Human Sex Reversal Due to Impaired Nuclear Localization of SRY

A CLINICAL CORRELATION

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SRY, an architectural transcription factor encoded by the sex-determining region of the Y chromosome, initiates testicular differentiation in mammalian embryogenesis. The protein contains a high-mobility group (HMG) box, a DNA-bending motif conserved among a broad class of nuclear proteins. Mutations causing human sex reversal (46, XY pure gonadal dysgenesis) are clustered in this domain. Basic N- and C-terminal regions of the HMG box are each proposed to provide nuclear localization signals. The significance of the C-terminal basic cluster (SRY residues 130–134) is uncertain, however, as its activity in cell culture varies with assay conditions. To test its importance, we have investigated a C-terminal sex-reversal mutation (R133W, position 78 of the HMG box). This de novo mutation impairs nuclear localization but not specific DNA binding or sharp DNA bending. Correlation between these properties and the phenotype of the patient suggests that nuclear localization of SRY is required for testicular differentiation and directed in part by the C-terminal basic cluster. To our knowledge, these results provide the first example of impaired organogenesis due to a nuclear localization signal mutation.

SRY, the testes-determining factor encoded by the human Y chromosome (1), contains a high-mobility group (HMG) box (2–4), a conserved motif of DNA bending (Fig. 1, A and C, and Ref. 5). Mutations in SRY are associated with 46, XY pure gonadal dysgenesis leading to failure of testicular differentiation and female somatic phenotype (XY sex reversal; Refs. 3 and 6–8). Clinical mutations cluster in the HMG box and most commonly impair specific DNA binding (7, 9, 10). SRY is a nuclear protein (11) expressed in the primordial Sertoli cells of the differentiating gonadal ridge (12–14). Although SRY is presumed to function as an architectural transcription factor (9, 15, 16), its downstream genetic pathway is not well characterized (for a review, see Ref. 17).

Immunohistochemical studies of murine and human embryos have demonstrated that SRY is a nuclear protein (11, 18). Nuclear localization signals (NLSs) in human SRY have been defined in cell culture. Berta and colleagues (11), using microinjection of proteins in adult human fibroblastic cells, identified an NLS in the N-terminal region of the human HMG box (Fig. 2A; SRY residues 59–75). This NLS comprises two sets of basic amino acids separated by 12 residues (Fig. 2B), features characteristic of bipartite NLS motifs in diverse proteins (19, 20).

An isolated N-terminal SRY peptide (residues 58–78) was shown to be sufficient to direct nuclear translocation of coupled rabbit IgG (protein SRY21 in Fig. 2A). By contrast the remainder of the HMG box (residues 74–137) was unable to direct nuclear translocation of coupled rabbit IgG (protein SRY64 in Fig. 2A). Although these findings appear to exclude a second NLS in SRY, Sündbeck and Scherer (21) subsequently used a complementary methodology (transient transfection of SRY-β-galactosidase fusion genes in COS-7 cells; Fig. 2B) to identify a basic cluster NLS (residues 130–134; underlined in Fig. 1, B, top sequence) in the C-terminal tail of the HMG box (highlighted in red in Fig. 1, A–C). Whereas the microinjection assay suggested that the N-terminal NLS is sufficient to direct complete nuclear localization, in the transient transfection assay both N- and C-terminal NLS motifs are required (21). These differences may be due to assay procedures and/or cell lines. The function of the NLSs of SRY has not been established in vivo as to date no clinical mutations have been shown to impair nuclear localization.

In this communication we provide evidence for the physiological importance of the putative C-terminal NLS. Experimental design exploits a de novo sex-reversal mutation in the C-terminal basic cluster (R133W; Fig. 1B and Ref. 22) to distinguish between DNA binding and nuclear localization. The site of mutation is disordered in the NMR structure of a specific SRY-DNA complex (Fig. 1C and Ref. 10). Although the variant SRY domain exhibits essentially native DNA recognition, the mutation impairs nuclear translocation in a rat embryonic gonadal ridge cell line. Control substitution R133A also impairs NLS function, suggesting that mislocalization is due to

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1 The abbreviations used are: HMG, high-mobility group; GMSA, gel mobility shift assay; LeF-1, lymphoid enhancer factor 1; NLS, nuclear localization signal; PBS, phosphate-buffered saline; PGE, permutation gel electrophoresis; SRY, sex-determining region of Y chromosome; TCP-1, T-cell factor 1; bp, base pair(s); X-gal, 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside; WT, wild type.

2 Of the greater than 30 clinical mutations found in SRY to date only four map outside of the HMG box. One is a familial point mutation N-terminal to the HMG box (S18N) associated with partial gonadal dysgenesis (57); the second and third are nonsense mutations at codons 2 and 4 (22, 58), respectively; the fourth causes deletion of the C-terminal 41 residues (59), which includes a potential PDZ-binding site (11).

3 Although residues in the N-terminal NLS of SRY contribute to the minor groove DNA-binding surface of the domain, NLS activity does not require DNA binding: a sex-reversal mutation elsewhere in the HMG box (Y127C; Fig. 1B) impairs specific DNA binding but not nuclear localization (11). In the structure of an SRY-DNA complex (43)/Tyr-127 (residue 72) contacts the DNA backbone.

4 It is possible that in a fibroblastic cell line the C-terminal NLS functions too weakly to be detected given the low peptide-IgG coupling ratio used in the microinjection assay (11).
loss of the native side chain rather than specific interference by tryptophan. Correlation between impaired nuclear localization and phenotype (XY sex reversal) strongly suggests that the C-terminal basic cluster contributes to the nuclear localization of SRY in vivo and that such localization is required for testicular differentiation. To our knowledge, this is the first example of impaired organogenesis associated with mutation of an NLS.

EXPERIMENTAL PROCEDURES

Protein Purification

Native and variant SRY HMG box domains (84 amino acid; residues 57–140) were expressed in Escherichia coli as thrombin-cleavable fusion proteins and purified as described previously (23). The final SRY fragment (86 residues) contains two additional N-terminal residues (Gly-Ser) derived from the thrombin site. Purity was >98% as assessed by SDS-polyacrylamide gel electrophoresis and reverse-phase high-performance liquid chromatography. Predicted molecular masses of SRY fragments were verified by mass spectrometry.

Circular Dichroism

Spectra were obtained using an Aviv spectropolarimeter in 50 mM KCl and 10 mM potassium phosphate (pH 7.4) as described previously (24).

DNA Binding Studies

The sequence specificity of the SRY HMG box has previously been described (6, 7, 9, 10, 23). For use in the gel mobility shift assay (GMSA), a 15-bp DNA probe (23) was prepared containing sequence 5′-GTGATTGTGCAG-3′ and complement (core target site in bold). The probe was labeled with 32P and annealed. Each reaction contained 6.25–50 nM protein (see caption to Fig. 2C) and <1 nM labeled DNA in 10 mM potassium phosphate (pH 7.0), 50 mM NaCl, 4 mM dithiothreitol, and 2.5 mM MgCl2; the reaction was incubated for 1 h on ice. In control GMSA studies using a heterologous DNA target site (phage λ operator site O1; 17 bp) the SRY HMG box did not form a shifted band. Gels were repeated in triplicate.

DNA Bending Studies

For permutation gel electrophoresis (PGE), DNA probes of equal length (147 bp) with an SRY binding site (5′-GTGATTGTGCAG-3′ and complement) at varying distance from ends (distances of bend center from 5′-end are 120, 95, 79, 51, 47, and 27 bp) were generated by polymerase chain reaction from vector pBend2 as described previously (25) and 5′-labeled with [32P]ATP using T4 polynucleotide kinase. 10-μl binding reactions contained 50 mM NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 20 ng of poly(dI-dC), 400 ng of bovine serum albumin, 10% glycerol, ~1 nM 32P-labeled DNA probe, and 200 nM protein. After incubation on ice, samples were run on a 10% polyacrylamide gel with (29:1 ratio bisacrylamide) in 0.5X (0.045 M) Tris borate buffer containing EDTA (TBE) at ~10 V/cm. Gels were repeated in triplicate. Induced DNA bend angles were calculated as described previously (26). In multiple PGE studies of the wild-type protein inferred DNA bend angles were in general ~1°. Wild-type and variant proteins were run on the same gel.

Optical Microscopy

Confocal analysis was performed using a Zeiss LSM-410 laser scanning confocal microscope equipped with barrier filter for fluorescein (argon 488 nm as light source). An ×40 oil-immersion objective (numerical aperture, 1.3) was used for imaging of fluoroscence-labeled samples. Images were captured from the confocal optical section. Image analysis was performed using vendor software.

Cell Culture

COS-7 (American Type Culture Collection) and CH34 cell lines (kindly provided by Prof. P. K. Donahoe, Massachusetts General Hospital, Boston, MA) were cultured in 12-well plates at 60% confluency in Dulbecco’s modified Eagle’s medium containing 5% heat-inactivated fetal bovine serum and 1× penicillin/streptomycin (Life Technologies, Inc.) at 37 °C under 5% CO2.

Cellular Localization Studies

Constructions SRY-WT and pSRY-D3 (Fig. 2B) were kindly provided by Dr. Gerd Scherer (Institute of Human Genetics, University of Freiburg, Germany). SRY-R133W and SRY-R133A were obtained from SRY-WT by site-directed mutagenesis using the QuikChangeTM kit (Stratagene, Inc.). Transfections were performed using LipofectAMINE 2000 kit (Life Technologies, Inc.). Following transfection, fresh Dulbecco’s modified Eagle’s medium containing 5% heat-inactivated fetal bovine serum and 1× penicillin/streptomycin was added. Cells were evaluated after 48 h by 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) staining or immunohistochemistry as follows.

Enzymatic Staining—Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and stained for 2 h with X-gal as described by the vendor (Invitrogen, Inc.).

Immunocytochemistry for Fixed Cells—Cells were fixed with 3.5% formaldehyde in PBS on ice for 20 min, treated with successive ethanol solutions at ~20 °C (70% ethanol for 7 min, 100% ethanol for 7 min, and 70% ethanol for 5 min), incubated for 45 min with anti-β-galactosidase antibody (at 1:100 dilution), and incubated for 45 min with fluorescein isothiocyanate-conjugated anti-mouse antibody (at 1:500 dilution). Antisera were obtained from Molecular Probes, Inc. (Eugene, OR). After two PBS washes, specimens were observed by LSM 410 confocal microscopy.

Live Labeling—Cells at 37 °C were treated with 1 mM 5-chloromethyl-fluorescein-di-β-galactopyranoside (CMFDA) in hypertonic loading medium at 37 °C for 10 min and incubated in hypotonic loading medium for 2 min and culture medium for 30 min.

a Inferred DNA bend angles depend somewhat on gel composition (the present gel system yields an estimate of 79°, for example, whereas 8% acrylamide gel with the same ratio of bisacrylamide yields an estimate of 72°; A. Jancso-Radek and M. A. Weiss, unpublished results). Such apparent effects are thought to reflect limitations of data analysis (26) rather than physical differences in DNA bending. Changes in bend angle (Δθ) induced by native and variant SRY domains are robust to changes in gel composition.
Nuclear Localization of SRY

RESULTS

DNA Binding Studies—Effects of the R133W mutation on structure, DNA binding, and DNA bending were evaluated in an SRY domain containing the HMG box and C-terminal tail (residues 56–140). CD spectra of native and variant domains are indistinguishable (data not shown). The mutation has no detectable effect on specific DNA binding as evaluated using the GMSA (Fig. 2C). Likewise, native and variant domains induce similar anomalies in permutation gel electrophoresis (Fig. 2D). The dependence of electrophoretic mobility on flexure displacement corresponds to apparent DNA bend angles of 79° and 73°, respectively. Although these values are model-dependent (26), the data indicate that the variant domain retains sharp DNA bending activity. Its small difference in bend angle, although of biophysical interest (see “Discussion”), would not be associated with attenuation of transcriptional activation or repression by homo- or hetero-HMG box factors (27, 28).

Nuclear Localization—Studies used an SRY-β-galactosidase fusion plasmid (kindly provided by G. Scherer; Ref. 21). In accord with previous studies SRY directs nuclear localization of β-galactosidase enzymatic activity (Fig. 3, A and B, column 1; Refs. 11 and 21). A previously characterized five-residue deletion in the C-terminal basic cluster (residues 130–134; construction SRY-D3 in Fig. 2B; Ref. 21) was used as a control for loss of C-terminal NLS activity. Transient transfection experiments were performed using COS-7 cells to enable comparison with the results of Sübeck and Scherer (21) and a male rat gonadal ridge embryogenic cell line (CH34 cells; Refs. 9 and 29) to provide a physiologically appropriate milieu. Cellular localization of β-galactosidase activity was quantitatively evaluated by two complementary methods: staining of fixed cells using X-gal (rows a and a’ in Fig. 3, A and B, respectively) and confocal immunofluorescence analysis of fixed cells (rows b and b’). To obtain meaningful statistics, ~100 cells were counted by X-gal staining, and ~20–50 fixed cells were visualized by confocal microscopy. Representative images are shown in Fig. 3. As a control for possible confounding factors related to cell fixation, live labeling and confocal immunofluorescence analysis of
transfected cells were used to verify our interpretation of subcellular localization (rows c and c’).

Control construction SRY-WT directs exclusive nuclear localization of β-galactosidase in ~90% of COS-7 cells (114 of 127 cells counted) or CH34 cells (115 of 123 cells counted) (Fig. 3, column 1), whereas SRY-D3 yields a broad distribution of β-galactosidase in the nucleus and cytoplasm in ~95% of COS-7 cells (108 of 111 cells counted) and ~80% of CH34 cells (124 of 154 cells counted) (Fig. 3, column 4). Similar results were obtained by the immunohistochemical assay. Like the SRY-D3 negative control, R133W and R133A constructions (Fig. 3, columns 2 and 3, respectively) gave rise to pancellular distribution in more than 80% of cells analyzed by either assay. β-Galactosidase staining in COS-7 cells was pancellular in 83 of 101 cells counted (R133W) and 108 of 122 (R133A); corresponding values in CH34 cells were 57 of 69 (R133W) and 114 of 124 (R133A). Similarly, immunoreactive staining in COS-7 cells was pancellular in 39 of 42 cells counted (R133W) and 21 of 22 (R133A); corresponding values in CH34 cells were 19 of 21 (R133W) and 19 of 22 (R133A). Any differences in occurrence between cell lines (Fig. 3, A versus B) or among methods of visualization (Fig. 3, rows a and b and a’ and b’) are not of statistical significance. Impairment of nuclear localization due to mutations R133W or R133A is in each case similar to that due to deletion of the basic cluster. Their partial nuclear import presumably reflects continued activity of the N-terminal NLS.

**DISCUSSION**

SRY was originally identified by genetic analysis of patients with intersex abnormalities (4). In the absence of a priori biochemical information, the mechanism of action of SRY was hypothesized on the basis of homology between its HMG box and a conserved family of architectural transcription factors. This hypothesis has been corroborated in part by a striking correlation between sites of sex-reversal mutations (3, 6–8) and sites in the HMG box required for folding or DNA binding (7, 9, 30, 31). Almost all clinical mutations in SRY cluster in the HMG box, and almost all de novo mutations impair DNA binding (9). The present study highlights a mutation in the C-terminal tail of the HMG box (R133W; Ref. 22) that impairs nuclear localization but not DNA binding. The mutation alters an invariant arginine in a basic cluster previously proposed as an accessory NLS (21). That an alanine substitution likewise impairs nuclear localization suggests that the defect is due to loss of the basic side chain rather than specific interference by the bulky Trp-133 side chain. Because alterations in the C-terminal basic cluster leave intact the N-terminal NLS, effects of C-terminal substitutions are incomplete, leading to a broad pattern of cytoplasmic and nuclear localization.

Genetic corroboration of a putative NLS in a transcription factor can be confounded by the location of such signals within DNA-binding domains (32, 33). SRY contains NLSs within or adjoining its HMG box (32, 33). A classical bipartite NLS occurs (19, 20, 34–36) at the N terminus of the HMG box (11). This region spans the first β-strand and α-helix (10). Sex reversal mutations in the N-terminal NLS occur at arginines (R62G and R76S; Refs. 37 and 38). If these mutations should impair both DNA binding and nuclear localization (Arg-62 contacts the DNA backbone; Ref. 10), then a selective correlation with one or the other activities would not be possible. The present study has focused on the C-terminal basic cluster because its NLS activity has been inconsistently observed in cell culture (11, 21). We chose to investigate the sex-reversal mutation R133W because this residue is disordered both in the SRY-DNA complex (10) and in the homologous lymphoid enhancer factor 1 (Lef-1)-DNA complex (39). We therefore hypothesized that the de novo substitution might exhibit native DNA binding properties but impaired nuclear localization.

An extended basic tail in the homologous Lef-1-DNA complex binds within the compressed major groove (39). The basic side chains not only provide an NLS (40) but are also proposed to function as an “electrostatic clamp” to stabilize the sharply bent DNA structure (39, 41). Truncation of the Lef-1 tail leads to a marked loss of DNA affinity and marked attenuation in DNA bending (42). It is possible that an analogous clamp exists in the SRY complex but was not observable in its NMR structure (10, 43) due to truncation of the fragment after residue 133 (arrowhead in Fig. 1B). It is interesting that the R133W SRY domain exhibits a decrement in induced DNA bend angle (Fig. 2D). Perhaps the several basic residues in the SRY tail together provide an analogous electrostatic clamp. Although we cannot exclude altered protein-DNA architecture (44) as a mechanism of R133W-associated sex reversal, the following considerations suggest that a small decrement in bending is unlikely to be of physiological significance. (i) Similar such decrements in Lef-1-induced DNA bending (obtained by variations in its DNA target site) are not associated with changes in transcriptional activation in cell culture (42). (ii) Similar such decrements due to mutations in yeast hypoxic repressor Rox1 (28) (also a specific HMG box protein) are not associated with changes in transcriptional repression in vivo; changes in activity correlate instead with specific DNA affinity. (iii) The R133W-associated decrement is smaller than variations in bend angle observed among mammalian SRY complexes and in complexes between human SRY and closely related DNA target sites (27). To our knowledge, no sex-reversal mutation has been identified in SRY with a selective defect in DNA bending. A physiological requirement for a sharp DNA bend without precise calibration in mammalian development would be consistent with studies of bacterial gene regulation: the 180° “U-turn” bend of initiation host factor may be functionally replaced by the 130° bend induced by Lef-1 (45, 46). In the future this issue may be explored through studies of the phenotypes of XX mice engineered to contain Sry transgenes (1) with mutations in the HMG box designed to affect the bend angles of the Sry-DNA complex to determine whether sex reversal occurs.

Diverse human diseases are associated with mislocalization of proteins: impaired display of receptors or channels on the cell surface, impaired secretion of hormones or growth factors, and impaired nuclear import. Such abnormalities are often a secondary consequence of other processes (47). Identification of a primary disorder of localization requires demonstration of otherwise native biochemical activities. An outstanding example is provided by a diabetes-associated mutation in human proinsulin (48, 49). Similarly, primary disorders of nuclear localization have been proposed in Fanconi anemia (50, 51) and the Li-Fraumeni (p53) cancer susceptibility syndrome (52–54). Such
mutations affect sites of protein-protein interaction (rather than primary NLS sequences) required for nuclear import of a cytoplasmic assembly. To our knowledge, the present study provides the first example of a mutation in an NLS associated with failure of organogenesis. Because SOX-9 and SRY contain homologous NLSs (21), SOX-9 sequences in campomelic dysplasia should be screened for similar mutations (55, 56, 61). Similar studies of NLS mutations in cell culture may establish the physiologic relevance of these and other NLS sequences in disease states.

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