure, mainly in helix 3. Both SOX9 mutations are located close to helix 1/helix 2 tryptophan residues. In the related Sox5 protein, the concept of “multidomain unfolding” has been explored whereby unfolding of helix 1/helix 2 arm leads to large (15 nm) changes in tryptophan fluorescence (45).

Thus a complete loss of structure in the helix 1/helix 2 wing is an unlikely consequence of the mutations since the tryptophan fluorescence properties of the two mutant SOX9 proteins are largely unaffected, but small changes could be through “local effects” of the substitutions. This leaves the possibility that helix 3 itself is perturbed in local structure. Threonine is
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known to cause loss of secondary structure (46) and to bend α-helices (47). Furthermore, thermally induced unfolding of helix 3 as a separate domain from helix 1 and helix 2 has been observed in the HMG domain of Sox5 (45).

Helix 3 of the SOX9 HMG domain possesses a functional NLS, as does helix 1 (28) (Fig. 1D). We show that SOX9 is recognized by the nuclear transport receptor importin-β and not by the more widely reported NLS receptor, importin-α. Given that the NLSs are highly conserved among SOX proteins (shaded in Fig. 1D), we predict that all SOX proteins utilize an importin-β-mediated nuclear import pathway. The NLS located at the end of helix 3 is a conventional basic amphipathic helix which, in SRY, mediates nuclear import via direct interaction with importin-β. In SOX9, the A158T mutant showed decreased nuclear accumulation. This mutation is more proximal to the helix 3 NLS than F154L (whose nuclear import was normal) and might disrupt the function of this NLS. However, A158T bound with wild type affinity to importin-β suggesting that while this recognition step is normal, other components of the importin-β-mediated nuclear import pathway could be affected. Alternatively, the A158T mutation could indirectly affect the function of the helix 1 NLS, whose import mechanism has not been defined, but may involve calmodulin which has been implicated in import and which recognizes helix 1 of SRY (31). The two NLSs appear to be able to function independently but are close together in three-dimensional space, and the dramatic conformational changes to SRY HMG domain that occur upon calmodulin binding might regulate NLS activity. Further studies are also required to elucidate the component of nuclear import that presumably fails to recognize efficiently the A158T mutant. The demonstration here that a mutation outside the NLS regions affects nuclear localization raises the possibility that a large number of SRY, SOX9, and SOX10 clinical mutations could affect nuclear import in addition to, or distinct from, DNA binding and bending. In support of this, the A158T mutation R62G, a mutation outside the helix 3 NLS, showed decreased nuclear accumulation and reduced importin-β binding.

Substitutions at the two positions in SOX9 studied here have also been reported at the corresponding positions in the HMG domain of SRY in two XY gonadal dysgenesis patients. The first example is SRY-A113T, analogous to SOX9-A158T, which showed reduced DNA binding like the SOX9-A158T mutant but also showed altered DNA bending (48). This suggests that altering the secondary structure in this region of the HMG domain of SRY and SOX9 (which show 70% amino acid similarity) has different consequences. Thus DNA bending by wild type SRY and SOX9 HMG domains is not identical, despite their ability to bind DNA of the same sequence to the same angle (27, 29). Key determinants of DNA bending appear to reside on helix 1 consistent with the observation that helix 1/2 is unperturbed and DNA bending is normal in both SOX9 mutants in this study. Helix 1/2 wing contributes two key helix 1 amino acids, Met111 (Ile68 in SRY), which acts as a wedge intercalator and adjacent Met107 (Met64 in SRY; two sex-reversing SRY mutations at Met64 alter bend angle (8)), which interacts with the DNA backbone at the site of the bend. The second example is the SRY-F109S substitution in an XY gonadal dysgenesis patient, which corresponds to SOX9-F154L and is a familial mutation inherited by the fertile father (49). The structure of SRY shows that the Phe109 is buried within the hydrophobic core and packs against Ala111, Val114, and Trp115 of helix 1 and Phe155 of helix 2 (SOX9 numbering; Fig. 1C) and might be expected to affect protein stability. Although

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SRY-F109S protein showed no significant change in DNA binding affinity compared with wild type (49), the substitution of Phe for Leu in SOX9 caused a 20-fold reduction in DNA binding. The simplest explanation is that serine substitution would be less disruptive to the hydrophobic core than leucine substitution in either context.

This study presents data for the first time on the effect of point mutations in CD upon transactivation activity in cultured cells and allows us to correlate this with DNA binding activity in vitro. In the A158T mutant, a 6-fold loss of DNA binding activity together with a 2-fold loss of nuclear import led to only a 26% loss of transcriptional activation. Similarly, in the F154L mutant, a 20-fold loss of DNA binding activity led to only a 62% loss of transcriptional activation activity. Our binary in vitro system is simplistic given that SOX9 acts in the context of a multiprotein complex in vivo. During testsis formation, SOX9-SF1 and WT1-SF1 are required on the MIS promoter (11), and the Sox5/Sox6 heterodimers are required for maximal activation of the Col2a1 promoter (18). Furthermore, reduced DNA binding affinity may still permit transient occupancy of a SOX9-binding site by SOX9 in vivo at levels sufficient to activate transcription once bound, provided the correct DNA architecture is established (50, 51). Also, it might be that other functions of the HMG domain, for example interactions with co-activators, are at play. Our data are consistent with that for yeast ROX1, the only other HMG domain protein for which in vitro DNA binding activities have been reported. In ROX1, substitutions causing a large reduction in DNA binding activity in vitro produce a small effect upon ANB1 repressor activity in vivo (52). For example the analogous change to SOX9 A158T in ROX1 affects DNA binding 10-fold and repression in vivo 4-fold. In ROX1 Phe154 is Trp and a substitution to Leu affects DNA binding 1000-fold but repression only 50-fold. Thus a reduction in DNA binding in vitro produces only a small effect in vivo, but this is presumably sufficient to account for the phenotypic effects. On this basis, small changes in DNA binding activity of SOX9 mutants may show undetectable changes in transactivation and lead to wild type phenotype. In SRY, small changes in DNA binding activity with partial penetrance of SRY were observed to occur in familial cases, i.e. when a weak allele is inherited by a fertile father (7, 49). However small changes in SOX9 DNA binding activity in vitro do not seem to correlate with milder symptoms, in these cases it could be that alterations of non-DNA binding functions of the HMG domain underlie the defect. Our data show that A154T mutant has 62% of wild type activation function in cultured cells. Given that this observation reflects the situation in vivo in CD/SRA1 where one allele is mutant for SOX9 and the other is wild type, our study raises the possibility that a high level of SOX9 transactivation activity is normally required for proper testis and bone formation. It is likely that interactions with transcriptional co-activators or components of the basal transcriptional machinery may attenuate the effect of mutation.

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