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The Role of SOX9 in Autosomal Sex Reversal and Campomelic Dysplasia [and Discussion]

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The role of *SOX9* in autosomal sex reversal and campomelic dysplasia

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SUMMARY

In eutherian mammals, the Y-chromosome gene *SRY* is required for induction of testis development. Although the Y chromosome is sex determining, loci located elsewhere in the genome participate in the complex cascade of genetic interactions required to form a testis. Male to female sex reversal (46,XY females) occurs at a high frequency in individuals afflicted with the skeletal malformation syndrome campomelic dysplasia. Chromosomal translocations in individuals with both syndromes had localized an autosomal sex reversal locus (*SRA1*) and a campomelic dysplasia locus (*CMPD1*) to the long arm of human chromosome 17. The molecular cloning of a translocation breakpoint in a sex reversed campomelic dysplasia patient revealed its proximity to *SOX9*, a gene which is related to *SRY*. Analysis of *SOX9* in patients without chromosomal rearrangements demonstrated single allele mutations in sex reversed campomelic individuals, linking this gene with both bone formation and control of testis development. Identification of *SOX9* as *SRA1/CMPD1* and the role of *SOX9* mutations in sex reversal and campomelic dysplasia are discussed.

1. INTRODUCTION

The sexual phenotype of a mammalian embryo is determined by the sex chromosome content established at fertilization. In the presence of a Y chromosome, the indifferent gonads are induced to form as testes; in its absence, ovarian development occurs. The developmental decision to form testes is pivotal: the differentiation of male internal ducts and external genitalia are secondary, resulting from endocrine functions of the testes. In the absence of testes, female internal and external genitalia develop, regardless of the presence or absence of ovaries. Sex determination can thus be equated with determination of testis formation. The cloning of the gene *SRY* (*s*ex determining *r*egion *Y* gene; *Sry* in mouse) from the minimum known testis determining region of the mammalian Y chromosome (Sinclair *et al*. 1990) was soon followed by evidence for its identity as the Y chromosome testis determining factor. Expression patterns of *Sry* in mice were shown to be consistent with a role in initiation of testis development and *de novo* mutations of *SRY* were found in XY females (Goodfellow & Lovell-Badge 1993). A convincing demonstration of *SRY* as the primary signal for testis determination was the male development of karyotypically female transgenic mice carrying a mouse *Sry* transgene (Koopman *et al*. 1991). The sex reversed mice contained testis and were indistinguishable physically and behaviourally from normal XY male mice. Infertility of these mice can be attributed to the absence of additional Y loci necessary for spermatogenesis or to the inability to sustain spermatogenesis as a result of the breakdown of the germinal epithelium in the presence of two X chromosomes. The protein encoded by *SRY* contains a DNA-binding motif, initially identified by sequence similarity to the DNA-binding domains of the human upstream binding factor and *h*igh *m*obility *g*roup proteins such as the chromatin-associated protein HMG1. The *SRY* 'HMG box' binds to specific DNA sequences *in vitro* suggesting that it affects transcription of genes downstream in the testis development pathway. A large number of genes have subsequently been identified that encode proteins containing regions related to the *SRY* HMG box. Genes which encode proteins with greater than 60% amino acid similarity to the *SRY* HMG box region have been defined as *SOX* genes (Goodfellow & Lovell-Badge 1993).

The *Sry* induced male development of XX transgenic mice indicates that it is the sole gene on the Y

chromosome required for testis determination in mice, and the occurrence of human XX males with *SRY* as their only detected Y chromosome derived gene suggests that this is true in humans as well (Fukutani *et al.* 1993; Jäger *et al.* 1990; Sinclair *et al.* 1990). Gonad development undoubtedly involves many genes, with *SRY* acting as a differential in a complex developmental pathway, the components of which are encoded on chromosomes other than the Y. Genetic evidence suggests that some of these genes are regulatory. The regulated developmental pattern of *Sry* expression in mice indicates that at least one gene acts upstream of *Sry*. In humans, many XY sex reversals cannot be accounted for by mutations in *SRY* and some XX males are not the result of the presence of *SRY* (Schafer 1995). These individuals are presumed to have mutations in an X chromosome or autosomal gene required for testis formation. Some of these loci have been defined and several genes involved in testis formation have been cloned. *SF-1*, located on chromosome 9q, is a key regulator of enzymes involved in steroid production (including the sex hormones), and is also necessary for the development of gonads in mice (Luo *et al.* 1994). Chromosomal rearrangements associated with aberrant sexual phenotypes were invaluable in the identification and cloning of *SRY* and continue to serve as a guide to identifying genes involved in sex determination. The gene *WT1* was originally localized by examining deletions of chromosome 11 in children with WAGR syndrome, (characterized by Wilms tumour, aniridia, genitourinary abnormalities, gonadoblastoma and mental retardation). This gene is essential for mouse gonad development (Kreidberg *et al.* 1993), and dominant negative mutations of human *WT1* result in XY female sex reversal (Mueller 1994). Duplications of a 160 kilobasepair (k.b.) region of Xp21 in XY individuals leads to female development (Bardoni *et al.* 1994). Sex reversal occurs in these individuals because of the presence of two active copies of the *DSS* (*d*osage *s*ensitive *s*ex reversal gene) locus. Sex-reversal has also been reported in a number of subjects with terminal deletion of 9p (Bennett *et al.* 1993), and in individuals with terminal deletion of 10q (Wilkie *et al.* 1993). In each case, the cytogenetically observed distances between the breakpoints makes it unlikely that they are interrupting a common gene, suggesting that the deletion results in monosomy of a critical gene (or genes), or uncovers heterozygous mutations responsible for the phenotype.

An additional autosomal sex reversal locus is associated with campomelic dysplasia (CD), a congenital skeletal malformation syndrome. The most conspicuous feature of CD is bowing and angulation of the long bones which is found combined with other skeletal and extraskeletal defects. Affected individuals usually die of respiratory distress in the first week of life, however, the severity of the phenotype is variable and a few patients survive into adult life. Campomelic dysplasia occurs with an incidence of 0.5–1 in 100 000 births (Camera & Mastroiacovo 1982; Connor *et al.* 1985), although the syndrome may be under-diagnosed or occur at higher frequencies in some populations (Normann *et al.* 1993). Over a hundred cases of CD appear in the literature. Of those that have been karyotyped, 26 are 46,XX females, 15 are 46,XY males and 38 are 46,XY females with a variety of genital defects. The remaining 47 non-karyotyped cases show a skewed sex ratio of 31 : 16 in favour of females. In the majority of 46,XY patients, male development is compromised or absent. Sexual development can range from normal male with descending testis to female genitalia accompanied by dysgenetic gonads, but various degrees of masculinization between these extremes can be found in sex reversed patients.

Reports of two *de novo* chromosome rearrangements of the long arm of chromosome 17 implicated this chromosome in CD and in the associated sex reversal (Maraia *et al.* 1991; Young *et al.* 1992). Subsequently, using *de novo* reciprocal translocations, Tommerup *et al.* (1993) localized the campomelic dysplasia locus (*CMPD1*) and an XY sex reversal locus (*SRA1*) to human chromosome 17q24.3-q25.1 with the genes *GH* and *TK* as flanking markers. This mapping formed the basis for experiments we undertook to clone a chromosome 17 translocation breakpoint from a sex reversed CD patient in hopes of identifying the molecular basis of the *SRA1* and *CMPD1* phenotypes (Foster *et al.* 1994).

2. IDENTIFICATION OF AN *SRA1*/*CMPD1* CANDIDATE GENE

The karyotype of the sex reversed campomelic dysplasia individual (patient E.) we chose to investigate was 46,XY,t(2;17) (q35;q23-24) (Young *et al.* 1992). The chromosome 17 breakpoint appears cytogenetically across the region containing *SRA1* and *CMPD1*, and corresponds to a position between the genes *GH* and *TK*. Our initial goals were to create a map within this region using normal human chromosome 17 and to identify proximate markers flanking the breakpoint close enough to initiate a physical map across the region. The distance between *GH* and *TK* had previously been determined by linkage analysis to be separated by 25 centimorgans (sex-averaged) (Haines *et al.* 1990), a length too great to easily span with cloned DNA sequences. It was necessary to position additional markers at higher density within the region to facilitate physical mapping. A number of markers existed which were located in the region of interest, but independent linkage maps using subsets of the presently available markers had been created and could not be integrated. We used whole genome radiation-fusion hybrids (Walter *et al.* 1994) to integrate the existing markers into a single map. Radiation hybrid mapping does not require polymorphic markers, which permitted the use of markers which could not be mapped by other methods, and facilitated the inclusion of DNA sequences isolated during subsequent physical mapping. Using a polymerase chain reaction (PCR) assay, we screened DNA samples from a panel of 129 whole genome radiation-fusion hybrids to map 38 markers across the region from *GH* to *TK* on chromosome 17. We next

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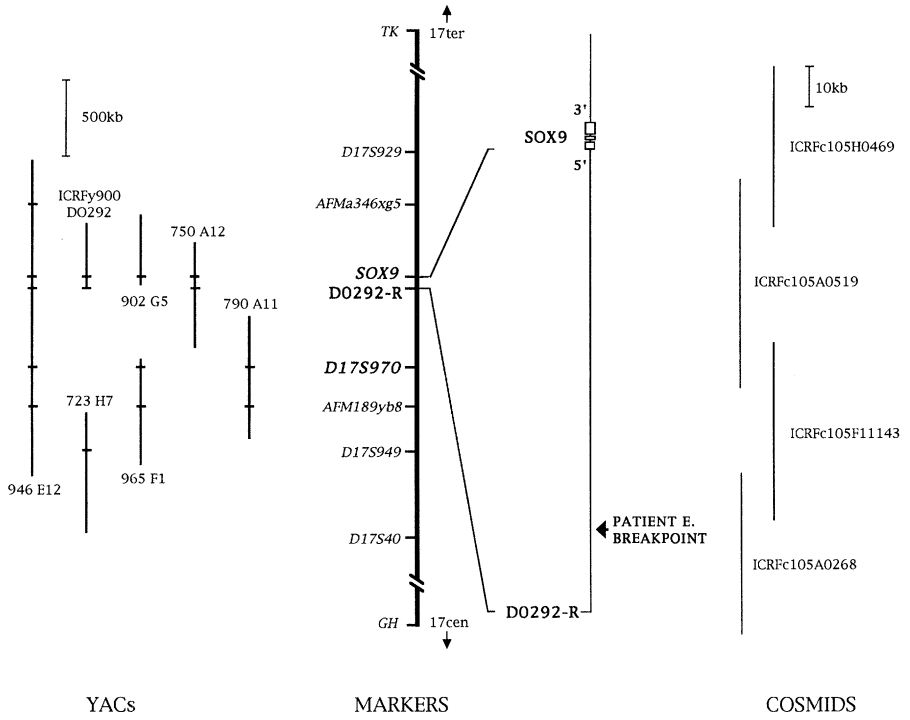

Figure 1. Relation between the chromosome 17 radiation hybrid map, YAC contig and cosmid contig for the region of the patient E. translocation breakpoint. Markers are indicated along the central solid bar representing genomic DNA. YACs are positioned to the left with the length of each bar indicating size of the YAC. Overlap with genomic markers indicates confirmed content; terminal regions of each YAC may include non-chromosome 17 sequences present due to chimerism. The cosmid walk is shown to the right of an expansion of the breakpoint region genomic DNA. The organisation and orientation of *SOX9* are indicated.

determined the position of the markers relative to the breakpoint. The markers were tested on the somatic cell hybrid B1, which retained the patient E. translocation chromosome 2pter-q35:17q23-qter in the absence of the normal human chromosome 17 and in the absence of the reciprocal translocation chromosome. Chromosome 17 markers present in B1 must be located distal to the breakpoint (i.e. between the breakpoint and the end of the long arm of chromosome 17), whereas markers not present in the hybrid must be located proximal to the breakpoint. This analysis allowed us to position the microsatellite marker *D17S970* as the closest proximal marker relative to the breakpoint and the gene *SOX9* as the flanking distal marker (see figure 1). We estimated that a distance of 1–2 Mb. separated the two markers and initiated physical mapping of this region. The markers flanking the translocation breakpoint were used to identify yeast artificial chromosome (YAC) clones containing normal chromosome 17 sequences corresponding to the breakpoint region. Based on marker content, the YACs were used to construct a contig across the region, with several YACs spanning the breakpoint (see figure 1). Chromosome 17 sequences from one YAC provided a proximal marker, D0292-R, located closer to the breakpoint than *D17S970*, and reduced the interval to 105–120 k.b. Using *SOX9* and D0292-R as starting points, a cosmid walk was performed resulting in a contig of normal chromosome 17 sequences which physically linked the two markers and spanned the breakpoint region. Subclones of the cosmids were used as probes on Southern blots to test for rearranged chromosome 17 sequences in patient E. and hybrid B1. Sequences 88 k.b. from *SOX9* identified the patient E. translocation breakpoint (figure 1).

Transcripts from the human *SOX9* gene had previously been isolated in our laboratory from a testis cDNA library in a screen for genes sharing homology to a *SOX* gene HMG box (Stevanovic *et al.* 1993). The identity of the isolated cDNAs as *SOX9* was established by comparison with the published mouse *Sox-9* HMG box region (Wright *et al.* 1993). A composite transcript of 3934 b.p. was assembled using sequence obtained from cDNA clones. Comparison of this sequence with genomic DNA revealed the presence of two introns, the first reported in a *SOX* gene. The composite cDNA contained an open reading frame (ORF) with an HMG box which, when translated using the most 5′ methionine as the translation initiation site, is predicted to code for a polypeptide of 509 amino acids. The presence of an in-frame stop codon located 125 b.p. upstream of the methionine strongly suggests that the complete ORF is contained within the cloned cDNA sequences. The *SOX9* HMG box amino acid sequence is 71% similar to the *SRY* HMG box and exhibits specific binding *in vitro* to the same DNA target sequences as *SRY* (V. R. Harley, P. N. Goodfellow, personal communication). The C-terminal end of the predicted protein contains a glutamine and proline rich region similar to that found in some transcriptional activators. These features suggest that *SOX9* is likely to function as a transcription factor. Using human monochromosomal somatic cell hybrids and chromosome 17 deletion hybrids, we initially mapped *SOX9* to

17q23-qter, a location refined to 17q24 by fluorescence *in situ* hybridization. Because of its location, *SOX9* was used as a marker in the breakpoint mapping and cloning experiments. After establishing its proximity to the translocation breakpoint, a role for *SOX9* in CD and sex reversal was investigated.

3. MUTATION ANALYSIS OF *SOX9*

To test the hypothesis that *SOX9* is involved in the aetiology of campomelic dysplasia and the associated sex reversal, we examined DNA samples from male, female and sex reversed CD patients without detectable chromosome aberrations. Initial screening was performed using a single-strand conformation polymorphism (SSCP) assay across all of the *SOX9* coding sequences and intron/exon junctions. Fragments which showed altered SSCP patterns were cloned and sequenced. Heterozygous mutations of *SOX9* were found in six patients: three XY females, two XX females and one XY male (Foster *et al.* 1994). DNA from parents of two of the patients was available for testing and in these cases the *SOX9* mutations were shown to be *de novo*. Based on the expression pattern and location of the mouse *Sox-9* gene (Wright *et al.* 1995), Wagner *et al.* (1994) independently examined *SOX9* for mutations in CD patients. Heterozygous mutations were found in four CD XY females, including one shown to not be present in parental DNA. The *de novo* appearance of mutations in sex reversed CD patients establishes that alterations in *SOX9* can cause both campomelic dysplasia and autosomal sex reversal.

4. DISCUSSION

(*a*) *Relation of* SOX9 *to the translation breakpoints*

The causal relation between the translocation breakpoints and the phenotypes in CD patients is currently unresolved. The breakpoints which have been mapped relative to *SOX9* are located 50 k.b. or greater 5′ of the apparent *SOX9* transcriptional start site and do not appear to be clustered (Foster *et al.* 1994; Wagner *et al.* 1994). A single 5′ end of *SOX9* has been defined by RACE experiments (Wagner *et al.* 1994) and RNase protection (S.G., unpublished observations). The 3′ end polyadenylation site is 3947 b.p. from the 5′ transcription start site defining a transcript which contains the complete open reading frame. However, Northern analysis identifies a *SOX9* transcript of approximately 4.3 k.b. This discrepancy suggests that additional transcript sequences may remain unidentified and it is possible that 5′ exons are interrupted by the chromosome breaks. Alternatively, the translocations may interrupt *SOX9* control elements, or exert position effects induced by sequences brought adjacent to the region containing *SOX9*. Position effects induced by chromosomal rearrangements acting at distances comparable to those observed in CD translocation patients have been reported for several genes (Bedell *et al.* 1995; Fantes *et al.* 1995). It is noteworthy that most CD translocation patients have survived early childhood and often the disease is less severe in these individuals than in patients without translocations (Mansour 1994).

(*b*) *Genetics*

Translocations of chromosome 17q24.3-q25.1 defined a region containing *SRA1* and *CMPD1* but it was not known if the sex reversal and bone malformations were caused by mutation of a single gene or mutation of linked genes in a contiguous gene syndrome (Tommerup *et al.* 1993). The detection of *SOX9* mutations in CD patients with sex reversal identifies *SOX9* as both *SRA1* and *CMPD1*, and indicates that the pleiotropic effects are the result of mutations in a single gene.

Campomelic dysplasia has previously been described as an autosomal recessive or even X-linked disease, although some reported cases were consistent with a dominant disorder. Our results indicate that CD is an autosomal dominant disease. We have not detected mutations in both *SOX9* alleles of any patient, despite having performed SSCP across the entire open reading frame including intron–exon boundaries. The predicted loss of protein function resulting from these mutations together with changes in single alleles implies that the dominance is the result of haploinsufficiency and not because of gain of function. One patient has been described with sequence changes in both *SOX9* alleles: one allele has a frameshift mutation predicted to disrupt protein function but the other allele has a 9 b.p. deletion which does not alter the reading frame of the protein (Wagner *et al.* 1994). This deletion may be a rare polymorphism which does not contribute to the phenotype.

(*c*) SOX9 *expression*

Northern blot analysis has detected *SOX9* transcripts in foetal brain, liver and kidney. Adult expression appears to be greatest in testes with *SOX9* RNA found in nearly all adult tissues tested with the exceptions being leukocytes, spleen and thymus (Foster *et al.* 1994; Wagner *et al.* 1994). The widespread distribution of *SOX9* expression may be indicative of a general role for *SOX9* or may be occurring in the mesenchymal cells common to the tissues tested. The absence of expression of *SOX9* in peripheral blood leukocytes reported (Wagner *et al.* 1994) is consistent with this idea, although we have detected *SOX9* expression in transformed human lymphoblastoid cells.

In situ hybridization experiments have shown *SOX9* expression in developing bone of seven week human embryos and in the area of the rete testis and seminiferous tubules of an eighteen week human male foetus (Wagner *et al.* 1994). Such experiments have also shown that the gene is expressed predominantly in mesenchymal condensations throughout the mouse embryo before and during cartilage deposition (Wright *et al.* 1995). These expression patterns provide additional evidence for a primary role of *SOX9* in skeletal formation.

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Table 1. SOX9 *mutations identified in patients with campomelic dysplasia*

genotype–phenotype	mutation	location	reference
XY female	frameshift	3′ of HMG box[a], 5′ of poly Q/P[b]	(Foster *et al.* 1995)
XY female	frameshift	3′ of HMG box, 5′ of poly Q/P	(Foster *et al.* 1995)
XY female	splice acceptor	1st exon/HMG box	(Foster *et al.* 1995)
XY female	splice donor	2nd exon	(Wagner *et al.* 1995)
XY female	nonsense	HMG box	(Wagner *et al.* 1995)
XY female	nonsense	3′ of poly Q/P	(Wagner *et al.* 1995)
XY female[c]	frameshift	5′ of poly Q/P	(Wagner *et al.* 1995)
	9 bp deletion	poly Q/P	
XY male	missense	HMG box	(Foster *et al.* 1995)
XX female	missense	HMG box	(Foster *et al.* 1995)
XX female	nonsense	immediately 3′ of HMG box	(Foster *et al.* 1995)

[a] DNA binding domain located at amino acids 104–181 of the 509 amino acid predicted protein.
[b] Glutamine–proline rich region located at amino acids 339–379 of the 509 amino acid predicted protein.
[c] Mutations found in both *SOX9* alleles of this patient.

(*d*) SOX9 *and testis determination*

The demonstration that mutations in *SOX9* are associated with both abnormal skeletal formation and abnormal testis formation raises a question about the role of *SOX9* in normal development. How do the pleiotropic affects in such divers tissues as cartilage and gonads arise from mutation of a single gene? The cell lineages from which these tissues arise diverge early embryonically, prior to the obvious differentiation of these tissues. Interestingly, another gene involved in gonad formation appears to have distinct roles in tissue development. The gene *WT1* is necessary for both development of gonads and kidneys. Mutations in *WT1* can result in kidney tumours or in XY sex reversal. These cases differ from the present situation in that there is a direct embryological relation between gonads and kidneys. One explanation for pleitropic effects caused by mutations in a single gene is that one phenotype occurs secondarily in response to the effects of the mutation on another tissue. Expression patterns strongly support the view that *SOX9* is directly involved in skeletal development, but such evidence does not exist for *SOX9* and testis development. It is possible that the sex reversal phenotype is solely caused by secondary effects resulting from the developmental structural aberrations which occur in the formation of the skeleton. Although this possibility cannot be excluded at this time, given the sequence similarity of *SOX9* and *SRY*, it seems improbable that the disruption of a testis formation which is induced to form by *SRY*, a *SOX9* related gene, is coincidental. The similarity of the *SOX9* and *SRY* HMG box suggests another mechanism by which disruption of testis formation by *SOX9* could be the result of secondary effects. *SOX9* may not normally be expressed in developing testis, but mutations cause an inappropriate spatial expression such that *SOX9* interferes with or competes with *SRY*. This is unlikely as there is no indication that the mutations observed in nontranslocation patients would alter expression of the transcript (see below). A final possibility for secondary effects of *SOX9* mutations in testis development is that *SOX9* is normally co-expressed in developing testicular tissue with *SRY*, but not normally important in testis formation, and the mutations of *SOX9* are dominant gain of function, i.e. exert their effect because of an altered function of the mutant protein, such as competing for an *SRY* binding site. We have screened for mutations in the *SOX9* open reading frame in two CD patients with chromosomal 17 translocations without detecting mutations. In these cases, no mutation of *SOX9* is present to act in a dominant negative manner. It seems likely that *SOX9* has a direct role in testis formation, with a loss of protein function leading to the failure to propagate the appropriate developmental signal. A wide variation of phenotype is often seen in haploinsufficiency syndromes (Fisher & Scambler 1994). No obvious correlation is seen between the sex reversal phenotype and severity of the campomelic dysplasia syndrome. Variability of the CD associated sex reversal phenotype may be the result of variable loss of *SOX9* activity or allelic differences at other loci (see below).

During development of the testis, mesenchymal cells of the mesonephros migrate into the male gonad and are required for testicular cord formation (Buehr *et al.* 1993). Given the importance of *SOX9* in bone formation, and that cartilage and bone are derived from mesenchymal cells, these cells may express *SOX9* during or after migration into the gonad. Interaction between these cells could be the link between the gonadal and skeletal phenotypes associated with *SOX9* mutations.

Another locus involved in sex determination, *DSS*, exhibits dosage sensitivity. The presence of two *DSS* copies in 46,XY individuals causes male to female sex reversal involving varying degrees of masculinization. XY individuals deleted for *DSS* develop as males, but it is not known if nullisomy for *DSS* is compatible with female development in 46,XX individuals. Because of the importance of *SOX9* in bone formation, it is likely that nullisomy for *SOX9* is lethal. Individuals monosomic for *SOX9* can develop ovaries, and trisomy for 17q including the region containing *SOX9* has not been associated with sex reversal. Gonadal abnormalities in XX female CD patients have not been reported.

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(*e*) SOX9 *mutations*

Table 1 lists the published *SOX9* mutations detected in ten campomelic dysplasia patients. The mutations are varied in type and location. Eight of the mutations described would be expected to interfere with or destroy the normal function of the protein because of the introduction of a premature stop codon, by disrupting splicing, or by inserting or deleting short sequences which result in a frameshift mutation. Two missense mutations are both located in the conserved HMG box, suggesting that this DNA binding domain contains functionally critical sequences.

SOX9 mutations have been described in six XY female CD patients, four in subjects with ovaries containing follicles (Foster *et al.* 1994; Wagner *et al.* 1994). The limited number of *SOX9* mutations presently identified do not provide insight into a relation between mutation type and the degree of sexual abnormalities. It is worth noting, however, that the single non-sex reversed XY patient has a missense mutation located in the *SOX9* HMG box. As an amino acid substitution, it may not result in as severe a disability of protein function as the truncated proteins predicted in the XY females, although it does cause CD. It is possible that there are differing sensitivities to reductions in the protein function such that levels which lead to skeletal malformation may not necessarily lead to failure to form normal testis. A second possibility is that different regions of the *SOX9* protein are involved in the bone development function and the testis development function of *SOX9*. The non-sex reversing XY male mutation located in the HMG box does not lead to truncation or disruption of other regions of the protein, whereas the sex reversing mutations each result in the predicted loss of the carboxyl terminal region of the *SOX9* protein.

It is feasible that *SOX9* mutations could be responsible for XY sex reversal without inducing the skeletal abnormalities of CD. If bone development is more sensitive to reduced *SOX9* function than is testis formation, mutations in *SOX9* leading to sex reversal should always be accompanied by CD. If different domains of the protein are important in different developmental functions, XY females resulting from *SOX9* mutations and unaccompanied by CD should exist. Analyses of XY females without bone malformations are required to address these questions.

(*f*) *Evolution of* SOX9

The dosage sensitivity of *SOX9* in sex determination and its sequence similarity to *SRY* suggest a possible evolutionary relation between the two genes. It is plausible that before establishment of heteromorphic sex chromosomes, a primordial sex determination system operated via dosage control of *SOX9* or another *SOX* gene (Foster & Graves 1994). Two doses of this gene led to testis formation and male development, whereas a single dose, achieved by chromosomal inactivation or local imprinting, resulted in development of ovaries and the female phenotype. Establishment of a dominant induction system could then occur by duplication of this gene and mutation to constitutive or over-expression and thus, when present, increase dosage above a threshold required to induce testis development.

Alternatively, both *SOX9* and *SRY* (and other *SOX* genes) may have arisen from a common progenitor which possessed the basic properties of a *trans*-acting regulatory factor. Duplication and mutation of this gene would provide a source of new alleles for the control of developmental processes.

(*g*) *Other* SOX *genes and sex determination*

The similarity between the *SOX9* gene and *SRY* might suggest *a priori* a role in testis determination. But in the past few years, many *SOX* genes have been isolated, including family members with greater HMG box similarity to *SRY* than *SOX9*. Examination of sequences outside of the HMG box of the *SOX* genes revealed high divergence and no significant similarity to *SRY*. Expression patterns of some of these *SOX* genes have been investigated and are suggestive of divers roles in differentiation (Denny *et al.* 1992; Farr *et al.* 1993; Stevanovic *et al.* 1993; van de Wetering *et al.* 1993), but none until *SOX9*, had been implicated in sex determination. The involvement of *SOX9* in sex determination was unexpected. The association of *SOX9* with the development of two different tissues suggests that other *SOX* genes, including those which have known expression patterns, should be carefully assessed for a role in sex determination. Further characterization of *SOX9* expression of targeted mutations in transgenic animals will contribute to our understanding of the role of *SOX9* in skeletal development and testis determination.

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Discussion

P. Burgoyne (*NIMR, The Ridgeway, Mill Hill, London, U.K.*). I remain to be convinced that *SOX9* is part of the sex determination pathway. I still think it more likely that the mutant *SOX9* alleles associated with sex reversal are acting as dominant negatives which compete with *Sry*, although the campomelic dysplasia may well be due to haploinsufficiency as Dr Schafer suggests. The translocation patients Dr Schafer mentioned, with breakpoints 5′ of *SOX9* may have upregulated *SOX9* expression or caused inappropriate expression in the supporting cell lineage. I will probably remain a sceptic until there are mutations associated with sex reversal which lie within the HMG box and which clearly reduce or abolish binding.

A. J. Schafer. One mutation found in a sex reversed (XY female) campomelic dysplasia patient is predicted to interfere with correct splicing of the HMG box, resulting in a protein which is unlikely to retain any DNA binding activity (Foster *et al.* 1994). This remains to be tested though, and Dr Burgoyne's proposition remains a possibility. We hope that examination of *SOX9* in additional campomelic dysplasia patients (both with and without sex reversal), and studies of *SOX9* expression from the translocation chromosomes will establish the mechanism by which *SOX9* mutations result in campomelic dysplasia and XY sex reveral.

J. A. M. Graves (*University of La Trobe, Melbourne, Australia*) Are there deletions of 17q? What is the phenotype of heterozygotes?

A. J. Schafer. The suggestion that campomelic dysplasia (and accompanying sex reversal, when present) is caused by hap insufficiency of *SOX9* predicts that chromosomal deletions resulting in monosomy of 17q should cause campomelic dysplasia. These deletions are very rare, presumably due to an associated lethality, and have almost always been reported associated with a ring chromosome. In a single reported 17q

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deletion not associated with a ring chromosome, the XX female patient exhibited many of the manifestations of campomelic dysplasia: dysmorphic facies, respiratory distress, and dysplastic changes throughout the pelvic and lower limbs including angulation of the femora (Bridge *et al.* 1985). Cytogenetically, the deletion removes the region containing *SOX9* and this patient may be an example of campomelic dysplasia resulting from haploinsufficiency of *SOX9* as a result of monosomy of distal 17q.

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