# Mutation analysis of the 2 kb 5′ to SRY in XY females and XY intersex subjects

C Kwok, C Tyler-Smith, B B Mendonca, I Hughes, G D Berkovitz, P N Goodfellow, J R Hawkins

**Abstract**

**Mutations in the Y linked testis determining gene SRY cause 46,XY sex reversal. However, only about 15% of cases of 46,XY sex reversal are accounted for by mutations in SRY. In this study we have investigated the possibility that mutations affecting the expression of SRY might cause some of the cases of sex reversal in which the coding sequence of SRY is normal. We have screened 2 kb of DNA immediately 5′ to the SRY coding sequence in 49 subjects with varying degrees of 46,XY sex reversal. Two variant bases were identified, one of which was determined to be a polymorphism and the other is unique, but familial.**

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**Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK**
C Kwok
P N Goodfellow
J R Hawkins

**CRC Chromosome Molecular Biology Group, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK**
C Tyler-Smith

**Department of Endocrinology, University Hospital, Caixa Postal 8091, Sao Paulo, Brazil**
B B Mendonca

**Department of Paediatrics, Addenbrooke's Hospital, Cambridge CB2 2QQ, UK**
I Hughes

**Division of Pediatric Endocrinology, Department of Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, USA**
G D Berkovitz

Correspondence to:
Dr Hawkins.

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SRY is the Y located male sex determining gene. In the presence of SRY, the undifferentiated embryonic gonads develop as testes, whereas in the absence of SRY the gonads develop as ovaries.[1 2] Mutations in SRY lead to failure of testis development.[3] In such cases, the gonads develop as ovaries and the affected subjects develop as females. The ovaries in these patients are dysgenetic, appearing as fibrous streaks, devoid of follicles and germ cells. Mutations in the WT1 and SOX9 genes can cause varying degrees of sex reversal, but do so in association with other abnormalities.[4–6]

The stage at which testis development is disrupted is thought to correlate with the resultant phenotype. Failure of testis determination disrupts testis development at its earliest stage, causing complete gonadal dysgenesis, which results in a full sex reversed phenotype. Abnormalities at later stages of testis development cause partial gonadal dysgenesis, the extent of testis differentiation corresponding with the extent of virilisation.

Approximately 15% of subjects diagnosed as having XY complete gonadal dysgenesis harbour mutations in SRY.[7] The majority of these patients are therefore mutant for unknown genes in the testis determining pathway or have mutations affecting the regulation of SRY.

To date 24 mutations in SRY have been described. All but two cause complete gonadal dysgenesis.[8] One patient is a true hermaphrodite. The phenotype in this case was caused by a postzygotic somatic mutation, so that the patient is mosaic.[9] The other patient has one streak gonad and one "normal looking" gonad.[10] Surprisingly, seven of the 24 SRY mutations are inherited. In some cases the fathers are likely to be germline mosaics. In three cases, however, the fathers were shown not to be mosaic and the mutations are familial.[10–15] How normal males can carry and transmit mutations in SRY is unclear.

In this study we investigated if SRY regulatory mutations might account for XY females who do not harbour mutations in the coding region of SRY. Two kilobases of 5′ flanking DNA sequences were screened for mutations in 49 subjects with a spectrum of XY sex reversal phenotypes using the single strand conformation technique (SSCP). This region was chosen because it includes the postulated transcription initiation sites and is the region in which the major regulatory elements are most likely to be concentrated.[16–18] Part of this region is disrupted by a deletion of at least 33 kb which begins not less than 1·7 kb 5′ to SRY in one XY female patient.[19] In addition, a 310 bp DNA sequence upstream from the first ATG is capable of supporting CAT reporter gene expression in heterologous cell lines.[17] We chose not to limit this study to patients with complete gonadal dysgenesis, as it is conceivable that SRY regulatory mutations might reduce the levels of SRY expression rather than abolishing it altogether, possibly permitting some degree of testis differentiation. We identified two variant bases, one of which was determined to be a polymorphism, and the other was not found on any control Y chromosome.

## Materials and methods

### PATIENTS

A total of 49 patient samples were included in this study. In all cases, the coding region of SRY had previously been shown to be normal. The gonadal phenotypes of these patients range from complete and partial gonadal dysgenesis through to well formed testes. Six patients have complete gonadal dysgenesis and a further eight patients with the same phenotype but lacking in gonadal histological data are classified as having gonadal dysgenesis. Four patients have gonadal dysgenesis with additional abnormalities: two patients have mental retardation, one has Turner's syndrome, and one has hydrocephalus and cardiac defects. Twenty subjects have partial gonadal dysgenesis, and a further seven patients also have testicular regression. Four patients are incompletely masculinised.

### METHODS

Owing to the limitation of the amount of DNA available, the 2 kb DNA fragment was amplified

in two 1 kb DNA fragments using primers P3-Bam + P2 and P4 + P1 as shown in fig 1. The PCR products were diluted accordingly to allow for reamplification. Twenty six pairs of primers were designed to reamplify short, overlapping DNA fragments from the two 1 kb DNA templates for SSCP analysis. Ten μl PCR reactions contained 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 9·0 (at 25°C), 1·0% Triton X-100, 1·5 mmol/l $MgCl_2$, 0·2 mmol/l of dGTP, dATP, dTTP, 0·02 mmol/l of non-radioactive dCTP, 0·05 μl [$\alpha$-$^{33}$P] dCTP (1000–3000 Ci mmol$^{-1}$, 10 mCi ml$^{-1}$), 0·2 mmol/l each primer, 50 ng genomic DNA, and 0·1 U *Taq* DNA polymerase (Promega). The PCR cycling profile was as follows: preheating to 94°C for three minutes followed by 94°C, one minute; 40°C, 30 seconds; 72°C, 30 seconds for 35 cycles and a final extension at 72°C for five minutes. All PCR reactions were performed in 96 well microtitre dish format, using a Hybaid DNA thermal cycler.

Ten μl formamide dye (95% formamide, 20 mmol/l EDTA, 0·05% bromophenol blue, 0·05% xylene cyanol) were added to 10 μl of PCR product. The reactions were denatured at 100°C for five minutes, then placed on ice. Two μl were loaded onto 8% acrylamide: bisacrylamide (37·5:1), 5% glycerol gels. Electrophoresis was carried out at 25 W at 4°C for eight hours in 0·5 × TBE. Dried gels were exposed to X-OMAT AR films (Kodak) overnight at room temperature.

Following detection of an altered mobility pattern in SSCP analysis, duplicate non-radioactive PCR reactions were performed. PCR products were end repaired with T4 DNA polymerase, phosphorylated with T4 polynucleotide kinase, and ligated to pBluescript (Stratagene) linearised with *Eco*RV. Multiple clones derived from each PCR reaction were sequenced by dideoxy terminator chain termination sequencing reaction (Sequenase 2.0) following the manufacturer's protocols.

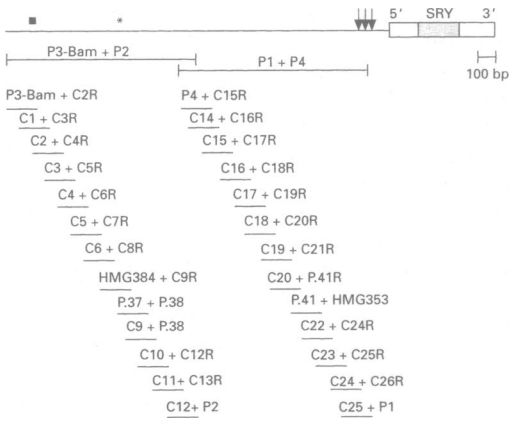


*Figure 1 PCR primers and amplification products used in the SSCP analysis of 2 kb DNA sequences 5′ of SRY. The genomic structure of SRY and the 5′ flanking region is shown schematically with the open reading frame represented by a box, with the HMG box shaded. The line represents the 5′ flanking sequence of SRY. The three arrows above the line indicate putative transcriptional start sites. The black square shows the position of the unique variant and the DNA polymorphism is shown by the asterisk. The two primary PCR products used as DNA templates are shown below by two overlapping lines, with the primer pairs indicated. The secondary overlapping PCR products are represented below. Primer pairs used to produce each fragment are indicated. Primer sequences are available on request.*

## Results

We have attempted to maximise the efficiency of SSCP in several ways. One of the major factors influencing the sensitivity is the size of the fragment analysed,[20] so we have designed PCR primer pairs which amplify small (<200 bp) fragments. The context of the mutation also plays a role in SSCP detectability. The overlapping of the PCR fragments such that each base pair of sequence is tested twice in different contexts should further enhance the mutation detectability. Finally, the gel running conditions (8% acrylamide, 2·6%C, 5% glycerol gels running at 4°C) in combination with the small overlapping fragments should allow the detection of well over 90% of point mutations.[20 21] The region analysed is from −2185 to −140 bp 5′ of the first ATG of the SRY open reading frame (fig 1). Previously, the region −140 to +646, containing the entire ORF of SRY, had been analysed by SSCP and DNA sequencing, and no mutations were identified (C Kwok, unpublished observation). Forty nine XY sex reversed or intersex patients were screened. Abnormally migrating SSCP bands were investigated by repeating the SSCP analysis to confirm the band shift pattern and by sequencing multiple clones derived from duplicate PCR reactions.

Five XY female patients (B16, cam174, F8, PT229, PT232) have identical SSCP shifts using primers HMG384 + C9R as well as primers P.37 + P.38. Upon cloning and se-

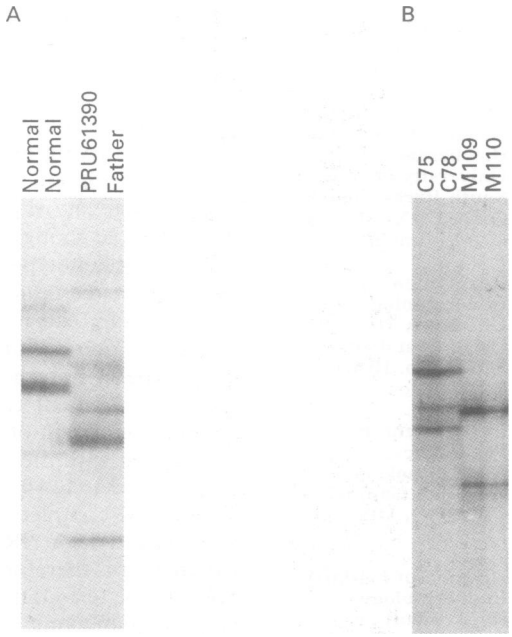


*Figure 2 (A) SSCP analysis of patient PRU61390, her father, and unaffected controls. The PCR amplification was performed using primers C1 + C3R. (B) SSCP analysis of unaffected subjects for the −1533 G to A variant. C75 and C78 (corresponding to samples 75 and 78 of Mathias et al[25]) are Kenyan negroids, having the −1533G genotype. M109 and M110 (corresponding to samples 109 and 110 of Jobling et al[26]) are Indian, and have the −1533 A genotype. The PCR amplification was performed using primers HMG384 + C9R.*

quencing the PCR products from these patients using primers HMG384+C9R, a G to A transition was identified at position −1533 (numbering refers to the sequences by Behlke *et al*[22]). This also corresponds to position 10831 of the cAMF cosmid sequence.[23] One hundred and forty three unaffected males from different ethnic origins were also tested (92 whites, 34 mongoloids, 11 negroids, four oceanics, one Amerindian, and one unknown ethnic origin). Eleven samples were found to have identified SSCP shifts with these patients (fig 2B).

A unique SSCP shift was identified in patient PRU61390 using primers C1 and C3R (fig 2A). Upon cloning and sequencing the PCR product, an A to G transition was observed at position −2027. The father was also found to carry the variant. The variant was not found among the 143 control Y chromosomes.

## Discussion

It is generally assumed that the phenotype of a patient with failed or incomplete testis development indicates the stage at which gonadal differentiation was disturbed. SRY is considered to be at, or close to, the top of the genetic hierarchy of gonadal differentiation and, as such, mutations in SRY result in complete gonadal dysgenesis. 46,XY patients with partial gonadal dysgenesis and only mild virilisation are considered to be mutant for genes below, but close to, SRY in the hierarchy, whereas patients with greater degrees of virilisation are considered to be mutant for genes further down the hierarchy.

It is unclear, however, what phenotype to expect for SRY regulatory mutations. If a threshold level of SRY protein is required for testis differentiation, the expected phenotype for such mutants would be normal male or fully sex reversed XY female, depending on whether the level of protein exceeded the threshold or not. Alternatively, reduced levels of normal SRY protein might permit some degree of testicular development, resulting in patients in whom the extent of virilisation reflects the level of SRY protein produced. It is thus conceivable that a phenotype consistent with a mutation in a gene well "downstream" of SRY could in fact be the result of an SRY regulatory mutation. In this study to investigate the possibility of SRY regulatory mutations, we made no previous judgement as to the likely phenotype by including DNA samples from patients with both complete and partial gonadal dysgenesis as well as other gonadal abnormalities.

In the only other study of a similar kind, no variants were found in the 380 bp immediately 5′ to the SRY open reading frame in a cohort of 46 patients.[10] In this study of 49 patients, we have analysed a much larger region (2 kb) 5′ to SRY and have used an SSCP approach designed for maximum sensitivity. This 2 kb 5′ flanking sequence of SRY encompasses all the putative transcription initiation sites published and also includes the minimal DNA fragment that could activate transcription of a reporter gene in a heterologous cell culture system.

The identification of non-coding mutations would help to define the regulatory region of SRY. Until now, cell transfection studies and cross species homology searches have failed to identify possible promoter elements. The identification of promoter elements would potentially enable the identification/cloning of genes upstream of SRY in the testis determining pathway.

In this study, five patients were found to share a single base change. This change was also found on 11 control sample Y chromosomes, all of which are "group 3" Y chromosomes.[24] The variant has been shown to be common in the Indian population, but rare elsewhere. Such single base dimorphisms are rare on the Y chromosome and are very useful in the study of Y chromosome evolution and population migration.[24–26] Details of the population genetics of this variant will be described elsewhere by C Tyler-Smith and M Jobling.

One patient (PRU 61390), a fully sex reversed XY female, has a variant not found in the 48 other patients or the 143 Y chromosomes of varied ethnic origins. This patient has a normal SRY coding sequence. The variant may be a rare or "private" polymorphism or may cause sex reversal. It is known that SRY mutations can exhibit variable penetrance. The presence of a mutation in a patient's father does not necessarily mean that the mutation is not the cause of sex reversal. It is also possible, albeit unlikely, that the father is mosaic for the variant, with a low level of mosaicism in his gonads. The status of the variant may only be resolved by promoter-reporter analysis in cultured cells or by DNA analysis of additional relatives of the patient along the patrilineal line.

The paucity of mutations found in this region of DNA in the genomes of these patients indicates that SRY regulatory mutations are not a major cause of sex reversal or that the regulatory elements of SRY lie elsewhere.

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