Loss of DNA-dependent dimerization of the transcription factor SOX9 as a cause for campomelic dysplasia

Elisabeth Sock1, Roberta A. Pagon2, Kathelijn Keymolen3, Willy Lissens3, Michael Wegner1 and Gerd Scherer4,*

1Institut für Biochemie, Universität Erlangen, D-91054 Erlangen, Germany, 2Children’s Hospital and Regional Medical Center, Seattle, WA 98105-0371, USA, 3Center for Medical Genetics, University Hospital VUB, B-1090 Brussels, Belgium and 4Institute of Human Genetics and Anthropology, University of Freiburg, D-79106 Freiburg, Germany

Received December 5, 2002; Revised and Accepted April 23, 2003

Campomelic dysplasia (CD) is a semilethal osteochondrodysplasia, characterized by skeletal anomalies that include bending of the long bones, and by XY sex reversal. CD results from haploinsufficiency for the transcription factor SOX9, a key regulator at various steps of cartilage differentiation and of early testis development. Two functional domains are so far recognized for SOX9, a high-mobility group (HMG) DNA-binding domain and a C-terminal transactivation domain. We present two CD patients with de novo mutations in a conserved region preceding the HMG domain. A long-term survivor with the acampomelic form of CD has an A76E amino acid substitution, while a severely affected CD patient had an in-frame deletion of amino acid residues 66–75. The conserved domain has been shown to function in the related transcription factor SOX10 as a DNA-dependent dimerization domain. We show that, like SOX10, SOX9 also binds cooperatively as a dimer to response elements in regulatory regions of some target genes such as the cartilage genes Col11a2 and CD-Rap. Dimerization and the resulting capacity to activate promoters via dimeric binding sites is lost in both mutant SOX9 proteins while other features involved in SOX9 function remained unaltered. These findings establish the dimerization domain as the third domain essential for SOX9 function during chondrogenesis.

INTRODUCTION

Campomelic dysplasia (CD, MIM 114290) is a severe malformation syndrome that is characterized by skeletal anomalies including bowing of femora and tibiae, hypoplastic scapulae, 11 pairs of ribs, lack of mineralization of thoracic pedicles, pelvic malformations and bilateral clubfeet. Malformations such as Robin sequence, narrow airways resulting from tracheobronchial cartilage defects, hypoplastic lungs and a bell-shaped thorax cause severe respiratory problems that usually lead to death during the neonatal period. A second characteristic feature of CD is the male-to-female sex reversal that occurs in about two-thirds of XY cases (1,2). Like the sex reversal and the various skeletal symptoms, the eponymous feature campomelia, the bending of the long bones, is not an obligatory feature and is absent in about 10% of CD cases, referred to as acampomelic CD (ACD) cases. De novo heterozygous loss-of-function mutations in the SOX9 gene have been identified in CD cases with XY sex reversal, identifying haploinsufficiency for SOX9 as the cause for both phenotypes and assigning a role for SOX9 in chondrogenesis and in testogenesis (3,4). In line with this, expression of Sox9 has been documented at sites of ensuing cartilage deposition throughout embryogenesis (5,6) and in the developing testis (7,8). Recent mouse models for CD have substantiated the role of SOX9 as a key regulator at various steps of cartilage differentiation (9–11). As direct targets for SOX9 during chondrogenesis, the collagen genes Col2a1 (6,12) and Col11a2 (13), as well as CD-Rap (14) and Aggrecan (15) have been identified. With respect to testis development, the direct involvement of Sox9 in the expression of the Anh gene, an early marker for Sertoli-cell differentiation, has been documented both in vitro (16) and in vivo (17).
SOX9 is a member of the SOX family of transcription factors, belonging to the subgroup E that also includes SOX8 and SOX10 (18,19). Like all SOX proteins, SOX9 contains a 79-amino acid DNA-binding motif known as the high-mobility-group (HMG) domain. In addition to this domain, that recognizes typical SOX binding sequences (6,12,20), SOX9 has a second domain essential for its function, a proline/glutamine/serine-rich C-terminal transcription-activation domain (21). A proline/glutamine/alanine (PQA)-rich motif enhances the potency of the C-terminal transactivation domain, but is unable to activate transcription alone (20). Recently, we have identified and characterized a dimerization domain in a conserved region immediately preceding the HMG domain in the related transcription factor SOX10 and presented initial evidence that SOX9 can also dimerize on SOX10 target sites (22).

In the present study, we describe two CD patients with de novo heterozygous SOX9 mutations in the conserved region mentioned above. A milder affected ACD case has a single amino acid substitution, the first found outside the HMG domain. A severely affected CD patient shows a deletion of 10 amino acid residues. Functional studies of the mutant SOX9 proteins reveal that both mutations selectively abrogate DNA-dependent dimerization and thus interfere with promoter activation via natural target sites that require binding of SOX9 dimers. These results establish dimerization as an essential component of SOX9 function and the corresponding region as the third essential domain of SOX9.

RESULTS

De novo mutations in a conserved region preceding the HMG domain

Patient E.R., now 6 years old, presented at birth with respiratory distress and clinical and radiographic signs typical for CD, such as hypoplasia of scapulae and of pelvic bones, but without overt bending of the long bones, representing an acampomelic case (Fig. 1A). He walked at 20 months of age. He has normal male external genitalia and a 46,XY karyotype. (See Case Report for details.) Sequencing of the entire SOX9 ORF revealed a heterozygous point mutation in codon 76, leading to substitution of an alanine by a glutamic acid residue (A76E; GCG→GAG; Fig. 2A). This amino acid replacement affects a residue located within a region directly preceding the HMG domain that is not only well conserved between SOX9 proteins from human, mouse, chicken, alligator and the pufferfish Fugu, but also between the SOX subgroup E members SOX9, SOX10 and SOX8 (Fig. 2D).

Baby girl M., who died soon after birth from respiratory failure, had all the hallmarks of CD (see Case Report), including bending of femora, tibiae and fibulae (Fig. 1B). A rare finding in CD is the absence of toenails and a broad gap between the first and second toes, combined with syndactyly between the second, third and fourth toes (Fig. 1C). Her karyotype was 46,XX. She had a heterozygous in-frame deletion, causing loss of amino acid residues 66–75 in the conserved domain of SOX9 (Fig. 2B and D). The 30 bp deletion occurred between two hexanucleotide repeats, retaining one copy (Fig. 2C).

Both mutations were shown to be de novo in origin, by sequence analysis of the corresponding region in DNA from

Figure 1. Selected radiographic and clinical findings in the two CD cases. (A) Radiograph of pelvis and of lower extremities of patient E.R. at 5 weeks of age. The pelvis shows tall iliac and absent pubic bones. Note straight long bones. (B) Radiograph of pelvis and of lower extremities of patient M. taken shortly after birth. Note hypoplastic iliac bones, and marked bowing of femora, tibiae and fibulae. (C) Left foot of patient M. shows absent toenails, a very short first toe and syndactyly between the second, third and fourth toe, with a broad gap between the first and second toe. Note also bulging above the ankle at the position of the tibial bend. Picture taken shortly after birth.

Figure 2. SOX9 mutations in conserved region preceding the HMG domain. (A) Sequence chromatogram for patient E.R. The heterozygous point mutation C→A in codon 76 results in substitution of an alanine by a glutamic acid residue (A76E). (B) Sequence chromatogram of the cloned deletion allele of patient M. The 30 bp deletion joins codon 65 to codon 76 (see C). (C) Thirty base pair in-frame deletion in patient M. As indicated by the grey bars, one of the two hexanucleotide repeats and all of the intervening DNA is deleted, leaving one copy of the repeat and resulting in removal of amino acids 66–75. (D) Comparison of amino acid sequences (single-letter code) of vertebrate SOX9 proteins (upper part) and of SOX subgroup E proteins from human (lower part) N-terminal to and at beginning of the HMG domain. Identical residues are indicated by dashes, gaps by blanks. The amino acid substitution A76E from patient E.R. and the first and second residue 66 from patient M. are indicated at the top. The region constituting the dimerization domain in SOX10 (22) is indicated at bottom. SOX9 sequence references: human (accession no. AAB32870); mouse (NP_035578); chicken (AAB09663); alligator (AAD17974); Fugu (AAL32172); hSOX10 (NP_008872); hSOX8 (NP_365402).
the parents and by the documentation of paternity through microsatellite marker analyses (not shown).

**Mutant SOX9 proteins exhibit unchanged cellular localization, stability and DNA-binding affinities**

To analyze the consequences of these mutations on SOX9 function, we introduced both mutations into the SOX9 cDNA. For better detection, we used a shortened, tagged version of SOX9 (21) for most studies. Wild-type as well as both mutant SOX9 proteins were detected by western blot in extracts from transiently transfected cells at the expected size (Fig. 3A). The shortened SOX9 proteins lack the transactivation domain, but contain all sequences necessary for nuclear localization (23). Correspondingly, the wild-type SOX9 protein used in our study exhibited the same, primarily nuclear localization in transiently transfected CV1 cells as a nuclear version of the enhanced green fluorescent protein (Fig. 3B). The same subcellular localization was also observed for both the A76E substitution mutant and the Δ66–75 deletion mutant (Fig. 3B), in accord with the fact that none of the two previously mapped nuclear localization signals is affected by the two mutations. Equally unaffected is the nuclear export signal recently identified in this group of SOX proteins (24, 25).

We also compared stability of wild-type and mutant SOX9 proteins in transfected COS cells by analyzing clearance rates of 35S-pulse-labeled full-length proteins (Fig. 4A). Both wild-type and mutant SOX9 proteins had comparable half-lives of ~4 h, arguing that the two SOX9 mutations do not have a dramatic effect on protein stability.

Using the well-known high-affinity binding site 3 from the *Col2a1* intronic enhancer (12), we next compared DNA binding abilities of wild-type and mutant SOX9 proteins in electrophoretic mobility shift assays (Fig. 4B). Both mutant SOX9 proteins recognized this site, yielding a complex of identical mobility as the wild-type protein. When SOX9–DNA complexes were challenged by increasing amounts of competitor oligonucleotides, DNA binding was reduced. Approximately 100-fold molar excess was needed to reduce binding of wild-type and mutant proteins to residual levels. As evident from this identical behavior in DNA binding assays, the two SOX9 mutants bind DNA with affinities comparable to the wild-type. Similar results were obtained in experiments with site B, another high-affinity binding site for SOX proteins from the *Protein zero* gene promoter (26) (data not shown).

**Mutant SOX9 proteins show a specific loss of DNA-dependent dimerization**

As the region affected by both mutations has previously been implicated in DNA-dependent dimerization in the related SOX10 protein, we next analyzed whether dimerization was altered in the mutants. Although we had previously shown that SOX9 is able to form dimers upon DNA binding, we had done so on a natural target site for SOX10 (22). Whether DNA-dependent dimerization occurs on binding sites for SOX9 in any of its target gene promoters has not been analyzed so far. For that reason, we reassessed various previously identified SOX9 binding sites (Fig. 5A). As evident from Figure 5B, some of these sites such as the BC and DE sites from the *Col11a2* enhancer or the target site from the *CD-Rap* promoter
and extracts from transfected COS cells expressing WT or mutant Col2a1 monomeric binding site 3 from the Col2a1 against the chase period. (A76E, Δ66–75) SOX9 proteins (amino acids 1–75) SOX9 proteins (amino acids 1–75) relative amounts were then plotted using a phosphoimager, with levels of radioactivity obtained for each protein without a competitor, and a competitor added; m, bound monomer.

Figure 4. Comparison of stability and DNA-binding affinities of wild-type and mutant SOX9 proteins. (A) Wild-type (WT) and mutant (A76E, Δ66–75) SOX9 proteins were immunoprecipitated from extracts of 35S-pulse-labeled COS cells after various chase periods and analyzed on 10% SDS–polyacrylamide gels. The amount of immunoprecipitated radioactivity was quantified using a phosphoimager, with levels of radioactivity obtained for each protein without a chase period arbitrarily set to 100%. The relative amounts were then plotted against the chase period. (B) Electrophoretic mobility shift assay with the monomeric binding site 3 from the Col2a1 enhancer as probe (for sequence, see Fig. 5A) and extracts from transfected COS cells expressing WT or mutant (A76E, Δ66–75) SOX9 proteins (amino acids 1–304) as indicated above the lanes. Increasing amounts (20-, 50-, 100-, 200-fold molar excess) of unlabeled Col2a1 oligonucleotide were added as competitor (comp). —, No competitor added; m, bound monomer.

The SOX9-responsive intronic enhancer from the Col2a1 intronic enhancer or the site from the AMH promoter, only bind SOX9 in its monomeric form. This simultaneous use of both monomeric as well as dimeric binding is similar to that reported for the related SOX10 (22,26). When binding of the A76E substitution mutant or the Δ66–75 mutant was analyzed on this set of SOX9 target sites by electrophoretic mobility shift assays, it became immediately obvious that all complexes indicative of the DNA-bound dimer were no longer present or drastically reduced (see C/C2, Col11a2 BC, Col11a2 DE and CD-Rap in Fig. 5B). Instead of the slow-migrating high affinity complexes, weaker complexes with higher mobility were obtained, characteristic of a monomer bound with lower affinity. Binding to monomeric sites, in contrast, was unchanged for the mutant proteins (see C/C2 mut, Col2a1 site 3 and AMH in Fig. 5B).

When increasing amounts of proteins were used with probes that support dimeric binding such as the site from the CD-Rap promoter, two molecules of the mutant SOX9 proteins could be bound to a single probe (Fig. 6A). However, this was evoked by independent binding of two SOX9 monomers rather than cooperative binding of one SOX9 dimer as evident from the binding kinetics. The above data thus document a selective, complete and identical loss of cooperative DNA binding in both SOX9 mutants.

The SOX9-responsive intronic enhancer from the Col2a1 gene has previously been shown to contain four binding sites for SOX9 (27) which are jointly bound by a SOX9-containing CSEP complex (28). While recognition of site 3 from the Col2a1 enhancer was indistinguishable between wild-type and mutant SOX9 proteins (see Fig. 5B), significant differences became apparent on the complete 48 bp intronic enhancer with its four sites (Fig. 6B). At low protein concentrations, wild-type SOX9 bound the enhancer both as monomer and as dimer, as evident from the almost simultaneous appearance of protein–DNA complexes with characteristic corresponding mobilities (complexes 1 and 2 in Fig. 6B). At high protein concentrations, the monomer band disappeared completely. In addition to the dimer, a new complex was obtained whose mobility indicated occupancy of all four sites (complex 4 in Fig. 6B) arguing for cooperativity between all bound SOX9 proteins. When the A76E or the Δ66–75 mutant proteins were used instead, monomer binding remained predominant at all concentrations. With higher amounts of mutant protein, more than one site within the 48 bp enhancer became bound, as reflected by the progressive appearance of protein–DNA complexes containing two, three or four mutant SOX9 proteins. Arguing for non-cooperative binding of the mutants, the protein–DNA complex containing two mutant SOX9 molecules has a lower mobility than the wild-type SOX9 dimer. Interestingly, formation of the low mobility complex with four SOX9 proteins was barely visible for both mutants, indicating an additional loss of higher-order cooperativity.

Mutant SOX9 proteins have altered transactivation potential

To analyze whether the specific loss of DNA-dependent dimerization translates into changed promotor activation, we performed co-transfection experiments in Neuro2A cells. Full-length wild-type SOX9 or either of the two full-length SOX9 mutants were transfected with luciferase reporters under the control of various SOX9-responsive promoters, such as the AMH promoter (16) or the CD-Rap promoter (14). On average, we obtained a 9- to 10-fold induction by wild-type SOX9 (Fig. 7A and B). The two promoters, however, behaved completely different in co-transfections with the mutant SOX9 proteins. Whereas similar activation rates were obtained for wild-type and mutant SOX9 proteins on the AMH promoter (Fig. 7A), the CD-Rap promoter failed to respond to both the A76E and the Δ66–75 mutant (Fig. 7B), in agreement with the fact that the main SOX9-responsive site in the CD-Rap promoter is a dimer site, whereas SOX9 binds to the AMH promoter as a monomer. This failure of both mutant SOX9 proteins to specifically activate promoters with sites that support dimeric binding was also corroborated by the analysis of a construct in which four copies of the dimeric DE site from the Col11a2 gene enhancer were used to drive SOX9-dependent activation of a minimal promoter (29). This construct was efficiently activated by wild-type SOX9 (Fig. 7C), while the minimal promoter itself was not activated (data not shown), indicating that the observed SOX9 effect was mediated by the DE enhancer. However, the construct remained completely unresponsive when SOX9 mutants were co-transfected instead of wild-type SOX9 (Fig. 7C).

When four copies of the Col2a1 intronic enhancer were used to drive SOX9-dependent activation of the same minimal promoter (12), we again observed a robust response to wildtype SOX9 (Fig. 7D). On this construct, the mutant SOX9 proteins exhibited reduced activity, with 38-fold (for A76E) and 18-fold induction rates (for Δ66–75) relative to the 215-fold promoter activation obtained with wild-type SOX9. This partial loss of transactivation capacity for the mutant SOX9 proteins might have been expected from the fact that the Col2a1 enhancer contains monomeric as well as dimeric binding sites. Thus, there is a good overall correlation between the loss of
cooperative binding in the two SOX9 mutants and their inability to activate promoters through sites that require such cooperative binding.

**DISCUSSION**

Important functions and/or functional domains of a protein are most convincingly revealed by a mutant phenotype, but the relevant mutations may be rare. This is the case for the two SOX9 mutations reported here. Since the identification of SOX9 as the gene causing CD when mutated (3,4), about 20 amino acid substitutions have been identified (30) (G.S., unpublished), all of which locate to the HMG domain and lead to reduction or loss of DNA-binding. The same holds true for amino acid substitutions in the related SOX10, which cause Waardenburg–Hirschsprung disease. The A76E mutation in SOX9 is thus a rare exception. Likewise, the 30 bp deletion removing amino acids 66–75 is the first in-frame *de novo* deletion found in SOX9. The only other SOX9 in-frame deletion was detected in a patient with a frameshift mutation in the second SOX9 allele (4). It removed three residues from the PQA motif, turned out to be inherited and probably represents a rare, neutral variant (G.S., unpublished data). Slipped mispairing during replication involving the misalignment of the hexanucleotide repeats is the likely mechanism for the generation of the 30 bp deletion (31).

As in many dominant disorders, genotype/phenotype correlations are difficult to establish in CD due to incomplete penetrance and variable expressivity. We have noted, however, that long-term survivors (i.e. patients living for more than 2 years, accounting for about 10% of all cases) (2) and ACD cases are overrepresented in the group of patients where the respective mutant SOX9 allele is a hypomorph with proven or

**Figure 5.** DNA-dependent dimerization of SOX9 proteins on natural target sites. (A) SOX9 binding sites used in this study as previously identified in chondrocyte or Sertoli cell-specific promoters/enhancers of target genes: C/C and C/C’mut from the promoter of the rat P0 gene (26), Col2a1 site3 from the intronic enhancer of the mouse Col2a1 gene (12), CD-Rap from the promoter of the mouse cartilage-derived retinoic acid-sensitive protein gene (14), BC and DE from the mouse Col11a2 gene (13), and AMH from the promoter of the human anti-Müllerian hormone gene (16). (B) Electrophoretic mobility shift assays with nuclear extracts from transfected COS cells expressing wild-type SOX9 (aa 1–304) or the two mutants (A76E, Δ66–75). The oligonucleotides shown in (A) were used as probes as indicated below the lanes. —, Extract from mock-transfected COS cells; m, bound monomer; d, bound dimer.
likely residual SOX9 activity, such as amino acid substitutions in the HMG domain retaining some DNA-binding activity (32), partial truncation of the C-terminal transactivation domain with residual transcription activation capacity (32), or translocations interrupting cis-acting regulation of SOX9 (33). Because the A76E missense mutation in patient E.R. goes with acampomelia and long-term survival, it also probably represents a hypomorph. In line with such an assumption, we found no evidence for altered nuclear export or increased protein turnover due to reduced stability of the mutant proteins. Interference with the transactivation domain of SOX9 appears equally unlikely, as mutation and transactivation domain reside in completely different parts of the protein. Further discounting such a general interference with transactivation capacity, the AMH promoter was efficiently transactivated by both mutant SOX9 proteins in our co-transfection studies.

The subtlety of the A76E substitution indicates that only a single feature of the protein is affected. The respective alanine residue is located in a highly conserved region which in the related SOX10 protein was shown to mediate DNA-dependent dimerization (22) and is itself conserved between SOX8, SOX9 and SOX10. Thus we reasoned that the A76E substitution might interfere with formation of SOX9 dimers on DNA. Although dimer sites have been shown to occur in natural target gene promoters of SOX10 (26,34,35), it was unclear whether such sites are also present and functional in regulatory regions of SOX9 target genes. Here we show that SOX9, like its relative SOX10, shows dimer formation on some of the previously identified natural response elements. In particular, dimeric binding was detected to target sites within the Col11a2 enhancer and CD-Rap promoter. Importantly, dimer formation is indeed abolished for the A76E mutation, causing a consecutive inability of the mutant protein to
transactivate promoters which are SOX9-responsive via dimer sites. For the 48 bp enhancer of the Col2a1 gene the situation is more complex. In accord with the ability of the enhancer to support both monomeric as well as dimeric binding, we observed a partial loss of Col2a1transactivation for the A76E mutant.

Some promoters of identified SOX10 target genes contain functionally important binding sites for SOX10 monomers and dimers. Removal of the dimer site or elimination of the protein’s ability to dimerize, did not completely abolish responsiveness of the target gene promoter to SOX10, but led to a partial reduction (22,36). Thus, it is likely that a comparable loss of dimerization capacity in SOX9 leads to altered expression of target genes with effects depending on the relative contribution of dimer sites versus monomer sites to regulation of the respective promoter. In agreement with this, we detected complete loss of transactivation capacity for the A76E mutant on the CD-Rap promoter (dimer site only), partial loss on the Col2a1 enhancer (dimer and monomer sites), and fully retained transactivation capacity on the AMH promoter (monomer site only). We would therefore assume that a mutation which specifically abrogates dimerization such as the A76E mutation causes a somewhat milder phenotype than a loss-of-function allele and that this is seen in ACD patient E.R.

Although similarly defective in dimerization, removal of residues 66–75 in patient M. resulted in a severe CD phenotype with campomelia and neonatal death. The severity of the phenotype in this patient as compared with patient E.R. may simply be attributable to a higher expressivity of the mutant trait. Alternatively, the deletion mutant may exhibit other defects in addition to a lost dimerization capacity, although these were not revealed in the current study. Nevertheless, the assumption of additional defects is supported by our recent finding for the related SOX10 protein that mutation of several strongly conserved amino acids within the dimerization domain does not affect DNA-dependent dimerization (36).

With respect to sex reversal, patient M. is uninformative, being an XX female. Patient E.R. on the other hand is a non-sex-reversed XY male. In line with an unaltered male sex, binding to the monomer response element within the AMH promoter and SOX9-dependent activation remain unaffected by the A76E mutation in patient E.R. The lack of sex reversal in E.R. may, however, also be due to incomplete penetrance, as only two-thirds of XY cases are born as sex-reversed females, and as identical, independent SOX9 mutations are known to lead to XY sex reversal in one case, but not in the other (37,38). Although SOX9-dependent activation of the AMH promoter does not appear to require SOX9 dimers, the role of DNA-dependent dimerization in testogenesis remains unclear. We cannot rule out that additional, as yet unidentified target genes of SOX9 during testogenesis have response elements with an arrangement similar to that seen in the collagen genes Col11a2 and CD-Rap and may thus bind SOX9 dimers. Nevertheless, the identification of these two mutations in CD patients not only defines a new important domain in the transcription factor SOX9, but also proves that the dimerization capacity of this group of SOX proteins, which has previously been only described in vitro, is indeed essential for their in vivo function.

MATERIALS AND METHODS

Case reports

E.R. (Seattle) was born at 32 weeks gestation by cesarean section to healthy, unrelated parents. His twin sister and his older half-sister are healthy. He underwent an urgent tracheostomy at 24 days of life. He had characteristic facies, subglottic stenosis and bronchomalacia, right clubfoot and normal male external genitalia. Radiographic findings included small scapulae, under-mineralization of vertebrae C3–C7, tall iliac bones, absent pubic bones, and straight long bones. He walked at 20 months of age. He remains dependent on his tracheostomy. He has chronic respiratory insufficiency with restrictive lung disease. He does not swallow and required gastrostomy then jejunostomy in infancy. He relies on total parenteral nutrition for all caloric intake. He has intestinal dysmotility which required ileostomy and colectomy. He also underwent cholecystectomy for gallstones. He has had recurrent septicemia with multiple organisms. He has chronic otitis media and hearing loss requiring myringotomy tubes. He underwent anterior and posterior spine fusion at about 3 years of age and a revision about 6 months later. Subsequently, his congenital kyphosis progressed to an 85° curve. He intermittently complains of back and leg pain. Presently, he is 6 years old. Height is 87 cm (50th centile for a 2 year old); weight is 14.1 kg (50th centile for a 3 year old). Karyotype is 46,XY.

Baby girl M. (Brussels) is the second child of healthy, non-consanguineous parents of Indian origin. The elder brother is in good health. During pregnancy, prenatal ultrasound disclosed severe limb anomalies, suggestive for campomelic dysplasia. Amniocentesis was performed, showing a normal fetal karyotype (46,XX with G-banding). The parents were counseled and conservative management of the pregnancy was elected. The girl was delivered prematurely at a gestational age of 28 weeks and she died a few hours after birth. She presented with low-set, posteriorly rotated ears, short nose with flat nasal bridge and long philtrum, micrognathia and a short neck. The lower limbs were bowed with a bulge at the position of the tibial bend, bilateral clubfeet, absent toenails, a very short first toe and syndactyly between the second, third and fourth toes, with a broad gap between the first and second toes. The external genitalia were female. X-rays revealed hypoplastic cervical vertebrae, hypoplastic scapulae, hypoplastic iliac bones and bowing of femora, tibiae and fibulae.

PCR and sequence analysis of CD cases

The three exons and the exon/intron boundaries of SOX9 were amplified by PCR as described (32) and used directly for DNA sequencing. The 30 bp deletion in baby girl M. was verified by sequencing of the PCR product cloned into the TOPO TA cloning vector (Invitrogen). Sequence reactions were performed with the Thermosequenase II Dye Terminator Cycle Sequencing kit (Amersham Pharmacia) and analysed on an ABI Prism 310 automated DNA sequencer (Applied Biosystems). Paternity was tested using theProfilerPlus kit and Genotyper version 3.7 software from Applied Biosystems.
Plasmids

The pcDNA3-based expression vector for human SOX9 (either full-length or amino acids 1–304) with aminoterminial FLAG epitope has been described before (21). The two SOX9 mutations identified in patients E.R. and M. (Ala76→Glu; Δ66–75) were introduced into these constructs using PCR-dependent site-directed mutagenesis. All expression cassettes were verified by DNA sequencing. As luciferase reporter plasmids we used: AMH promoter from positions −154 to +10 (16), gift of F. Poulat, CD-Rap promoter from 2.2 kb of the 5′ flanking region from the mouse CD-Rap gene (14), gift of L. Sandell, 4×(DE) Col11a2 luc [containing four copies of the DE enhancer from the mouse Col11a2 gene in front of the Col2a1 minimal promoter (29), gift of L. Bridgewater], and 4×48 Col2a1 p89luc [containing four copies of the mouse Col2a1 intronic enhancer in front of the Col2a1 minimal promoter (12), gift of B. de Crombrugghe]. The nGFP expression plasmid was as reported (25).

Cell culture, transfections, western blots, and immunofluorescence studies

COS and CV1 cells were maintained in DMEM supplemented with 10% fetal calf serum. Forty-eight hours after transfection (10 μg DNA per 10 cm plate) with DEAE-dextran (500 μg/ml) and subsequent chloroquine treatment, COS cells were harvested and extracts were prepared as described (26). Polyclonal rabbit antisera originally prepared against bacterially expressed SOX10, but cross-reactive with all class E SOX proteins (39), served as primary antibody (1:3000 dilution), horseradish peroxidase-coupled protein A as secondary detection reagent in western blots using the ECL detection system. For immunofluorescence studies, CV1 cells were transfected with expression plasmids for SOX9 proteins and a GFP protein with nuclear localization signal on cover slips using Superfect reagent. After fixation of cells in 3% paraformaldehyde 48 h post-transfection, GFP expression was detected by its auto-fluorescence, whereas SOX9 expression was detected using the above-mentioned antiserum and Cy3-coupled anti-rabbit IgG antibodies.

For stability studies, COS cells were transfected with expression plasmids for SOX9 proteins using Superfect reagent (Qiagen). Forty-eight hours post-transfection, cells were starved in cysteine/methionine-free DMEM and metabolically labeled with [35S] cysteine/methionine for 1 h each, and harvested after increasing periods of incubation in DMEM supplemented with 10% FCS as described (40). Following immunoprecipitation with a monoclonal antibody directed against the FLAG epitope (Sigma), SOX9 proteins were separated on SDS–10% polyacrylamide gels before detection and quantification on a phosphoimager (Molecular Dynamics).

Electrophoretic mobility shift assays

COS cell extracts expressing the various SOX9 proteins (amino acids 1–304) were incubated with 0.5 ng of 32P-labeled probe (for sequences, see Figs. 5A and 6B) for 20 min on ice in a 20 μl reaction mixture as described, using poly(dGdC) as unspecific competitor (22). In some reactions, specific competitor was added in the range of 20- to 200-fold molar excess. Samples were loaded onto native 5% polyacrylamide gels and electrophoresed in 0.5× TBE (45 mM Tris/45 mM boric acid/1 mM EDTA, pH 8.3) at 120 V for 1.5 h. Gels were dried and exposed for autoradiography.

Luciferase assays

Neuro2A cells were maintained in DMEM supplemented with 10% FCS and transfected in duplicates on 35 mm plates by the calcium phosphate precipitation method using 100 ng of pcDNA3-based effector plasmids and 100 ng of luciferase reporter plasmids per plate. The amount of plasmid was kept constant by addition of empty pcDNA3 vector. Cells were harvested for luciferase assays 48 h post-transfection as described (35).

ACKNOWLEDGEMENTS

We thank Darci Sternen for genetic counseling of the E.R. family, the parents of the patients for their interest and support, and Christine Zeschnigk for expert technical assistance. F. Poulat, T. Schomber, L. Sandell, L. Bridgewater and B. de Crombrugghe are acknowledged for providing plasmids. This work was supported by grants from the Deutsche Forschungsgemeinschaft to M.W. (We1326/7-2) and to G.S. (Sch 194/11-3 and 194/15-1).

NOTE ADDED IN REVISION

While this manuscript was under review, Bridgewater and colleagues (29) independently published the existence of paired SOX9 binding sites in the Col11a2 enhancer, in close agreement with our findings.

REFERENCES


