Am. J. Hum. Genet. 57:1028–1036, 1995

Mutations in *SOX9*, the Gene Responsible for Campomelic Dysplasia and Autosomal Sex Reversal

Cheni Kwok,[1] Polly A. Weller,[1] Silvana Guioli,[1] Jamie W. Foster,[1] Sahar Mansour,[2,5] Orsetta Zuffardi,[3] Hope H. Punnett,[4] Marina A. Dominguez-Steglich,[5,6] J. David Brook,[5,6] Ian D. Young,[5] Peter N. Goodfellow,[1] and Alan J. Schafer[1]

[1]Department of Genetics, University of Cambridge, Cambridge; [2]Department of Clinical Genetics, St. George's Hospital Medical School, London; [3]Dipartimento di Patologia Umana ed Ereditaria, Biologia Generale e Genetica Medica, Pavia; [4]Genetics Laboratory, St. Christopher's Hospital for Children, Philadelphia; [5]Centre for Medical Genetics, City Hospital, and [6]Department of Genetics, Queens Medical Centre, University of Nottingham, Nottingham

Summary

Campomelic dysplasia (CD) is a skeletal malformation syndrome frequently accompanied by 46,XY sex reversal. A mutation-screening strategy using SSCP was employed to identify mutations in *SOX9*, the chromosome 17q24 gene responsible for CD and autosomal sex reversal in man. We have screened seven CD patients with no cytologically detectable chromosomal aberrations and two CD patients with chromosome 17 rearrangements for mutations in the entire open reading frame of *SOX9*. Five different mutations have been identified in six CD patients: two missense mutations in the *SOX9* putative DNA binding domain (high mobility group, or HMG, box); three frameshift mutations and a splice-acceptor mutation. An identical frameshift mutation is found in two unrelated 46,XY patients, one exhibiting a male phenotype and the other displaying a female phenotype (XY sex reversal). All mutations found affect a single allele, which is consistent with a dominant mode of inheritance. No mutations were found in the *SOX9* open reading frame of two patients with chromosome 17q rearrangements, suggesting that the translocations affect *SOX9* expression. These findings are consistent with the hypothesis that CD results from haploinsufficiency of *SOX9*.

Introduction

Campomelic dysplasia (CD) is a rare and usually lethal congenital skeletal malformation syndrome. The reported incidence of this syndrome is 0.5–1/100,000 (Camera and Mastroiacovo 1982; Connor et al. 1985), although this condition may be underdiagnosed or the incidence may vary between different populations (Normann et al. 1993). Major diagnostic radiological features of CD include hypoplastic scapulae, shortened and angulated femora and tibiae, and underdeveloped or nonmineralized thoracic pedicles. Common facial features include micrognathia, cleft palate, flat nasal bridge, and low-set ears. In addition, 11 pairs of ribs, abnormal pelvic bones with dislocation of the hips, small chest, pretibial dimples, and talipes equinovarus are frequently observed (Mansour et al. 1995). Nonskeletal anomalies such as the absence of olfactory bulbs and tracts, dilatation of cerebral ventricles, and a variety of cardiac and renal defects are also associated with the disease. Death usually occurs in the neonatal period, following respiratory distress. Male-to-female sex reversal (XY female) frequently accompanies CD, occurring in approximately three quarters of affected XY cases (Hovmoller et al. 1977; Houston et al. 1983; Mansour et al. 1995). A gradation of genital defects has been found in these 46,XY CD individuals, ranging from normal male genitalia with descended testes, through ambiguous genitalia of varying male and female sexual differentiation, to normal female genitalia. Histological examination of the gonads from the sex-reversed patients reveals variable testicular development, ranging from gonads with variable degrees of testicular differentiation to poorly differentiated ovarian tissue with primary follicles (Hoefnagel et al. 1978; Houston et al. 1983; Cooke et al. 1985). The most common reported phenotype of sex-reversed CD patients is of normal female external genitalia with gonads positioned as ovaries, composed of ovarian-like stroma with a few primordial follicles.

Characterization of three independent de novo chromosome 17 translocations in CD patients assigned a locus *CMPD1* to 17q24.3-q25.1 (Tommerup et al. 1993). Two of these translocation patients displayed XY sex reversal, placing an autosomal sex-determining locus, *SRA1*, in the same region. *SOX9*, a gene related to the mammalian Y chromosome sex-determining gene *SRY*, was mapped to this region and found to be near the translocation breakpoint of a sex-reversed CD patient

Received June 30, 1995; accepted for publication August 14, 1995.

Address for correspondence and reprints: Dr. Alan J. Schafer, Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, United Kingdom. E-mail: ajs1003@mole.bio.cam ac.uk

0002-9297/95/5707-0007$02.00

(Foster et al. 1994). Subsequent identification of de novo mutations in sex-reversed CD patients demonstrated that *SOX9* is the gene responsible for both CD and autosomal sex reversal (Foster et al. 1994; Wagner et al. 1994). Physical mapping of chromosome 17 translocation breakpoints in sex-reversed CD patients has shown the closest to occur 50 kb 5′ of the start of initiation of the *SOX9* transcript, as defined by 5′ RACE (Wagner et al. 1994) and RNase protection assays (S. Guioli, unpublished observations). A causative relationship between the translocations and the CD and sex-reversal phenotypes has not been established.

SOX9 belongs to the family of *SRY*-related box (*SOX*) genes. The SRY protein has a DNA-binding domain (the HMG box) that is shared within a superfamily of genes that includes both the nonspecific DNA-binding HMG proteins and sequence-specific transcription factors (Laudet et al. 1993). *SOX* genes are defined as containing a DNA binding domain of ⩾60% similarity to the amino acid sequence of the SRY HMG box (Goodfellow and Lovell-Badge 1993). In addition to the HMG box, SOX9 contains a stretch of 41 amino acid residues rich in proline and glutamine, similar to activation domains found in some transcription factors (Mitchell and Tjian 1989), suggesting that SOX9 functions as a *trans*-acting regulatory factor. Human *SOX9* is widely expressed, having been detected in adult tissues including heart, brain, kidney, pancreas, prostate, and testis (Foster et al. 1994; Wagner et al. 1994). No expression of *SOX9* is detected in leukocytes, spleen, and thymus by Northern analysis (Wagner et al. 1994). *SOX9* is expressed in human fetal brain, testis, and within the perichondrium and chondrocytes of resting, proliferative, and upper hypertrophic zones of developing long bones and ribs (Wagner et al. 1994). The mouse *Sox-9* open reading frame (ORF) has 96% identity at the DNA level with human *SOX9*. Using an interspecific backcross, *Sox-9* has been mapped to distal mouse chromosome 11 in a region of synteny shared with human chromosome 17q24.3-q25.1, which contains *SOX9*. The expression pattern of mouse *Sox-9* is similar to that of human *SOX9*, and *Sox-9* has been shown to be highly expressed at sites of chondrogenesis (Wright et al. 1995).

In this study, we describe a systematic SSCP analysis of the ORF of *SOX9* in seven CD patients with no detectable chromosomal aberrations and in two patients with chromosome 17q24.3-q25.1 rearrangements known to be located 5′ of the *SOX9* ORF. Mutations were identified in six of the patients of normal karyotype, including 46,XX females, 46,XY males, and sex-reversed 46,XY females. A variety of mutation types and positions has been identified, including missense mutations in the HMG box, frameshift mutations, and a splice-acceptor mutation. Mutations were not detected in the *SOX9* ORF of the CD patients with chromosome 17 rearrangements.

Patients and Methods

Patients

All patients described in this study were evaluated as being afflicted with CD on the basis of the clinical, radiological, and pathological diagnostic criteria of Mansour et al. (1995). The subjects tested in this study are listed below with a description of ancillary phenotype, karyotype, and sexual phenotype. Appropriate informed consent was obtained for subjects used in this study.

Patient 242/87.—The patient was delivered at term with a characteristic CD phenotype but died within a few days from respiratory distress. Cytogenetic studies showed a 46,XY karyotype. The patient had external male genitalia, a bifid scrotum with palpable testes, and a small penis with hypospadias.

Patient 2/82.—This patient was delivered at term with stigmata of CD and died after 12 d because of respiratory distress. Cytogenetic studies showed a 46,XX karyotype, and the patient had normal external female genitalia.

Patient 1196/89.—The patient was delivered at term with classic features of CD and other abnormalities including epicanthal folds, obstruction of the nasolacrimal ducts, sparse hair, brachydactyly. The karyotype of the patient was 46,XY, but the external genitalia were normal female. This individual is sex reversed. Both parents appear normal, and DNA samples were obtained from each.

Patient S-294.—The patient was delivered at term and died shortly after birth. Phenotypic features were typical of CD. Splayed toes and an absence of knee epiphyses were observed. The karyotype of the patient was 46,XY. The external male genitalia exhibited hypospadias. DNA was obtained from both parents of this patient.

Patient B.—This individual was delivered at term and died after 6 h. She had typical features of CD, brachydactyly, flexion deformity of fingers, and normal female external genitalia, although her karyotype was 46,XY. This patient was sex reversed. DNA was obtained from the subject's mother.

Patient GM04329.—This patient presented with multiple congenital anomalies typical of CD and died shortly after birth because of upper-respiratory-tract obstruction and acute pneumonia. She had a karyotype of 46,XX, with normal external and internal female genitalia. Multiple small cysts were found, on histological examination of the ovaries. DNA was obtained from both parents of this patient.

Patient C.—The patient was delivered at term but died within 1 h. This patient had typical features of CD with

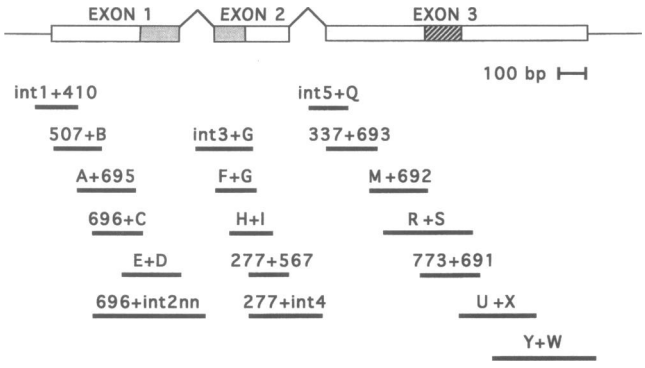

Figure 1 PCR primers and amplification products used in *SOX9* SSCP analysis. The genomic structure of *SOX9* is shown schematically, with the ORF represented by three boxes separated by lines representing the introns. The HMG box is shaded, and the polyproline/glutamine tract is hatched. The overlapping PCR products used in SSCP analysis of *SOX9* are represented by bars below. Primer pairs used to produced each fragment are indicated. (See table 1 for detailed primer information.)

posterior midline cleft palate and external female genitalia. This patient had a 46,XY karyotype and was sex reversed.

Patient E.—This fetus was terminated at 18 wk gestation, following ultrasonography showing bowing of the lower limbs. Classic features of CD were observed along with normal female external genitalia. Autopsy showed no visceral abnormalities and a normal uterus, fallopian tubes, and ovaries. Cytogenetic studies revealed a 46,XY karyotype with an apparently balanced reciprocal translocation of t(2;17)(q35; q23-24) (Young et al. 1992). This individual was sex reversed.

Patient V.—The patient has radiological and clinical features of CD, although she does not have bowing of her femora and has only mild bowing of her tibiae. This patient is alive at 21 mo, is developing kyphoscoliosis, and is suspected to have bilateral conductive hearing loss (Mansour et al. 1995). The karyotype of the patient is 46,XY inv(17)(q11.2:q24.3-25.1) with normal female external genitalia. This individual is sex reversed.

PCR Amplification

The *SOX9* ORF was amplified from genomic DNA in 18 overlapping fragments of 124–363 bp (fig. 1). Primer sequences, annealing temperatures, and PCR fragment sizes are shown in table 1. Amplifications were performed in a total volume of 10 μl containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0 (at 25°C), 0.1 % Triton X-100, 1.5 mM $MgCl_2$, 0.2mM each of dGTP, dATP, dTTP, 0.02 mM of nonradioactive dCTP, 0.05 μl [α-^{33}P] dCTP (1,000–3,000 Ci mmol^{-1}; 10 mCi ml^{-1}), 0.2 μM each primer, 50 ng genomic DNA, and 0.1 U *Taq* DNA polymerase. The PCR cycling profile was as follows: preheating to 94°C for 3 min, followed by 94°C for 1 min, annealing temperature for 30 s, 72°C for 45 s for 35 cycles, and a final extension at 72°C for 5 min. Amplifications using Vent *Taq* polymerase (New England Biolabs) were performed in a total volume of 10 μl, using the buffer provided by the manufacturer with 1 mM each of dGTP, dATP, dTTP, 0.02 mM dCTP, 0.25 μl [α-^{33}P] dCTP (1,000–3,000 Ci mmol^{-1};10 mCi ml^{-1}), 0.2 μM of each primer, 50 ng genomic DNA, and 0.1 U Vent *Taq* DNA polymerase. Samples were heated to 98°C for 5 min prior to addition of enzyme and nucleotides. The PCR cycling profile was 98°C for 1 min, annealing temperature for 45 s, 72°C for 1 min for 35 cycles, followed by a final extension at 72°C for 5 min. All PCR reactions were carried out in a Perkin Elmer Cetus DNA thermal cycler TC1.

SSCP

Ten microliters of denaturing solution (0.2% SDS, 20 mM EDTA) and 10 μl formamide dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) were added to 10 μl of radioactive PCR products. The reactions were denatured at 100°C for 5 min and were then placed on ice. Two microliters were loaded onto 8% acrylamide:bisacrylamide (37.5:1), 0.5 × Tris-borate EDTA (TBE) (45 mM Tris-borate, 1 mM EDTA), 5% glycerol gels. Electrophoresis was carried out at 25 W at 4°C for 8 h in 0.5 × TBE. Dried gels were exposed to X-OMAT AR films (Kodak).

DNA Sequence Analysis

Following detection of an altered mobility pattern in SSCP analysis, duplicate nonradioactive PCR reactions were performed. PCR products were end repaired with T4 DNA polymerase, treated with polynucleotide kinase, and ligated into the *Eco*RV site of pBluescript II (Stratagene). Multiple clones derived from each PCR reaction were sequenced by Dye Deoxy Terminator Cycle Sequencing (ABI) following the manufacturer's protocols.

Parental Haplotyping

In cases where DNA was obtained from the parents of CD patients, DNA profiling of each family was performed to confirm maternity and paternity, using 12 fluorescently labeled PCR primer pairs that amplify microsatellite markers (heterozygosity >70%) located on human chromosome 8. Polyacrylamide gel electrophoresis of PCR products was carried out on the 373A ABI DNA sequencer (Applied Biosystems). Results were analyzed using the Genescan 672 software kit and Genotyper program (Applied Biosystems). The parental haplotypes are concordant with the sib for all the families tested (data not shown).

Table 1

PCR Primers and Conditions for SSCP Analysis of *SOX9*

Primers[a]	Sequence[b]	Annealing Temperature (°C)	Size (bp)
EXON 1:			
int1 (F)[c]	GCTTCTCGCCTTTCCCGGCC	70	139
410 (93R)	CGCGGAGTCCTCGGACATGGT		
507 (10F)	CTGGACCCCTTCATGAAGATGA	62	156
B (165R)	CTTGGGGAACGTGTTCTCCTG		
A (83F)	AGGACTCCGCGGGCTCGC	62	196
695 (278R)	ACCGGCATGGGCACCAGCGT		
696 (145F)	CAGGAGAACACGTTCCCCAAG	58	161
C (305R)	TTGTTCTTGCTGGAGCCG		
E (238F)	GTGCTCAAAGGCTACGACTG	62	193
D (430R)	TCCAGAGCTTGCCCAGCGTC		
696 (145F)	CAGGAGAACACGTTCCCCAAG	65	364
int2nn (R)[c]	GGGGCAAATCAGCCCTGACCAG		
EXON 2:			
int3 (F)[c]	GGATTTCACTGACCCCTCTC	65	185
G (558R)	CTGCCCGTTCTTCACCGACTTC		
F (433F)	CTTCTGAACGAGAGCGAGA	60	126
G (558R)	CTGCCCGTTCTTCACCGACTTC		
H (483F)	GCGCGTGCAGCACAAGAAGG	70	138
I (620R)	AGCGCCTTGAAGATGGCG		
277 (534F)	GAGGAAGTCGGTGAAGAAC	65	126
567 (659R)	TCGCTCATGCCGGAGGAGGAG		
277 (534F)	GAGGAAGTCGGTGAAGAAC	70	238
int4 (R)[c]	CCACGAAGAATCTCCCAGGC		
EXON 3:			
int5 (F)[c]	GTCTGCACAGCCCTTGTTG	65	124
Q (757R)	TCAGGTCAGCCTTGCCCGGC		
337 (687F)	GCAATCCCAGGGCCCACCGAC	70	168
693 (854R)	TTGGAGATGACGTCGCTGCTC		
M (836F)	GCAGCGACGTCATCTCCAAC	65	183
692 (1018R)	GCTGCTTGGACATCCACACGTG		
773 (997F)	CACGTGTGGATGTCCAAGCAGC	62[d]	203
691 (1199R)	TCCGTCTTGATGTGCGTTCGC		
R (873F)	CAACGAGTTTGACCAGTACCT	62[d]	306
S (1178R)	TGGGACTGGCCCGGCTCGCTG		
U (1129F)	CCACAGGCGCACACGCTGAC	67	252
X (1380R)	GGTGCCCTGGCCTGCCGCGTG		
Y (1239F)	CTCGCCCCAACAGATCGCCTA	67	322
W (R)[c]	GGCCATCGTCGCCCTTCGTGG		

[a] Numbers in brackets are the 5′ base position in the *SOX9* open reading frame (Foster et al. 1994; GenBank accession no. S74152). F refers to forward primer, and R refers to reverse primer.

[b] Primer sequences are written 5′ to 3′.

[c] Primers in noncoding sequences.

[d] PCR was performed using Vent *Taq* polymerase (See Patients and Methods).

Results

SSCP and DNA Sequence Analysis

To perform a sensitive SSCP analysis, we designed PCR primer pairs that amplify small (124–364 bp), overlapping fragments such that the majority of the *SOX9* ORF is tested twice in different amplified fragments. The regions analyzed span the entire ORF of *SOX9*, the intron-exon boundaries and portions of the 5′ and 3′ untranslated sequences (53 bp upstream of the first ATG of the ORF and 30 bp downstream of the stop codon) (fig. 1). Seven CD patients with no apparent chromosome 17 anomalies and two with known chromosome 17 rearrangements were screened by using this approach. Aberrant SSCP patterns were investigated further by repeating the SSCP analysis to confirm the abnormal shift pattern, followed by sequencing of multiple clones derived from at least two independent PCR amplifications. The identity and position of primer pairs used for SSCP analysis are shown in figure 1. Details

Table 2

***SOX9* Mutations Identified in This Study in Campomelic Dysplasia Patients**

Patient	Genotype and Sexual Phenotype	Primer Pair(s) Detecting SSCP Shift	Nucleotide Position[a] and Mutation	Type of Mutation and Codon Position[b]
242/87	46,XY male	D+E, 696+int2nn	334; T to C	Missense Phe to Leu at codon 112
2/82	46,XX female	D+E, 696+int2nn	356; C to T	Missense Ala to Val at codon 119
1196/89	46,XY female	int 3+G	Intron 1 in HMG box; splice-acceptor dinucleotide AG to CG	Splice-acceptor mutation
S-294	46,XY male	R+S, 773+691	Following nucleotide 1103; single A insertion	Frameshift at codon 368
B	46,XY female	R+S, 773+691	Following nucleotide 1103; single A insertion	Frameshift at codon 368
GM04329	46,XX female	Y+W	Following nucleotide 1519; 4-bp insertion	Frameshift at codon 507
C	46,XY female	No shift	No mutation detected	No mutation detected
E	46,XY t(2;17)(q35;q23–24) female	No shift	No mutation detected	No mutation detected
V	46,XY inv (17)(q11.2;q24.3–25.1) female	No shift	No mutation detected	No mutation detected

[a] Nucleotides are numbered with respect to the A of the first ATG of the ORF (Foster et al. 1994) (GenBank accession no. S74152).
[b] Codons are numbered with respect to the first ATG of the ORF.

of primer pairs detecting SSCP shifts and the types of sequence variant found for each patient are listed in table 2 and described below.

Patient 242/87.—Analysis of PCR products from the 5′ end of the HMG box of *SOX9* (exon 1), using primers 696+int2nn and D+E, revealed abnormal SSCP patterns (SSCP shifts) in this patient (fig. 2). A thymine-to-cytosine transition at nt position 334 was found following cloning and sequencing of the PCR product amplified from the patient, using primers D+E. This mutation results in an amino acid substitution of leucine for phenylalanine in the HMG box at codon position 112.

Patient 2/82.—An SSCP shift was detected in this patient by using primers 696+int2nn and D+E (fig. 2). The sequence of the cloned PCR product from the patient revealed a cytosine-to-thymine base transition at nt position 356 of the *SOX9* ORF. This results in a change of alanine to valine in the HMG box at codon position 119.

Patient 1196/89.—An SSCP shift was detected in this patient, using primers int3+G (fig. 2), which amplify the 5′ end of exon 2, including the intron/exon boundary. Sequencing of the int3+G PCR product revealed a mutation of adenine to cytosine found at the 3′ splice-acceptor site of the first intron, which interrupts the HMG box. This results in a change of the invariant splice-acceptor consensus dinucleotide AG to CG and is predicted to abolish splicing. No abnormal SSCP patterns were observed when the parents of patient 1196/89 were tested, demonstrating that this is a de novo mutation.

Patient S-294 and Patient B.—These two patients were found to have identical abnormal SSCP patterns by using primers 773+691 and primers R+S (fig. 2). Cloning and sequencing of the 773+691 PCR product for both patients revealed in both instances a single adenine insertion following nt position 1103 in the region containing the poly-proline/glutamine stretch. This results in a frameshift that creates an altered reading frame terminating at TAA at nt position 1671. The predicted SOX9 protein contains wild-type amino acid sequence for the first 368 residues, followed by 208 amino acid residues of new sequence at the C-terminus. No SSCP shift was detected in this region of *SOX9* in the parents of S-294, showing that this is a de novo mutation. SSCP analysis of patient B maternal DNA revealed a normal SSCP pattern for this region of *SOX9;* paternal DNA of patient B was not available for testing.

To ensure that the patient S-294 and patient B samples were distinct and to rule out the possibility of cross-contamination, we tested new samples from both patients and obtained identical SSCP shifts. In addition, a *SOX9* polymorphism (see below) was identified in one allele from patient S-294 but not in patient B (data not shown). It is interesting that, although these two patients share the same *SOX9* mutation, S-294 was a normal male and B was sex-reversed (XY female).

Patient GM04329.—An abnormal SSCP pattern was detected in DNA from this patient at the 3′ end of *SOX9*, using primers Y+W (fig. 2). This shift resulted from an

insertion of 4 bp following nt 1519, which is only 2 amino acids from the end of the putative protein. The altered reading frame terminates at the same position as in patients S-294, and B and would result in a mutant SOX9 protein consisting of wild-type sequences for the first 507 residues of the normal 509 amino acid sequences, followed by 51 amino acid residues of mutant sequence at the C-terminus. No SSCP shifts were detected in the parents of GM04329, identifying this as a de novo mutation.

A Polymorphism within the SOX9 ORF

Six of the nine patients tested showed similar altered SSCP patterns with the primer pairs F+G, int3+G, and H+I, which produce PCR amplification products sharing a common region of overlap in the *SOX9* gene. The altered pattern was also seen in DNA from unaffected individuals and was found to be due to a C-to-T transition at nt position 507, a substitution that does not alter the amino acid (histidine) at this position. Examination of 252 chromosomes from unaffected (Caucasian) individuals by SSCP gave a frequency of 20% for this polymorphic allele.

Patients with No Detected Mutations

No SSCP shifts were detected in patient C. In addition, no abnormal SSCP patterns were detected in the two patients with chromosome 17 anomalies: patient V, carrying a paracentric inversion at chromosome 17q, and patient E, with a balanced reciprocal translocation between chromosomes 17q and 2q.

Discussion

Previous studies using SSCP for mutation screening have been of variable success, detecting 35%–100% of known mutations (Dianzani et al. 1993). The mutation-detection efficiency of SSCP is dependent on a number of factors. One is the length of the PCR fragment tested: DNA fragments shorter than 200 nt have been used to achieve mutation-detection efficiencies of >90% in SSCP analysis (Hayashi and Yandell 1993). Various studies have shown that the efficiency of detection may also depend on the sequence context surrounding the specific variant in the PCR product (Glavac and Dean 1993; Sheffield et al. 1993). In our screening of *SOX9*, we sought to optimize the efficiency of mutation detection, taking both of these factors into account, by using short, overlapping PCR products for SSCP analysis (fig. 1). In addition, we have used gel electrophoresis conditions that have been shown to increase the mutation-detection rate (Glavac and Dean 1993; Leren et al. 1993). In this study, we identified *SOX9* mutations in six of seven CD patients with no detectable chromosomal aberrations (table 2). Using the SSCP strategy described, we have found mutations in a total of 9 of 10 CD patients without chromosome 17 rearrangements (Foster et al. 1994; this study), demonstrating the effectiveness of this approach. In each instance where a mutation occurred in a region of *SOX9* that was spanned by two PCR amplification products, both displayed an altered SSCP mobility. The single subject (patient C) in whom a mutation was not found may have a *SOX9* ORF mutation not detected by the SSCP analysis or may have a mutation outside the *SOX9* ORF. This individual is heterozygous for the polymorphism at nt position 507 (data not shown), demonstrating that two *SOX9* alleles are present. Although the karyotype of this individual showed no obvious chromosomal aberrations, it is pos-

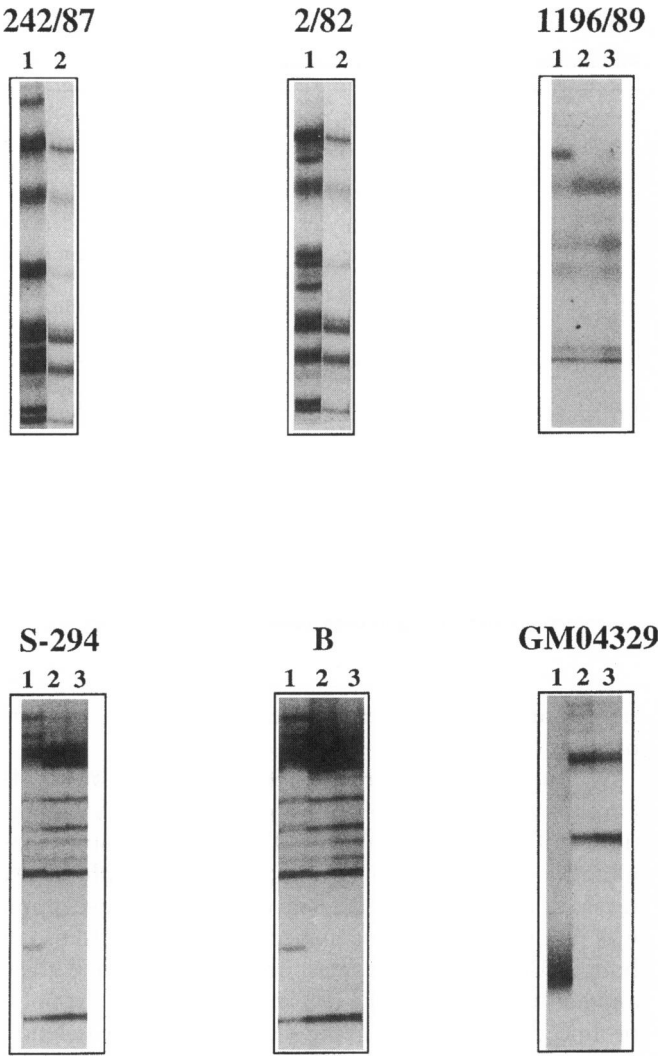

Figure 2 SSCP shifts. In each case, lane 1 is the patient indicated, and lane 2 is an unaffected individual. An additional unaffected individual is shown for 1196/89, S-294, B, and GM04329 (lane 3). PCR amplification products shown: 2/82 and 242/87, D+E; 1196/87, int3+G; S-294 and B, 773+691.

Table 3

Previously Identified *SOX9* Mutations

Patient	Genotype and Sexual Phenotype	Codon Position and Mutation[a]	Type of Mutation	Reference
M.A.	46,XY female	148; Glu to Stop	Nonsense	Wagner et al. 1994
S.H.	46,XX female	195; Gln to Stop	Nonsense	Foster et al. 1994
F.S.	46,XY female	Intron 2; 5′ splice-donor dinucleotide GT to AT	Splice mutation	Wagner et al. 1994
A.H.	46,XY female	Insertion of G following codon 261	Frameshift resulting in truncation of protein	Foster et al. 1994
G.	46,XY female	Insertion of 4 nt following codon 286	Frameshift resulting in truncation of protein	Foster et al. 1994
J.J.[b]	46,XY female	(1) Insertion of G following codon 329	Frameshift resulting in extension of protein	Wagner et al. 1994
		(2) In-frame deletion of nine amino acids in poly proline/glutamine tract	Deletion of three amino acids	
S.P.	46,XY female	440; Tyr to Stop	Nonsense	Wagner et al. 1994

[a] Codons are numbered with respect to the first ATG of the ORF.
[b] Mutations were found in both alleles of this patient (see text).

sible that a small rearrangement or deletion may be present.

We and others (Foster et al. 1994; Wagner et al. 1994) have previously described a total of seven *SOX9* mutations in CD patients (table 3). These studies found that mutations that result in CD (both with and without sex reversal) occur in a single allele of *SOX9*. An exception was reported in which sequence variants were found in both *SOX9* alleles: a frameshift mutation in one allele and a 9-bp deletion in the region that codes for the polyproline/glutamine tract in the second allele. The deletion does not alter the reading frame and has been suggested to be a rare polymorphism (Wagner et al. 1994). All other patients had one normal and one mutant *SOX9* allele. In this study, each of the mutations found was heterozygous; sequencing of cloned PCR products from shifted regions always yielded clones with normal sequence and clones containing a mutation. This is consistent with CD (and the accompanying sex reversal) having a dominant mode of inheritance.

Eleven of the *SOX9* mutations identified to date (tables 2 and 3) would be predicted to interfere with or destroy the normal function of the protein by introducing a premature stop codon, by disrupting splicing, or by insertion of nucleotides that result in frameshift mutations. The presence of inactivating mutations in *SOX9* suggests that the CD and sex-reversal phenotypes are due to haploinsufficiency of the *SOX9* gene. Although these mutations can be postulated to produce proteins with a gain of function that causes CD (and sex reversal, when present), our SSCP analysis did not detect mutations in the two sex-reversed CD chromosome 17–translocation patients tested, nor was a mutation found in the *SOX9* ORF of an additional translocation patient (Wagner et al. 1994). In these patients, presumably no mutated SOX9 protein would be present to act in a gain of function manner. At least seven CD patients with chromosome 17q rearrangements in the region of *SOX9* have been reported. (Maraia et al. 1991; Young et al. 1992; Tommerup et al. 1993; Mansour et al. 1995). The breakpoints that have been physically mapped lie at a distance from *SOX9* (Foster et al. 1994; Wagner et al. 1994). The translocation breakpoint in patient E has been mapped to 88 kb 5′ of the ORF of *SOX9* (Foster et al. 1994), and the patient V translocation breakpoint is located further 5′ of *SOX9* than the translocation breakpoint in E (M. A. Dominguez-Steglich and J. D. Brook, unpublished data). The apparent absence of mutations in the open reading frame of *SOX9* in patients with chromosome 17q translocations strongly supports haploinsufficiency as the cause of CD (and of the associated sex reversal, when present) and suggests that the chromosome 17q rearrangements may cause CD and sex reversal by interfering with the normal expression of *SOX9*. Analysis of *SOX9* expression from the rearranged chromosome is required to test this hypothesis.

We have identified a variety of *SOX9* mutations with regard to both the type of mutation and their distribution across the ORF (fig. 3). Mutations throughout the *SOX9* ORF result in campomelic dysplasia, although, to date, no mutations have been identified 5′ of the SOX9 HMG box. In this study, we provide the first detailed description of missense mutations associated with CD. Both of these amino acid changes occur in the putative DNA-binding domain (HMG box) of SOX9, demonstrating the importance of this region in normal

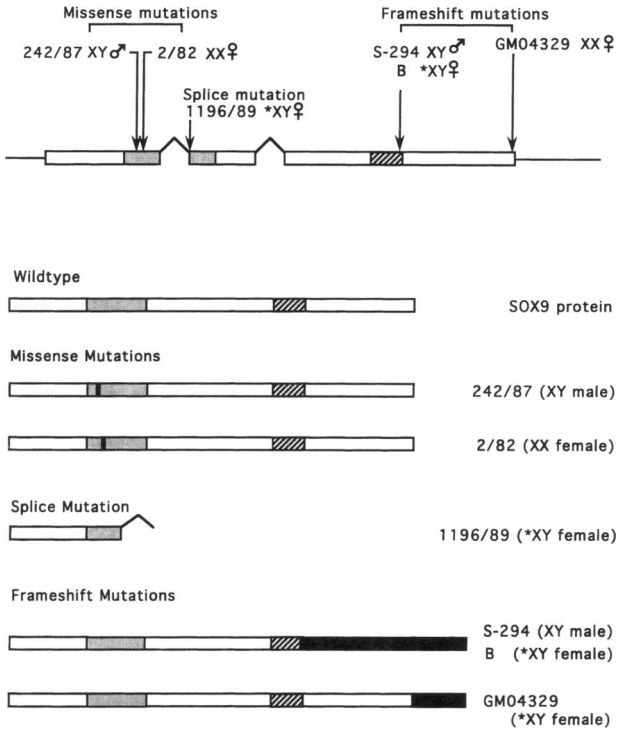

Figure 3 Distribution of mutations found in the *SOX9* gene. *Top,* Location of mutations found in this study relative to the *SOX9* ORF. Boxes represent coding regions distributed over the three *SOX9* exons. The HMG box is shaded, and the hatched box represents the poly-proline/glutamine tract. Arrows indicate the location of mutations detected in this study and are labeled with the type of mutations found, the patient's identifier, genotype, and phenotype. An asterisk (*) indicates sex-reversed XY females. *Below,* Conceptual translation of the predicted *SOX9* protein for each mutation. Patient identifier, genotype, and phenotype are on the right of each mutant protein. An asterisk (*) indicates sex-reversed XY females. Frameshift mutations in patients B, S-294, and GM04329 and mutant sequences at the C-terminus are indicated by black boxes.

SOX9 function. The HMG-box mutation in patient 242/87 would change a phenylalanine that is invariant at this position in all reported human and mouse *SOX* genes. Single amino acid changes can severely impair or abolish sequence-specific in vitro binding of the SRY HMG box (Nasrin et al. 1991; Jäger et al. 1993; Harley and Goodfellow 1994), and nearly all *SRY* missense mutations found in sex-reversed XY females occur in the HMG box (Hawkins 1994). Both the 242/87 and the 2/82 *SOX9* amino acid changes occur within a region of the HMG box likely to contain a nuclear localization signal (Poulat et al. 1995) and may interfere with targeting of the SOX9 protein to the nucleus. An HMG-box mutation in a third patient (1196/89) occurs in the splice-acceptor dinucleotide of intron 1 and is predicted to disrupt the normal splicing of the HMG box (fig. 3). The *SOX9* mutations we found in three patients and six of the previously reported *SOX9* mutations occur 3′ of the HMG box. (table 1; fig. 3). The mutation found in patients S-294 and patient B introduces a frameshift in the middle of the poly-proline/glutamine tract, while the GM04329 mutation causes a change in reading frame just 2 amino acids from the normal *SOX9* stop codon, adding 2 mutant amino acids and 47 amino acids not present in the normal SOX9 predicted protein (fig. 3). The C-terminal region of the SOX9 protein may normally interact with other proteins, and mutations in this region could alter SOX9 domains or disrupt the SOX9 protein conformation required for interaction with other proteins.

The *SOX9* mutations identified to date do not reveal an obvious correlation between mutation type or location and the presence or absence of sex reversal. All mutations found in CD XY females are predicted to severely disrupt SOX9 protein structure (tables 2 and 3), while the non-sex-reversed XY male patient 242/87 has a missense mutation located in the SOX9 HMG box. It is possible that this mutant protein retains enough function for normal sexual development but not for normal skeletal development. However, the 46,XY male patient S-294 shares the same frameshift mutation as the sex-reversed 46,XY female patient B. The difference in sexual phenotype of these two 46,XY individuals demonstrates variable penetrance of the disease and may result from differences in genetic background. Analysis of XY females without skeletal abnormalities will reveal if mutations in *SOX9* can cause sex reversal in the absence of CD. The identification of additional *SOX9* mutations from CD patients and experiments with transgenic animals will be valuable in furthering our understanding of mammalian skeletal development and sex determination.

Acknowledgments

We thank D. Donnai, C. Bennett, and C. Garrett for subject materials, John Todd for providing microsatellite markers used in parental haplotyping, and are grateful for the contribution made by patients and their parents. We appreciate comments on the manuscript from M. Stevanovic, J. R. Hawkins, R. Korn and T. Ebersole. This work was supported by a Peterhouse research studentship (to C.K.), by the Wellcome Trust (grant 035201/Z/92 to P.A.W., J.W.F., P.N.G., and A.J.S.; grant 039876/Z/93 to P.N.G.), and by a Telethon research fellowship (to S.G.).

References

Camera G, Mastroiacovo P (1982) Birth prevalence of skeletal dysplasias in the Italian Multicultural Monitoring System for Birth Defects. In: Papadatos CJ, Bartsocas CS (eds) Skeletal dysplasias. Alan R Liss, New York, pp 441–449

Connor JM, Connor RAC, Sweet EM, Gibson AAM, Patrick WJA, McNay MB, Redford DHA (1985) Lethal neonatal chondrodysplasias in the West of Scotland 1970–1983 with

a description of a thanatophoric, dysplasialike, autosomal recessive disorder, Glasgow variant. Am J Med Genet 22:243–253

Cooke CT, Mulcahy MT, Cullity GJ, Watson M, Sprague P (1985) Campomelic dysplasia with sex reversal: morphological and cytogenetic studies of a case. Pathology 17:526–529

Dianzani I, Camaschella C, Ponzone A, Cotton RGH (1993) Dilemmas and progress in mutation detection. Trends Genet 9:403–405

Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M, Weissenbach J, et al (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene. Nature 372:525–530

Glavac D, Dean M (1993) Optimization of the single strand conformation polymorphism (SSCP) technique for detection of point mutations. Hum Mut 2:404–414

Goodfellow PN, Lovell-Badge R (1993) SRY and sex determination in mammals. Ann Rev Genet 27:71–92

Harley VR, Goodfellow PN (1994) The biochemical role of SRY in sex determination. Mol Reprod Dev 39:184–193

Hawkins JR (1994) Sex determination. Hum Mol Genet 3:1463–1467

Hayashi K, Yandell DW (1993) How sensitive is PCR SSCP? Hum Mut 2:338–346

Hoefnagel D, Wurster-Hill DH, Dupree WB, Benirschke K, Fuld GL (1978) Camptomelic dwarfism associated with XY-gonadal dysgenesis and chromosomal anomalies. Clin Genet 13:489–499

Houston CS, Opitz JM, Spranger JW, Macpherson RI, Reed MH, Gilbert EF, Herrmann J, et al (1983) The campomelic syndrome: review, report of 17 cases, and follow-up on the currently 17-year-old boy first reported by Maroteaux et al. in 1971. Am J Med Genet 15:3–28

Hovmoller ML, Osuna A, Eklof O, Fredga K, Hjerpe A, Lindsten J, Ritzen M, et al (1977) Camptomelic dwarfism: a genetically determined mesenchymal disorder combined with sex reversal. Hereditas 86:51–62

Jäger RJ, Harley VR, Pfeiffer RA, Goodfellow PN, Scherer G (1993) Familial mutation in the testis determining gene *SRY* shared by both sexes. Hum Genet 90:350–355

Laudet V, Stehelin D, Clevers H (1993) Ancestry and diversity of the HMG box superfamily. Nucleic Acids Res 21:2493–2501

Leren TP, Solberg K, Rodningen OK, Ose L, Tonstad S, Berg K (1993) Evaluation of running conditions for SSCP analysis: application of SSCP for detection of point mutations in the LDL receptor gene. PCR Methods Appl 3:159–162

Mansour S, Hall CM, Pembrey ME, Young ID (1995) A clinical and genetic study of campomelic dysplasia. J Med Genet 32:415–420

Maraia R, Saal HM, Wangsa D (1991) A chromosome 17q de novo paracentric inversion in a patient with campomelic dysplasia; case report and etiologic hypothesis. Clin Genet 39:401–408

Mitchell PJ, Tjian R (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA. Science 245:371–378

Nasrin N, Buggs C, Kong XF, Carnazza J, Goebl M, Alexander-Bridges M (1991) DNA-binding properties of the product of the testis-determining gene and a related protein. Nature 354:317–320

Normann EK, Pedersen JC, Stiris G, van der Hagen CB (1993) Campomelic dysplasia: an underdiagnosed condition? Eur J Ped 152:331–333

Poulat F, Girard F, Chevron M-P, Gozé C, Rebillard X, Calas B, Lamb N, et al (1995) Nuclear localization of the testis determining gene product SRY. J Cell Biol 128:737–748

Sheffield VC, Beck JS, Kwitek AE, Sandstrom DW, Stone EM (1993) The sensitivity of single strand conformation polymorphism analysis for the detection of single base substitutions. Genomics 16:325–332

Tommerup N, Schempp W, Meinecke P, Pederson S, Bolund L, Brandt C, Goodpasture C, et al (1993) Assignment of an autosomal sex reversal locus (SRA1) and campomelic dysplasia (CMPD1) to 17q24.3-q25.1. Nat Genet 4:170–174

Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantes J, et al (1994) Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the *SRY*-related gene *SOX9*. Cell 79:1111–1120

Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, Gangadharan U, et al (1995) The *Sry*-related gene *Sox-9* is expressed during chondrogenesis in mouse embryos. Nat Genet 9:15–20

Young ID, Zuccollo JM, Maltby EL, Broderick NJ (1992) Campomelic dysplasia associated with a *de novo* 2q-17q reciprocal translocation. J Med Genet 29:251–252