Use of probes for ZFY, SRY, and the Y pseudoautosomal boundary in XX males, XX true hermaphrodites, and an XY female

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Abstract

Three XX males, two XX true hermaphrodites, and an XY female were studied for possible deletions using probes for the recently characterised SRY gene and the pseudoautosomal boundary. The XX males and true hermaphrodites were negative for all three probes, while the XY female was positive. One XX male and one XX true hermaphrodite were sibs. A previous sib pair of an XX male and an XX true hermaphrodite have been shown to be positive for Y chromosomal material near the pseudoautosomal boundary. Thus, both phenotypes can be produced from different mutations, some involving the SRY gene and others not.

The search for a gene that induces the first event in testis differentiation has been directed to the sequences of the short arm of the Y chromosome present in XX males.

The smallest amount of that region was recognised in three XX males and one XX true hermaphrodite who were negative for more proximal Y sequences and for the ZFY gene.¹ This finding led to a chromosome walk spanning the 35 kb segment proximal to the pseudoautosomal boundary sequence in the Y chromosome, and a recognition of

the SRY gene with the characteristic of a transcription factor.² From 50 consecutive sequences recognised in this search, only one, PY53.3, was found to be present in some XX males and conserved in all eutherian mammals tested. Its homologue in the mouse (p4.2-2) was found in the mutant SXr¹ that contains the minimum portions of the mouse Y chromosome known to contain the testis determining factor. Transcripts of the SRY mouse homologue were found in the embryonic gonad during the state of Sertoli cell differentiation.³ This evidence makes SRY the best candidate to date for the testis determining factor (TDF) on the Y chromosome in man.

The ZFY gene, previously considered as a possible TDF, has now been shown to map 60 kb proximal to the Y pseudoautosomal boundary and is excluded as a candidate for TDF.^{4,5} Nevertheless, ZFY has been recognised in about 70% of the XX males tested⁶ and, in conjunction with probes for the pseudoautosomal boundary region, it can be used to localise segments of the Y chromosome missing or transferred in persons with sex chromosome anomalies.

In view of the discovery of the SRY gene, we used probes spanning the region of the Y chromosome from the pseudoautosomal boundary to the ZFY gene to examine the DNA from six subjects: three XX males, two XX true hermaphrodites, and one XY female. Two of the patients, one XX male and one XX true hermaphrodite, reared as a male, are brothers and were studied previously with the ZFY probe.⁷

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Materials and methods

PATIENTS

The sporadic cases have not been published previously. The XY female is of Caucasian origin and the other three subjects are Brazilian.

FAMILIAL CASES

Case 1 was 14 years old. The karyotype was 46,XX in blood lymphocytes and skin fibroblasts (G and C

banding) and he has been reared as a male. There was a uterus, bilateral fallopian tubes, a vagina, a left ovotestis, right perineal hypospadias, and a right testis with epididymus and deferens. The ovarian portion contained stroma, follicles, and ova, and the testicular tissue showed seminiferous tubules, hyalinisation, and fibrosis.

Case 2 was 17 years old. Karyotype analysis was the same as in the brother, case 1. There was perineal hypospadias, small bilateral testes normally placed, and absence of Müllerian structures. Testicular biopsy showed seminiferous tubules, hyalinisation, and fibrosis.

SPORADIC CASES

Case 3 was 2 years old. The karyotype was 46,XX in blood lymphocytes (G and C banding) and he has been reared as a male. There was perineal hypospadias, bilateral cryptorchidism, and absence of Müllerian structures. Testicular biopsy showed seminiferous tubules and Sertoli cells.

Case 4 was 4 years old. The karyotype was 46,XX in blood lymphocytes (G and C banding) and he has been reared as a male. There was normal male external genitalia with right cryptorchidism and absence of Müllerian tubules.

Case 5 was 13 years old at the time of diagnosis. The karyotype was 46,XX and she was negative for Y centromeric specific probe Y-97 and probe Y190. She has been reared as a female. There was hypertrophy of the phallus, a right ovotestis, a left ovary, bilateral fallopian tubes, a uterus, and a vagina. Ovarian tissue with stroma follicles and ova was present as well as testis tissue with seminiferous tubules. The left ovary was considered normal and was left in its place. The ovotestis was removed. The patient underwent normal puberty and four pregnancies. The first pregnancy ended at 6 months with a stillbirth of undefined sex. Then she had two normal sons with normal 46,XY karyotypes. The last pregnancy was continuing normally at the last medical visit at the age of 27.

Case 6 was 4 years old. The karyotype was normal, 46,XY (G and C banding) and she has been reared as a female. There was normal external genitalia, bilateral fallopian tubes, a uterus, a vagina, and bilateral streak gonads.

SOUTHERN BLOTTING

DNA was extracted using the method of Kunkel *et al.*⁸ Restriction enzymes were used as recommended by the manufacturer (Anglian). One percent agarose gels were made up in TBE buffer (electrophoresis was carried out in the same buffer) and DNA was transferred from the gel to nylon membranes using the method of Southern.⁹ Either Hybond N or

Hybond N⁺ membranes from Amersham were used. In the former case blotting was overnight using 20 × SSC, for N⁺ the process took five hours using 0.4 mol/l NaOH. Filters were prehybridised with 0.5 mol/l sodium phosphate/7% SDS with 200 µg/ml denatured salmon sperm DNA. Prehybridisation and hybridisation were performed at 65°C in a Bachofner hybridisation oven. The hybridisation solution was the same as that used in the prehybridisation, plus a radioactive probe. Prehybridisation times were one hour for Hybond N and three hours for N⁺. Hybridisation was overnight. Autoradiographs were developed overnight using Amersham Hyperfilm MP.

LABELLING OF PROBES

Probes SRY and pMF-1, which detects the ZFY gene, were kindly provided by Dr Peter Goodfellow. The plasmids were grown in *E coli* strain RR1 and purified using Qiagen columns as recommended by the manufacturer (Hybaid). Insert DNA was cut with the appropriate restriction enzymes, separated from vector DNA in a 1% agarose gel, and electrophoresed; 100 ng of insert was used in a random hexanucleotide labelling reaction (Amersham) with ³²P-dCTP.

PCR

Primers specific for the X and Y chromosomal sequences and a primer for the pseudoautosomal region were provided by Dr Peter Goodfellow and their sequences are given in Ellis *et al.*¹⁰ Amplification was performed using a Techne PHC-2 block with 30 cycles at 94°C for one minute, 54°C for one minute, and 72°C for one minute; 500 ng DNA was used per reaction, with one unit of Amersham *Taq* polymerase in manufacturer's buffer. All three primers were used in each reaction. Products were separated in a 2% agarose gel in TBE buffer and stained with ethidium bromide.

Results

The three XX males and two true hermaphrodites produced a female pattern of bands using the ZFY probe. The XY female showed the same pattern as a normal male. Fig 1 shows the results obtained using the ZFY probe. Controls were a normal male and female and a male with a 46,Y,t(X;Y)(Xp22.3;Yq11) karyotype.

Fig 2 illustrates the amplification products using primers for the pseudoautosomal boundary. Those samples which gave a female pattern with ZFY produced no bands with the pseudoautosomal primers, while the XY female was positive with PCR. Thus, the XX males and true hermaphrodites

Figure 1 Southern blot of *EcoRI* digests probed with PMF.1 (detects ZFY gene). Track (1) normal male, (2) XX male, case 4, (3) XY female, case 6, (4) XX male, case 3, (5) 46,Y,t(X;Y) male, (6) XX true hermaphrodite, case 1, (7) XX male, case 2, sib of case 1, (8) XX true hermaphrodite, case 5. The additional band in track 3 is a partial digest and was not apparent in this sample on other blots.

gave the same bands as normal females when analysed with ZFY and pseudoautosomal probes. To determine whether there had been a small amount of Y chromosomal DNA transferred to the XX males, or deleted from the XY female, the SRY probe was used on *HindIII* digests of DNA. The results are shown in fig 3. Once again, the XX males and true hermaphrodites were negative for the SRY probe and the XY female gave the same band pattern as the male controls.

Discussion

The present study showed absence of the Y specific boundary sequence and the SRY and ZFY genes in two sporadic XX males, one sporadic true hermaphrodite, and two sibs, one XX male and one XX true hermaphrodite. The same Y sequences were present in one XY female.

The hypothesis of an X;Y translocation in the paternal meiosis, proposed by Therkelsen¹¹ in 1964 and Ferguson Smith¹² in 1966, as the origin for some XX males has been largely confirmed,¹³⁻¹⁸ with the high rate of recombination in the pseudoautosomal region predisposing to unequal crossover and translocations.¹⁹ Unlike these cases, the XX male patients in the present study belong to the Y negative XX

male group that comprises a third of the cases studied up to now.¹⁷ This category usually has variable degrees of hypospadias and gynaecomastia.^{6,20}

True hermaphroditism in humans shows genotypic heterogeneity. In 60% of cases the karyotype was 46,XX and in 12% 46,XY; 13% were chimeras 46,XX/46,XY and the remainder were mosaics with normal or abnormal Y chromosomes.^{21,22} At first sight, this picture suggests that either the XX cases have some Y chromosome material, or that in most of the cases the abnormality in the differentiation of the bipotential gonad occurs at a stage after expression of the putative TDF gene. It is interesting to note that in the structural abnormalities of the Y chromosome with or without a 45,X lineage, the patients usually develop dysgenetic testes²³ instead of ovotestes.

In families where XX males and XX true hermaphrodites occur as sibs,^{7,17} or in families where sibs exhibit the XX male condition and abnormal genitalia,²⁰ subjects have mostly been negative for Y chromosome specific probes. The isolated cases of Y negative XX males usually have ambiguous genitalia and this has been taken as evidence for a common origin with XX true hermaphrodites.^{7,20}

To date, only two XX true hermaphrodite patients showing hybridisation with Y chromosome material have been reported by Palmer *et al.*¹ and Jäger *et al.*²⁴ The patient described by Palmer *et al.*¹ had an XX male brother with hypospadias. Both sibs were positive for the Y specific boundary sequence¹ and the SRY gene.

Our familial cases, one XX true hermaphrodite and an XX brother, showed no hybridisation with either of the two proposed TDF genes (SRY or ZFY), which agrees with previous reports^{20,25} for the familial or sporadic cases of XX true hermaphrodites. Our results, together with the family described by Palmer *et al.*¹ imply that even in the familial cases the two conditions are heterogeneous with respect to the presence of Y chromosome material.

Mittwoch²⁶ proposed that the Y chromosome DNA sequences concerned with 'testis determination' may act by enhancing cell division in the relevant gonadal cells, thus accelerating gonadal development. On the assumption that testes need to differentiate early, the Y chromosome could thus function to minimise the chance element in XY fetuses of the gonad entering the ovarian pathway and to maximise the probability of their developing as males. The proposition does not exclude the presence of a Y directive gene, the best candidate being the SRY gene, functioning as the first switch event in normal males and absent in XX normal females.

Among those patients who are Y negative XX males and XX true hermaphrodites, there may be

Figure 2 PCR amplification across the X and Y pseudoautosomal boundaries. Track (1) negative control, (2) normal female, (3) normal male, (4) 46,Y,t(X;Y) male, (5) normal male, (6) XX male, (7) XX male, (8) XX male, case 2, (9) blank, (10) XX true hermaphrodite, case 1, sib of case 2, (11) XX true hermaphrodite, case 5, (12) XY female, case 6, (13) negative control, (14) 1 kb ladder. Primer sequences are given in reference 10.

activation of a mechanism downstream in the pathway from the TDF gene which leads to the first switch of Sertoli cell differentiation.

From the familial cases presented here and elsewhere,^{7,17} it is apparent that the same mechanism that can induce complete testis formation in an XX male is not necessarily enough to differentiate all the indifferent gonad cells into one testis in the XX true hermaphrodites. Which endpoint is reached seems to be a matter of chance or the result of action on the embryonic cells of one or more as yet unidentified factor(s).

In the cases presented here and by Palmer *et al.*,¹ there is a variation in the gonadal differentiation. The Y positive hermaphrodite described by Palmer *et al.*¹ had bilateral ovotestes. Our familial cases had one fully developed testis and one ovotestis, while our sporadic case of true hermaphroditism had one fully developed ovary with an ovotestis.

At least 11 XY female patients have been described with deletions including the TDF region of the Y chromosome.^{16,27-32} However, the XY female studied here did not show deletions of the Y chromosome, including SRY, as has also been described in other cases.⁴ The presence of the SRY gene in an XY female obviously does not show that the gene is functional; there could be microdeletions or point mutations in these subjects leading to a lack of gene product or an inactive protein.

The absence of the SRY gene in XX males and XX true hermaphrodites shows that this gene is not

essential for testis determination. These are very rare cases, however, where a mutation in the proposed pathway controlled by the product of the SRY gene may have allowed testis development in the absence of that gene product. In normal males, the SRY gene seems likely to be the initial switch in the process leading to the production of testis. Demonstration of this gene in those cases of XX males with known translocation of Y material will increase the probability that SRY is indeed TDF. Sequence analysis of the DNA from XY females who are positive for SRY, as in the case presented here and those of Ferguson-Smith *et al.*,⁴ should show whether these subjects have an inactive gene or perhaps a mutation elsewhere in the pathway of gonadal differentiation. In this respect, it is of interest that two XY females have recently been reported as showing mutations in the SRY gene.^{33,34} Elucidation of the structure of the SRY gene product and, from that, its possible mode of action, will provide more information on the starting point in this pathway.

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Figure 3 Southern blot of HindIII digests probed with SRY cDNA. Tracks 1 to 8 are as for fig 1. Track 9 is a normal male, track 10 a normal female.

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- 1 Palmer MS, Sinclair AH, Berta D, *et al.* Genetic evidence that ZFY is not the testis-determining factor. *Nature* 1989; **342**:937-9.
- 2 Sinclair AH, Berta P, Palmer MS, *et al.* A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* 1990; **345**:240-4.
- 3 Gubbay J, Collignon J, Koopman P, *et al.* A gene mapping to sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature* 1990; **346**:245-50.
- 4 Ferguson-Smith MA, North MA, Affara NA, Briggs H. The secret of sex. *Lancet* 1990; **336**:809-10.
- 5 Page DC, Fisher EM, McGillivray B, Brown LG. Additional deletion in sex-determining region of human Y chromosome resolves paradox of 46X,t(Y;22) female. *Nature* 1990; **346**:279-81.
- 6 Ferguson-Smith MA, Cooke A, Affara NA, Boyd E, Tolmie JL. Genotype-phenotype correlations in XX males and their bearing on current theories of sex determination. *Hum Genet* 1990; **84**:198-202.
- 7 Abbas N, Boucekkine C, Moreira-Filho CA, Ternes Pereira E, Cabral de Almeida JC, Fellous M. Familial cases of XX males: argument for a common origin of Y negative 46,XX male and true hermaphrodites. *Cytogenet Cell Genet* 1989; **51**:948.
- 8 Kunkel LM, Smith KD, Boyer SH, *et al.* Analysis of human Y-chromosome specific reiterated DNA in chromosome variants. *Proc Natl Acad Sci USA* 1977; **74**:1245-9.
- 9 Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975; **98**:503-17.
- 10 Ellis N, Kidd J, Goodfellow PJ, Kidd K, Goodfellow P. Strong linkage disequilibrium between the XY274 polymorphism and the pseudoautosomal boundary. *Am J Hum Genet* 1990; **46**:950-5.
- 11 Therkelson AJ. Sterile male with chromosomal constitution, 46,XX. *Cytogenetics* 1964; **3**:207-18.
- 12 Ferguson-Smith MA. X-Y chromosomal interchange in the aetiology of true hermaphrodites and XX Klinefelter's syndrome. *Lancet* 1966; **i**:475-6.
- 13 Magenis RE, Casanova M, Fellous M, Olson S, Sheeny R. Further cytologic evidence for Xp-Yp translocations in XX males using in situ hybridization with Y-derived probe. *Hum Genet* 1978; **75**:228-33.
- 14 Page DC, de la Chapelle A, Weissenbach J. Chromosome Y-specific DNA in related human XX males. *Nature* 1985; **315**:224-7.
- 15 Ferguson-Smith MA, Affara NA, Magenis RE. Ordering of Y-specific sequences by deletion mapping and analysis of X-Y interchange in males and females. *Development* 1987; **101**(suppl):41-50.
- 16 Muller U, Donlon T, Schmid M, *et al.* Deletion mapping of the testis determining locus with DNA probes in 46,XX males and in 46,XY and 46,X,dic(Y) females. *Nucleic Acids Res* 1986; **14**:6489-504.
- 17 de la Chapelle A. The Y-chromosomal and autosomal testis-determining genes. *Development* 1987; **101**(suppl):33-8.
- 18 Affara NA, Ferguson-Smith MA, Tolmie J, *et al.* Variable transfer of Y-specific sequences in XX males. *Nucleic Acids Res* 1986; **14**:5375-87.
- 19 Weissenbach J, Le Villiers J, Petit C, Rouyer F, Simmler MC. Normal and abnormal interchanges between the X and Y chromosome. *Development* 1987; **101**(suppl):67-74.
- 20 Abbas NE, Toubanc JE, Boucekkine C, *et al.* A possible common origin of Y-negative human XX males and XX true hermaphrodites. *Hum Genet* 1990; **84**:356-60.
- 21 Polani PE. Hormonal and clinical aspects of hermaphroditism and the testicular feminizing syndrome in man. *Philos Trans R Soc (Lond) (B)* 1970; **259**:187-204.
- 22 Simpson JL. True hermaphroditism: etiology and phenotypic considerations. Sex differentiation and chromosomal abnormalities. *Birth Defects* 1978; **XIV**(6):9-35.
- 23 Daniel A, Lyons N, Casey JH, Gras L. Two dicentric isochromosomes, one without the Yq heterochromatic segment. Review of the Y isochromosomes. *Hum Genet* 1980; **54**:31-9.
- 24 Jäger R, Ebensperger C, Fraccaro M, Steinbach P. A ZFY-negative 46,XX true hermaphrodite is positive for the Y pseudoautosomal boundary. *Hum Genet* 1990; **85**:666-9.
- 25 Damiani D, Billerbeck AEC, Goldberg ACK, Setian N, Fellous M, Kalil J. Investigation of the ZFY gene in XX true hermaphroditism and Swyer syndrome. *Hum Genet* 1990; **85**:85-8.
- 26 Mittwoch U. Sex, growth and chance. *Nature* 1990; **344**:389-90.
- 27 Boyd E, Cooke A, Jamieson D, Kwok K, Mitchell N, Snadden L. Mapping the testis determinants by an analysis of Y-specific sequences in males with apparent XX and XO karyotypes and females with XY karyotypes. *Nucleic Acids Res* 1987; **15**:7325-42.
- 28 Disteche CM, Casanova M, Saal H, *et al.* Small deletions of the short arm of the Y chromosome in 46,XY females. *Proc Natl Acad Sci USA* 1986; **83**:7841-4.
- 29 Magenis RE, Tochen NL, Holahan KP, Carey T, Allen L, Brown MG. Turner syndrome resulting from partial deletion of Y chromosome short arm: localisation of male determinants. *J Pediatr* 1984; **105**:916-19.
- 30 Rosenfeld RG, Luzzatti L, Hintz RL, Miller OJ, Koo GC, Wachtel SS. Sexual and somatic determinants of the human Y chromosome: studies in a 46 XYp⁻ phenotypic female. *Am J Hum Genet* 1979; **31**:458-77.
- 31 Le Villiers J, Quack B, Weissenbach J, Petit C. Exchange of terminal portions of X and Y chromosomal short arms in human XY females. *Proc Natl Acad Sci USA* 1989; **86**:2296-300.
- 32 Blagowidow N, Page DC, Huff D, Mennuti MT. Ulrich-Turner syndrome in one XY female fetus with deletion of the sex-determining portion of the Y chromosome. *Am J Med Genet* 1989; **34**:159-62.
- 33 Berta P, Hawkins JR, Sinclair AH, *et al.* Genetic evidence equating SRY and the testis determining factor. *Nature* 1990; **348**:448-50.
- 34 Jäger RJ, Anvret M, Hall K, Scherer G. A human XY female with a frame shift mutation in the candidate testis-determining gene SRY. *Nature* 1990; **348**:425-54.