Jpn J Human Genet 37, 311–320, 1992

Case Report

SEX-DETERMINING REGION Y (SRY) IN A PATIENT WITH 46,XX TRUE HERMAPHRODITISM

Akito TANOUE,[1] Toshiro NAKAMURA,[1] Fumio ENDO,[1]
Soroku NISHIYAMA,[1] Hitoshi SAKIYAMA,[2] and Ichiro MATSUDA[1,*]

[1]*Department of Pediatrics and* [2]*Department of Urology, Kumamoto University Medical School, Honjo, Kumamoto 860, Japan*

Summary Using a polymerase chain reaction method, a search for Y-specific DNA sequences was made in samples derived from tissues of a 46,XX true hermaphrodite. We found a sequence of SRY in the ovotestis, skin and leukocytes. Other DNA sequences, which covered the pseudoautosomal boundary region, amelogenin gene and DYZ1 locus of Y-chromosome were not detected. The SRY gene detected in the patient by the polymerase chain reaction was not detected by Southern blot analysis, using the SRY fragment as a probe. These findings suggest that in the patient there is a mosaicism of cells with and without part of the Y chromosome, including the SRY sequence. As the SRY sequence was responsible for the development of the gonadal primordium to the ovotestis, SRY seems essential for gonadal differentiation in testis development.

Key Words true hermaphroditism, SRY, mosaicism

INTRODUCTION

A true hermaphrodite is an individual possessing both testicular and ovarian tissues. In more than half of the reported cases there was a 46,XX karyotype, and the remainder had a 46,XX karyotype or sex-chromosome mosaicism (Donahoe *et al.*, 1978).

The mechanisms that govern gonadal differentiation, a critical step in the development of internal structures and external genitalia, have been the subject of wide discussion. The role of the Y chromosome was emphasized when it became clear that independently of the number of X chromosomes present, the Y chromosome determines development of the testicle, even if dysgenetic. It has been reported

Received September 7, 1992; Revised November 10, 1992.
*To whom correspondence should be addressed.

that the testis-determining factor gene (TDF) lies on the Y chromosome and that it is responsible for initiating male sex determination. Sinclair *et al.* (1990) determined a candidate sequence for TDF and used the term SRY (for sex-determining region Y), because this sequence has many of the properties expected for TDF. It was also reported that some sex-reversed XY females without Y chromosome deletions had mutations in the SRY, thereby suggesting that the SRY is responsible for sex determination (Berta *et al.*, 1990; Jäger *et al.*, 1990a). Koopman *et al.* (1991) demonstrated that SRY is indeed the TDF gene.

In other reports, true hermaphrodites with the 46,XX karyotype were analyzed for Y specific regions, including ZFY, which is ~200 kb apart from SRY (Palmer *et al.*, 1990), and such sequences were not detected in peripheral leukocytes by Southern blot analysis (Abbas *et al.*, 1990; Damiani *et al.*, 1990). We report here evidence for the presence of SRY in tissues from a 46,XX true hermaphrodite, determined using the polymerase chain reaction method (PCR) and Southern blot analysis.

PATIENT AND METHOD

Patient. The patient, 13 months old, was born with ambiguous genitalia, consisting of clitoromegaly, a single vaginal opening, the absence of labia minora and the presence of a hernia sac resembling scrotum. The family history was unremarkable. Laboratory investigation revealed normal 24-hr urinary 17-ketosteroid (0.3 mg/day) and 17-hydroxycorticosterone (1.3 mg/day) values, and normal serum electrocyte concentrations. Gonadotropin levels before and after LH-RH infusion tests were within the normal range. The serum estradiol (E_2) level was <10.0 pg/ml and was elevated to 42 pg/ml after the human menopausal gonadotropin (HMG) infusion test. Serum testosterone and 5*α*-dihydroxy testosterone (5*α*DHT) levels were <5.0 ng/dl and <0.02 ng/ml, respectively. After the human chorionic gonadotropin (hCG) infusion test, the testosterone level was elevated to 80.2 ng/dl and the 5*α*DHT was 0.29 ng/ml. Chromosome analysis performed on peripheral blood leukocytes revealed a 46,XX karyotype.

As one gonad was palpable in the scrotum on the right side, the patient was assumed to be a true hermaphrodite. At exploratory laparotomy, the uterus was palpable and one fallopian tube was identified. Gonads were present on the right side, one in the hernia sac which resembled a scrotum and the other was in the inguinal canal. The intraoperative pathology assessment was that the gonad in the hernia sac was testis and the one in the inguinal canal was ovotestis with a clear boundary. The whole gonad in the hernia sac and a half of the inguinal canal were excised. Detailed histological examination on the excised gonads revealed that both were ovotestis. In the testicular tissue, the seminiferous tubules were occupied only with Sertoli cells. The interstitial tissues were fibrous and there were no Leydig cells. In the ovarian zone, primordial follicles and a few primary

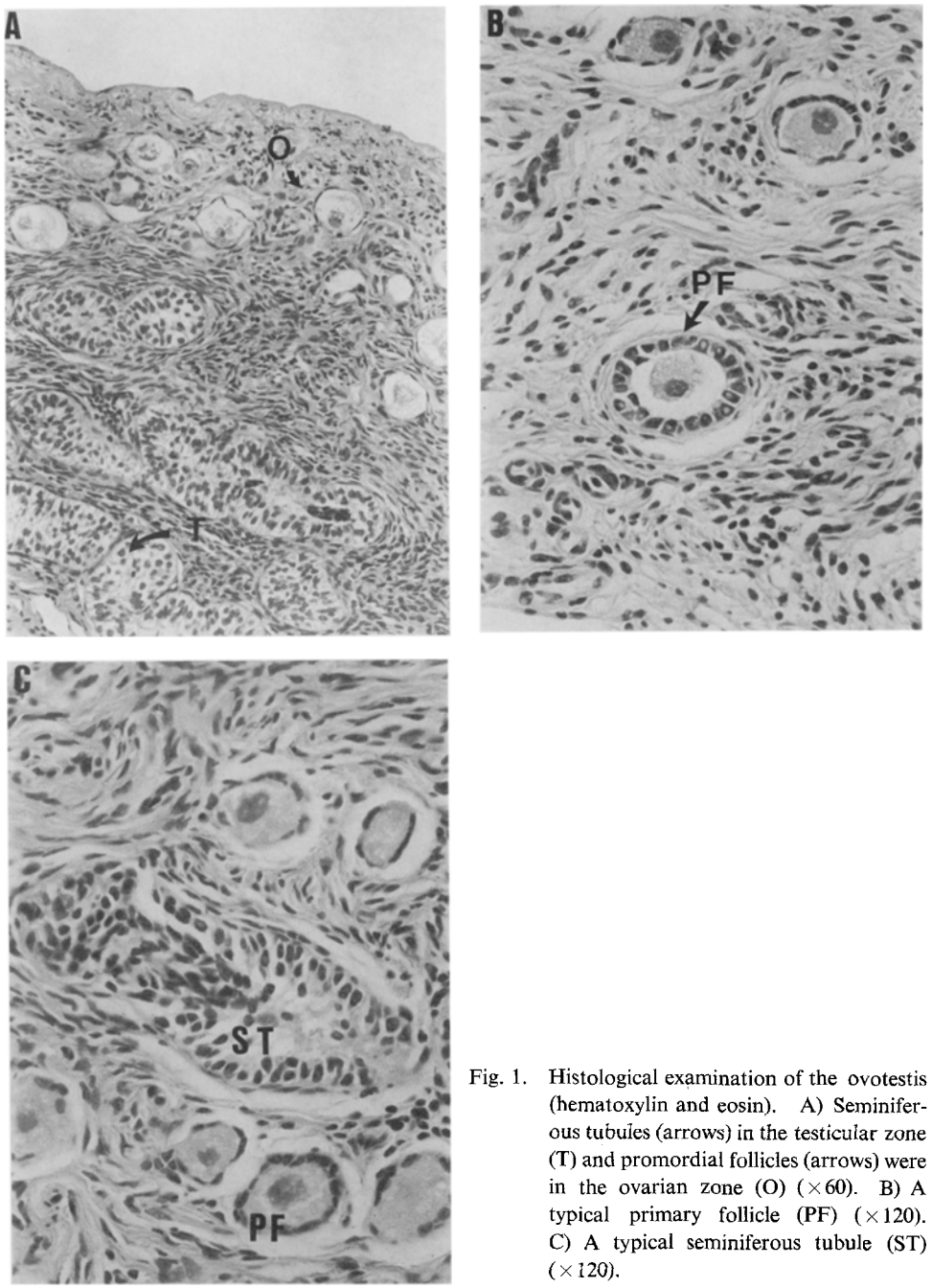

Fig. 1. Histological examination of the ovotestis (hematoxylin and eosin). A) Seminiferous tubules (arrows) in the testicular zone (T) and promordial follicles (arrows) were in the ovarian zone (O) (×60). B) A typical primary follicle (PF) (×120). C) A typical seminiferous tubule (ST) (×120).

Table. 1 Sequences of oligonucleotide primers.

Region	Primer	Fragment[a] (chromosome)	Annealing temnerature (°C)
Sex-determining region Y	AP-1: 5′ GAATATTCCCGCTCTCCGG3′	422 bp (Y)	57
	AP-2: 5′ ACAACCTGTTGTCCAGTTGC3′		
Pseudoautosomal boundary region	PAR-A: 5′ GTACTACCTTTAGAAAACTAGTATTTTCCC3′	947 bp (Y)	54
	PAR-B: 5′ CTGCAGAAACAAGCTCATCAGCGTGACTAT3′	771 bp (X)	
	PAR-C: 5′ GAATTCTTAACAGGACCCATTTAGGATTAA3′	(X and Y)	
Amelogenin	AMXY-1F: 5′ CTGATGGTTGGCCTCAAGCCTGTG3′	1.0 kb (X)	55
	AMXY-2R: 5′ TAAAGAGATTCATTAACTTGACTG3′	0.8 kb (Y)	
B fragment of DYZ1	Y1-6F: 5′ AATTTGAGCATTCGTGTCCATTCT3′		
	Y1-4R: 5′ AATGCCCTTGAATTAAATGGACT3′	1,024 bp (Y)	55
Exon 10 of peptidase D	10E5: 5′ AAGATTCGGCCGAGGGCCTTCCCTTGGG3′	214 bp (19)	
	10E3: 5′ TTGGATCCCACTGGTCACCCCCAGG3′		

[a] The size of fragment amplified with the primers is indicated and is specific for the chromosome.

follicles were visible (Fig. 1).

DNA analyses. High molecular weight DNA was prepared from the genital skin, ovotestis and peripheral leukocytes, as described (Tanoue *et al.*, 1990). Control DNA was obtained from peripheral leukocytes of normal females and males. Oligonucleotide primers for AP-1 and AP-2, PAR-A, -B, and -C, AMXY-1F and AMXF-2R, Y1-6F and Y1-4R located within the SRY (Jäger *et al.*, 1990b), pseudoautosomal boundary region (Ellis *et al.*, 1990), amelogenin gene (Nakahori *et al.*, 1991) and DYZ1 (Nakagome *et al.*, 1991a), were synthesized for PCR amplifications. Each nucleotide sequence of the primers is described in the Table 1. PCR was performed using ~100 ng/50 μl mix of genomic DNA and Taq polymerase (Takara Shuzo Co.) using DNA Thermal Cycler (Takara Shuzo Co.) as described elsewhere (Saiki *et al.*, 1985). Reactions conditions were 1 min denaturation at 94°C, 1 min annealing at 54–57°C and 3 min extension at 72°C for 30 cycles. One-fifth of the products were gel-electrophoresed and stained with ethidium bromide. The amplified DNA fiagment corresponding to SRY was sequenced by the di-deoxynucleotide chain termination method using radio-isotope (Sanger *et al.*, 1977). Southern blot analysis was performed as described (Southern, 1975).

RESULT

Genomic DNA from the ovotestis, leukocytes or skin was analyzed to investigate whether these tissues contained a Y-specific sequence, especially SRY. The results of PCR amplification of the sequences are shown in Fig. 2. With the primer set within the open reading frame of SRY, a 422 bp frgament was amplified from DNAs from a control male, patient's ovotestis, leukocytes and skin, but not from a control female (Fig. 2A). To monitor the PCR, a 214 bp fragment of peptidase D gene was co-amplified (Tanoue *et al.*, 1990), and was successfully amplified in all samples. Sequencing of the 422 bp fragments from the control male and patient's tissues revealed that the nucleotide sequence of both fragment was identical to the SRY sequence described in previous reports (dafa not shown) (Sinclair *et al.*, 1990; Jäger *et al.*, 1990a).

Next we examined whether the patient's tissues contained other Y specific regions. A pseudoautosomal region of Y is known to be located at the distal area of SRY on the short arm of the chromosome, an area where recombinations sometimes occur between X and Y chromosome (Ellis *et al.*, 1990). To amplify part of a pseudoautosomal boundary region, PCR was performed on DNAs using a 30 mer oligonucleotide primer which corresponded to the pseudoautosomal sequence of X and Y (PAR-C), in conjunction with a 30 mer oligonucleotide corresponding Y-specific (PAR-A) or X-specific (PAR-B) sequences. Products were identified as a 947 bp fragment from the Y chromosome, and a 771 bp fragment from the X chromosome (Ellis *et al.*, 1990). Both fragments were found in products from the control male, while only the 771 bp fragment derived from X chromosome was

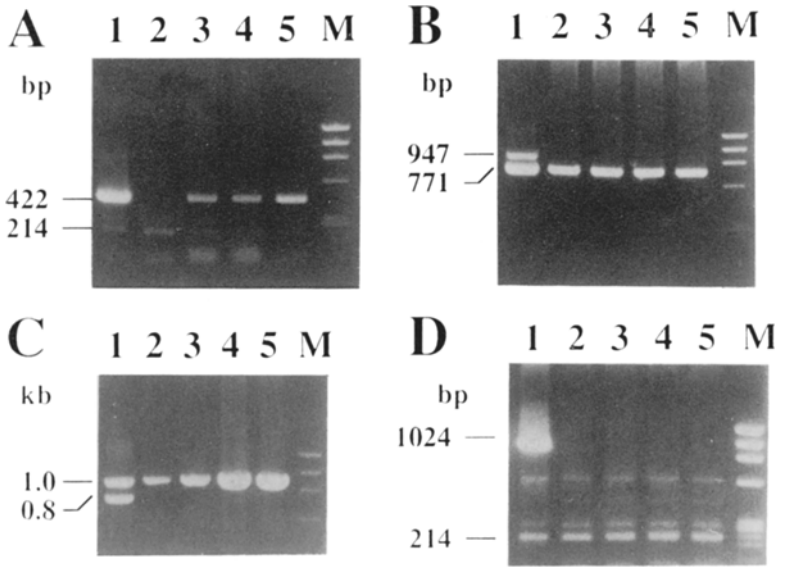

Fig. 2. PCR analysis of SRY (A), pseudoautosomal boundary region (B), amelogenin gene (C), and B fragment of DYZ1 (D). Samples from a control male (lane 1), control female (lane 2), patient's leukocytes (lane 3), patient's genital skin (lane 4), and patient's ovotestis (lane 5). Marker, ΦX174/*Hae*III digested fragment. PCRs were performed as described in METHODS. A 10 μl aliquot (of 50 μl) was electrophoresed on a 1.5% agarose gel. A) PCR with the primers within the open reading frame of SRY (AP-1, AP-2). The SRY specific fragment is a 422 bp. A 214 bp fragment was co-amplified with a primer set of peptidase D gene (10E5, 10E3) as internal standard for PCR. B) PCR analysis of X and Y boundaries. Oligonucleotides specific for X- or Y-chromosomal sequence were used together with an oligonucleotide from the pseudoautosomal regions as primers (PAR-A, B, and C). The Y specific fragment is 947 bp and the X-specific fragment, 771 bp. C) PCR with the primers within the amelogenin locus (AMXY-1F, AMXY-2R). The Y-specific fragment is 0.8 kb and the X-specific fragment, 1.0 kb. D) PCR with the primer within the B tragment of the DYZ-1 locus (Y1-6F, Y1-4R). The Y-specific fragment is a 1,024 bp. A 214 bp fragment was co-amplified with primer set of the peptidase D gene (10E5, 10E3) as the internal standard for PCR. The bands visible between 214 bp and 1,024 bp were nonspecific.

found in products from control female, patient's leukocytes, patient's skin or patient's ovotestis (Fig. 2B).

An amelogenin gene is located at centromere on the short arm of Y and X chromosomes (Nakahori *et al.*, 1991). With the primer set within the amelogenin gene, a 1.0 kb fragment was amplified from the X chromosome and an 0.8 kb fragment from Y chromosome (Nakahori *et al.*, 1991). The fragment from the Y chromosome was not detected in any sample from the patient's DNA (Fig. 2C). The B fragment of DYZ1 was specific on the Y chromosome (Nakagome *et al.*, 1991a).

A 1,024 bp fragment was amplified on DNA from the control male, but not on those from the control female, patient's leukocytes, skin and ovotestis. The peptidase D gene was co-amplified in all samples (Fig. 2D). Thus, tissues from the patient contained part of the Y chromosome including SRY, but not long arm of the Y chromosome.

Since it was possible that the pseudoautosomal boundary PCR assay was less sensitive than the SRY PCR assay, we performed PCR of SRY and Y specific pseudoautosomal region using mixed peripheral leukocytes derived from the father and the mother of the patient changing by a ratio 1:1 to 1:10,000. Amplified DNA fragment corresponding to Y specific pseudoautosomal region was visible up to a 1:500 mixed sample, whereas that of SRY was clearly shown in a 1:10,000 mixed sample (Fig. 3).

Next, we analyzed DNA from control male, control female and the true hermaphrodite, by Southern blot analysis after *Eco*RI or *Hin*dIII digestion, using the 422-bp SRY fragment as a probe. As shown in Fig. 4, the probe hybridized with the 13 kb fragment generated by *Eco*RI digestion in the control male (black arrow), whereas no hybridization band, including the corresponding band, was observed in the DNA sample from the control female and from the patient, even after a longer exposure. When the filter was re-hybridized with a 4.0 kb-*Bam*HI-*Eco*RI fragment of genomic DNA of peptidase D gene, a 10 kb fragment was visualized, in all lane (white arrow), when the same samples were digested with *Hin*dIII and subjected to Southern hybridization with the 422 bp SRY probe, a 2.1 kb fragment, corresponding to the SRY gene (Sinclair *et al.*, 1990), was visualized only in case

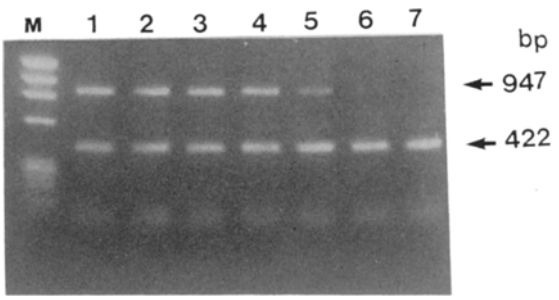

Fig. 3. PCR analysis of SRY and pseudoautosomal boundary region of Y with primers within SRY (AP-1, AP-2) and pseudoautosomal boundary region of Y (PAR-A, PAR-C). DNAs are derived from leukocytes from the father and the mother of the patient. The SRY fragment is a 422 bp and the Y specific pseudoautosomal fragment is a 947 bp. Lane 1, the father's DNA only; lane 2, DNA from mixed peripheral leukocytes with a ratio 1:1 of the father and the mother, respectively; lane 3, a ratio with 1:50 of the father and the mother; lane 4, a ratio with 1:100 of the father and the mother; lane 5, a ratio with 1:500 of the father and the mother; lane 6, a ratio with 1:1,000 of the father and the mother; lane 7, a ratio with 1:10,000 of the father and the mother; lane M, DNA marker (ΦX-174/*Hae*III).

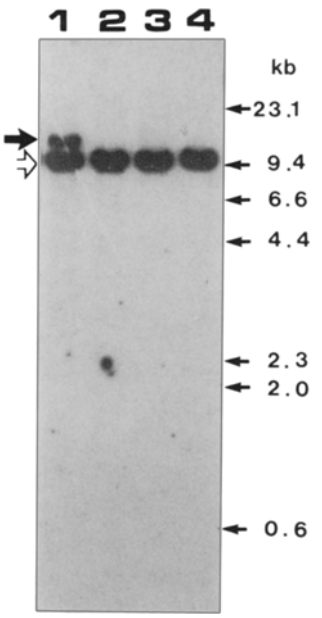

Fig. 4. Southern blot analysis of genomic DNA from a true hermaphrodite, control female and control male, using a 422-bp SRY fragment and a 4.0 kb fragment of the peptidase D gene (Tanoue *et al.*, 1990). A 10 μg DNA sample was digested with *Rco*RI, electrophoresed on 0.8% agarose gel, and transferred to a nitrocellulose filter. The filter was first hybridized with the 422-bp SRY fragment labeled with ^{32}P. The filter was washed and exposed to X-ray film for 7 days. The same filter was re-hybridized with the 4.0 kb fragment of peptidase D gene, as an internal standard. The SRY gene is indicated by a black arrow and peptidase D gene by a white arrow. Molecular weight marker (λ/*Hin*dIII) is shown at right side. Lane 1, leukocytes from a control male; lane 2, ovotestis from the patient; lane 3, leukocytes from the patient; lane 4, leukocytes from a control female.

of the control male (not shown).

DISCUSSION

We obtained evidence for the presence of SRY in tissues from a true hermaphrodite whose peripheral leukocytes showed a 46,XX female karyotype. As the SRY sequence was detected by PCR but not Southern blot analysis, the SRY-positive-cell population is scanty compared to that of the SRY-negative-cell. Thus, the hybridization band was not visible by the latter procedure which is less sensitive than PCR.

Although the mechanism by which the SRY exerts its influence on gonadal development is a matter of speculation, the ovotestis found in our patient was potentially composed of ovarian tissue without SRY and testicular tissue with

SRY, and this mosaicism. It is possible that in the SRY-positive cells, part of Y chromosome including SRY was translocated to the X chromosome or to another autosome. Because of the small population of SRY-positive cells and the small size of the amplified SRY sequence (less than 1.0 kb), an *in situ* hybridization study on chromosome was not feasible.

There has been no adequate explanation for the etiology of true hermaphroditism. Familial 46,XX males co-existing with familial 46,XX true hermaphrodites in the same pedigree suggests that both represent alternative expressions of the same genetic defects (Skordis *et al.*, 1987). The X-Y chromosome interchange has occurred in many XX males and a Y-chromosome-specific sequence has been detected, while in XX true hermaphroditism such sequences seem to be rare. Nakagome *et al.* (1991b) found that in a true hermaphrodite without the ZFY locus, SRY was detected by PCR. Mosaicism might relate to the development of true hermaphroditism in such cases.

We could not exactly determine the region of Y chromosome that the patient had. Since SRY is located only a few kilobases proximal to the Y pseudoautosomal boundary, it is unlikely that SRY would be present and the pseudoautosomal boundary absent. As shown in our examination, the pseudoautosomal boundary PCR assay is less sensitive than the SRY PCR assay and the presence of the Y pseudoautosomal boundary region in the patient can't be excluded. However, Jäger *et al.* (1990b) reported that only one case out of seven 46,XX true hermaphrodites who lack ZFY had Y pseudoautosomal boundary. This data combined with our data suggests that there may be small part of Y chromosome in patients with true hermaphroditism.

In summary, hermaphroditism in the patient is apparently caused by mosaicism of SRY-positive and -negative cells. The SRY is essential for testis differentiation as a trigger leading the undifferentiated gonads to the testis.

Acknowledgments We thanks Dr. Y. Nakagome (National Children's Medical Research Center) for information of sequences within amelogenin, Dr. Y. Shibata (Department of Surgical Pathology, Kumamoto University) and Dr. K. Yoshinaga (3rd Department of Anatomy, Kumamoto University) for advice on the histology, Dr. S. Karashima for the photographs, Dr. H. Awata for technical assistance, M. Ohara for helpful comments, and M. Hayashi for secretarial services.

REFERENCES

Abbas NE, Toublanc JE, Boucekkine C, Toublanc M, Affara NA, Job JC, Fellous M (1990): A possible common origin of "Y-negative" human XX males and XX true hermaphrodites. Hum Genet **84**: 356–360

Berta P, Hawkins JR, Sinclair AH, Taylor A, Griffiths BL, Goodfellow PN, Fellous M (1990): Genetic evidence equating SRY and the testis-determining factor. Nature **348**: 448–450

Damiani D, Billerbeck AEC, Goldberg ACK, Setian N, Fellous M, Kalil J (1990): Investigation of the ZFY gene in XX true hermaphroditism and Swyer syndrome. Hum Genet **85**: 85–88

Donahoe PK, Crawford JD, Hendren WH (1978): True hermaphroditism: a clinical description and a proposed function for the long arm of the Y chromosome. J Pediatr Surg **13**: 293–301

Ellis N, Taylor A, Bengtsson BO, Kidd J, Rogers J, Goodfellow PN (1990): Population structure of the human pseudoautosomal boundary. Nature **344**: 663–665

Jäger RJ, Anvret M, Hall K, Scherer G (1990a): A human XY female with a frame shift mutation in the candidate testis-determining gene SRY. Nature **348**: 452–454

Jäger RJ, Ebensperger C, Fraccaro M, Scherer G (1990b): A ZFY-negative 46, XY true hermaphrodite is positive for the Y pseudoautosomal boundary. Hum Genet **85**: 666–668

Koopman P, Gubbay J, Vivian N, Goodfellow PN, Lovell-Badge R (1991): Male development of chromosomally female mice transgenic for Sry. Nature **351**: 117–121

Nakagome Y, Nagafuchi S, Seki S, Nakahori Y, Tamura T, Yamada M, Iwaya M (1991a): A repeating unit of the DYZ1 family on the human Y chromosome consists of segments with partial male specificity. Cytogenet Cell Genet **56**: 74–77

Nakagome Y, Seki S, Fukutani K, Nagafuchi S, Nakahori Y, Tamura T (1991b): PCR detection of distal Yp sequences in an XX true hermaphrodite. Am J Med Genet **41**: 112–114

Nakahori Y, Takena O, Nakagome Y (1991): A human X-Y homologous region encodes "Amelogenin." Genomics **9**: 264–269

Palmer MS, Sinclair AH, Berta P, Ellis NA, Goodfellow PN, Abbas NE, Fellous M (1990): Genetic evidence that ZFY is not the testis-determining factor. Nature **342**: 937–939

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985): Enzymatic amplication of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**: 1350–1354

Sanger F, Nicklen S, Coulson AR (1977): DNA sequencing with chain termination inhibitors. Proc Natl Acad Sci USA **74**: 5463–5467

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R, Goodfellow PN (1990): A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature **346**: 240–244

Skordis NA, Stetka DG, MacGillivray MH, Greenfield SP (1987): Familial 46,XX males coexisting with familial 46,XX true hermaphrotides in same pedigree. J Pediatr **110**: 244–248

Southern EM (1975): Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol **98**: 503–517

Tanoue A, Endo F, Matsuda I (1990): Structural organization of the gene for human prolidase (peptidase D) and demonstration of a partial gene deletion in a patient with prolidase deficiency. J Biol Chem **265**: 11306–11311