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The aetiology of XX True Hermaphroditism in the southern African black population

Carolyn Jane Padoa

A dissertation submitted in fulfilment of the requirements for the degree of Master of Science in Human Genetics in the Faculty of Medicine, at the University of the Witwatersrand, Johannesburg

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ABSTRACT

A true hermaphrodite is an individual in whom both testicular and ovarian tissue is present. The tissues may be present in the same gonad, an ovotestis, or as separate gonads, an ovary and testis. Twenty nine southern African black patients were investigated in an attempt to determine the molecular aetiology of this disorder. The most common karyotype observed in 20 subjects tested was 46,XX (19/20). Roughly half the hermaphrodites were reared as males and the other half as females. The testicular tissue of the hermaphrodites was histologically immature, but in contrast, the appearance of the ovaries was normal. Nearly 80% of the 24 true hermaphrodites (on whom histopathology reports were available) had an ovotestis on one or both sides of the body. The ovotestis was the most common gonad (58.70%) while the testis was found least frequently (13.04%). The ovotestis and testis occurred more frequently on the right side of the body whereas the ovary was predominantly situated on the left side. A minimum incidence of 1 in 40 070 was calculated for XX true hermaphroditism in the South African black population with a minimum of 27 babies expected to be born each year with the disorder.

Four different hypotheses were tested in an attempt to unravel the molecular basis of XXTH in South African blacks. A number of autosomal and X-linked loci have been implicated in sex reversal, including chromosomes 9, 10 and 17. Abnormalities in SOX9, a gene located on chromosome 17, can lead to both XX and XY sex reversal. Analysis of the SOX9 gene, by Southern blot, failed to conclusively implicate a role for this gene in the aetiology of XX true hermaphroditism. Another hypothesis is that there may be a mutation in an X-linked gene followed by non-random X chromosome inactivation in true hermaphrodites. Here, a mutated gene on an active X chromosome may lead to inappropriate testis development. The human androgen receptor gene was investigated by methylation sensitive restriction enzyme digestion, PCR and polyacrylamide gel electrophoresis to determine the pattern of X-inactivation. Non-random X-inactivation of the opposite parental X chromosome alleles in the testicular and ovarian tissue would suggest that a gene, necessary for normal female sex determination, is mutated and present on the active X chromosome which results in testis formation in individuals with a 46,XX karyotype. In these cases, the normal copy

of the gene would be on the inactive X chromosome and, therefore, not expressed. Due to the poor quality of the DNA extracted from archival formalin-fixed paraffin-embedded gonadal tissue, no conclusion could be reached regarding this hypothesis and its possible role in the aetiology of XX true hermaphroditism. The DAX1 gene is close to or within the dosage sensitive sex reversal (DSS) locus on the X chromosome. When present in duplicate, this gene has been shown to result in male to female sex reversal. The DAX1 gene was screened for mutations by SSCP analysis. Although band shifts were identified in three regions of the gene, only one region, DAX1.3, revealed the presence of a possible mutation upon sequencing. The change observed was a T to C transversion at nucleotide 660 of the DAX1 gene causing an amino acid substitution (cysteine to serine). Fourthly, it is believed that mosaicism or chimerism with a Y bearing cell line in the gonads of true hermaphrodites may be responsible for the presence of testicular tissue. As a result, our patients were scored for the presence or absence of three Y-specific loci by PCR. Three individuals were positive for all three loci. No cytogenetic analyses had been performed on these samples and it was, therefore, not known what their karyotypes were. They could have been 46,XY males or XX/XY mosaics with minor sexual ambiguity which would explain the results. One XXTH patient with a mosaic cell line was positive for SRY and the amelogenin locus on the short arm of the Y chromosome, but not for 49a on the long arm of the Y chromosome. As most of the genes involved in testicular development on the Y chromosome are found on the short arm, this finding is compatible with the phenotype observed in this true hermaphrodite. Mosaicism/chimerism in the gonads, however, cannot yet be ruled out as a possible/probable explanation for testicular differentiation in these patients. In conclusion, the aetiology of XX true hermaphroditism remains elusive and further investigations are required.

DECLARATION

I, Carolyn Jane Padoa, do hereby declare that this dissertation, submitted in fulfilment for the requirements for the degree of Master of Science in the Department of Human Genetics at the University of the Witwatersrand, Johannesburg is my own unaided work. Any assistance that I have received is stated in the acknowledgements. It has not been submitted before for any degree or examination in any other university. I declare that the protocol has been cleared by the Committee for Research on Human Subjects (Clearance certificate protocol number M960915).



Carolyn Jane Padoa

15 day of December 1999.

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LIST OF ABBREVIATIONS

α	Alpha
A	Adenine
ACD	Acid citrate dextrose
AG	Ambiguous genitalia
AHC	Adrenal hypoplasia congenita
AMEL-Y	Amelogenin like sequence on the Y chromosome
AMH	Anti-Müllerian Hormone
APS	Ammonium persulfate
ARE	Androgen Receptor Element
bp(s)	Base pair(s)
Bkm	Banded krait minisatellite
BSA	Bovine serum albumin
χ^2	Chi-squared
°C	Degrees Celsius
C	Cytosine
CAH	Congenital adrenal hyperplasia
CD	Campomoelic dysplasia
Ci	Curie
Cl	Chloride
CMPD1	Campomoelic dysplasia locus 1
cpm	Counts per minute
dATP	Deoxyadenine triphosphate
DAX	DSS-AHC critical region on the X chromosome
dCTP	Deoxycytidine triphosphate
ddH_2O	Deionised distilled water
ddNTP	Dideoxyribonuleotide triphosphate
dGTP	Deoxyguanine triphosphate
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

dNTP	Deoxyribonuleotide triphosphate
dpc	days post coitum
dRhodamine	Dichlororhodamine
DSS	Dosage sensitive sex reversal
DTT	Dithiothreitol
dTTP	Deoxythymine triphosphate
dUTP	Deoxyuridine triphosphate
E.Coli	*Escherisha coli*
EDTA	Ethylenediaminetetra-acetic acid
FPG	Female pathway gene
FS	Stoffel Fragment
g	Gram
G	Guanine
^{3}H	Tritium
HCl	Hydrochloric acid
HGT	High gelling temperature
HH	Hypogonadotropic hypogonadism
HMG	High mobility group
HUMARA	Human androgen receptor assay
Kb(s)	Kilobase(s)
KCl	Potassium chloride
λ	Lambda
l	Litre
LA	Luria agar
LB	Luria broth
LMP	Low melting point
M	Molar
mA	Milliampere
MDE	Mutation detection enhancement
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MIS	Müllerian inhibiting substance

ml	Millilitre
mM	Millimolar
MPG	Male pathway gene
mRNA	Messenger RNA
μg	Microgram
μl	Microlitre
μM	Micromolar
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OD	Optical density
%	Percentage
p	Short arm of a chromosome/probability
α-^{32}P	Phosphorus-32 at the α position of the carbon chain
PAB	Pseudoautosomal boundary
PABY	Pseudoautosomal boundary on the Y chromosome
PAGE	Polyacrylamide gel electrophoresis
PAR	Pseudoautosomal region
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
PGC	Primordial germ cell
pmol	Picamole
PVP	polyvinylpyrrolidone
q	Long arm of a chromosome
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
Σ	Sum of
s	Seconds
SAIMR	South African Institute for Medical Research
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulphate
SF	Steroidogenic factor

SOX	Sry-related HMG box-containing gene
SRA	Autosomal sex reversal locus
SRY	Sex determining region of the Y
SSCP	Single stranded conformational polymorphism
ssDNA	Salmon sperm deoxyribonucleic acid
T	Thymine
Taq	*Thermophilus aquaticus*
TBE	Tris borate EDTA
TDF	Testis determining factor
TE	Tris EDTA
TEMED	N,N,N',N'-tetramethylethylene diamine
Tris	Tris - (hydroxymethyl) - aminomethane
TSB	Transformation and storage buffer
U	Units
UV	Ultraviolet
V	Volts
VNTRs	Variable number tandem repeats
vs.	Versus
WT	Wilm's tumour
Xic	X-inactivation centre
Xist	X-inactivation specific transcript
XXTH	XX true hermaphrodite
ZFY	Zinc finger Y

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CHAPTER 1

1. INTRODUCTION

1.1. XX True Hermaphroditism

Diverse disorders of sexual development exist in man, many of them having been recognised since ancient times and indeed some of them conferring on their possessors positions of dubious privilege in society. These disorders fall into the category termed intersexuality. Intersexual conditions arise from errors in sexual development of the early embryo which has the potential to develop as either a male or a female. This results in various degrees of intermediate sex. Intersexuality is, therefore, defined as the possession, at birth, of both male and female characteristics in the gonads and/or other internal organs and/or external genitalia (Jacobs, 1969).

The incidence of intersexed individuals in the population is relatively low. Nonetheless, since the availability of suitable cytogenetic techniques in the late 1950s, a very large variety of abnormal sex chromosome constitutions have been reported in man. Many, but not all, have been shown to be associated with varying degrees of abnormality of primary and secondary sex characteristics. The most common intersexual anomaly reported in the world is that of male pseudohermaphroditism with the true hermaphrodite being one of the less common variants. However, in van Niekerk's (1974) experience, true hermaphroditism is the most common type of intersexuality occurring among the Bantu-speaking people of South Africa.

True hermaphrodites are individuals who have both ovarian and testicular tissue. The two tissues may be present in either the same gonad, an ovotestis, or as separate gonads (an ovary or a testis). Some false hermaphrodites, or pseudohermaphrodites, have testes and are called male pseudohermaphrodites; others have ovaries and are known as female pseudohermaphrodites.

i. Male pseudohermaphrodites

These individuals have a 46,XY chromosome complement. They are characterised by the presence of testicular tissue and either female or ambiguous genitalia, with or without a vagina. The testes fail to exert an adequate influence on the differentiation of the secondary sexual organs, i.e., there is insufficient secretion of testicular androgens or defective testosterone and 5α-dihydrotestosterone at target tissues due to receptor insensitivity to androgens. The paramesonephric duct may fail to regress and a uterus may develop (Rösler *et al*, 1996).

ii. Female pseudohermaphrodites

These individuals have a 46,XX chromosome constitution. The most common single cause of female pseudohermaphroditism is the congenital adrenogenital syndrome, resulting from virilizing hyperplasia. They are characterised by the presence of ovaries and masculinisation of the external genitalia due to the excessive adrenal production of androgens during the foetal period (Seaver *et al*, 1994). This varies from enlargement of the clitoris to almost masculine genitalia.

iii. True hermaphrodites

Hermaphroditism is a genetically heterogeneous condition (McElreavey *et al*, 1992) that may be a model for studying testicular determination. Although true hermaphroditism shares many features with other conditions of testicular dysgenesis, this syndrome remains a distinct clinical entity based on the histological findings (Hadjathanasiou *et al*, 1994). The most frequent karyotype in these patients is 46,XX or various forms of mosaicism, whereas 46,XY is very rarely found (van Niekerk & Retief, 1981a; Ramsay *et al*, 1988).

Salén described the first true hermaphrodite reported in the literature in 1899. Since then several hundred cases have been reported.

1.1.1. Historic and legal aspects of hermaphroditism

1.1.1.1. Hermaphrodites through the ages

The concept of bisexuality has existed through the ages. Long before the advent of science, the question of what determines whether an organism shall develop into a male or a female has been debated. Some pre-scientific accounts contain implications

that are not immediately obvious. In the book of Genesis we are told that Eve was formed from one of Adam's ribs. There is, however, strong evidence that this interpretation is based on a mistranslation.

The Hebrew word *zela*, which is usually translated as 'rib', also means 'side' and this is apparently the correct rendering as written in the story of the creation in the book of Genesis. According to this rabbinical tradition, Adam and Eve were not originally a couple but an hermaphrodite being, who was subsequently bisected. There is both internal and comparative evidence in favour of this interpretation. Following the account of Eve's creation, we have the verse: 'Therefore shall a man leave his father and his mother and cleave unto his wife' (Genesis ii, 24). This is a logical conclusion of Adam's bisection, but is clearly not intelligible if one assumes that he merely lost a rib (Mittwoch, 1986).

In Babylonian times, the birth of a baby with ambiguous genitalia was considered a bad omen. Many of the Greek and Roman philosophers, such as Heroditus and Plato, make reference to ambisexual beings in their writings (cited in van Niekerk, 1974). In a symposium by Plato (427 – 347 BC) (translated by Jowett, 1871), Aristophanes describes three sexes – males, females and androgens – each split in half by an angry Zeus and each forever seeking its partner. Apollo was bidden to heal their wounds. Once this was accomplished, the two halves came together in mutual embrace – a neat and attractive way to explain homosexual and heterosexual attraction (reviewed by Hunter, 1995). It was Theoprast and Alkiphron (300 BC), however, who first used the term hermaphrodite.

During the Hellenic period the hermaphrodite was thought to possess supernatural powers. An example of a truly bisexual god is Hermaphroditus. Hermaphroditus was believed to be the son of Hermes, god of athletics, invention, secrets and occult philosophy, and Aphrodite, goddess of love. According to Greek mythology, when Hermaphroditus was 15 years, he came upon a crystal pool where a beautiful nymph, Salmacis, dwelt. She was taken by his beauty and made advances to him 'but the boy blushed rosy red, for he knew not what love is.' When he threatened to run away, Salmacis withdrew and hid in a nearby thicket. Hermaphroditus thought he was alone. He removed his garments to bathe in the clear water, whereupon Salmacis could no

longer contain her passion. She threw off her own clothes and leapt into the water, enwrapping him in a serpentine embrace. 'Strive as you may, wicked boy,' she cried, 'you shall not escape me.' Salmacis asked the gods to grant her one prayer: that 'no day ever come that shall separate him from me or me from him'. The gods granted her prayer. Their bodies were fused, and from two, they became one, with one face and one form, neither male nor female, but having the features of both. They were no longer two, nor such as to be called one woman and, one man. They seemed neither and yet both (Adapted from *Metamorphoses* by the Roman storyteller Ovid as reported by van Niekerk, 1974).

'Some say that Hermaphroditus is a god... has a body which is beautiful and delicate like that of a woman, but has the masculine quality and vigour of a man, but some declare that such creatures of two sexes are monstrosities' as quoted by Diodoros (60 BC) on the god Hermphroditus (reviewed by van Niekerk, 1974).

To classify an individual as a 'true hermaphrodite', Victorian medical men insisted upon histological proof of ovarian and testicular tissue. However, because biopsies of gonads were not done until the 1920s, the only 'true hermaphrodites' tended to be dead or autopsied hermaphrodites. With the advent of new technologies – specifically laparotomies and biopsies – in later years, it became possible to label some living people as 'true' hermaphrodites. Disturbed physicians noted that no one knew what to do with such people. There was no place, socially or legally, for true hermaphrodites.

1.1.1.2. Hermaphrodites and the law

Much has been written, and many laws enacted concerning hermaphrodites in different nations. In the Corpus Iuris Civilis, Ulpian was asked in the first book to Sabinus: 'With whom shall we compare a hermaphrodite? I believe that we should rather determine his sex in accordance with that which manifests itself the strongest'. Since then, many laws have been passed governing the status of the hermaphrodite. During the Middle Ages, babies with ambiguous genitalia were considered monsters and drowned or burned as a result. Later, it was ruled that hermaphrodites could marry. The choice of partner, whether male or female, was left to personal selection. They could not, however, become judges or priests. Some European countries allow hermaphrodites to

choose male or female status. Other countries require that a doctor make this decision (reviewed by van Niekerk, 1974).

The fundamental legal problem is 'to which sex does a hermaphrodite belong?' Hindu law is interesting in this respect because it stipulates a special provision for the hermaphrodite with regard to inheritance. A son will inherit 100%, a daughte inherits only half, and a hermaphrodite, as decreed by Hindu law, inherits three-quarters (reviewed by van Niekerk, 1974). Blackstone, on the other hand, is of the opinion that every heir is either male or female. Hence, a hermaphrodite, which is both male and female, must inherit according to the predominant sex (reviewed by van Niekerk, 1974).

In a legal sense, the predominant sex of a hermaphrodite has never been defined. Sexuality has many facets: chromosomal, gonadal, hormonal, phenotypic, as well as the psychological or assumed gender role. Which of these criteria should carry the most weight in any legal classification of the hermaphrodite? Often, the most important factor in gender role is the sex of rearing and not the chromosomal composition, gonads or appearance of the hermaphrodite. To change the legal sex to the predominant anatomic sex, in such cases, could be psychologically disastrous to the patient (van Niekerk, 1974).

1.1.2. Classification of XX true hermaphrodites

There are four methods that have been used to classify true hermaphrodites. These are all based on the appearance of the gonads found in the patients following histological examination.

i. Klebs Classification (1876) (reviewed by van Niekerk, 1974):
   This method for classifying true hermaphrodites included eighteen possible variations. Although Sauerbeck later modified this method, it was found to be impracticable, complex and incomplete. It is, therefore, no longer used.

ii. Hinman Classification (1935) (Hinman, 1935 reviewed in van Niekerk, 1974):
Most authors use this classification which is divided into three groups:

a. Group I, bilateral
An individual, who has an ovary and a testis, either separate or united as an ovotestis, on both sides.

b. Group II, unilateral
Any person having a testis and an ovary, either separate or united as an ovotestis, on one side, and either a testis or an ovary on the opposite side.

c. Group III, lateral
Any individual with a testis on one side and an ovary on the opposite side.

iii. Jones and Scott Classification (1958) (Jones & Scott, 1958 reviewed in van Niekerk, 1974):
This method of classification is essentially identical to the Hinman classification. The only difference being the subdivision of the three varieties into groups.

GROUP	GONAD ONE SIDE	GONAD OPPOSITE SIDE
Alternating or lateral variety		
• Group I	Ovary	Testis
Bilateral varieties		
• Group IIa	Ovotestis	Ovotestis
• Group IIb	(Ovary, Testis)	(Ovary, Testis)
Unilateral varieties		
• Group IIIa	Ovary	*Ovotestis*
• Group IIIb	Ovary	(Ovary, Testis)
• Group IIIc	Ovary	Two ovotestes
• Group IV	Testis	Ovotestis
• Group V	(Ovary, Testis)	No gonad
• Group VI	Ovotestis	Not examined

iv. Sasaki and Makino Classification (1960) (Sasaki & Makino, 1960 reviewed in van Niekerk, 1974):

This method of classification is not commonly used because, although it lists the different combinations of gonads, they are not grouped into the three main categories, namely: lateral, bilateral and unilateral.

GROUP	GONAD ONE SIDE	GONAD OPPOSITE SIDE
Group 1	Ovary	Testis
Group 2	Ovotestis	Ovotestis
Group 3	Ovary	Ovotestis
Group 4	Testis	Ovotestis
Group 5	Ovotestis	No gonad
Group 6	Ovotestis	Not examined

1.1.2.1. The clinical picture of XX true hermaphrodites

The clinical picture of the 24 XXTH patients described by van Niekerk (1974) and the majority of those cited in the literature appear to be remarkably constant.

1.1.2.1.1. Presenting symptoms

There are two symptoms that true hermaphrodites commonly present with:

i. ambiguous external genitalia

ii. breast development in patients that have been reared as males (van Niekerk, 1974; Damiani *et al*, 1997).

Other presenting symptoms include hematuria in a patient reared as a male, cryptorchidism, amenorrhea in a patient reared as a female, lower abdominal pain because of endometriosis, inguinal hernia, a slight degree of hypospadias, and cliteromegaly (van Niekerk, 1974; Hadjiathanasiou *et al*, 1994).

1.1.2.1.2. The types and distribution of gonads

There are three types of gonads that can be found in true hermaphrodites. These are an ovary, testis and ovotestis.

i. The ovotestis

The ovotestis (Figure 1.1) is one of the most arresting features of true hermaphrodites. The location of the ovotestis, i.e., in the ovarian or scrotal position, seems to be influenced by the ratio of ovarian to testicular tissue. The greater the mass of testicular tissue present, the greater the chances that the ovotestis will descend from the ovarian position to the inguinal or scrotal region (van Niekerk, 1974).



Figure 1.1. Cross section of the ovotestis of a patient showing infantile testicular tissue on the left and numerous primordial follicles on the right (van Niekerk, 1974). A similar appearance is observed in the separate ovarian and testicular gonadal tissue of true hermaphrodites.

ii. The ovary and testis

In the majority of cases the histological appearance of the ovarian tissue was characterised as well developed and essentially normal, even when it was part of an ovotestis. Most ovaries found in true hermaphrodites do show signs of ovulation (van Niekerk & Retief, 1981b). This is substantiated by reports in the literature of true hermaphrodites giving birth to normal children with normal appearing external genitalia (Narita *et al*, 1975; Mayou *et al*, 1978; Kim *et al*, 1979; Talerman *et al*, 1990). In contrast, the testicular tissue, from the testis and ovotestis, of true hermaphrodites is immature with interstitial fibrosis and no, or rare, spermatogonia (Hadjiathanasiou *et al*, 1994). Spermatogenesis was found and proven in only two cases both times occurring in a separate testis. One of these patients, with 46,XX/46,XY mosaicism, is reported to have fathered a child (Krob *et al*, 1994).

When the gonadal distribution was looked at in 694 true hermaphrodites (data collected from six papers), the unilateral true hermaphrodite with an ovary on one side and an ovotestis on the opposite side was present in 33.0% of the cases sited in the literature. The bilateral (ovotestis on both sides of the body) and lateral (an ovary on one side and a testis on the other) true hermaphrodite each accounted for approximately 25% of cases (refer to Table 1.1)

Table 1.1: Gonadal distribution in 694 true hermaphrodites (van Niekerk, 1974 [n=24]; van Niekerk, 1976 [n=3]; van Niekerk & Retief, 1981b [n=382]; Ramsay *et al*, 1988[n=37]; Hadjiathanasiou *et al*, 1994 [n=22]; Krob *et al*, 1994 [n=226])

TYPE OF DISTRIBUTION	NUMBER OF PATIENTS	PERCENTAGE (%)
Ovary-Testis	169	24.35
Ovotestis-Ovary	229	33.00
Ovotestis-Ovotestis	176	25.36
Ovotestis-Testis	78	11.24
Ovotestis-?	19	2.74
Others	23	3.31
TOTAL	**694**	**100**

If one were to extract, from the above data (Table 1.1), the cases of true hermaphrodites reported in the South African black population, a very different distribution frequency of the gonads would be observed. The 27 patients of van Niekerk (1974, 1976), together with the 37 cases of Ramsay *et al* (1988), showed that the unilateral (ovotestis – ovary/testis) true hermaphrodite accounted for 62.50% of the cases. The second most frequent gonadal distribution was that of the bilateral ovotestis (17.19%). The differences observed for the gonadal distribution are statistically significant at the 5% level ($p = 0.0196$). This may imply a different cause of hermaphroditism in the South African black population.

Of the 694 true hermaphrodites examined in Table 1.1, the location of the gonad on either the left or right hand side of the body is given for 1363 of the gonads. This information is presented in Table 1.2.

Table 1.2: The incidence of gonads occurring on the left or right side in true hermaphrodites

GONAD*	LEFT (%)	RIGHT (%)	TOTAL NUMBER OBSERVED (%)
Ovary	288 (43.11%)	135 (19.42%)	423 (31.03%)
Testis	105 (15.72%)	159 (22.88%)	264 (19.37%)
Ovotestis	275 (41.17%)	401 (57.70%)	676 (49.60%)

* Data extrapolated from Table 1.1.

From these data it is clear that the ovary is more prevalent on the left side, while the testis and ovotestis occur more frequently on the right side of the body. It is also evident that the ovotestis is the most common gonad in the true hermaphrodite accounting for 49.60% of the gonads. The second most common gonad is the ovary (31.03%) and, lastly, the testis (19.37%).

Upon examination of 195 confirmed true hermaphrodite cases (refer to Table 1.3), it became clear that 46,XX was the most common karyotype, occurring in 59.5% of the cases; 12.8% of the hermaphrodites had a 46,XX/46,XY mosaic pattern while the 46,XY

karyotype was found in 12.3% of the patients analysed. The remaining 15.3% of cases showed different mosaic cell lines (van Niekerk and Retief, 1981b). It is assumed that karyotype analysis was performed on peripheral lymphocytes although this is not stipulated.

The most common chromosomal complement found in true hermaphrodites is a 46,XX karyotype. Upon reviewing 283 cases of human true hermaphrodites published from 1980 to 1992, Krob *et al* found the 46,XX karyotype to occur in 70.6% of cases. Chromosomal mosaicism (20.2%) was the second most common group and the 46,XY karyotype accounted for 7% of cases. Interestingly, 87 of the 88 patients, of black or mixed black origin (based on the references from van Niekerk and Retief, 1981b [n=27]; Ramsay *et al*, 1988 [n=37]), on whom karyotyping was performed had a 46,XX chromosome complement. In cases reported from other regions, as summarised by Krob *et al*, 1994, only 52% of the hermaphrodites had a 46,XX karyotype This and the fact that the distribution of gonads differs to that in the rest of the world may be an indication that the aetiology of true hermaphroditism in the South African black population is different to that in European hermaphrodites.

The important observation to note from these data is that out of the group with the 46,XX chromosomal complement, the gonadal distribution was such that the majority of cases fall into the unilateral group of true hermaphrodites, i.e., an ovary on one side and an ovotestis on the opposite side of the body. This occurred in 37% of the cases listed in Table 1.3. The second most common gonadal distribution of hermaphrodites with a 46,XX karyotype was a bilateral ovotestis in 28.4% of the cases (van Niekerk & Retief, 1981b).

Table 1.3: Gonadal distribution and karyotype in 195 true hermaphrodites (van Niekerk & Retief, 1981b)

DISTRI-BUTION	46,XX	46,XY	46,XX/ 46,XY	46,XY/ 47,XXY	45,X/ 46,XY	OTHER	TOTAL (%)
Ovary-Testis	23	13	10	5	4	5	60 (30.77)
Ovary-Ovotestis	43	3	7	3	1	2	59 (30.26)
Ovotestis-Ovotestis	33	2	6	2	0	2	45 (23.08)
Ovotestis-Testis	12	5	2	0	1	2	22 (11.28)
Ovotestis-Unknown	5	1	0	1	1	1	9 (4.62)
TOTAL (%)	116 (59.49)	24 (12.31)	25 (12.82)	11 (5.64)	7 (3.59)	12 (6.15)	195

In Table 1.3 the gonadal distribution is compared for true hermaphrodites with a Y chromosome in their chromosomal complement and those without a Y chromosome. In the 60 hermaphrodites with an ovary on one side and a testis on the opposite side, a Y chromosome was found in 37 (61.7%) of cases. Among the 79 true hermaphrodites with a Y chromosome, a testis was present in 47 (59.5%) [37 with the ovary-testis distribution and ten with the testis-ovotestis gonadal distribution] (van Niekerk & Retief, 1981b). When comparing this to the overall prevalence of a testis (193 in 921 [21.0%]) in the 406 true hermaphrodites (Table 1.2), it would seem that the presence of a Y chromosome increases the possibility that a testis is one of the gonads present.

1.1.2.1.2.1. Gonadal tumours in true hermaphrodites

Van Niekerk (1974) states the incidence of dysgerminoma among true hermaphrodites to be between 1 and 2%. In contrast, Verp & Simpson (1987) give an incidence of 4% among patients with a 46,XX karyotype and 10% among patients with a 46,XY and mosaic 46,XX/46,XY karyotype. Where a dysgerminoma occurs and the tumour

replaces one gonad, it is very difficult to judge whether the patient is a true hermaphrodite unless the other gonad is an ovotestis.

1.1.2.1.3. External genitalia

The development of the external genitalia is generally believed to be an effect of dihydrotestosterone. This effect on the foetal gonad is correlated with the fact that only 12% of patients with true hermaphroditism cited in the literature had a penile urethra. This indicates that most patients with true hermaphroditism are deficient in foetal dihydrotestosterone production or are insensitive to this hormone. The testicular tissue in the true hermaphrodite seems to be capable of stabilising the Wolffian duct, but incapable of fully differentiating male type external genitalia. It is significant, however, that the sex of rearing of 126 case reports of true hermaphroditism in the literature, are specifically mentioned. Amongst these, 73% were reared as male and 27% as female. The apparent reason for this is that most true hermaphrodites have a phallus at birth (van Niekerk & Retief, 1981b).

The majority of patients reported in the literature had a phallus measuring between two to eight centimetres. The question of terminology arises, whether to call the structure an enlarged clitoris or small phallus. There is a tendency to use the term 'enlarged clitoris' if the patient is psychologically a female and 'phallus' if the patient is psychologically a male. Absence of either clitoral enlargement or a phallus in true hermaphrodites is an exception to the rule. The vast majority of patients have one or the other (van Niekerk, 1974).

Overzier classified various abnormal external genitalia found in intersex cases as Types I, II, III, IV, and V. Type I corresponds to normal female external genitalia. In Type II a common external orifice is present, while in Type III a urogenital sinus is situated interior to the clitoris or phallus. Type IV shows an internal urogenital sinus and Type V shows normal male external genitalia. External genitalia of the majority of true hermaphrodites reported in the literature belonged to Types III and IV (van Niekerk, 1974).

1.1.2.2. The diagnosis of XX true hermaphroditism

Whenever a patient with ambiguous genitalia is being examined, the possibility of true hermaphroditism should be considered. The differential diagnosis in such a case would

be female pseudohermaphroditism, male pseudohermaphroditism, true hermaphroditism, and penoscrotal hypospadias with associated cryptorchidism.

Van Niekerk (1974) mentions the value of palpating the gonad which may be present in one of the labioscrotal folds. In the true hermaphrodite it would usually be present in the right labioscrotal fold or in the right hemiscrotum. It is also important to feel carefully for any difference in consistency when palpating a gonad. In an ovotestis, the testicular portion will feel soft but the ovarian portion is quite firm. Hormonal and cytogenetic evaluations should be performed. The diagnosis of a true hermaphrodite, however, can only be established once ovarian and testicular tissues have been histologically demonstrated (Donahoe *et al*, 1978).

1.1.2.2.1. Age at diagnosis

The age at which true hermaphrodites are diagnosed varies considerably. There have been reports of patients being diagnosed at only 2 weeks of age (van Niekerk, 1974) and the diagnosis of an 85 year old individual by post mortem (Vaugh & Gonzalez-Angulo, 1961).

Ten or twenty years ago, the majority of patients (75%) were diagnosed by the age of 20. This was most probably due to the abnormal appearance of the external genitalia and abnormal breast development, amenorrhea or failure to undergo secondary sex changes when patients reached puberty (which prompted parents to seek medical advice) (van Niekerk, 1976). Increased awareness of the implications of ambiguous genitalia has resulted in detection and referral as new-borns (Donahoe *et al*, 1978).

Some true hermaphrodites never seek medical advice and are therefore only detected at routine medical examination or at autopsy (van Niekerk, 1974). In a study by Ramsay *et al* (1988) only 16 of the 38 (42%) patients were below five years of age when first investigated. To assign the appropriate sex and to undertake correctional surgery it is critical to diagnose true hermaphrodites before the age of five, by which time gender identity is firmly established (reviewed in Ehrhardt & Meyer-Bahlburg, 1981). This was confirmed when seven patients (diagnosed after five years of age) and their families, were interviewed. The emotional problems due to confused gender

identity in these individuals attest to the tragic consequences of late diagnosis (Ramsay *et al*, 1988).

1.1.2.2.2. Sex of rearing

The gender to which a patient is assigned is a controversial issue with no general consensus. In the past, there was a tendency for diagnosed true hermaphrodites to be reared as males since most true hermaphrodites have a phallus at birth. Of 146 true hermaphrodites reported in the literature, approximately 75% were reared as males (van Niekerk, 1974). However, based on the appearance of the external genitalia and the ability to reconstruct a functional genitourinary system, most of these children are presently raised as females (Luks *et al*, 1988). Hadjiathanasiou *et al* (1994) generally chose the female gender because it was easier to reconstruct functionally satisfactory female genital anatomy and because of the presence of normal ovarian tissue, as well as a uterus and a vagina, in most cases. The choice of sex assignment for raising the patients was made according to the age of the patient at diagnosis, the clinical and hormonal data, the degree of virilization of the external genitalia, and the familial and cultural context.

Luks *et al* (1938) believed the appearance of the external genitalia alone allowed a prompt and definitive gender assignment in infancy. They thus developed a classification of external genitalia based on the following characteristics. Class I represents normal female genitalia, class II an enlarged clitoris, class III partially fused labioscrotal folds, class IV fused labioscrotal folds, class V a hypoplastic scrotum and penoscrotal hypospadias, and class VI represents normal male genitalia. This classification, based on the appearance of the external genitalia, may aid gender assignment in infants identified as true hermaphrodites.

Whatever the sex assignment, any gonadal tissue that does not agree with the assigned sex should be removed and plastic surgery undertaken to repair the external genitals. Whenever possible, it is desirable to conserve gonadal tissue relevant to the assigned sex because spontaneous pubertal development can take place and an evaluation of the gonadal function can be made at puberty. However, because of the ill-defined risk of gonadal tumour in adults, periodic clinical and ultra sonographic assessment of the

testicular tissue is recommended in patients reared as males (Hadjiathanasiou *et al*, 1994).

1.1.2.3. Treatment of XX true hermaphrodites

In order to treat a hermaphrodite effectively, the first prerequisite is to decide on the gender of rearing. Elaborate surgical and hormonal treatments have been developed to make the assigned sexual anatomy more believable, that is, more 'typical' of the gender decided upon. If the patient is younger than two years of age, the external genitalia should be carefully assessed in order to determine what the sex of rearing should be. The decision should be based solely on the appearance of the external genitalia, without regard to the type of gonad, chromosomal pattern or structure of the internal genital tract (van Niekerk, 1974; van Niekerk, 1976).

When necessary, surgical reconstruction can be done. If the child is to be raised as a female, clitoral recession, labioscrotal reductions and enlargements, and vaginoplasties for exteriorization can be accomplished in early infancy as an extensive one-stage procedure. In preparing a patient for a female gender role, care should be taken to remove all testicular tissue present. If patients are to be raised as males, then various types of hypospadias repair can be done, gonads can be replaced with prostheses, the prepenile scrotum can be reconstructed, and Müllerian structures can be removed with the goal of preserving the vas deferens. In the patient assigned a male gender role, care should be taken to identify and remove all ovarian tissue.

In those instances where gonadal tissue consistent with the gender role of the patient cannot be retained, substitution hormone therapy should be instituted and maintained indefinitely (van Niekerk, 1974; van Niekerk, 1976). Replacement therapy with glucocorticoids and mineralocorticoids must be precisely managed to permit proper growth, and testosterone, oestrogen, and progesterone replacement must be carefully considered and managed. A most important element in the care of these patients is the psychological support that the families and the patient require. This must be delivered with sensitivity (Federman & Donahoe, 1995).

1.1.2.3.1. Ethical issues regarding treatment and correctional surgery of XX true hermaphrodites

The recent publication of the unexpected, long term outcomes in the classic and well known 'John/Joan' case have roused substantial public interest in intersexuality and 'reconstructive' genital surgery. At eight months, a doctor accidentally ablated John's (a normal 46,XY male twin) penis during a circumcision. Upon consultation with a team of physicians and sexologists at the Johns Hopkins Hospital, it was decided John should be medically reconstructed and raised as a girl – 'Joan' (Diamond & Sigmundson, 1997).

For decades, Hopkins sexologist John Money and others, reported the alleged success of this particular sex reassignment as proof that physicians could essentially create any gender out of any child, provided that the cosmetic alteration was performed early, the genitals looked 'right' and that everyone agreed to agree on the child's assigned gender. Diamond and Sigmundson (1997) briefly summarised the postulates of this approach which arise from the original work of Money and colleagues: '(1) individuals are psychosexually neutral at birth and (2) healthy psychosexual development is dependent on the appearance of the genitals.'

Although the John/Joan case was not an intersexuality case, many clinicians have nevertheless suggested that, in intersex cases, the same postulates should hold.

But reports of the success of John/Joan were premature. In fact, at age fourteen Joan, after many years of anguish and confusion, had chosen to resume life as John.

Diamond and Sigmundson later conceded that 'the evidence seems overwhelming that normal humans are not psychosocially neutral at birth but are, in keeping with their mammalian heritage, predisposed and biased to interact with environmental, familial, and social forces in either a male or female mode' as demonstrated by the classic John/Joan case. In other words, the above two postulates do not hold. Rather, gender identity arises from biology (nature) and not from psychosocial rearing (nurture). They also comment on the lack of long term follow-up of cases and the lack of ethical medical treatment of intersexed patients. Many parents, especially those unfamiliar with sex development, are bothered by their children's intersexed genitals and receptive to offers

of 'normalising' medical treatments. Many also actively seek guidance about gender assignment and parenting practices (reviewed by Dreger, 1998).

In the United States today, upon the identification of an 'ambiguous' or intersexed baby, teams of specialists (geneticists, paediatric endocrinologists, paediatric urologists, and parents) are assembled (Reiner, 1997). Together, a decision is made as to which sex/gender a given child will be assigned. The individual then undergoes reconstructive surgery to 'normalise' the external genitalia to fit the assigned sex and hormone treatments. It is also important to foster, among the child's family and community, the conviction that the child is indeed the sex decided – 'psychosocial' rearing of the child according to the norms of the chosen sex. Doctors typically take charge of the first two kinds of activities and hope that the child's family and community will successfully manage the all critical third.

In an effort to forestall, or end, any confusion about the child's sexual identity, clinicians try to see to it that an intersexual's sex/gender identity is permanently decided by specialist doctors within 48 hours of birth. Unfortunately, the present treatment of intersexuality in the United States seems to be deeply informed by the 'monster' approach, that is, ethical guidelines that would be applied in nearly any other medical situation are, in cases of intersexuality, ignored. Patients are not always fully informed; risky procedures are performed without follow-up; consent is not fully informed and autonomy and health are risked because of unproven (and even disproven) fears that atypical anatomy will lead to psychological disaster.

As new guidelines for dealing with persons with ambiguous genitalia are further developed, it will be critical to take seriously two tasks. First, intersexed individuals must not be subject to different ethical standards from other people simply because they are intersexed. Second, the experiences and advice of adult intersexuals must be solicited and taken into consideration (reviewed by Dreger, 1998).

1.1.3. Prevalence of XX true hermaphroditism

In southern Africa ambiguous genitalia seems to be much more common in blacks than in whites. In a study by Ramsay *et al* (1988) 152 black and only 31 white patients were

referred to their laboratory, over a four year period, for chromosome analysis because of ambiguous genitalia. During the period under review, there were twice as many white as black patients referred. All thirty eight of the black patients who were histologically examined, were proven to be true hermaphrodites; the remaining 114 had an XX karyotype but were not histologically examined. None of the 31 white patients was confirmed to be a true hermaphrodite on histological examination. The majority were affected with congenital adrenal hyperplasia.

This type of hermaphroditism (46,XX) is, thus, unusually prevalent in southern African blacks. This leads to the conclusion that there must be some unique factor(s) causing the high incidence of this sexual anomaly in blacks and, it seems likely that the condition has a common aetiological and pathogenetic basis in this population. True hermaphroditism is the least common intersexual anomaly in the world, however, according to van Niekerk (1974), true hermaphrodotism is the most common type of intersexuality in the Bantu-speaking people of South Africa. Thus, there seems to be a special predisposition in them that may account for the high incidence of this disorder.

1.1.3.1. An estimated incidence of XX true hermaphroditism in South Africa

Because XX true hermaphroditism appears to be very common in the South African black population, we set out to estimate the incidence of hermaphroditism in this population. Information was gathered from two sources, namely:

i. The Chris Hani-Baragwanath hospital birth defects data base. This data base keeps a record of all patients born at the hospital with some form of congenital abnormality.
ii. Individuals that had been referred to the South African Institute for Medical Research Cytogenetics laboratory because of a sexual anomaly/ambiguity.

1.1.3.2. Familial incidence

In general, true hermaphroditism occurs sporadically. However, the occurrence, although not common, of 46,XX true hermaphroditism in siblings has been reported in the literature (Fraccaro *et al*, 1979; Raine *et al*, 1989). Despite the low occurrence of true hermaphroditism in siblings, it is important insofar as it suggests a possible genetic aetiological factor for this condition (van Niekerk, 1974). 46,XX males (patients with a female karyotype, male phenotype and testicular development) sometimes co-exist in the same family with true hermaphrodites (Kuhnle *et al*, 1 3; Slaney *et al*, 1998).

Four families have so far been reported where 46,XX true hermaphrodites co-exist with 46,XX males suggesting that both disorders are variations of the same genetic defect (Fraccaro *et al*, 1979; Skordis *et al*, 1987). The presence of male sexual development in genetic females, with transmission through normal male and female parents, indicates that the critical genetic defect may be a new mutation manifesting as an autosomal dominant trait. The different phenotypic manifestations arise from variable expressivity (Slaney *et al*, 1998) or an X-linked mutation (limited by the presence of the Y chromosome) (Ramos *et al*, 1996). In these cases a common origin of the sex determining disorder has to be postulated (Krob *et al*, 1994).

1.1.4. The aetiology of XX true hermaphroditism

The genetic mechanism(s) responsible for 46,XX true hermaphroditism remains elusive. True hermaphroditism is a genetically heterogeneous condition with several possible causes. Three major hypotheses have been proposed to explain the aetiology of true hermaphroditism and the phenomenon of testicular differentiation that occurs despite the fact that approximately 60% of these individuals have an XX karyotype and lack the Y chromosome:

i. Mosaicism, with a loss of the Y containing cell line, presumably after it had initiated gonadal differentiation into a testis at an early embryonic stage, is the oldest explanation proposed (Miro *et al*, 1978; Abbas *et al*, 1990; Berkovitz *et al*, 1992).

ii. Translocation of chromosomal material encoding the testis determining factor (TDF) from the Y chromosome to an autosome or X chromosome during meiotic division is another possibility. If this were the correct explanation, a high familial incidence would be expected, but few families with XX true hermaphroditism have been described (Ferguson-Smith, 1966).

iii. An autosomal or X-linked dominant mutation that permits testicular determination in the absence of TDF thus allowing XX zygotes to assume a male phenotype (Petit *et al*, 1987; Abbas *et al*, 1990; Berkovitz *et al*, 1992). There is no direct evidence that a single gene mutation such as this occurs in humans.

The unusually high prevalence of XX true hermaphroditism in southern African blacks suggests that the condition has a common cause and pathogenesis in this population. A study by Spurdle *et al* (1995) of 16 XXTH patients failed to reveal the presence of the

SRY locus and the pseudoautosomal boundary of the Y chromosome (PABY). Y chromosome sequences are thus very unlikely to be involved in the cause of the disorder in southern African Bantu-speaking blacks. Ramsay *et al* (1988) obtained detailed family histories for 11 of their 38 XXTH patients. From the information gathered they concluded that it is unlikely that mutation of a single gene, either autosomal or sex linked, is responsible for the occurrence of hermaphroditism in the South African black population.

As twinning is common in blacks (personal communication, Prof. J. Kromberg [SAIMR]), the possibility that true hermaphrodites may be chimeras from a resorbed twin was explored. No siblings of the true hermaphrodites were twins. It therefore seems unlikely that twinning is an aetiological factor of true hermaphroditism in blacks. The rarity of cytogenetically detectable XX/XY chimerism among the black hermaphrodites (one of 39 cases) also argues against twinning being a significant factor. As the twinning rate is declining in this population, it will be of interest to see whether the rate of true hermaphroditism also declines (Ramsay *et al*, 1988).

An environmental aetiology is extremely difficult to exclude. Three of the 11 mothers had taken differing 'traditional' remedies of unknown composition. The above findings do not point to any discreet genetic or environmental aetiology, but the strikingly higher frequency of XX true hermaphroditism in southern African blacks indicates that there is likely to be some form of predisposition in this population (Ramsay *et al*, 1988).

1.2. Sex determination and gonadal differentiation

The most obvious distinction in the human species is that of gender. Since the days of Aristotle, both scientists and non-scientists, have been fascinated and perplexed by the biological processes that determine the sex of an individual (Smith, 1994).

1.2.1. The determination of sex

Sexual dimorphism in humans has been the subject of wonder for centuries. In 355 BC, Aristotle postulated that sexual dimorphism arose from differences in the heat of semen

at the time of copulation. In his scheme, hot semen generated males, whereas cold semen made females (reviewed by Haqq & Donahoe, 1998). The Greeks believed it was the level of male excitement during intercourse that determined whether the child would be male or female – the more excited the man, the greater the likelihood of siring a boy (Roberts, 1988). In medieval times, there was great controversy about the existence of a female pope, who may have in fact had an intersex phenotype (New & Kitzinger, 1993). Recent years have seen a resurgence of interest in mechanisms controlling sexual differentiation in mammals. Sexual differentiation relies on the establishment of chromosomal sex at fertilisation, followed by the differentiation of the gonads, and ultimately the establishment of phenotypic sex in its final form at puberty. Each event in sexual determination depends on the preceding event, and normally, chromosomal, gonadal, and somatic sex all concur. There are, however, instances where they don't agree, and sexual differentiation is ambiguous, with male and female characteristics combined in a single individual. Analysis of such individuals has permitted identification of some of the genes involved in sex determination (Haqq & Donahoe, 1998).

Today it is known that a cascade of complex molecular and morphological events must occur at the appropriate time and in the correct sequence in order for an organism to develop into a 'normal' male or female. This cascade of events depends on a switch mechanism.

In mammals, a gene on the Y chromosome appears to function as this switch, diverting development from the female pathway and turning on a succession of male determining genes. This gene is termed the testis determining factor, or TDF. In other animals the switch may be environmental, as in alligators, where the temperature at which the embryo is incubated is important; or chromosomal as in *Drosophila melanogaster*, where sex determination is controlled by a gene dosage mechanism i.e., it is dependent on the number of X chromosomes relative to sets of autosomes in a cell (the X:autosome ratio) (Anon, 1988; Gurbay *et al*, 1990; Parkhurst & Meneely, 1994).

The sex of an individual is the outcome of two distinct processes: sex determination and sex differentiation. Sex determination is the initial event that determines whether the gonads will develop as testes or ovaries; and sexual differentiation is the subsequent

events that ultimately produce either the male or the female sexual phenotype i.e., development of the genital ducts, external genitalia, and secondary sexual characteristics (Mittwoch, 1992; Ramkissoon & Goodfellow, 1996, Parker *et al*, 1999).

1.2.1.1. Chromosomal, gonadal and phenotypic sex

Sexual development in the mammalian embryo involves three sequential processes. The first step, which occurs at fertilisation, is to establish chromosomal sex (by the content of the sex chromosomes). Females will normally be XX and males XY, but the number of X chromosomes is irrelevant as the Y behaves as a dominant male determinant. Secondly, the indifferent gonads begin to differentiate into either testes or ovaries (gonadal sex). Ovarian differentiation can be considered the default pathway, which is 'overridden' in males by the testis determining gene, TDF, found on the Y chromosome; and thirdly, the differentiation of internal and external genitalia, that is, the translation of gonadal sex into phenotypic sex (Nordqvist & Lovell-Badge, 1994; Ramkissoon & Goodfellow, 1996).

1.2.2. Gonadal development

The presence or absence of hormones, which are produced by the male and female gonads in the developing foetus control male and female developmental pathways in mammalian sexual differentiation (Werner *et al*, 1996). Thus, sex determination can be reduced to its essence: the initiation of gonadal development into either a testis or an ovary.

During the fifth week of human embryological development, the gonads appear as thickenings of the coelomic epithelium along the median surface of the mesonephros. These thickenings are known as the genital (or gonadal) ridges. The genital ridges increase in size and gradually separate from the mesonephric ridge, developing its own mesentery, the mesorchium in the male and the mesovarium in the female. At the same time, cells of the coelomic epithelium proliferate to form finger-like epithelial cords, called primary sex cords.

Primordial germ cells (PGCs) migrate from the hind gut to the gonadal ridges and become incorporated into the primary sex cords. The germ cells can become either

spermatozoa or ova. The somatic cells of the gonad are composed of cells characteristic of both the ovary and testis: the supporting cells (Sertoli cells in the male and follicle cells in the female), steroidogenic cells (Leydig cells in the male and theca cells in the female), and connective tissue cells (reviewed by Bogan & Page, 1994). It is here that the paths diverge as gonads with a Y chromosome develop as testes whereas those where the Y chromosome is absent form an ovary.

The genetically determined female gonad continues its indifferent phase by growing further until ovarian follicles are formed. Each follicle contains a single egg cell, in the primary oocyte stage, surrounded by somatic cells termed follicular or granulosa cells. Between the follicles there is another type of somatic cell, the interstitial or theca cells, which will eventually produce oestrogens.

The testis is composed of a multitude of seminiferous tubules, each containing a large number of primordial germ cells that differentiate into the precursors of spermatozoa i.e., prespermatogonia. The somatic cells found within the tubules are known as Sertoli cells. The interstitial somatic cells, which form between the tubules, are known as Leydig cells. Leydig cells are responsible for the production of androgens (Mittwoch, 1986).

Two pairs of genital ducts associated with the undifferentiated gonads develop. The Müllerian ducts have the potential to differentiate into the fallopian tubes, uterus, cervix and upper vagina of the female reproductive tract. The Wolffian ducts become the vas deferens, epididymis and seminal vesicles of the male reproductive tract. The embryo, therefore, has the forerunners of both male and female reproductive tracts, of which one is destined to differentiate while the other must regress (Werner *et al*, 1996). Which of these two alternative routes is taken depends on the presence or absence of testes (Mittwoch, 1986).

1.2.2.1 Development in the male

The development of the testes:

The first identifiable step in the male sex determination pathway is testicular differentiation. This is controlled by the presence of three hormones: the Müllerian inhibiting substance (MIS; also known as anti-Müllerian hormone, AMH), testosterone

and dihydrotestosterone (Nordqvist & Lovell-Badge, 1994). MIS is secreted by Sertoli cells early after the onset of sex determination. MIS mediates regression of the female reproductive tract, the Müllerian ducts, which would otherwise give rise to the uterus and oviducts. Differentiation of the Wolffian ducts to form the male internal genitalia i.e., epidiymis, vas deferens and seminal vesicles, is induced by testosterone. Dihydrotestosterone, a conversion product of testosterone, promotes the formation of the prostate and male urethra as well as the external genitalia, the penis and scrotum (Nordqvist & Lovell-Badge, 1994; Lovell-Badge & Hacker, 1995; Werner *et al*, 1996). The Leydig cells secrete the latter two enzymes (Figure 1.2.).

The importance of the male hormone is seen in freemartin cattle in which a genetically female twin sharing a placental circulation with a male twin becomes masculinised as a result of circulating testicular hormone (Hunter, 1995).

1.2.2.2. Development in the female

The development of the ovary:

The female phenotype can be considered as the default pathway as no specific factors have been found, so far, which are required for the developing ovary. In the absence of testes, and therefore in the absence of the male hormones, the Wolffian ducts passively regress, creating a free environment for the Müllerian ducts to realise their potential in the female pathway (Werner *et al*, 1996). The primitive gonad in the XX individual differentiates into an ovary. Oogonia arise in the cortex of the gonads, and primary oocytes form before birth (Figure 1.2.) (Rothwell, 1993).

1.2.2.3. Gonadal differentiation and growth in the true hermaphrodite

In true hermaphroditism the normal regulation of gonadal differentiation is missing and the bipotentiality of the embryonic gonad persists, with the result that both ovarian and testicular tissues are formed. Gonadal differentiation may revert to a more primitive evolutionary level at which the rate of growth of the gonad determines its sexual direction. When this exceeds a certain threshold a testis may develop despite the absence of the TDF gene (Blyth & Duckett, 1991).

In true hermaphrodites there are a number of different combinations of gonads e.g., ovary and testis, bilateral ovotestis, etc. In patients who have two types of gonads

present, they are distributed in a bilateral asymmetric fashion. The ovary is more commonly situated on the left side of the body, whereas the testis is more common on the right side of the body. One might, therefore, expect the ovotestes, being part ovary and part testis, to be distributed in a bilaterally symmetrical fashion, but this is not the case. The distribution of the ovotestes resembles that of the testes occurring predominantly on the right hand side.

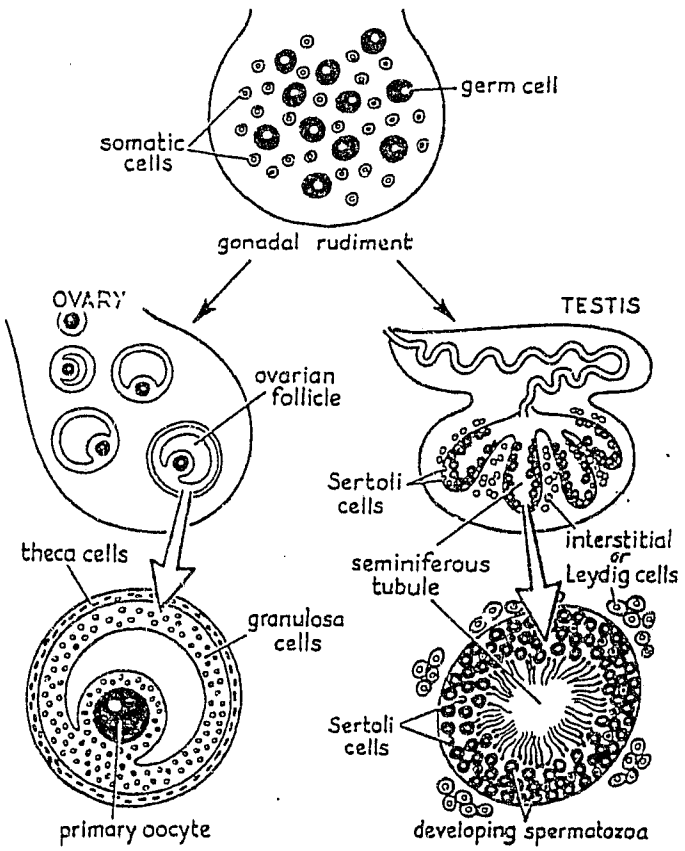


Figure 1.2. Development of ovary and testis from gonadal rudiment (Mittwoch, 1986).

The reason becomes apparent from an analysis of the distribution of gonads in patients with ovotestes (from van Niekerk & Retief, 1981b). Approximately a third of the cases had two ovotestes. In the remaining cases, an ovotestis and ovary were two and a half times as common as an ovotestis and testis occurring together (Mittwoch, 1986). An ovotestis, therefore, represents a testicular gonad more often than an ovarian one and, like a testis, it is preferentially situated on the right side.

The unequal distribution of ovaries and testes in true hermaphrodites cannot be easily explained genetically, as the TDF would be expected to be distributed evenly, and in equal quantities, in the cells of the body. If, however, gonadal differentiation is ultimately based on rates of growth, the question arises whether the asymmetry of gonadal growth in hermaphroditism can be related to an underlying asymmetry of the human body. By comparing growth characteristics in right and left gonads of normal human foetuses this possibility was tested (Mittwoch & Kirk, 1975). In the three variables investigated, i.e. weight, protein and DNA contents, the right gonads, on average, exceeded the left gonads. By combining the two sets of data, the relationship between growth and differentiation in human gonads can be deduced.

Ovaries are smaller and do not grow as fast as testes; left gonads are smaller and grow slower than right gonads. If there was any casual relationship between these phenomena, we would expect left gonads to have a tendency to become ovaries and right gonads to differentiate into testes. In normal development this relationship is of no consequence, since the sex chromosome constitution will override all other tendencies. However, in the development of hermaphrodites, we witness the breakdown of the sex-chromosomal mechanism, and we also see the bilaterally asymmetrical development of ovaries and testes in accordance with the basic asymmetry of normal gonadal development (Mittwoch, 1986).

The significance of these results is twofold. Firstly, the observed bilateral asymmetry of gonadal growth in human foetuses provides an explanation for the asymmetrical distribution of ovaries and testes in human hermaphrodites. Secondly, the weak asymmetries observed in the growth of foetal gonads link the human situation to that observed in other classes of vertebrates, some of which exhibit very marked asymmetries. Birds provide a striking example.

1.2.2.4. Sex determination in birds

In birds, the chromosomal mechanism of sex determination is the opposite of that in mammals: the female is the heterogametic sex with a chromosome constitution ZW, where the Y chromosome is referred to as the W chromosome. The male, on the other hand, is the homogametic sex i.e., two equal sex chromosomes which are generally referred to a ZZ (Klug & Cummings, 1991).

Sexual differentiation in birds is accompanied by a very marked degree of bilateral asymmetry in favour of the left side. At five days, the gonads of ZZ embryos are larger than the female ZW gonads. In both sexes though the left gonad is larger than the right gonad. However, the pattern changes in embryos that are seven to eight days old. The left gonad of ZW embryos increases in size and outgrows all other gonads, developing into an ovary. The bilateral asymmetry is therefore greatly increased in female embryos. Thus, as sexual differentiation occurs, the gonads become more symmetrical in males and less symmetrical in females. In most species of birds, only the left gonad becomes a functional ovary, while the right gonad of females remains rudimentary. If, however, the ovary is removed or becomes diseased, the right gonad will hypertrophy but, instead of developing into an ovary, it will develop into a more or less normal testis resulting in phenotypic sex reversal. If this were to take place, then the hen may become masculinised and assume the habits of a cock. Such hens are unlikely to inseminate other hens, because the germ cells from the right gonad will have disappeared long ago. There is experimental evidence, however, that active sperm production can take place in the right gonads of female chickens if the ovary has been removed within a month of hatching. Evidentially the potentially hermaphrodite state can be more easily activated in birds than in mammals (Mittwoch, 1986).

1.3. The search for the testis determining factor

1.3.1. The sex determining region of the Y (SRY)

1.3.1.1. Studies that gave clues to the existence of SRY

Our understanding of mammalian sex determination derives from two pieces of information. In a series of experiments, some 40 to 50 years ago, Alfred Jost and

colleagues surgically removed the genital ridges from rabbit foetuses and then allowed them to develop to term. They found that if the genital ridges were removed before the onset of ovary/testis differentiation, male embryos failed to develop internal and external reproductive organs, while females developed normally. These results implied that the basic mammalian body plan is female, but the presence of testes determines whether the individual will develop as a male or a female (reviewed by Hawkins, 1994).

The second piece of information concerns the chromosomal basis of sex determination. In 1923, the existence of X and Y chromosomes in humans was demonstrated cytologically. Until 1959, it was assumed that sex in mammals, like the fruit fly *Drosophila melanogaster*, was decided by the number of X chromosomes. It was then discovered that human individuals and mice with a single X chromosome (e.g. Turner's syndrome) – instead of the normal two X chromosomes of females or the X and Y chromosome of males – develop as females. In contrast, individuals carrying a Y chromosome develop as males – regardless of the number of X chromosomes (e.g. Klinefelter's syndrome) (Page *et al*, 1987). These data proved that the presence of a Y chromosome in mammals is normally associated with development of testes in the embryo, leading to the formation of a male. These observations imply that the Y chromosome carries a gene required for testis development. In humans this gene was named the testis determining factor (TDF) because of the uncertain nature of the genetic material involved. In the mouse, however, it was called the testis determining Y gene (Tdy) (Hawkins, 1994). The classical view holds that this gene(s) may function by encoding or controlling the production of a specific determining factor, such as a protein, which acts to induce testicular differentiation. In the absence of the Y related gene, ovaries are formed and female characteristics develop (Hunter, 1995).

The Y chromosome has since been subjected to extensive genetic and molecular analysis in an attempt to find TDF. An early sequence thought to play a role in sex determination was the Bkm (banded krait mini satellite) repeat sequence (Jones & Singh, 1981). Although these sequences were found on the sex chromosomes of many mammals, they were poorly represented on the human Y chromosome. This left the stage open for the most enduring candidate, the male specific minor histocompatibility antigen, H-Y (Wachtel *et al*, 1975). For more than ten years, many believed that H-Y was the product of TDF. The finding of male mice and men with normal testicular

development but lacking H-Y antigen excluded the *Hya* gene as a possible candidate (Palmer, 1990).

The mapping of the testis-determining gene on the Y chromosome was aided by mutations that interfere with normal gonadal differentiation and reverse the sex of individuals, as in 46,XX males and 46,XY females.

By studying individuals such as XY females, who had deletions of their Y chromosomes, it was possible to localise TDF to the short arm of the Y chromosome. A more precise localisation was, however, made possible by examining the chromosomes of XX sex reversed males. These XX males appear to lack the Y chromosome but have a male phenotype with infertile testes. Sex reversed XX males arise from an abnormal recombination event that occurs during meiosis when the X and Y chromosomes pair. Ordinarily, an obligate recombination event occurs within the homologous regions on the tips of the short arms of the X and Y chromosomes. The recombination is restricted to this pseudoautosomal region (PAR) to maintain the chromosomal basis of sex determination. However, the recombination event occasionally extends beyond this region into the Y-specific sequences and results in transfer of TDF to the X chromosome (Gubbay *et al*, 1990; Hunter, 1995).

It was these sex reversed individuals that led David Page, in 1987, to his close encounter with the TDF. He described an XX male with the smallest segment of the Y then known, just 280kb of DNA proximal to the pseudoautosomal boundary. Of this, 140kb overlapped with a deletion in a sex reversed XY female. Within this 140kb overlap a new exon encoding a domain of 13 zinc-finger motifs, was found and called ZFY for zinc finger on the Y chromosome (Page *et al*, 1987). However, the candidate gene, ZFY, fell out of favour with the discovery of a number of facts thought to be incompatible with the characteristics of a 'testis determining' gene. Chief among these was the finding of DNA from three XX males and one true hermaphrodite that lacked ZFY and yet who had Y chromosome specific DNA sequences mapping to the region very close to the pseudoautosomal boundary border. It was also shown that, although *Zfy* was active in the mouse at the right time, it was in the incorrect cell type (Palmer *et al*, 1989; Cherfas, 1991; Mittwoch, 1992).

1.3.1.2. The precise mapping of SRY

In 1990 the hunt for the ultimate genetic source of masculinity was over. Scientists announced that the 30 year search for the gene that switches mammalian development from its usual destination – the female – to the male had ended (Cherfas, 1991). When ZFY was excluded as a candidate for TDF, Sinclair *et al* (1990) conducted a more detailed search for breakpoints in the three XX males and one hermaphrodite. They found that the smallest portion of the Y chromosome DNA able to induce maleness extended 35kbs adjacent to the pseudoautosomal boundary (Figure 1.3.). It was within this small region that the human Y located gene SRY (sex-determining region of the Y chromosome) was found. At the same time, a corresponding sequence, *Sry*, was cloned from the mouse Y (Gubbay *et al*, 1990).

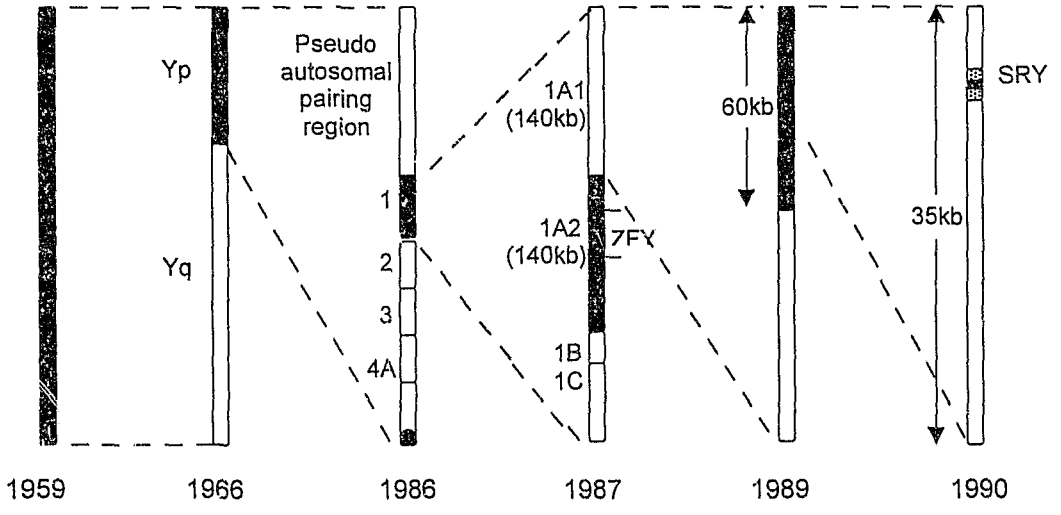


Figure 1.3. The hunt for the testis determining factor from 1959 when the Y chromosome was shown to be male determining to 1990 and the identification of the sex-determining region SRY (McLaren, 1991).

Three lines of evidence indicate that the SRY gene is necessary and sufficient for male sex determination and is the TDF:

i. The SRY gene maps to the smallest region of the Y chromosome known to be male determining in humans and in the mouse; namely, the 35kb interval just proximal to the PAR of the Y chromosome (Cherfas, 1991);

ii. XY females with gonadal dysgenesis and with the Y chromosome intact have mutations in the SRY gene (Schmitt-Ney *et al*, 1995);

iii. In a remarkable set of experiments, with transgenic mice, the final proof that SRY was in fact TDF was obtained. These mice contained the normal female XX chromosomes in addition to 14kb of Y chromosome bearing the *Sry* gene. The gene was found to induce testicular differentiation and subsequent male development in three out of eleven transgenic XX mice (Koopman *et al*, 1991). In contrast, XY mice differing from normal XY males only by the deletion of 11kb of Y chromosome DNA, including the *Sry* gene, developed as phenotypic females. These XY female mice are fertile and can transmit the mutant Y chromosome to their offspring (Lovell-Badge & Robertson, 1990; Gubbay *et al*, 1992).

The candidacy of *Sry* was further strengthened by the observation that it was expressed in the developing mouse gonad at the expected time for testis determination and it did not require the presence of germ cells (Koopman *et al*, 1990).

1.3.1.3. The molecular genetics of SRY and its role in sex determination

Clues to the mechanism by which SRY/*Sry* causes testis determination have come from the direct study of SRY sequences and the analysis of *Sry* expression in the mouse. SRY, about 1kb in size, encodes a protein that comprises a 56 amino acid N-terminal domain, a 79 amino acid central domain and a 68 amino acid C-terminal domain (Gubbay *et al*, 1990; Sinclair *et al*, 1990). The 79 amino acid motif has homology with the DNA-binding motif of high mobility group (HMG) proteins (and is thus referred to as the HMG-box), a human RNA *polymerase* I transcription factor, and also to the Ma protein of the yeast *Schizosaccharomyces pombe*, known to be involved in yeast mating type and meiosis (reviewed by Palmer, 1990; Ramkissoon & Goodfellow, 1996). Sequence analyses of SRY proteins from a variety of mammals indicate that there is little sequence similarity, even among primate species, outside the DNA-binding HMG

domain. Within the DNA-binding domain, however, sequence identity is greater than 60% (reviewed by Gahmberg & Tolvanen, 1996).

In vitro binding assays demonstrate that the SRY protein binds preferentially to the sequence A/TAACAAT via its HMG box (Harley *et al*, 1994). The SRY protein has been shown to bind to the minor groove of the double helix in a sequence specific manner and is predicted to bend the target DNA by at least 85º, and perhaps as much as 130º, by means of the concave surface of the α-helical HMG box (Nasrin *et al*, 1991; Giese *et al*, 1992). Thus it is possible that DNA bending is the sole mechanism by which SRY acts in sex determination. By bringing together distant DNA sequences or altering chromatin structure, SRY-induced bending might activate or repress target gene expression (reviewed by Bogan & Page, 1994).

1.4. The role of autosomal or X-linked loci in sex determination

The discovery that SRY on the Y chromosome is the switch, or rate-limiting step, that initiates the differentiation of the bipotential gonad into a testis promised to answer the age-old question about how mammalian sex is determined. In 1970 Ford wrote 'The male determining action of the human Y chromosome could be effected in either of two ways. It could, itself, be the bearer of genetic information necessary for the development of testes (and consequently masculine genitalia), or this information could be located on one or more different chromosomes and normally demand the presence of a Y chromosome for activation'.

Nearly thirty years down the line, however, it is still quite unclear how SRY acts to trigger testis development. SRY is believed to regulate downstream genes in the sex determination cascade, although no direct targets of SRY are clearly known (Capel, 1998).

1.4.1. Sex reversed individuals

The study of sex reversed individuals has played an extensive role in furthering our knowledge of genes involved in sex determination.

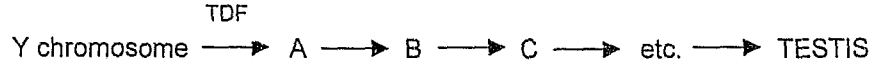
1.4.1.1. XX males

de la Chapelle et al (1964) first described males with a 46,XX female karyotype in 1964. This phenomenon of sex reversal that leads to the development of testes in individuals with a XX chromosome constitution has now been extensively documented and reviewed, even though the incidence of this condition is only about 1 in 20 000 new-born males (Vergnaud *et al*, 1986). XX males have small testes devoid of germ cells and they are therefore sterile. 46,XX males resemble patients with Klinefelter's syndrome in a number of ways, except that sex reversed males are shorter in stature (Hunter, 1995).

The genetic basis of XX sex reversed individuals:
Ferguson-Smith (1966) proposed that an abnormal crossover event was responsible for sex reversal in humans, accounting for both XX males and XY females. In other words, Y DNA positive XX males arise as a result of an asymmetrical exchange of the pseudoautosomal region plus SRY between the X and Y chromosomes during male meiosis (Tar *et al*, 1995).

The majority of XX males have Y chromosome material present and are referred to as Y(+) XX males. The remaining patients in which no Y chromosome specific DNA can be demonstrated are referred to as Y(-) XX males. The development of testes in the second class of XX males would seem to violate a fundamental principle of genetics. Several ideas have, however, been put forward to explain the aetiology of Y(-) XX males.

i. The existence of putative genes, downstream from SRY, that are able to trigger testis determination when they undergo a mutation. If the testis determining pathway is switched on (initiated) by a gene on the Y chromosome (TDF) and it proceeds through a cascade of steps involving autosomal or X-linked genes,



then, a constitutive mutation at any of these downstream loci would result in testicular development, even in the absence of the Y chromosome. For example, induction of gene A in the absence of the normally required signal from the Y.

Conversely, a deleterious mutation at any of these loci could block development of the testis, even when the Y chromosome is intact (Wachtel & Tiersch, 1994).

ii. Mosaicism has also been proposed, with a prevalent XX cell lineage and a scarce or hidden Y chromosome bearing lineage (Hunter, 1995).

iii. Many cases have been shown to result from the translocation of Y chromosome material containing the SRY gene to the X chromosome or one of the autosomes (Vergnaud *et al*, 1986).

1.4.1.2. XY females

According to Ohno (1967), the mammalian X and Y chromosomes were once a pair of homologous autosomes. The Y apparently lost most of its genetic material to become a specialised testis inducer. The X, on the other hand, remained more or less invariant. Traditionally, it has been assumed that the genes necessary for normal ovarian development were among those lost from the Y chromosome. When the Y chromosome, however, fails to induce testicular differentiation, the gonadal primordium initiates ovarian development. It can, therefore, be deduced that some of the genes necessary for ovarian development are retained. The result is a 46,XY dysgenetic gonad, a fibrous streak devoid of germ cells and follicles, that closely resembles the 45,XO streak gonad (reviewed by Hunter 1995).

The condition described above is called XY gonadal dysgenesis. The chief signs in 46,XY gonadal dysgenesis are delayed puberty and primary amenorrhea. A striking feature that distinguishes XY gonadal dysgenesis from 45,XO Turner syndrome is the high incidence of gonadal tumours – especially gonadoblastoma and dysgerminoma. The external genitalia are female but the clitoris may be enlarged in some cases.

The genetic basis of XY gonadal dysgenesis:

The most obvious explanation for the development of the XY streak gonad is deletion or mutation within the Y-linked testis determining gene. A total of 33 SRY mutations have been described that account for only 10-15% of XY gonadal dysgenesis females. A further 10-15% of these cases result from deletion of SRY due to aberrant X/Y interchange. The aetiology of the remaining 70-80% of XY gonadal dysgenesis cases is still enigmatic (Scherer *et al*, 1998). Possible explanations for these XY sex reversed cases are discussed below.

The most trivial explanation is that the underlying SRY mutation has been missed as researchers have focused their attention on the HMG domain of SRY. Of the 33 SRY mutations that have been identified, only three were not located in this region. In addition, the technique used in most instances to detect these SRY mutations was SSCP analysis which, at best, detects 90% of point mutations in a PCR fragment. These two sources of error, however, are not likely to be responsible for the high percentage of unexplained XY sex reversal cases (Scherer *et al*, 1998).

A more significant factor that may be resulting in a decreased prevalence rate of SRY mutations would be the inclusion of 46,XY females who may not have been cases of gonadal dysgenesis. If only XY gonadal dysgenesis cases that had been anatomically and histologically confirmed were studied, the 10-15% frequency quoted for SRY mutations causing sex reversal may be higher (Hawkins *et al*, 1992; Scherer *et al*, 1998).

It has also been postulated that mutations in sequences flanking SRY, and essential for its correct expression, are major causes of XY sex reversal. However, only two variant base substitutions and a point mutation were identified in 142 individuals with complete or partial XY gonadal dysgenesis. It would thus appear that mutations up or downstream of SRY are not a major cause of XY sex reversal (Schmitt-Ney *et al*, 1995; Kwok *et al*, 1996; Veitia *et al*, 1997; Poulat *et al*, 1998).

From the above information it is evident that the majority of XY gonadal dysgenesis cases will not be explained by mutations at the SRY locus but rather by mutations occurring in genes elsewhere in the genome that are suspected to cause XY sex reversal (Scherer *et al*, 1998). Some of these loci will be discussed in the proceeding section.

1.4.2. Loci involved in XY male to female sex reversal

1.4.2.1. Autosomal loci

Several autosomal loci in man are known to be associated with sex reversal, including deletion of the short arm of chromosome 9, deletion of the long arm of chromosome 10,

translocations involving the long arm of chromosome 17, and partial duplications of chromosome 22.

1.4.2.1.1. Deletions of chromosome 9p

A number of subjects, reported in the literature, with a Y chromosome and a terminal deletion of the short arm of chromosome 9, show sex reversal. All of these cases, however, involve translocations of chromosome 9 with other autosomes. Patients were therefore trisomic for part of one chromosome and monosomic for a region on the short arm of chromosome 9 (Bennet *et al*, 1993). Although these patients exhibited sex reversal, this could not be assigned to the deletion of chromosome 9 due to the presence of the other chromosomal anomaly. Patients have, however, been described with deletions of the distal short arm of chromosome 9 and ambiguous genitalia (Flejter *et al*, 1998). A number of cases of deletion of 9p in association with gonadal dysgenesis and sex reversal have also been reported (Bennett *et al*, 1993; Raymond *et al*, 1999). The latter cases suggest a role for chromosome 9 in testicular development. The interval containing this putative testis-determining gene(s) has been narrowed to the distal portion of 9p24.3 (Flejter *et al*, 1998).

1.4.2.1.2. Deletions of chromosome 10q

Although over 20 cases with terminal deletions of the long arm of chromosome 10 have been described, many of these have had additional chromosomal abnormalities. The number of XY sex reversed individuals due only to terminal deletions of chromosome 10 is small. The eight cases that do fall into this category, however, all show some form of abnormal genitalia (reviewed by Wilkie *et al*, 1993). It would, therefore, seem that this deletion is associated with abnormal sexual differentiation in the male, ranging from complete sex reversal to cryptorchidism. The molecular basis behind this variability is unclear.

Some homology is found between the distal portion of human chromosome 10 and chromosomes 7, 14 and 19 of the mouse. These loci have not yet, however, been shown to play a role in sex determination in the mouse. Nevertheless, the above findings implicate a gene on the long arm of chromosome 10 to be involved in the normal human male sex differentiation pathway (Wilkie *et al*, 1993).

1.4.2.1.3. Chromosome 17 and sex reversal

Translocations involving the long arm of chromosome 17 are associated with a severe dwarfism syndrome in humans termed Campomoelic dysplasia (CD). This disease is characterised by skeletal malformations that lead to death shortly after birth (Foster *et al*, 1994; reviewed by Marx, 1995). In addition, approximately 75% of XY individuals with CD, exhibit male to female sex reversal. The translocations in these patients were used to map the sex reversal locus, SRA1, linked with the CD locus, CMPD1, to the long arm of chromosome 17. An SRY related HMG-box containing gene, SOX, was identified as a candidate for both SRA1 and CMPD1 (da Silva *et al*, 1996). In all cases described so far, CD patients with or without sex reversal are heterozygous for mutations of this gene, SOX9. It therefore seems most likely that the phenotypes observed are due to haploid insufficiency rather than to dominant negative effects (reviewed by Ramkissoon and Goodfellow, 1996).

Mutation analysis gives no indication of how SOX9 fits into the testis determining pathway. It could be upstream of SRY, in a parallel pathway, or downstream. The expression of SOX9 during normal gonadal development in the mouse and chick has now been studied in detail. In mice, from about 9.5 to 10.0 dpc, each gonad begins to develop as a ridge of distinct cells. The genital ridges of XY and XX embryos are morphologically indistinguishable up until 11.5 dpc (Koopman *et al*, 1990). The genital ridges are comprised of primordial germ cells and one or more somatic cell types which give rise to supporting cells and steroidogenic cells. Other lineages of the gonad migrate into the genital ridge after 11.5 days, which coincides with the first morphological differences between the testis and ovary (Ginsburg *et al*, 1990).

Sox9 is expressed in the genital ridge of both XX and XY embryos from about 10.5 dpc. At the same time, Sry expression is only just being activated in XY embryos. By 11.5 dpc, Sox9 expression is very abundant in XY embryos but is absent from the genital ridges of XX embryos. At 13.5 dpc Sox9 transcripts are found exclusively within Sertoli cells (da Silva *et al*, 1996). This suggests a role for SOX9 in Sertoli cell differentiation and in the maintenance of Sertoli cell function.

da Silva *et al* (1996) also showed that Sox9 is expressed in male and female mice prior to SRY. However, with the onset of Sry transcription, Sox9 expression is down regulated in the genital ridges of XX embryos and up regulated in XY genital ridges.

More recent evidence suggests that duplication of SOX9 can cause XX sex reversal. Cytogenetic analysis of a new born, referred due to ambiguous genitalia, revealed a mosaic 46,XX,dup(17)(q23.1q24.3)/46,XX karyotype. Fluorescent *in situ* hybridisation with a clone containing the SOX9 gene showed it to be duplicated on the rearranged chromosome 17. The presence of SRY was ruled out by PCR and fluorescent *in situ* hybridisation. Thus, it would seem that individuals with a single copy of SOX9 exhibit XY sex reversal whereas three copies of the gene appear to be sufficient to initiate testis differentiation resulting in XX sex reversal (Huang *et al*, 1999).

1.4.2.1.4. Partial duplication of chromosome 22

The most recent autosome to be implicated in the aetiology of true hermaphroditism is chromosome 22. Aleck *et al* (1999) reported a true hermaphrodite that showed no evidence of Y chromosome material upon examination of DNA from blood, fibroblast lines from the ovarian and testicular gonads and DNA extracted from the right paraffin-embedded testis. The patient was, however, found to have an inverted duplication of the long arm of chromosome 22 from band q13.1 to the telomere and a deletion of the short arm from band p13 to the telomere. There have been other reports in the literature of intersex patients that involve chromosome 22 (Kim *et al*, 1977; Nicholl *et al*, 1994), but none have been diagnosed as true hermaphrodites until this case.

1.4.2.2. X-linked loci

A strong candidate that may explain some of the other causes of XY sex reversal is a gene called DAX1 (DSS-AHC critical region on the X chromosome, gene 1). Mutations in DAX1 lead to X-linked adrenal hypoplasia congenita (AHC) and hypogonadotropic hypogonadism (HH), a disorder in which the adrenal glands fail to develop normally (Muscatelli *et al*, 1994; Abe *et al*, 1999). DAX1 maps to a region on the X chromosome, Xp21, that has been previously shown to result in male to female sex reversal when present in duplicate (Bardoni *et al*, 1994). This locus was termed dosage sensitive sex reversal, DSS. The identification of XY male individuals deleted for DSS and, XY individuals with a duplication of Xp21 and an intact SRY gene that show male to female

sex reversal, suggest that this locus is not required for testis differentiation, but that it plays a role in ovarian development and/or functions as a link between ovary and testis formation (Bardoni *et al*. 1994).

The DAX1 gene encodes a member of the nuclear hormone receptor superfamily and displays a novel DNA-binding domain. In the human male, disruption of DAX1 does not prevent the initial stages of gonadal development but causes defects in adrenal cortex function. Cells of the adrenal cortex originate from the genital ridge (Swain *et al*, 1998). This suggests that DAX1 plays a role later in development than steroidogenic factor-1 (SF1, a member of the nuclear hormone receptor superfamily) which when disrupted prevents the development of adrenal glands and gonads (Muscatelli *et al*, 1994).

Studies by Swain *et al* (1996) show that the onset of Dax1 expression in XX and XY genital ridges, in mice, occurs at the same time as Sry in XY genital ridges. After 12.5 dpc, when Sry expression disappears, Dax1 expression decreases dramatically in the testis while it persists in the ovary. This suggests that Dax1 could be involved in sex determination where its prolonged expression is incompatible with testis development. Further evidence for the role of Dax1 in sex determination may be provided by transgenic experiments in which XY mice carry an extra copy of Dax1 (reviewed by Ramkissoon & Goodfellow, 1996).

These XY mice show delayed testis development when the gene is expressed at high levels, but do not normally show sex reversal. Complete sex reversal can result when weak alleles of Sry are tested against the transgene. This implies that Dax1 and Sry act antagonistically towards each other, with increased expression of Dax1 leading to female development and increased activity of Sry to male development. These results show that Dax1 is largely, if not solely, responsible for dosage sensitive sex reversal and provides a model for early events in mammalian sex determination, when precise levels and timing of gene expression are critical (Swain *et al*, 1998).

According to Ohno's Law, a gene that is X-linked in one mammalian species is likely to be X-linked in another. It may therefore be of interest to mention here the unusual mode of sex determination in the wood lemming, *Myopus schisticolor*, a small mouselike rodent. This animal has a remarkably skewed sex ratio of 1:4 (male:f

There are two kinds of female in this species – one produces male and female offspring, and the other produces only female offspring. The two kinds of female are phenotypically indistinguishable, but the former has a 32, XX karyotype, and the latter, a 32, XY karyotype. The female Y is the same as that of the male. In fact, it is the X that is rearranged (Wachtel, 1998). Two classes of X chromosome have been revealed by G-banding in this species: one (denoted X^*) is found in XY females (X^*Y), the other (denoted X) is found in XY males. This indicates occurrence of an X-linked gene, the mutation (rearrangement) of which blocks testicular differentiation and thereby leads to development of the XY ovary, which, in contrast to the situation in humans, is usually fertile in rodents (Lau *et al*, 1992).

1.5. Target genes involved in mammalian sex determination

In the fruitfly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, the male or female phenotype is specified by the sex chromosomes. However, most of the genes involved in sex determination in these organisms, are located on the autosomes (reviewed by Hodgkin, 1990; Parkhurst & Meneely, 1994). It is supposed that the same is likely true of mammals. Human XY sex reversed females in whom SRY appears to be intact support this supposition. It is, therefore, expected that mutations in autosomal or X-linked sex determining genes are involved. Similarly, although only about 30% of XX sex reversed males have no detectable portion of the Y chromosome, it is also suspected that autosomal or X-linked mutations result in the observed phenotype. These autosomal or X-linked genes may be upstream or downstream of SRY and may be on any chromosome except the Y. Genetic evidence indicates the presence of several genes involved in sex determination (Bogan & Page, 1994; reviewed by Hawkins, 1994).

If we are to understand how SRY functions to initiate testis differentiation, we need to identify the target genes it regulates. One problem is that it is unknown whether SRY acts as an activator, repressor or both (Lovell-Badge & Hacker, 1995).

1.5.1. The Müllerian inhibiting substance gene

One such target gene is MIS, a transforming growth factor β-like hormone that causes the breakdown of the Müllerian duct (Cate *et al*, 1986). MIS has been suggested as a possible target gene because of the function it performs in development of the male phenotype, and due to the fact that expression of MIS overlaps with that of SRY (Hacker *et al*, 1995). There is, however, a gap of approximately 22 to 48 hours between the onset of Sry and Mis expression (Münsterberg & Lovell-Badge, 1991). Thus, the possibility exists that one other regulatory step may be interposed. Even though MIS acts downstream of SRY in sex differentiation, it appears to have no critical function in testis determination as revealed by mutation studies in mice (Behringer *et al*, 1994) and humans (Knebelmann *et al*, 1991). It is, however, still important to study the gene's regulation. It has been suggested that another transcription factor, called steroidogenic factor 1 (SF1) may be responsible for the expression of the MIS gene (Luo *et al*, 1994; Shen *et al*, 1994).

1.5.2. The steroidogenic factor 1 gene

SF1 is an orphan nuclear receptor gene that plays a key role in regulating steroid hydroxylases and aromatases. The finding, by Shen *et al* (1994) that SF1 is expressed in Sertoli cells during embryogenesis suggests that it plays a broader role in gonadal development by regulating downstream target genes other than those involved in steroidogenesis. Examination of the expression of SF1 has revealed that, in addition to all primary steroidogenic tissue, it is also expressed in male and female urogenital ridges during embryonic development. This is the site at which the gonads will later develop (Ikeda *et al*, 1994). At 12.5 dpc when the bipotential gonad differentiates into an ovary or a testis, expression of Sf1 is extinguished in females, however, in males, expression persists in the Leydig and Sertoli cells of the testes. Evidence suggesting that SF1 has an important role in gonadal development (differentiation of the bipotential gonad into either ovary or testis), became apparent upon examination of mice in which the autosomal Ftz-f1 gene had been disrupted. This gene encodes two protein products, one of which is Sf1 (Lou *et al*, 1994). Analysis of the Sf1 knockout mice revealed the failure of adrenal glands and gonadal development in both male and female mice. Furthermore, both male and female mice have normal female internal

genitalia (Bogan & Page, 1994). The persistence of female genitalia in male mice lacking Sf1 is consistent with the proposal that Sf1 directly regulates MIS expression (Shen *et al*, 1994).

Spatial and temporal studies confirm that there is strong concordance between the expression of SF1 and that of MIS in the developing testes. It is still unclear how the expression of SRY in males is linked to SF1 regulation. Although it has been shown that SRY binds to, and bends, DNA in a sequence-specific manner (Harley *et al*, 1992), it does not contain any recognisable transactivation domain, suggesting that SRY may control gene expression by juxtaposing regulatory elements, rather than by transcriptional activation. The expression of SF1 may be maintained by the presence of SRY in the male genital ridge at the moment of differentiation. Or, SRY may be responsible for repressing a repressor of SF1 that is expressed at the time of gonadal differentiation (McElreavey *et al*, 1993), resulting in the lack of SF1 expression in the female genital ridge.

The involvement of SF1 in sex determination is further enhanced by the identification of a phenotypically female patient with a heterozygous FTZF1 mutation. This patient presented with primary adrenal failure in the first two weeks of life (Achermann *et al*, 1999). The mutation occurs in exon 3 of the FTZF1 gene which encodes part of the DNA-binding domain of SF1. This region is critical for the recognition of DNA binding sites and confers specificity to nuclear receptors in the regulation of target genes. This SF1 mutation causes complex XY sex reversal, including normal female external genitalia and retention of the uterus. These findings provide evidence that SF1 regulates the regression of Müllerian structures in humans, either through direct actions on the MIS promoter or secondary to an abnormality of Sertoli cell development or function. The presence of immature tubules in the gonad is consistent with SRY being the primary testis determining gene in mammals. The incomplete gonadal development observed here, however, suggests that SF1, and the genes that it regulates, are necessary for subsequent male sexual differentiation (Achermann *et al*, 1999).

1.5.3. The Wilm's tumour 1 gene

The arrest of gonadal development in SF1 deficient mice is reminiscent of the gonadal phenotype observed in Wilm's tumour (WT1) deficient mice (Kreidberg *et al*, 1993). The WT1 gene was originally isolated as an oncogene involved in the childhood kidney cancer, Wilm's tumour. Evidence that WT1 has a role in gonad formation comes from the following discoveries: first, the incidence of hypospadias and cryptorchidism is about ten times greater in patients with bilateral Wilm's tumour than in the general population. Second, high levels of WT1 mRNA expression are observed in the developing gonads early in foetal development. Thirdly, heterozygous mutations of WT1 in patients with Denys-Drash syndrome (Wilm's tumour in association with renal failure, gonadal and genital abnormalities) have been identified. In addition, WT1 is expressed in the mouse gonadal ridge at 9dpc (Pelletier *et al*, 1991). These results imply a central role for the WT1 gene product in genital system development.

The onset of SF1 and WT1 expression at an early stage in gonadal development, together with similar knockout phenotypes, suggests that both these zinc-finger containing proteins are active in a common pathway upstream of *Sry*, perhaps in the commitment and maintenance of the bipotential gonad (Ramkissoon & Goodfellow, 1996). Figure 1.4 illustrates where all of the above mentioned genes are believed to act in the sex determining pathway.

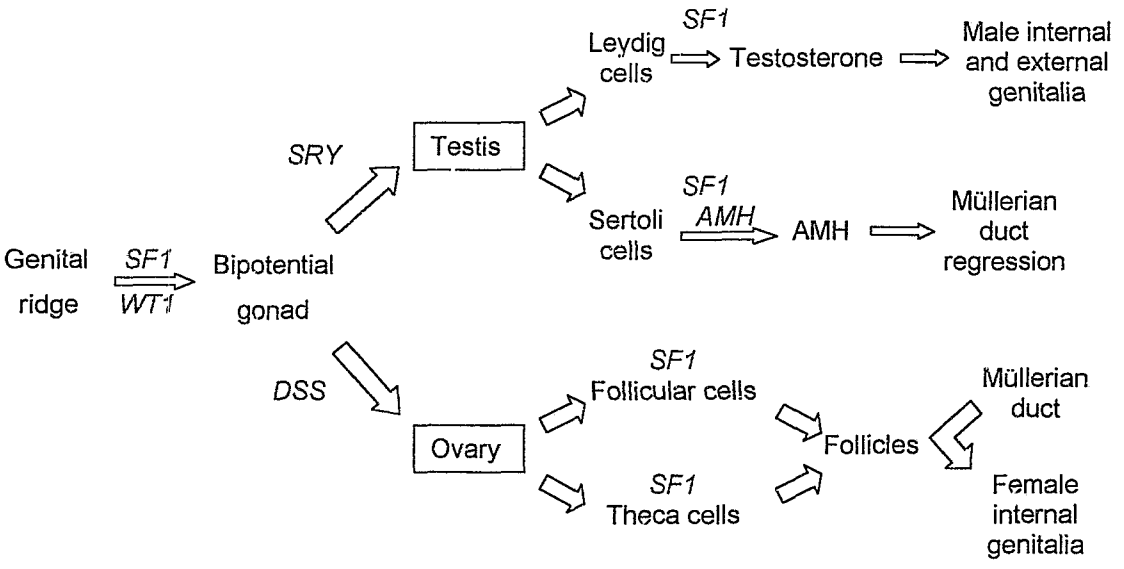


Figure 1.4. Gonadal development and sex determination. This figure illustrates the early steps in mammalian sex determination and the genes and proteins implicated at each stage (Ramkissoon & Goodfellow, 1996).

1.6. Models for mammalian sex determination

The molecular mechanisms by which SRY triggers testis development are unknown. It is possible for SRY to act as either an activator or repressor of the transcription of target genes. Current knowledge is inconclusive and sometimes contradictory. Genetic models (based on either of the two possibilities) for mammalian sex determination have been proposed.

1.6.1. The SOX model

Since haploinsufficiency of SOX9 promotes ovarian rather than testicular differentiation, it has been proposed that inhibition of SOX9 in the genital ridge must therefore suppress testis formation and lead to a female phenotype. Because the presence of

SRY promotes testis formation, a highly conserved gene, SOX3, has been suggested to provide the link between SRY and SOX9 (reviewed by Marshall Graves, 1998). Sox9 is expressed in the gonads of both XX and XY mice at 10.5dpc. At the onset of Sry expression, Sox9 is upregulated in XY embryos, and remains level or decreases in XX embryos. Sox3 is expressed at a low level in the gonad up to 11.5dpc, as is Sry. These patterns are compatible with competitive inhibition of Sox3 protein by Sry protein in binding to and regulating transcription of the Sox9 gene. In XX embryos, Sox3 protein binds to and inhibits Sox9 transcription. In XY embryos, Sry protein would compete with Sox3 binding and relieve this inhibition, permitting transcription of Sox9 and initiating testis differentiation (reviewed by Marshall Graves, 1998).

One of the more recent models proposed by McElreavey *et al* (1993) suggest that an autosomal testis repressor exists (the Z gene) that is active in females but that is itself repressed by SRY in males during sex determination.

1.6.2. The Z gene model

The Z gene model implies that a gene(s) other than SRY directly controls target genes in the male pathway, a view contradicted by the more usually accepted hypothesis that SRY directly activates a testis-determining cascade. This autosomal locus, Z, that confers male phenotypes when both copies of the recessive allele are present, even when SRY is absent, was the simplest way to interpret three pedigrees where the parents exhibited normal phenotypes, but in each family, two of the children were XX sex reversed males or 46,XX true hermaphrodites (McElreavey *et al*, 1993).

To explain the genetic observations – that SRY is not needed for male sex determination in the absence of Z – SRY has been proposed to antagonise or repress Z. In normal XX females, Z negatively regulates the expression of male specific genes. In normal XY males, SRY represses synthesis of Z or inhibits Z activity, thereby allowing expression of male specific genes, leading to testis development and a male phenotype. In XX patients who lack Z due to a mutation, inhibition of male specific genes does not occur, and, therefore, these XX individuals exhibit a male phenotype (McElreavey *et al*, 1993). XY individuals exhibiting a female phenotype can be explained in terms of a dominant mutation in Z, Z^i, which is insensitive to SRY action.

McElreavey *et al* (1993) proposed the existence of several 'leaky' Z mutants that conserve some residual activity, to explain the variability in sex phenotypes among XX sex reversed individuals (Figure 1.5).

1.6.2.1. A proposed cascade for sex determination

We have proposed a possible cascade of events, initiated by SRY, which results in testis formation. Included in this cascade is SOX9 (an autosomal gene that causes sex reversal in individuals with CD), a hypothetical negative regulator of male development, Z (McElreavey *et al*, 1993), and SF1 which binds to the MIS promoter and thus activates MIS. The proposed sequence of events in a normal XY male, are as follows: the expression of SRY triggers the cascade by activating a downstream gene, SOX9. SOX9, in turn, represses Z which (directly or indirectly) inhibits SF1 expression. In the absence of Z, SF1 expression is induced and hence MIS is activated. This results in regression of the Müllerian ducts and consequent formation of the testis (Figure 1.6).

1.6.3. The DSS-gene model

Jimenez *et al* (1996) propose that a single active copy of DSS may function to silence male pathway genes (MPGs) in normal XX females, permitting the induction of the ovarian pathway. This DSS gene model proposes that SRY inhibits the action of the copy of DSS present in XY individuals, thus permitting testis differentiation. This implies that the Z and DSS genes may be identical, and that Z is not autosomal as originally proposed, but X-linked as is DSS. A single copy of SRY, however, may be unable to counteract the effect of two active copies of DSS, so that male to female sex reversal would occur, as observed in humans with duplications of Xp21.

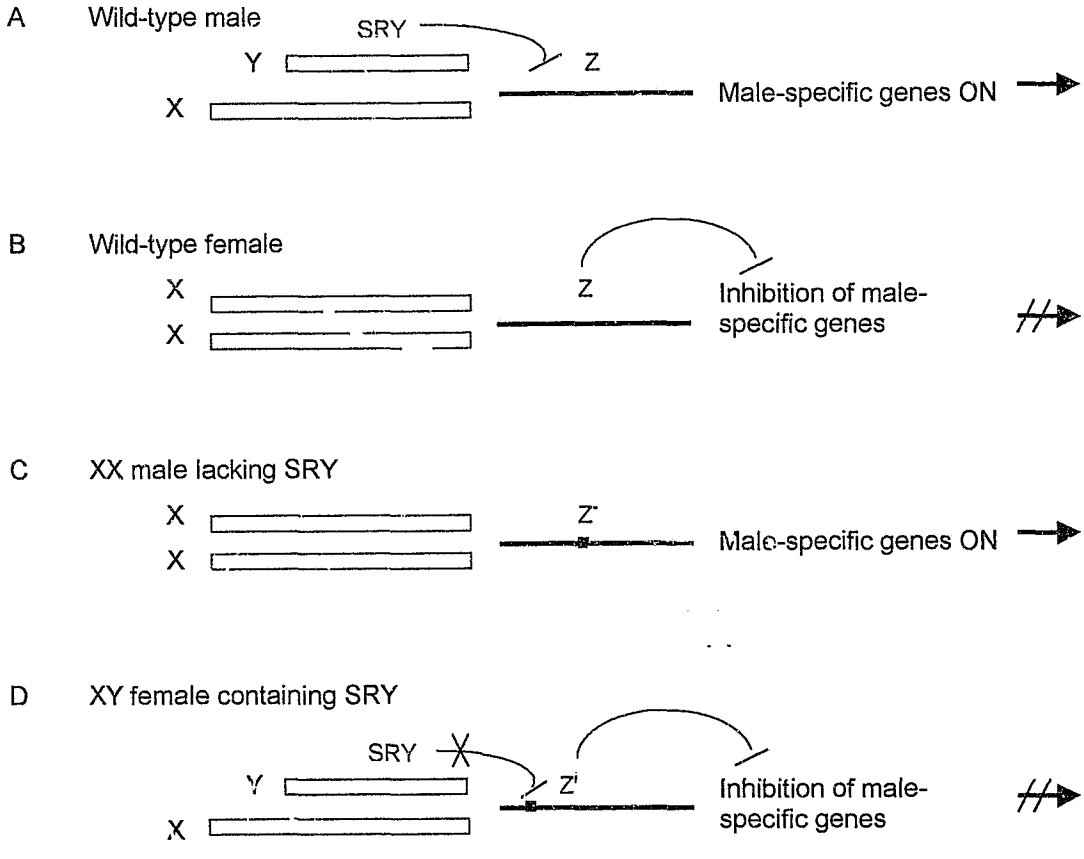


Figure 1.5. Z model regulatory cascade hypothesis for mammalian sex determination and an explanation for sex reversal in XX SRY^- and XY SRY^+ individuals. A. In normal XY males, SRY inhibits or represses synthesis of Z. This allows expression of male-specific genes and hence testis development and a male phenotype. B. In normal XX females, who lack SRY Z inhibits male sex determination and female sex determination can therefore proceed. C. In XX patients who lack Z due to a homozygous mutation, Z^- (■), inhibition of male-specific genes does not occur and they therefore exhibit a male phenotype. D. XY individuals with an intact SRY gene can exhibit a female phenotype if they carry a mutation in Z, Z^i (■), that is insensitive to SRY action (McElreavey *et al*, 1993).

As can be seen in Figure 1.7, SF1 and WT1 induce the formation of the bipotential gonads in the embryos of either sex chromosome constitution. In XY individuals SRY suppresses the repression by DAX1 of SF1. As a result, SF1 is able to transactivate MPGs, including SOX9 and AMH. The resultant differentiation of the testes leads to inhibition of the female pathway genes (FPGs) and hormonal induction of the male phenotype. In XX individuals, the active copy of DAX1 represses SF1 and, therefore, this gene cannot activate the MPGs. Later in development, activation of FPGs leads to the development of ovaries and the consequent hormonal induction of the female phenotype. Functional oocytes may be necessary to inhibit MPGs, thus precluding transdifferentiation of the ovarian tissue into testicular tissue as a consequence of late activation of MPGs (Jimenez & Burgos, 1998).

Evidence for the involvement of autosomal and X-linked genes in several sex reversed syndromes in humans and other mammals suggests that the pathway is more complex than the models described above and involves genes that have functions other than sex determination. This complexity and pleiomorphism should not be surprising by analogy with the sex determination pathways of *Drosophila melanogaster* and *Caenorhabditis elegans*. Indeed, it would be surprising if mammalian sex determination were really as simple as it was naively supposed a decade ago (reviewed by Marshall Graves, 1998).

SRY - activator

SOX9 - repressor

- repressor

SF1 – activator of MIS

MIS - mediates regression of the female reproductive tract, the Müllerian ducts

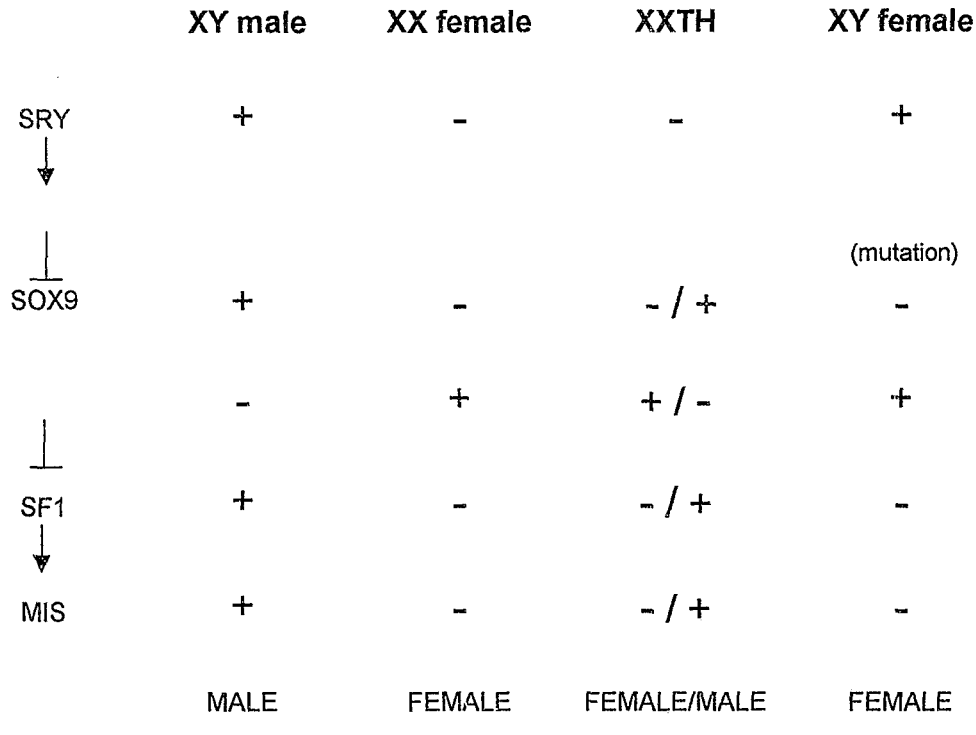


Figure 1.6. Schematic representation of the possible cascade of events that result in testis formation.

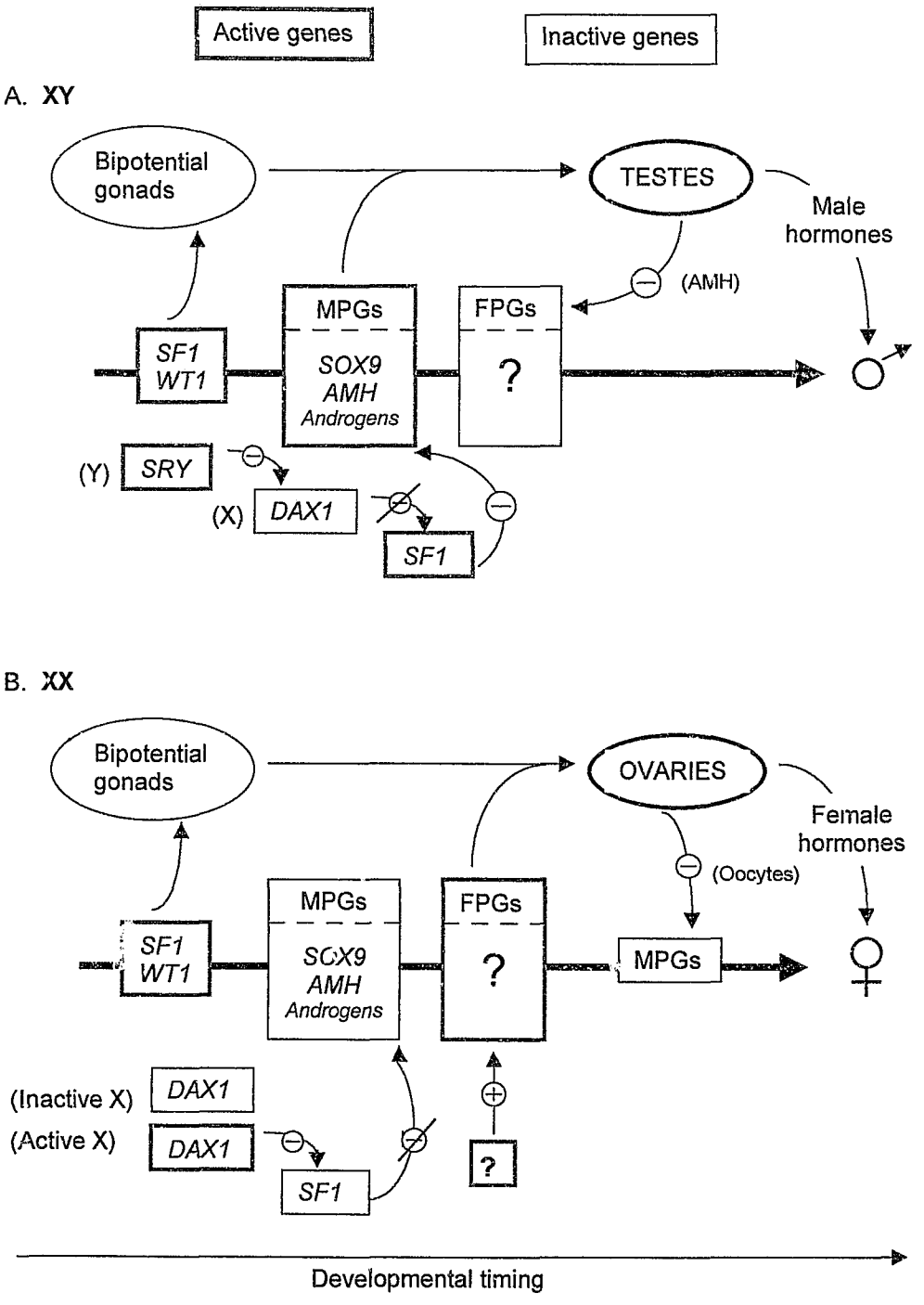


Figure 1.7. An updated model for mammalian sex determination based on the Z/DSS Model (Jimenez & Burgos, 1998).

1.7. An hypothesis on the molecular aetiology of XX true hermaphroditism

1.7.1. The X chromosome inactivation theory

In 1961, Mary Lyon postulated that dosage compensation for X-linked genes in mammals is accomplished by inactivating one of the two X chromosomes in females. X-inactivation occurs early in embryogenesis, at the 64 to 100 cell stage. It is thought to be a stochastic event; that is, each cell is equally likely to inactivate either the maternal or paternal chromosome. Neighbouring cells do not influence the chromosome 'choice'. Therefore, in normal females, approximately 50% of the cells transcribe genes on the maternal X chromosome (Schmidt & Du Sart, 1992). Some loci on the inactivate X chromosome are, however, transcribed. This late-replicating chromosome is relatively condensed during interphase and is visible as a Barr body at the nuclear membrane (Klug & Cummings, 1991; reviewed by Migeon, 1994).

The process of X-inactivation involves a regulatory gene, Xist (X-inactivation specific transcript). It is now known to represent the master switch locus regulating X-inactivation. Xist maps to the X-inactivation centre (Xic) and is transcribed exclusively from the inactive X chromosome in female somatic cells and at the time of X-inactivation during spermatogenesis in the male (Goto *et al*, 1998). The RNA remains in the nucleus and coats the chromosome *in cis*.

X chromosome inactivation is a complex developmental process that is likely to involve a variety of molecular mechanisms. In addition to the mechanisms that initiate inactivation, other mechanisms act to maintain it with high fidelity during cell division. Although several models have been proposed to explain the mechanism of X inactivation at a molecular level, experimental evidence in support of these theories is lacking (Mohandas *et al*, 1981).

An excellent candidate, however, for the mechanism that silences genes on the inactive X chromosome is DNA methylation. CpG islands on the active X are unmethylated whereas those on the inactive X are extensively methylated (Mohandas *et al*, 1981).

We thus propose that testis formation can occur in an individual with a 46,XX karyotype if a gene necessary for sex determination is mutated and is present and on the active X chromosome. The normal copy of the gene is found on the inactive methylated X chromosome and is therefore not expressed. Our hypothesis is thus based on whether X chromosome inactivation in the testicular and ovarian tissue of the gonads is random or non-random in XX true hermaphrodites. Non-random inactivation of either the maternal or paternal allele in either the testis or ovary would suggest that there is a gene(s) on the X chromosome which plays a role in testicular development in XX true hermaphroditism.

The basis of the X inactivation assay lies in the fact that one of the two X chromosomes in every female cell becomes inactivated in early foetal development. This is a random process that results in a mosaic pattern of X inactivation. The active and inactive X chromosomes may be distinguished from one another, in this case, on the basis of methylation. This X chromosome inactivation hypothesis can be assessed by examination of the methylation status of the CpG region near the polymorphic androgen receptor element (ARE) gene on the X chromosome.

The method relies on methylation-sensitive restriction enzyme digestion and PCR. Methylated DNA sequences i.e., inactive X chromosomes, are selectively amplified following digestion with *Hpa*II. This assay is able to distinguish between the paternal and maternal alleles and to identify their methylation status. Thus it is possible to determine whether there is random or non-random inactivation of the X chromosomes in XXTH subjects.

1.8. Aims of the study

The aims of the study were:

1. To determine the incidence of XX true hermaphroditism in the South African black population.

2. To determine whether X-inactivation is random or non-random in ovarian and testicular tissue of XXTH individuals.

3. To screen XXTH individuals for mutations in the DAX1 gene using SSCP analysis and sequencing of variant bands.
4. To screen XXTH individuals for deletions in their SOX9 genes, and dosage studies, by means of Southern blotting.

5. To test for the presence of a Y-bearing cell line in DNA extracted from gonadal tissue as well as from blood lymphocytes of the XXTH patients in an attempt to explain the aetiology observed.

CHAPTER 2

2. SUBJECTS AND METHODS

All reagents, suppliers and solutions are listed in Appendix 1

2.1. Subjects

The subjects used in this study can be divided into two groups:

i. Normal, healthy, random black individuals
ii. Confirmed and query black true hermaphrodites

2.1.1. Control samples

These samples consisted of between 8 to 12 healthy, black male and female individuals chosen at random from DNA samples that had been previously extracted, and were readily available, in the laboratory. Control individuals were included in every assay that was performed in this study.

2.1.2. Histologically confirmed XX true hermaphrodite patients

A total of 41 patients were used for this study. Detailed information on karyotype analysis and histology is given in section 3.1. The diagnosis of hermaphroditism had been confirmed in 34 patients with histopathology reports illustrating the presence of both ovarian and testicular tissue. The remaining seven clinically suspected patients had not been confirmed on histopathology. DNA samples were readily available for the majority of the affected patients and their family members, as research into the aetiology of XX true hermaphroditism has been an ongoing project in the department for many years. The samples were predominantly received from the Endocrine clinic and Paediatric ward at Chris Hani-Baragwanath hospital or from the genetic counselling clinic run by the Department of Human Genetics. In addition, paraffin-embedded blocks of gonadal tissue were available for a subset of the confirmed hermaphrodites. These

samples were obtained from the Histopathology Department at Chris Hani-Baragwanath hospital. During the course of my research, additional samples from patients with ambiguous genitalia, intersex or hermaphroditism were received from various hospitals and clinics in South Africa. These samples were batched and the DNA extracted when there were sufficient numbers.

Patients not wishing to participate in this research project were not, as a result, denied any kind of medical attention that they would have otherwise received. There was no cost incurred by those patients who made themselves available for this study and patient confidentiality was ensured.

2.1.3. An estimated incidence of XX true hermaphroditism in the South African black population

Because XX true hermaphroditism appears to be very common in the South African black population, we set out to estimate the incidence of this disorder in the black population. Information was gathered from two sources, namely:

i. The Chris Hani-Baragwanath hospital birth defects database. This database keeps a record of all patients born at the hospital with some form of congenital abnormality.
ii. Individuals that had been referred to the South African Institute for Medical Research (SAIMR) Cytogenetics laboratory for karyotyping because of a sexual anomaly/ambiguity.

All the sexual anomaly cases that had been entered on the birth defects database from 1989 to 1998 were retrieved. This included infants with ambiguous genitalia, hypospadias and any other ambiguity of the genitalia. With this information and the number of births at Chris Hani-Baragwanath hospital we were able to calculate a minimum estimate of the incidence of XX true hermaphroditism.

For the analysis of referrals to the cytogenetics laboratory, we obtained karyotype information for individuals sent to the department for chromosome analysis because of ambiguous genitalia, intersex or possible hermaphroditism from the period 1995 to 1998.

2.1.3.1. Statistical analysis

The data obtained from the two sources was statistically analysed by means of a computer programme SAS and excel. To measure the trend in the increase/decrease of the proportion of individuals diagnosed with a sexual anomaly to the number of babies with a congenital abnormality entered onto the database and the total number of births, logistic regressions were performed.

The chi-square (χ^2) analysis test was used to estimate how frequently the observed deviation can be expected to occur strictly as a result of chance. Once a χ^2 value was calculated, it must be interpreted in terms of a corresponding probability value (p). The p value indicates the probability of obtaining as great or even greater deviation by chance alone. A value less than 0.05 makes it unlikely that the observed results could be obtained by chance alone. Such a p value indicates substantial deviation from the predicted results.

The basic formula, $\chi^2 = \Sigma [(o - e)^2 / e]$, or a contingency table was used to calculate the χ^2 value when comparing data from two or more sources. The formula used for the latter method of χ^2 analysis is given in Table 2.1.

Table 2.1: The formula used to calculate the χ^2 test statistic

	CATEGORY 1	CATEGORY 2	SUB-TOTAL
STUDY 1	a	b	(a + b)
STUDY 2	c	d	(c + d)
SUB-TOTAL	(a + c)	(b + d)	TOTAL

$$\chi^2_{1df} = [(a + c) \times (a + b) / TOTAL] + [(a + c) \times (c + d) / TOTAL] + [(b + d) \times (a + b) / TOTAL] + [(b + d) \times (c + d) / TOTAL]$$

where 1df = one degree of freedom.

2.1.3.1.1. The Hardy-Weinberg equilibrium test

Hardy-Weinberg equilibrium describes the relationship between the allelic frequencies and the resulting genotype frequencies. The Hardy-Weinberg law states that both gene and genotype frequencies will remain in equilibrium in a population large enough such that sampling error is negligible, where mating occurs randomly, and there is absence of mutation, migration and selection (Klug & Cummings, 1991). To determine if the alleles in a population are in Hardy-Weinberg equilibrium, it is first necessary to calculate the allele frequency at the particular locus investigated. When considering two alleles (A and B) in a population, the frequency of A is represented by p and the frequency of B by q. The distribution of the possible genotype frequencies can, therefore, be expressed as:

$$p^2 + 2pq + q^2 = 1$$

Deviation from Hardy-Weinberg equilibrium can be tested using the χ^2 test. The basic χ^2 test uses the formula:

$$\chi^2 = \Sigma [(o - e)^2 / e]$$

where o = the observed value

e = the expected value

(o – e) = the standard deviation in each case

The probability value obtained from the χ^2 test indicates whether or not the null hypothesis (i.e., the observed genotypes are a product of a random union of gametes in the population) should be accepted or rejected.

2.2. Collection and storage of blood

Blood samples from random healthy black individuals were used as controls. They had been collected, and the DNA extracted, from previous field trips undertaken by staff at the SAIMR. Thus, these DNA samples were already available in the laboratory. The DNA samples for the majority of the XX true hermaphrodite patients were also available

in the laboratory, although some additional samples were collected during the study. All blood samples were collected in 5ml vacutainer tubes containing the anticoagulant ethylenediaminetetra-acetic acid (EDTA).

2.3. DNA extraction

The initial starting material, whole blood or paraffin-embedded tissue, influenced the method of DNA extraction to be used.

2.3.1. The salting out method

High molecular weight DNA was extracted from peripheral blood lymphocytes using the salting out method (Miller *et al*, 1988).

Because blood samples of the hermaphrodite patients were received at irregular intervals, buffy coats (which contain the white blood cells) were extracted and frozen (-20°C) until there was an adequate number of samples to perform the DNA extraction on. To obtain the buffy layer, blood samples were spun in the EDTA tubes at 2 300 rpm for five minutes in a Beckman (Model GS-6R) benchtop refrigerated centrifuge. This separated the blood into its three different components:

i. Plasma
ii. Buffy layer
iii. Red blood cells

A plastic pipette was used to carefully suck up the buffy layer which was then transferred to a 50ml Nunc tube. The buffy was frozen at –20°C until required.

When sufficient samples had been collected, the blood samples were thawed and total genomic DNA extracted. Approximately 45ml of cold sucrose-triton X lysing buffer was added to each Nunc. This hypotonic solution, and the cold temperature, causes swelling and lysis of the cell membrane of red blood cells. The contents of each Nunc were mixed well by inversion and centrifuged for 10 minutes at 2 300rpm in a Beckman (Model GS-6R) benchtop refrigerated centrifuge. After centrifugation, the supernatant containing lysed red blood cells and plasma was poured off, leaving behind a reddish-

pink pellet that contained mostly white blood cells but also a few red blood cells. The pellet was resuspended in 25ml of chilled sucrose-triton X lysing buffer. Vigorous shaking dislodged the pellet and the Nunc tubes were put on ice for 5 minutes. This helped to further lyse the remaining red blood cells. Next, the Nunc tubes were centrifuged for 5 minutes at 2 300rpm. The supernatant was again discarded and a pinkish-white pellet consisting of predominantly white blood cells was observed.
The next step involved the addition of the following to the pellet:

i. 3ml T20E5
ii. 200µl of 10% SDS
iii. 500µl of proteinase K solution

T20E5 contains EDTA, a chelating agent that binds divalent metal cations. Thus, endonucleases that require these ions as cofactors cannot function. SDS, a strong detergent, disrupts white blood cell membranes and unfolds proteins. This enables proteinase K to degrade proteins. After vigorous mixing by inversion, the solution was incubated at 42°C overnight.

The following morning, 1ml of saturated NaCl solution was added to each Nunc. The salt precipitates the protein in the mixture. Each tube was agitated vigorously and put on ice for 5 minutes. The tubes were spun at 4°C for 30 minutes at 2 300rpm. The resulting pellet contained protein precipitated by salt. The supernatant, which contained the DNA, was carefully poured into a new Nunc tube. Two volumes of absolute ethanol was added to each tube which was gently agitated by inversion to precipitate the DNA. The DNA was 'fished' out using a Gilson pipette and washed in ice-cold 70% ethanol. The 70% ethanol dissolves salts that remain after precipitation of the DNA. The DNA was transferred to a clean Eppendorf tube and allowed to air dry.

After air drying, the DNA was resuspended in the appropriate volume of TE, ranging from 200 to 1 000µl. TE is a more suitable storage buffer than water as it maintains the DNA in a more stable state. As mentioned previously, EDTA, present in the buffer, chelates metal ions thus preventing enzymatic DNA degradation. The DNA was stored in Eppendorf tubes at 4°C.

2.3.2. DNA extraction from paraffin-embedded tissue samples

The human androgen receptor assay, aimed at assessing methylation status of maternally and paternally inherited X chromosomes, required DNA from gonadal tissue of XX true hermaphrodites. DNA was therefore extracted from archival formalin-fixed paraffin-embedded gonadal tissue of six of the true hermaphrodites. The method described by Smith *et al* (1992) was used for the extraction process.

2.3.2.1. Microdissection of gonadal tissue from paraffin embedded samples

Paraffin-embedded tissue blocks were cut into 10 micron sections and mounted onto glass slides (the wax was removed so that the tissue samples were now water based). Using a dissecting microscope, the region to be microdissected was circled (by a histopathologist) on the coverslip of the original slide (which contained a stained section of the gonadal tissue of the true hermaphrodite) (slide A). The reverse side of slide A was wiped clean with ethanol to minimise the chances of contamination. Slide B, which contained the tissue to be microdissected, was then superimposed onto the bottom of slide A in such a way that the upper surface of slide B comes into contact with the bottom of slide A. The markings on slide A were then traced onto the underneath surface of slide B. Approximately 15µl (depending on the size of the tissue to be microdissected) of 20% ethanol was dropped onto the area of interest. With the aid of a dissecting microscope and using a sterile glass Pasteur pipette, or a sterile dissecting needle, the tissue was scraped off the slide and placed into a sterile Eppendorf.

DNA was extracted from 10 micron slices by incubating the paraffin-embedded tissue in approximately 150µl of incubation buffer. The buffer consisted of 10mM Tris pH 8.3, 50mM potassium chloride, 15mM magnesium chloride, 0.45% Polyoxyethylenesorbitan monolaurate (Tween 20) and 0.45% Nonidet P40. Tween 20 and Nonidet P40 are detergents that aid cell lysis by disrupting the cell membrane. The buffer was filter sterilised through a Millipore sterivex™-GS 0.22µm filter unit (without filling bell). This was followed by the addition of 10µg/ml bovine serum albumin (BSA) and proteinase K. BSA binds fatty acids and hydrophobic molecules while proteinase K digests proteins. The samples were incubated for about 16 hours at 55°C. They were then transferred to a heating block or boiling water bath at 95°C for 10 minutes and then cooled on ice.

Samples were centrifuged for 1 minute to pellet partially digested cell membranes. The supernatant containing the DNA was transferred to a clean, sterile tube and stored at 4°C (Smith *et al*, 1992).

2.4. Southern blotting

SOX9 appears to play some role in the sex determining pathway since individuals with mutations in their SOX9 gene show XY female sex reversal in approximately 70% of cases. Previous unpublished research suggested that exon 3 of the SOX9 gene may be deleted in XX true hermaphrodites. Southern blotting was therefore performed to test the involvement of this gene in the aetiology of XX true hermaphroditism.

In the human genome, frequent sequence differences (usually single base changes), or polymorphisms, occur from individual to individual once every few hundred base pairs. Each difference from the consensus sequence is present in a fraction of the human population, and every individual has some variation in their DNA. Some of the sequence changes alter the recognition sites of restriction endonucleases. This results in different sized DNA fragments when digested by particular enzymes. These size differences are known as restriction fragment length polymorphisms, or RFLPs. The detection of RFLPs relies on a specialised hybridisation procedure called Southern blotting (Lehninger *et al*, 1993). Genomic DNA is digested with one or more restriction endonucleases, and the resulting fragments are separated, according to size, by electrophoresis in an agarose gel. The DNA fragments are denatured and transferred to a nylon membrane. The relative positions of the DNA fragments are preserved during their transfer to the membrane. The DNA attached to the membrane is hybridised to a radioactively labelled DNA probe, and autoradiography is used to locate the positions of bands complementary to the probe.

2.4.1. Preparation of genomic DNA

The first step in this procedure involved digestion of genomic DNA from the patients and controls. The amount of genomic DNA needed to generate a detectable hybridisation signal depends on a number of factors:

i. The size of the genome
ii. The size of the probe
iii. The specific activity of the probe after labelling
iv. The amount of genomic DNA that would be transferred to the membrane (Sambrook *et al*, 1989)

2.4.1.1. Restriction endonuclease digestion of genomic DNA

Restriction endonucleases are endodeoxyribonucleases that recognise specific nucleotide sequences within double stranded DNA, and cleave the double stranded DNA within or adjacent to this site. These enzymes, isolated from bacteria, received their name because their biological function is to degrade or restrict foreign DNA molecules that enter the cell. Restriction endonucleases are classified into types I, II or III. Only type II enzymes were used in this study. Type II restriction enzymes recognise a specific palindromic sequence of 4 to 6 nucleotides in length and produce a double stranded break within the sequence. Cleavage results in either blunt-ended fragments or DNA fragments with 'sticky' ends (single stranded complementary tails) (Sambrook *et al*, 1989).

Restriction digestion followed the manufacturer's specifications. The DNA samples were digested with *EcoR*I and *Hind*III, in separate reaction tubes. The digest contained between 5 to 10µg of DNA, 5µl of the recommended 10x digestion buffer and 30U of the relevant restriction enzyme. The digestion mixture was made up to a final volume of 50µl with distilled water. The reactions were incubated at 37°C overnight.

The following day, a tenth of the sample was loaded onto a trial 0.8% agarose gel to check that the DNA had been completely digested. Digested DNA samples are represented as an even smear on the gel. If the reaction was incomplete, more enzyme was added and the samples incubated for a further 4 hours at 37°C.

The DNA fragments resulting from restriction endonuclease digestion of high molecular weight genomic DNA were size fractionated by electrophoresis through an agarose gel and transferred, *in situ*, to a solid support by the Southern blotting technique (Southern, 1975).

2.4.1.2. Agarose gel electrophoresis

Gel electrophoresis is a powerful technique used to separate, and characterise molecules according to their size. DNA fragments of 100bp to 25kb in length can be separated on agarose gels of varying concentrations. The higher the concentration of agarose used in a gel, the greater the efficient separation range between DNA fragments of a small size.

When an electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates toward the anode (positive pole). The rate of DNA migration in an agarose gel is influenced by a number of factors:

i. The molecular size of the DNA.
   Larger molecules migrate more slowly through the gel because of greater frictional drag and resistance experienced in trying to move through the pores of the gel. The rate of migration is therefore inversely proportional to the logarithm of the number of base pairs in the DNA molecule.
ii. The concentration of agarose.
   There is a linear relationship between the logarithm of the electrophoretic mobility of the DNA and the gel concentration. Thus, by using gels of different concentrations, it is possible to resolve a wide size range of DNA fragments.
iii. Other factors include conformation of the DNA, voltage, presence of intercalating dyes, and composition of the electrophoresis buffer (Sambrook *et al*, 1989).

Agarose gels were prepared as follows. A perspex plate and set of combs was cleaned with ethanol. The plate was taped with biohazard tape and the combs placed in position. An appropriate amount of Seakem high gelling temperature (HGT) agarose powder (the mass varies depending on the percentage gel required) was boiled in 1x TBE (Tris borate buffer) until the solution was clear and transparent. Ethidium bromide (10mg/ml) was added after the gel had cooled slightly. Ethidium bromide contains a planar group that intercalates between the stacked base pairs of DNA. DNA is visualised under UV light using this fluorescent dye. The dye bound to DNA displays an increased fluorescence compared to that of the dye in free solution. The gel mixture was poured into the prepared gel plates and allowed to set at room temperature. Agarose is a linear polymer, which upon hardening forms a matrix. The density of the

matrix depends on the concentration of the agarose used. The greater the concentration of agarose, the less porous the gel.

The remaining 45µl of digested DNA was mixed with Ficoll loading dye and loaded into individual gel slots on a 0.8% HGT agarose gel. Gel loading dyes, such as Ficoll, serve three purposes. They increase the density of the sample, ensuring that it sinks into the well; the colour allows visualisation of the sample thus simplifying loading; and the dye moves slightly ahead of small DNA fragments in an electric field. A commercially supplied 1kb ladder (Boehringer Mannheim; 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517, 506, 396, 344, 298, 220, 201, 154, 134, and 75bp) was loaded in the two outside wells of the gel. The marker serves as a molecular weight standard as it contains DNA fragments of known size. The gel was run overnight (approximately 17 hours) at 65mA. Digests were electrophoresed until the dye was 2cm from the end of the gel. After electrophoresis was complete, the gel was viewed on a UV transilluminator and a photograph taken with a Polaroid camera. The dimensions i.e., length and width, of the gel were recorded.

2.4.1.3. Southern gel transfer

The Southern blotting technique is the most common method used to transfer DNA digested with restriction endonucleases from an agarose gel to a solid support (usually a nylon membrane). The DNA fragments, which have been separated according to size by electrophoresis, are denatured *in situ* and transferred from the gel to a membrane where they are immobilised.

The gel was immersed in denaturing solution for 30 minutes at room temperature. This makes the DNA single stranded which is necessary for hybridisation to the probe. The denaturing solution was poured off and the gel washed twice in distilled water. The gel was then neutralised for 30 minutes in neutralising solution. This solution restores the gel to a neutral pH. The gel was then rinsed twice with distilled water and placed in 20x SSC (the transfer solution) for 10 minutes before blotting.

2.4.1.3.1. DNA transfer

A plastic trough was filled with approximately 500ml of 20x SSC. A glass plate, cleaned with ethanol, was placed across the trough. A single sheet of Whatman 3MM filter

paper (the width of the gel) soaked in 20x SSC was draped over the glass plate. The ends of the wick must be in contact with the transfer solution in the trough. Air bubbles were removed by rolling a sterile 10ml plastic pipette across the wick surface. The gel was inverted and placed on top of the wick. This is done so that the DNA will be in closer proximity to the nylon membrane. The area around the gel was covered with clingwrap. This prevents the wick from drying out and ensures capillary movement of the salt solution through the gel only.

The hybond-N nylon membrane was cut to the same dimensions of the gel, soaked in 20x SSC and carefully placed on top of the gel. Two pieces of Whatman 3MM filter paper, soaked in 2x SSC, were placed on the membrane, followed by a further four dry pieces of filter paper. A stack of absorbent paper towels, a glass plate and a telephone directory were added. It is important that there be an even distribution of weight over the gel area.

The transfer was allowed to proceed over two nights. The well positions were marked and the nylon membrane carefully removed and rinsed in 2x SSC. This is to remove residual agarose. The completeness of the transfer was detected by viewing the agarose gel under the UV transilluminator. The membrane was placed DNA side up on blotting paper and then baked for 1 hour at 80°C. The blot was stored in a dry area until it was required for hybridisation.

2.5. Plasmid transformation

The SOX9 clone, p26.2 (personal communication, S Guioli [United Kingdom]), was received as a DNA sample and needed to be transformed and regrown to obtain sufficient DNA for the experiment. Commercially supplied ampicillin resistant DH5α *Escherisha coli* (*E.coli*) competent cells were used for the transformation. The probe used in this study was p26.2. It was inserted into the plasmid vector pBS(+/-) at an *EcoR*I site. The size of the insert was approximately 2kb.

One to 2µl of plasmid DNA, p26.2, was added to 100µl of the competent cells. The cells were put on ice for 5 to 30 minutes. No heat shock was required. The

transformation mix was added to 900µl of transformation and storage buffer (TSB) containing 20mM glucose and incubated in a shaker (225 rpm) at 37°C for 60 minutes. A loop full of bacterial cells was streaked onto Luria agar (LA) plates containing 50µg/ml ampicillin for selection of transformants. The plates were incubated overnight at 37°C. The plasmid carries an ampicillin resistance gene. Only bacterial cells that have been transformed, i.e. taken up the plasmid, will therefore be resistant to ampicillin.

2.5.1. Plasmid DNA preparation

The plasmids were extracted from the bacterial cells using the Promega Wizard MaxiPlus preparation system. A single colony was selected from the LA plate and used to inoculate 250ml Luria broth (LB) containing 50µg/ml ampicillin. The flask was incubated overnight at 37°C with shaking. The inoculated broth was decanted into 50ml Nunc tubes. The cells were pelleted by centrifugation in a Beckman (Model GS-6R) benchtop centrifuge at 5 000g for 10 minutes at 22 to 25°C. The supernatant was discarded and the pellet resuspended in 15ml of cell resuspension solution. To aid resuspension, which is critical for optimal yields, the solution was pipetted up and down until no clumps were visible. Next, 15ml of cell lysis solution was added and the tube mixed gently by inversion. Cell lysis was complete when the solution became clear and viscous (approximately 20 minutes). This was followed by the addition of 15ml of neutralisation solution. The solution was mixed by gently inversion. The tubes were centrifuged at 14 000g for 15 minutes at room temperature. The supernatant was filtered through an autoclaved coffee filter into a clean graduate 100ml cylinder. The volume of the supernatant was measured before being transferred to a clean 50ml Nunc tube. The addition of 0.5 volume of isopropanol followed. The tube was then mixed by inversion. The tube was centrifuged at 14 000xg for 15 minutes at 22 to 25°C. The supernatant was discarded and the pellet resuspended in 2ml TE buffer.

2.5.2. Plasmid purification

10ml of Wizard™ Maxiprep DNA purification resin was added to the pelleted DNA resuspended in TE buffer. The resin/DNA mix was transferred into a Wizard™ Maxicolumn. A vacuum was applied to pull the resin/DNA mixture into the column. This

was followed by the addition of 25ml of column wash solution to the maxicolumn. A vacuum was applied to draw the solution through the column. To rinse the resin, 5ml of 80% ethanol was added to the column and a vacuum applied to draw the ethanol through the column. The vacuum was allowed to draw for an additional minute. The column was transferred to a 50ml Nunc tube and centrifuged at 1 300g for 5 minutes. The Nunc and liquid that collected were discarded, and the column attached to the vacuum. The resin was dried to completion by drawing a vacuum for an additional 5 minutes. The maxicolumn was then placed in a 50ml reservoir tube provided in the kit. Preheated water or TE (1.5ml) was added to the column. After one minute, the DNA was eluted by centrifuging the maxicolumn at 2 500 rpm for 5 minutes. The eluted plasmid DNA was then filtered through a 0.22 µm filter. An aliquot of plasmid DNA was checked on a 0.8% agarose gel.

2.5.3. Isolation of the SOX9 insert

An aliquot of the extracted plasmid DNA was digested with 30U of the restriction endonuclease *EcoR*I in a final volume of 70µl to confirm the sizes of the vector and the SOX9 DNA insert, p26.2. The reaction mix was incubated at 37°C for 3 hours. A tenth of the volume of the digested plasmid was run on a 0.8% agarose gel for approximately 1-2 hours at 100V to check the completeness of the digestion. The size of the plasmid and insert were 3kb and ± 2.2kb respectively (Figure 2.1).

2.5.4. Extraction of DNA fragments from gels using the phenol freeze method

A 1% low melting point (LMP) gel was prepared. The gel was poured and allowed to set at 4°C. The digestion reaction was loaded in a single well and separated by electrophoresis. A 1kb ladder was run in the adjacent lane. The gel was run overnight at 4°C at 30 to 40 mA. The insert (2.2kb fragment) was cut from the gel with a sterile blade, removing as much unnecessary gel as possible. The gel band, of approximately 0.3ml, was placed in an Eppendorf tube. An equal volume of phenol (up to 0.5ml) was added. The tube was vortexed to dissolve as much of the agarose as possible. The contents of the tube were frozen rapidly in an ethanol-ice-salt bath (-10°C) for 5 minutes. This was followed by a 5 minute spin in a microcentrifuge.

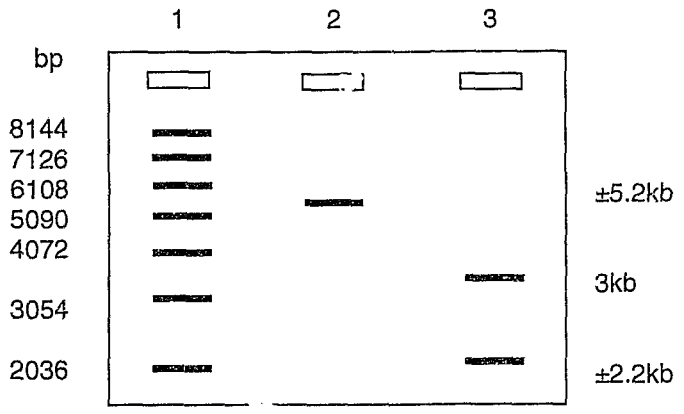


Figure 2.1. Schematic representation of the sizes of the plasmid DNA and excised SOX9 insert. Lane 1: 1kb molecular weight marker, Lane 2: linearised p26.2 plasmid DNA, Lane 3: digested p26.2 plasmid DNA.

The process of vortexing, freezing and spinning was repeated twice. After the third spin, the aqueous phase was removed. An equal volume of chloroform was added to this phase. The tube was then vortexed and spun in a microcentrifuge for 5 minutes. The aqueous phase was again removed and a tenth of the volume of 3M sodium acetate (pH 4.8) and two volumes of cold ethanol added.

The DNA was precipitated by placing the tubes at –70°C for 20 to 30 minutes or at –20°C overnight. The solution was spun in the microcentrifuge for 15 minutes to precipitate the DNA. The supernatant was discarded and the pellet washed in 70% ethanol. The pellet was dried and then resuspended in a small volume of TE (± 50µl if the pellet is not visible). The concentration of the extracted insert was determined by running 5µl on a 0.8% agarose gel.

The insert was radioactively labelled with the radioisotope α-^{32}P in the form of ^{32}P deoxycytidine triphosphate (dCTP) (5µCi/µl). The technique used is called oligolabelling.

2.6. Oligolabelling

The DNA to be labelled is first denatured by boiling to make it single stranded. It is then incubated with the Klenow fragment of DNA polymerase I (which lacks exonuclease activity) in the presence of a mixture of hexanucleotides and radioactive and non-radioactive deoxynucleoside triphosphates (dNTPs). Oligolabelling is a more effective technique of radioactively labelling DNA than that of nick translation for the following reasons:

i. Much higher specific activities are obtained
ii. Utilisation of label is more efficient, with up to 90% of the radioactive nucleotide being incorporated into the DNA under optimal conditions
iii. Both dissolved DNA and DNA in agarose gels can be labelled efficiently (without the removal of the agarose).

The Amersham multiprime kit was used for all oligolabelling reactions. In this method, random hexanucleotides bind to complementary sequences on the denatured DNA probe, and act as primers for extension by the Klenow fragment of DNA polymerase I. A 3µl aliquot of the purified probe, p26.2, was used for the labelling protocol. To the DNA, 5µl of primer (random hexanucleotides) and distilled water were added to reach a final volume of 34µl. The mixture was boiled at 95°C in a water bath for five minutes followed by a quick spin in the microfuge. After centrifugation, 10µl of buffer, 5µl of [α-^{32}P] dCTP and 2µl of enzyme (Klenow fragment) were added to give a total volume of 51µl. The mixture was gently pipetted up and down to mix and then spun briefly. The reaction mix was incubated at 37°C for 10 minutes. The reaction was stopped by the addition of 5µl of 0.2M EDTA. The labelled mixture was made up to 100µl with TE (warmed to 37°C) and then put through a spin column to remove the unincorporated [α-^{32}P] dCTP.

2.6.1. The removal of unincorporated nucleotides using spin columns

The spin columns were prepared as follows:

i. The bottom of a 1ml syringe was plugged, up to the 0.1ml mark, with glass wool
ii. The syringe was filled with Sephadex G-50 saturated in TE

iii. The syringe was placed in a 15ml centrifugation tube and spun for 5 minutes in the IEC benchtop centrifuge at full speed

iv. The syringe was refilled and spun until the column was packed to approximately 0.9ml

The top of an Eppendorf tube was cut off and the bottom placed in the 15ml tube under the syringe. This is to collect the probe that is eluted from the column. The 100µl of probe mix was loaded onto the column and spun at top speed for 5 minutes in the IEC centrifuge. An additional 100µl of TE was loaded onto the column that was again spun for 5 minutes. The Eppendorf containing the purified probe was retrieved from the bottom of the centrifuge tube.

The purified probe was made up to a final volume of 1ml with TE. Cherenkov counting was done on a 10µl aliquot using a Beckman LS 6000IC machine set to count in the tritium (^{3}H) window. The value obtained was corrected for the total volume of labelled solution and multiplied by a factor of three to obtain an estimate of the ^{32}P incorporated. A specific activity of 1×10^{7} was desired for good hybridisation. The following formula was used:

$$\text{Counts obtained} \times 100 \times 3 = \text{cpm/ml of } ^{32}P$$

2.6.2. Hybridisation of the DNA probe to the nylon membrane

The baked membrane was wet in a solution of 2x SSC and then placed in a hybridisation bottle. The bottle contained 20ml of a 1:1 mixture of prehybridisation solution and water that had been preheated to 65°C. Before the blot was added to the hybridisation bottle, 1ml of salmon sperm DNA was boiled at 95°C for 4 minutes, put on ice and then added to the prehybridisation mix. The blot was prehybridised at 65°C for one hour. The prehybridisation step is necessary to prevent the non-specific binding of the DNA probe to the membrane, decreasing background hybridisation.

The labelled DNA probe was denatured by boiling for 5 minutes at 95°C. It was then put on ice for a few minutes to prevent the single strands from reannealing. The probe was added to the hybridisation bottle that contained the blot and prehybridisation mix.

Hybridisation was carried out for two days. The membrane was removed from the bottle and washed to remove any non-specifically hybridised probe.

2.6.3. Post hybridisation washes

Washing protocols differ from probe to probe. It is also sometimes necessary to perform additional washes after autoradiography if the background is too high. The membrane was washed twice for 10 minutes at room temperature in a solution of 2x SSC and 0.1% SDS. This was followed by two washes at 65°C in a 1x SSC and 0.1% SDS solution for 20 minutes. The final wash was performed at 65°C for 10 minutes with a solution of 0.5x SSC and 0.1% SDS. The membrane was then rinsed three times in 0.1% SSC.

2.6.4. Autoradiography

After the blots were washed they were sealed in plastic making sure not to trap any air bubbles. The membrane was placed in a cassette and a Kodak biomax light film placed over the blot. The cassette was put at –70°C for 1 to 14 days depending on the intensity of the signal. The autoradiographs were developed using a Kodak M5 automatic developer.

Blots that were to be reused were soaked in denaturing solution for ten minutes followed by two washes, of ten minutes each, in special neutralising solution. All washes were carried out at room temperature. The blot was rinsed with distilled water between each wash. Stripped blots were stored in 2x SSC until required for hybridisation.

2.7. The Polymerase Chain Reaction

In 1984 Kary Mullis invented the Polymerase Chain Reaction (PCR) technique. Since then PCR analysis has found applications in a wide range of disciplines, including molecular biology, human genetics, evolution, development, and even forensics (Klug & Cummings, 1991).

PCR is a method for selective, specific and efficient amplification of DNA. It is a rapid and convenient alternative to traditional and labour intensive techniques such as Southern blotting. The PCR is an *in vitro* method for enzymatically synthesising defined sequences of DNA by means of two oligonucleotide primers. The primers are usually 18 to 30 bases long, complementary to sequences that lie on opposite strands of the template DNA, and flank the region that is to be amplified. The reactions are catalysed by a DNA polymerase enzyme isolated from the thermophilic eubacterium, *Thermus aquaticus*. This enzyme, termed *Taq* polymerase, is suitable for PCR because it is most active at temperatures around 75°C and it retains its activity after prolonged incubations at 95°C. Due to its heat tolerance, primer annealing and extension can be carried out at much higher temperatures. This increases the specificity of most reactions by reducing mispriming (Basic PCR: Protocols and optimization strategies).

A repetitive series of cycles involving template denaturation by heating to a temperature above its melting point, annealing of the primers to their target sequences at 50 to 65°C, and extension of the annealed primers by DNA polymerase at 72°C results in an exponential accumulation of a specific DNA fragment whose termini are defined by the 5' ends of the primers. Following the last cycle, there is often a final extension for ten minutes at 72°C to encourage completion of partial extension products and annealing of single stranded complementary products.

The temperature and length of time required for primer annealing depends upon the base composition, length, and concentration of the primer pairs. Thus, for most purposes, the annealing temperature has to be optimised experimentally.
There are a number of advantages to using PCR for genetic analysis:

i. only a small quantity (nanograms) of DNA is needed
ii. the assay only takes a few hours whereas Southern blotting take a minimum of five days
iii. the quality of DNA may be inferior i.e., slightly degraded, but it is still possible to get amplification of the target sequence
iv. it is a versatile technique

The disadvantages of PCR are:

i. it is a sensitive technique and therefore contamination may be a problem
ii. the DNA sequence surrounding the area of interest must be known so that specific primers can be designed and synthesised
iii. Only relatively small fragments of DNA (50 to 3 000bp) can be investigated with conventional PCR and traditional Taq polymerase. A new generation of DNA polymerases is now able to amplify much larger DNA fragments.

In this study, all PCR reactions were carried out in Cetus Perkin Elmer, Hybaid Omnigene and Touchdown thermal cyclers.

2.7.1. An assay to determine the methylation status of the androgen receptor element gene on the X chromosome

The second part of this study focused on X chromosome inactivation patterns in the XX true hermaphrodites to determine whether or not there is any correlation between X-inactivation and the type of gonadal tissue (testis or ovary) present. Random X-inactivation occurs early on in embryonic development and clones of cells which have either the maternal or paternal X chromosome inactivated, result. The inactivation is stably maintained in daughter cells by methylation at CpG dinucleotides. Non-random inactivation of the maternal or paternal allele in either the testis or the ovary would suggest that there is a gene(s) on the X chromosome which plays a role in testicular development in XX true hermaphrodites.

The human androgen receptor gene/element (ARE), located on the X chromosome at position q11-q12 (Brown *et al*, 1989), was studied to determine the pattern of X-inactivation. The human androgen receptor assay (HUMARA) was initially described and used to assess clonality of various tumours. It is now also used for prenatal diagnosis of X-linked disorders (Allen *et al*, 1992; Alterman *et al*, 1993; reviewed by Gale *et al*, 1996).

This assay takes advantage of a highly polymorphic trinucleotide repeat in the coding region of the first exon of the human androgen receptor gene. The CAG repeat is closely linked to four methylation sites. The methylation sites are unmethylated on the

active X chromosome and methylated on the inactive X chromosome (Busque *et al*, 1994). The HUMARA assay is, therefore, able to distinguish between the:

i. maternal vs. paternal X chromosome using the polymorphic triplet repeat and
ii. active vs. inactive X chromosome using differential methylation of the active vs. inactive X chromosome.

Sections of paraffin-embedded gonadal tissue were microdissected to obtain ovarian and testicular tissue and the DNA extracted. Patterns of X chromosome inactivation were determined by digestion of DNA with the methylation sensitive restriction endonuclease *Hpa*II, followed by PCR amplification using primers that flank the highly polymorphic HUMARA trinucleotide repeat. Because PCR does not maintain methylation patterns, the DNA was digested prior to PCR so that the unmethylated alleles were cleaved and consequently not able to be amplified whereas the methylated alleles were not cut and could be amplified (Figure 2.2).

2.7.1.1. Methylation sensitive digestion of samples

10 to 20µl (depending on the concentration) of microdissected DNA (0.5-5µg) was digested with the methylation sensitive restriction enzyme *Hpa*II (20U/reaction), together with 4µl of the recommended 10x buffer, in a final volume of 40µl. Digestion was carried out at 37°C overnight. The reaction was terminated by heating the samples for ten minutes at 95°C.

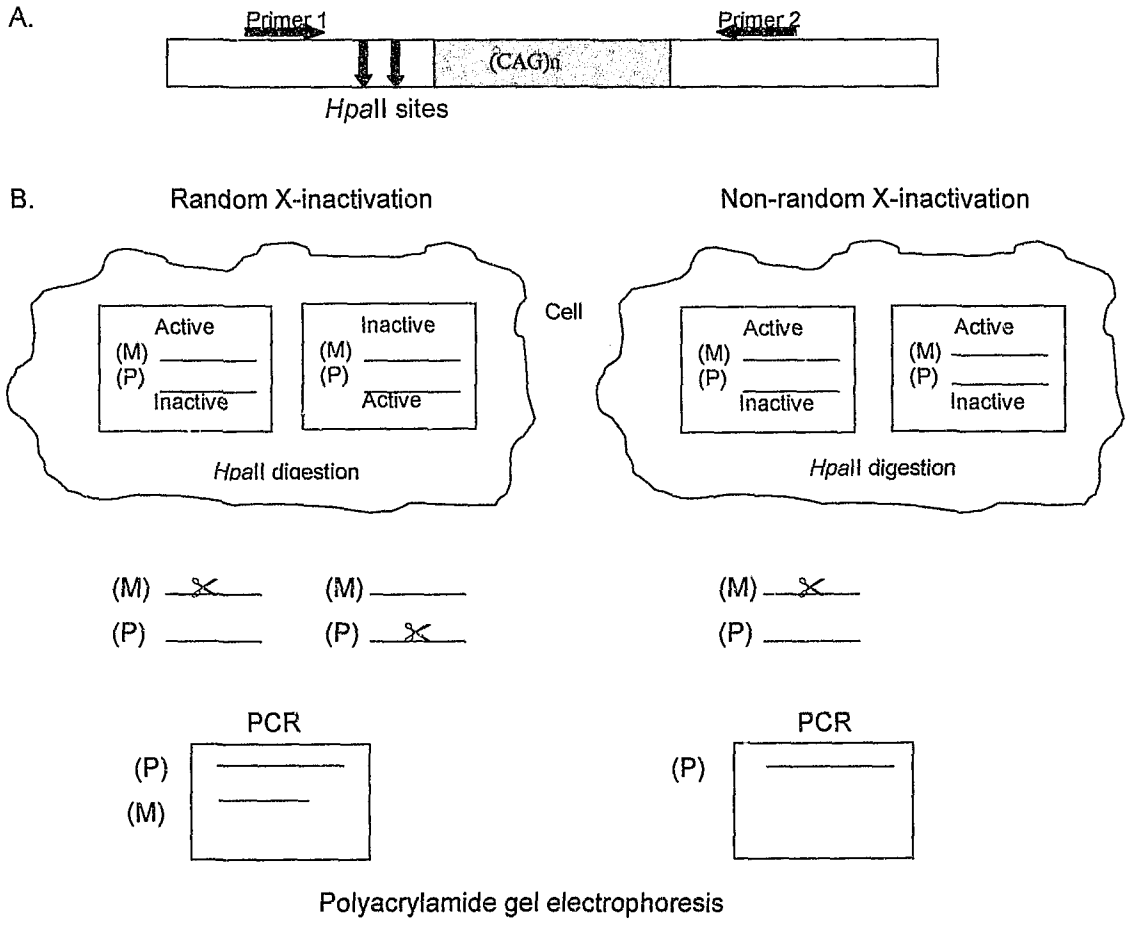


Figure 2.2. A. Simplified map of the X-linked human androgen receptor gene showing the positions of the primers relative to the *HpaII* sites and the CAG repeat. B. Schematic representation of results from the HUMARA assay. In the example the maternal (M) chromosome has a shorter CAG repeat than the paternal (P) chromosome. Random X chromosome inactivation results in inactivation (green) by methylation of either the paternal or maternal X chromosome in different cells. The active, non-methylated alleles (pink) are digested by *HpaII* (✂). Only the non-digested alleles, located on the inactivated paternal or maternal X chromosome, are amplified. The two alleles can be distinguished by their different lengths (adapted from Paradis *et al*, 1997).

2.7.1.2. PCR amplification of samples

The X-inactivation pattern was determined by PCR amplification (PCR product approximately 279bp) of digested and non-digested samples. Two primers, situated in exon 1 of the ARE, and flanking the trinucleotide repeat and the two *Hpa*II restriction sites were used (Figure 2.3):

ARE-2 5' TCC AGA ATC TGT TCC AGA GCG TGC 3' (24bp)
ARE-1 5' GCT GTG AAG GTT GCT GTT CCT CAT 3' (24bp)
(Gale *et al*, 1996).

PRIMER

1 TAGGGCTGGG AAGGGTCTAC CCTCGGCCGC CGTCCAAGAC
41 CTACCGAGGA GCTTTCCAGA ATCTGTTCCA GAGCGTGCGC ARE-2
81 GAAGTGATCC AGAACCCGGG CCCCAGGCAC CCAGAGGCCG
121 CGAGCGCAGC ACCTCCCGGC GCCAGTTTGC TGCTGCTGCA
161 GCAGCAGCAG CAGCAGCAGC AGCAGCAGCA GCAGCAGCAG
201 CAGCAGCAGC AGCAGCAAGA GACTAGCCCC AGGCAGCAGC
241 AGCAGCAGCA GGGTGAGGAT GGTTCTCCCC AAGCCCATCG
281 TAGAGGCCCC ACAGGCTACC TGGTCCTGGA TGAGGAACAG ARE-1
321 CAACCTTCAC AGCCGCAGTC GGCCCTGGAG TGCCACCCCG
361 AGAGAGGTTG CGTCCCAGAG CCTGGAGCCG CCGTGGCCGC
401 CAGCAAGGGG CTGC

CCGG = *Hpa*II restriction site; $(CAG)_n$ = the trinucleotide repeat

Figure 2.3. Partial sequence of exon 1 of the human androgen receptor gene showing locations of the primers, triplet repeat and *Hpa*II methylation sensitive restriction sites.

Ideally, dosage studies require a small number of cycles (18 to 23). In some cases, however, due to poor amplification, more cycles were performed. Five to 10μl of digested and undigested DNA was PCR amplified in separate reactions. The PCR reaction was carried out in a 25μl volume consisting of:

- 1μl of a10pmol stock of the forward and reverse primer

- 5µl of a 1.25mM stock of dNTPs
- 2.5µl of 10x *Taq* DNA polymerase buffer
- 2.5µl of 10% DMSO
- 2.5µl of a 2.5mM stock of spermidine (spermidine uncoils the DNA allowing easier access for the *Taq* DNA polymerase, thus facilitating the enzyme's activity)
- 1U of *Taq* DNA polymerase

The reaction mix was covered with mineral oil to prevent evaporation. The samples were initially denatured for two minutes at 95°C. Thereafter, each amplification cycle consisted of denaturation at 95°C for 45 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. These three steps were repeated for 28 cycles.

If no amplification was observed, a second round of PCR was performed with a 1µl aliquot from the first round of PCR amplification. With the exception of the addition of 5µl of a 1:40 dilution of the stock [α-^{32}P] dCTP (3 000Ci/mmol), the identical components and thermal profile were used for the second round of PCR amplification.

Following the final round of amplification, a 4µl aliquot of the radioactively labelled PCR product was mixed with 6µl of formamide loading dye. The samples were denatured at 95°C for three minutes and loaded on a 6% denaturing polyacrylamide gel. The gel was polymerised in the presence of urea.

2.7.2. Polyacrylamide gel electrophoresis

Polyacrylamide gels are poured between two glass plates that are separated by spacers 0.35mm wide. The gels are run in a vertical position and can range in length from 10cm to 100cm. The length of gel used will depend on the separation required. Throughout this study, gels of 45cm x 20cm were used.

Polyacrylamide gels have three advantages over agarose gels:

i. Greater resolving power e.g., DNA molecules that differ in length by 1bp in 500bp can be separated
ii. Larger quantities of DNA can be accommodated without significant loss of resolution

iii. DNA recovered from polyacrylamide gels is extremely pure.

There are two types of polyacrylamide gels:

i. Nondenaturing polyacryiamide gels for the separation and purification of fragments of double stranded DNA

ii. Denaturing polyacrylamide gels for the separation and purification of single stranded fragments of DNA (Sambrook *et al*, 1989).

A denaturing polyacrylamide gel is made with the addition of urea to the gel mixture. Urea suppresses base pairing of the DNA fragments. Subsequent migration therefore occurs at a rate that is not influenced by the base composition or sequence of the DNA fragment. The only factor influencing the rate of migration is the size of the fragment (Sambrook *et al*, 1989).

Amplified PCR products were electrophoresed for approximately four hours at 1 250V. A pBR322 ladder, digested with *Hind*III, was also loaded on the gel to orientate the gel. Following electrophoresis, the gel was removed and transferred to a piece of Whatman 3MM filter paper. This was covered with clingwrap and the gel dried at 80°C in a BioRAD model 583 gel dryer under vacuum. The dried gel was exposed overnight to Kodak biomax light film.

2.7.3. Fluorescent PCR of the HUMARA locus

The HUMARA assay was later modified for the ABI automated sequencer. The incorporation of 2'-deoxyuridine 5'-triphosphates (dUTPs) labelled with the fluorescent rhodamine dye R6G during the PCR reaction allowed for the accurate measurement of the peak intensities of each allele.

PCR was carried out as described in section 2.7.1.2. However, for the second round of amplification, instead of [α-^{32}P] dCTP, 0.15µl of 100mM fluorescent [R6G]dUTP was added to the reaction mix. After amplification, 3µl of loading buffer (2.5µl of 20mg dextran:1ml formamide dye and 0.5µl of ROX-350 size standard) was added to 1µl of PCR product. The fragment sizes of the ROX-350 size standard are 75, 100, 139, 150,

160, 200, 250, 300, 340, and 350bp. The samples were denatured at 95°C for 3 minutes and then put on ice to ensure that they remain single stranded. The samples were loaded on a 12cm x 24cm 4.3% polyacrylamide gel. The gel was run for 90 minutes at 1 600V.

As the fluorescently labelled DNA migrates through the gel, it passes a laser beam. The laser excites the fluorophores, which give off fluorescence at the appropriate wavelength. The fluorescent light that is given off is detected by a photomultiplier tube that converts the light into an electric signal. The electric signals are used to create a computer generated gel image. Using the Genescan 2.1 software the gel image is converted into an electropherogram for each lane. On the electropherogram, peaks are shown which represent the allele sizes of the sample.

2.8. Mutation detection in the DAX1 gene

Evidence that a dosage dependent sex determination mechanism may still exist in mammals comes from the finding that duplication of a region of the short arm of the X chromosome, in the presence of an intact Y, results in male to female sex reversal (reviewed by Ramkissoon & Goodfellow, 1996). Thus it appears that XY sex reversal can result from a double dose of this gene, DSS, in this region that is normally subjected to X-inactivation. Two copies of this gene disrupt testis formation but it is not essential for testis formation as shown by 46, XY individuals deleted for DSS but who still develop normal male genitalia. The DSS gene(s) is, therefore, predicted to be required for ovarian development (Werner *et al*, 1996). The two exons of the DAX-1 gene were screened by single stranded conformation polymorphism (SSCP) analysis to determine if this locus is somehow involved in the aetiology of hermaphroditism.

The DAX1 gene consists of two exons. Six primer sets were designed to span the DNA sequence of the entire exon regions, as well as the flanking intronic sequences (Figure 2.4). The gene was analysed in the XX true hermaphrodite patients by PCR amplification of each primer set followed by SSCP analysis. If a mobility shift was observed, the individual showing the aberrant band was sequenced using the ABI 377 automated DNA sequencer.

EXON 1 PRIMER

```
   1 ccgcgccccc ccgcacacac cgcccgcctc cgcgcccttg
  41 cccagaccga ggcggccgac gcgcctgcgt gcgcgctagg
  81 tataaatagg tcccaggagg cagccactgg gcagaactgg  DAX1.1
 121 gctacgggcg ccgcgggccA TGGCGGGCCA GAACCACCAG
 161 TGGCAGGGCA GCATCCTCTA CAACATGCTT ATGAGCGCGA
 201 AGCAAACGCG CGCGGCTCCT GAGGCTCCAG AGACGCGGCT
 241 GGTGGATCAG TGTTGGGGCT GTTCGTGCGG CGATGAGCCC
 281 GGGGTGGGCA GAGAGGGGCT GCTGGGCGGG CGGAACGTGG  DAX1.2
 321 CGCTCCTGTA CCGCTGCTGC TTTTGCGGTA AAGACCACCC
 361 ACGGCAGGGC AGCATCCTCT ACAGCATGCT GACGAGCGCA
 401 AAGCAAACGT ACGCGGCACC GAAGGCGCCC GAGGCGACGC
 441 TGGGTCCGTG CTGGGGCTGT TCGTGCGGCT CTGATCCCGG
 481 GGTGGGCAGA GCGGGGCTTC CGGGTGGGCG GCCCGTGGCA
 521 CTCCTGTACC GCTGCTGCTT TTGTGGTGAA GACCACCCGC  DAX1.3
 561 GGCAGGGCAG CATCCTCTAC AGCTTGCTCA CTAGCTCAAA
 601 GCAAACGCAC GTGGCTCCGG CAGCGCCCGA GGCACGGCCA
 641 GGGGGCGCGT GGTGGGACCG CTCCTACTTC GCGCAGAGGC
 681 CAGGGGGTAA AGAGGCGCTA CCAGGCGGGC GGGCCACGGC
 721 GCTTCTGTAC CGCTGCTGCT TTTGCGGTGA AGACCACCCG
 761 CAGCAGGGCA GCACCCTCTA CTGCGTGCCC ACGAGCACAA  DAX1.4
 801 ATCAAGCGCA GGCGGCTCCG GAGGAGCGGC CGAGGGCCCC
 841 CTGGTGGGAC ACCTCCTCTG GTGCGCTGCG GCCGGTGGCG
 881 CTCAAGAGTC CACAGGTGGT CTGCGAGGCA GCCTCAGCGG
 921 GCCTGTTGAA GACGCTGCGC TTCGTCAAGT ACTTGCCCTG
 961 CTTCCAGGTG CTGCCCCTGG ACCAGCAGCT GGTGCTGGTG
1001 CGCAACTGCT GGGCGTCCCT GCTCATGCTT GAGCTGGCCC
1041 AGGACCGCTT GCAGTTCGAG ACTGTGGAAG TCTCGGAGCC
1081 CAGCATGCTG CAGAAGATCC TCACCACCAG GCGGCGGGAG  DAX1.5
1121 ACCGGGGGCA ACGAGCCACT GCCCGTGCCC ACGCTGCAGC
1161 ACCATTTGGC ACCGCCGGCG GAGGCCAGGA AGGTGCCCTC
1201 CGCCTCCCAG GTCCAAGCCA TCAAGTGCTT TCTTTCCAAA
1241 TGCTGGAGTC TGAACATCAG TACCAAGGAG TACGCCTACC
1281 TCAAGGGGAC CGTGCTCTTT AACCCGGgta agggtactgg
```

1321 ccttaggcgc cggcttttcc cagctcacaa aagcatcggg
1361 cagtgcctat ctaggggcgc

EXON 2

```
   1 gctatttttа aaaagctagc aaaggactct gtaatgagct    DAX2.1
  41 gttttataac aaagagattt ttctccctcc agACGTGCCG
  81 GGCCTGCAGT GCGTGAAGTA CATTCAGGGA CTCCAGTGGG
 121 GAACTCAGCA AATACTCAGT GAACACACCA GGATGACGCA
 161 CCAAGGGCCC CATGACAGAT TCATCGAACT TAATAGTACC
 201 CTTTTCCTGC TGAGATTCAT CAATGCCAAT GTCATTGCTG
 241 AACTGTTCTT CAGGCCCATC ATCGGCACAG TCAGCATGGA
 281 TGATATGATG CTGGAAATGC TCTGTACAAA GATAtaaagt
 321 catgtgggcc acacaagtgc agtagtgcag ttcaccatga
 361 gggaagaata aagagctgtg ggcaaaagag tgtaaaatat
 401 tttaaaataa actttcttaa
```

Figure 2.4. Positions of the six PCR primer sets in the DAX1 gene.

2.8.1. PCR amplification of samples

PCR was optimised for each primer set. The primer sequences, PCR conditions and components of the reaction mix used for each primer set are listed in Table 2.2 and Table 2.3, respectively. All PCR reactions were carried out in a final volume of 25μl, with the exception of DAX1.1 and DAX1.4, which was carried out in 20μl. The sequences to be examined by SSCP analysis were all radioactively labelled by the incorporation of 5μl of a 1:40 dilution of the stock [α-^{32}P] dCTP (3 000Ci/mmol) into the PCR mix.

Table 2.2: DAX1 primer sequences*

PRIMER (product size)	PRIMER SEQUENCE (name of the primer)	LENGTH (bp)
DAX 1.1	5' CAC TGG GCA GAA CTG GGC TAC 3' (DAX1.1Fn)	21
(264bp)	5' CCT GCC GTG GGT GGT CTT T 3' (DAX1.1Rn)	19
DAX 1.2	5' GGG CGG AAC GTG GCG CTC CT 3' (DAX1.2Fn)	20
(308bp)	5' GCC ACG TGC GTT TGC TTT GAG 3' (DAX1.2Rn)	21
DAX 1.3	5' CCG CTG CTG CTT TTG TGG TGA A 3' (DAX1.3Fn)	22
(310bp)	5' GGC CCT CGG CCG CTC CTC CGG 3' (DAX1.3Rn)	21
DAX 1.4	5' GCC CAC GAC ACA AAT CAA G 3' (DAX1.4Fn)	19
(340bp)	5' CCC GGT CTC CCG CCG CCT GG 3' (DAX1.4Rn)	20
DAX 1.5	5' CAT GCT GCA GAA GAT CCT CAC CA 3' (DAX1.5Fn)	23
(257bp)	5' GGA AAA GCC GGC GCC TAA GG 3' (DAX1.5Rn)	20
DAX 2.1	5' GCT AGC AAA GGA CTC TGT GGT G 3' (DAX2.1Fn)	22
(349bp)	5' CCC TCA TGG TGA ACT GCA CTA C 3' (DAX2.1Rn)	22

* Primer sequences were designed using the computer programme DNAstar (see Appendix 2)

Table 2.3: PCR conditions and components

PRIMER	PCR COMPONENTS	PCR CONDITIONS
DAX 1.1	1µl of 10pmol forward and reverse primer	
	2µl of 10x buffer (-$MgCl_2$)	94°C for 5'
	2.5µl of a 1.25mM stock of dNTPs	94°C for 30s
	2.5µl of a 2.5mM stock of spermidine	58°C for 30s } x30
	2.5µl of 10% DMSO	72°C for 30s
	3µl of a 25mM stock of $MgCl_2$	72°C for 10'
	1U *Taq* DNA polymerase	

DAX 1.2	1µl of 5pmol forward and reverse primer 2.5µl of 10x buffer (-$MgCl_2$) 2.5µl of a 1.25mM stock of dNTPs 2.5µl of a 2.5mM stock of spermidine 2.5µl of 10% DMSO 1µl of a 25mM stock of $MgCl_2$ 1U *Taq* polymerase	94°C for 5' 94°C for 30s 63°C for 2' } x35 72°C for 10'
DAX 1.3	1µl of 5pmol forward and reverse primer 2.5µl of 10x buffer (-$MgCl_2$) 2.5µl of a 1.25mM stock of dNTPs 2.5µl of a 2.5mM stock of spermidine 2.5µl of 10% DMSO 2µl of a 25mM stock of $MgCl_2$ 1U *Taq* DNA polymerase	94°C for 5' 94°C for 30s 55°C for 30s } x35 72°C for 30s 72°C for 10'
DAX 1.4	1µl of 5pmol forward and reverse primer 2µl of 10x buffer (-$MgCl_2$) 2.5µl of a 1.25mM stock of dNTPs 2.5µl of a 2.5mM stock of spermidine 2.5µl of 10% DMSO 3µl of a 25mM stock of $MgCl_2$ 1U *Taq* DNA polymerase	94°C for 5' 94°C for 30s 52°C for 30s } x30 72°C for 30s 72°C for 10'
DAX 1.5	1µl of 5pmol forward and reverse primer 2.5µl of 10x buffer (-$MgCl_2$) 2.5µl of a 1.25mM stock of dNTPs 2.5µl of a 2.5mM stock of spermidine 3µl of a 25mM stock of $MgCl_2$ 1U *Taq* DNA polymerase	94°C for 5' 94°C for 30s 53°C for 30s } x40 72°C for 30s 72°C for 10'
DAX 2.1	1µl of 5pmol forward and reverse primer 2.5µl of 10x buffer 2.5µl of a 1.25mM stock of dNTPs 1U *Taq* DNA polymerase	94°C for 30s 53°C for 30s } x30 72°C for 45s 72°C for 10'

2.8.2. SSCP analysis of the DAX1 gene

The technique of SSCP analysis was first described by Orita *et al* (1989). Due to its simplicity, SSCP analysis is now widely used in the detection of mutations. This technique is based on the principle that under non-denaturing conditions, single stranded DNA fragments will fold into unique conformations determined by their primary sequence. The DNA strands will thus migrate differentially within the gel matrix. This secondary structure, stabilised by intramolecular interactions, is highly dependent on variables such as temperature and ionic concentrations. As a result, experimental conditions must be rigidly controlled. A single base substitution in a DNA fragment can induce a conformational change that is detected as a mobility shift in polyacrylamide gel electrophoresis (Figure 2.5) (Hayashi & Yandell, 1993; Sheffield *et al*, 1993).

Following PCR, 4µl of labelled PCR product was mixed with 6µl of formamide loading dye. Samples were denatured for three minutes at 95°C, cooled on ice, and then run on a mutation detection enhancement (MDE) gel. The MDE gel solution is a polyacrylamide like matrix that has a high sensitivity to DNA conformational differences. The unique structure of the gel causes DNA separation to occur on the basis of both size and conformation, thus increasing the probability of detecting sequence differences (MDE protocol - FMC).

Samples were electrophoresed in gels with and without 10% glycerol as it has been shown that low concentrations of glycerol in a gel frequently improve separation of mutated sequences (Hayashi, 1991). Gels were run for approximately 4 hours at 1 250V. When the run was complete, the plates were separated and the gel transferred onto Whatman 3MM filter paper. This was placed on a flat surface, with the gel side up, and covered with clingwrap. The gels were dried and exposed to Kodak biomax light film for 24 hours. When the film was developed, each individual was scored for the presence (+) or absence (-) of a mobility shift according to the banding pattern seen on the autoradiograph.

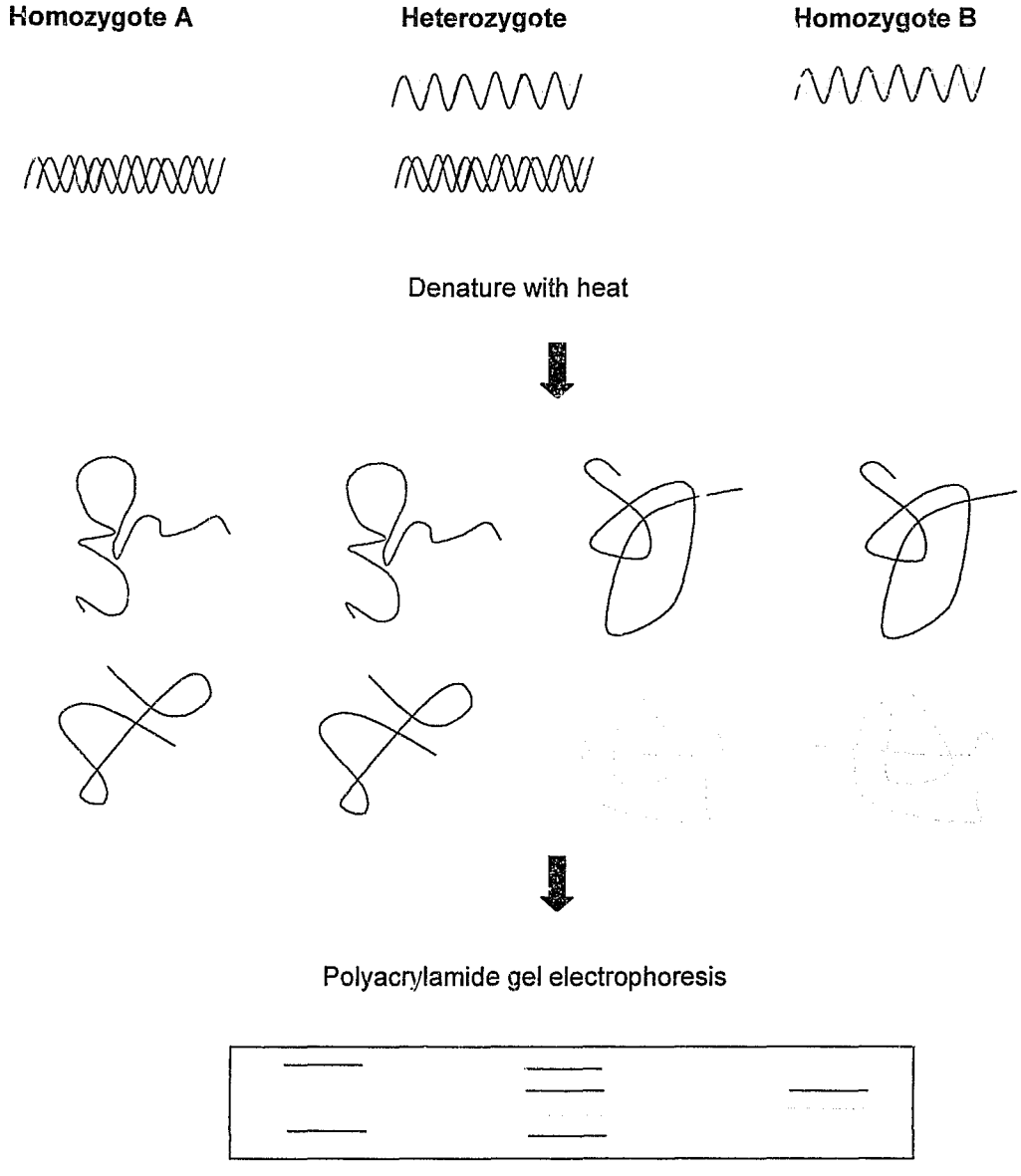


Figure 2.5. Diagram illustrating the principle of SSCP detection. In this method, sequences of interest are amplified by PCR. For efficient detection, the isotope ^{32}P dCTP is incorporated into the PCR product itself. An aliquot of the PCR product is mixed with formamide loading buffer, denatured to dissociate the strands and analysed by non-denaturing polyacrylamide gel electrophoresis.

2.9. DNA sequencing

One of the most important techniques available to molecular biologists is DNA sequencing. This technique allows the precise determination of the order of nucleotides in a segment of DNA. The sequencing of DNA has undergone vast improvement since the introduction of the chain-termination sequencing method of Sanger and the chemical degradation method of Maxam and Gilbert (Maxam & Gilbert, 1977; Sanger *et al*, 1977). For both these methods, four separate reactions, each with a different deoxynucleoside 5'-triphosphate (ddNTP) are set up. In the enzymatic method, labelled DNA fragments terminating in either adenosine (A), cytosine (C), guanosine (G) or thymidine (T) are produced. The chemical method produces fragments ending in G, G + A, C + T, or C. The four sets of reactions are loaded and run in adjacent lanes. A radioactively labelled nucleotide is also included in the synthesis so that the labelled chains of various lengths can be visualised by autoradiography after separation by high-resolution polyacrylamide gel electrophoresis. The resulting autoradiographic pattern is used to obtain the DNA sequence (Smith *et al*, 1986).

Although these techniques are highly effective, they are time consuming, labour intensive and involve the use of radioisotopes. As a result, an automated method of DNA sequence analysis, based on the Sanger sequencing protocol, was developed. The ABI 377 DNA sequencer, together with the Sequencing analysis software, is able to detect DNA fragments by fluorescence from four different dyes that are used to identify the A, G, C, and T extension reactions. Four chemically related, yet distinguishable, fluorescence-tagged ddNTPs are used to label the DNA with a suitable chain-extending DNA polymerase. The growing chain is, therefore, simultaneously terminated and labelled with the dye that corresponds to a particular base. Each dye emits light at a different wavelength when excited by the laser beam. All four colours can, therefore, be detected and distinguished in a single gel lane by polyacrylamide gel electrophoresis (Prober *et al*, 1987). Analysis of the fluorescent emission of each fragment allows one to identify the terminating nucleotide and assign the sequence directly in real time.

During electrophoresis, the laser is focused on a fixed position on the gel. As the DNA fragments migrate past this position, the dyes fluoresce. The fluorescence is detected

by a photomultiplier tube that converts the signal into an electric signal. The signals are recorded and the interpreted sequence is stored in a computer file (ABI PRISM^{TM} 377 DNA sequencer user's manual, 1995).

2.9.1. Pre-treatment of PCR products for sequencing

DNA samples to be sequenced were amplified using the same conditions as those described for SSCP analysis (as for section 2.8.2). To obtain high quality sequencing data that are easy to interpret, it is important that the PCR be as specific as possible. If PCR products smear, or if spurious bands are present on an agarose gel, inferior sequencing data will be obtained.

The quality and quantity of the PCR products were analysed by separation on a 3% agarose gel (Figure 2.6). A size standard should be run to check the correct size of the PCR product, and a product of known quantity can be used as a reference for quantitating the amount of PCR product. Alternatively, the quantity can be roughly estimated by eye.

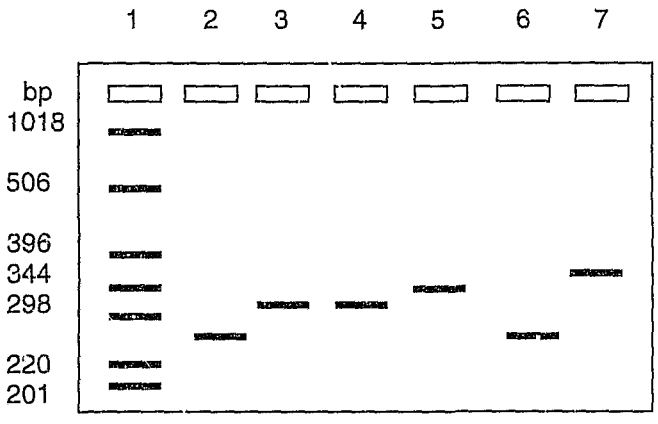


Figure 2.6. Schematic representation of the fragment sizes obtained (for cycle sequencing) by PCR amplification of the DAX1 gene using the six primer sets listed in Table 2.1. Lane 1: 1kb molecular weight marker, Lane 2: DAX1.1 PCR product (264bp), Lane 3: DAX1.2 PCR product (308bp), Lane 4: DAX1.3 PCR product (310bp), Lane 5: DAX1.4 PCR product (340bp), Lane 6: DAX1.5 PCR product (257bp), Lane 7: DAX2.1 PCR product (349bp).

PCR makes use of two primers, dNTPs, buffer and DNA polymerase to produce multiple copies of a specific DNA sequence. When complete, most of the dNTPs and primers remain intact. These will interfere with the cycle sequencing PCR, which also makes use of primers and nucleotides. Poor quality sequencing data will be obtained if the PCR products contain large amounts of these residual primers and nucleotides. The PCR product must, therefore, be purified prior to sequencing. One of two methods was used for purification depending on the purity of the amplified PCR product.

2.9.1.1. Enzymatic pre-treatment of PCR product for sequencing

In the absence of spurious bands, 5µl of PCR product was purified with two hydrolytic enzymes, exonuclease I (1U) and Shrimp alkaline phosphatase (SAP) (1U). Exonuclease I degrades residual single stranded primers and any extraneous single stranded DNA produced by the PCR. SAP dephosphorylates the excess nucleotides. The PCR product was incubated in the Hybaid omnigene for 15 minutes at 37°C

followed by a further 15 minutes at 80°C which inactivates the two enzymes (Comparative PCR sequencing: A guide to sequencing-based mutation detection, 1995).

2.9.1.2. Gel purification of PCR products

Because spurious bands interfere with sequencing, it is essential to separate and isolate the fragment of interest. To do this, the entire PCR product (as per 2.8.1) is loaded on a 3% NuSieve SGT agarose gel in 1x TAE buffer. Once separation has occurred, the band of interest was cut out and placed in an Eppendorf tube. The Wizard miniprep kit was used to extract the DNA from the gel. The tube was heated for 5 to 10 minutes at 70°C until the gel had melted completely. Next, 1ml of thoroughly mixed Wizard minipreps DNA purification resin was added. The DNA/resin mixture was mixed by pipetting up and down or by inversion for 20s. The DNA/resin mix was added to a syringe barrel attached to the luer-lock extension of a minicolumn. A vacuum was applied to pull the DNA/resin into the column. This was followed by the addition of 2ml of 80% isopropanol to the column. The vacuum was again applied to pull the isopropanol through the column. The vacuum was applied for an additional 1 to 2 minutes to dry the column. The syringe barrel was then removed and the column transferred to an Eppendorf tube. The column was spun in an Eppendorf microcentrifuge for 2 minutes at 12 000g to remove any excess isopropanol. The column was transferred to a clean Eppendorf tube and 50µl of distilled water was added. After 1 minute, the column was spun for 20s at 12 000g. The column was removed and discarded. An aliquot of the purified DNA was checked on a 3% agarose gel to ensure its extraction and purity. The DNA was stored at -20°C until required for sequencing.

2.9.2. Cycle sequencing PCR amplification

The ABI Prism™ dRhodamine terminator cycle sequencing ready reaction kit combines the unique properties of AmpliTaq DNA polymerase, FS (Stoffel Fragment) and the newly developed dRhodamine dye terminators. These new dichlororhodamine (dRhodamine) terminators provide more even peak patterns and less background noise than conventional rhodamine dye terminators. The result is higher accuracy, longer

read lengths and greater productivity in automated sequencing (dRhodamine terminator cycle sequencing ready reaction kit protocol - PE Applied Biosystems).

Samples were cycle sequenced in the forward and reverse directions. Two reactions were therefore performed for each sample being sequenced. The reaction was carried out in a 20µl volume which consisted of:

- 3 to 6µl cleaned PCR product
- 1µl of a 3.3pmol stock primer
- 8µl dye terminator cycle sequencing kit (contains dye-labelled terminators, dNTP mix, sequencing buffer, AmpliTaq DNA polymerase, FS).

The three steps involved in the amplification of the target sequences were denaturation at 96°C for 30s, annealing at 50°C for 15s, and extension at 60°C for 240s. This cycle was repeated 25 times.

2.9.3. Centri-sep purification of products

Following single stranded DNA amplification, centri-sep columns were used for the removal of excess dideoxy terminators from the completed DNA sequencing reactions. Centri-sep columns are used for the rapid and efficient purification of large molecules (nucleic acids, proteins, etc.) from small molecules (nucleotides, buffer, etc.). The column gel was hydrated, for at least 30 minutes, with 800µl distilled water. Air bubbles were removed by inversion and tapping of the column. Once the gel was free of bubbles, the fluid was drained by centrifugation for 2 minutes at 3 000rpm in an Eppendorf microcentrifuge. The PCR sample was then applied to the centre of the column, which was placed into a sample collection tube and spun for 2 minutes. The purified sample was collected at the bottom of the sample collection tube and then dried in a Savant speed vac concentrator vacuum centrifuge.

2.9.4. Electrophoresis of the PCR sequencing products

The DNA sequencing product was resuspended in 1.5µl of dye (20mg dextran:1ml formamide dye). The sample was heated at 95°C for approximately 3 minutes to

denature the DNA and then loaded onto a 4.3% polyacrylamide gel (41cm x 24cm) which was run for 7 hours at 1 600V in the ABI 377 automated DNA sequencer. The data were collected in computer files and analysed.

2.10. Detection of a Y-bearing cell line in the XX true hermaphrodites

The genetic aetiology of 46 XX true hermaphroditism, to date, is unknown. Previous studies have failed to identify SRY in both lymphocytic tissue and gonadal fibroblast culture. In an attempt to determine whether or not the presence of a Y-bearing cell line (mosaicism) could be responsible for the aetiology observed in our XX true hermaphrodites, three different regions of the Y chromosome were studied (Figure 2.7). DNA extracted from peripheral blood lymphocytes and paraffin embedded gonadal tissue were examined. Two of the loci are situated on the short arm of the Y chromosome (Yp): the sex determining region (SRY) which is located approximately 10kb from the pseudoautosomal boundary on Yp and the amelogenin locus (AMEL-Y) which is found at Yp11.1. The third system, the 49a low copy repeat, is located on the long arm of the Y chromosome (Yq) at Yq11.2.

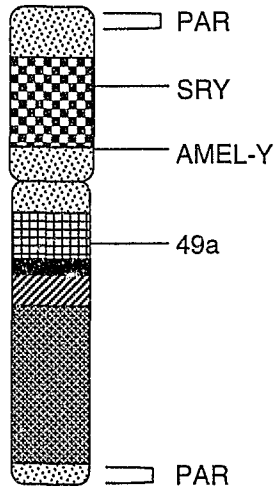


Figure 2.7. Physical map of the Y chromosome indicating the relevant positions of the three loci studied to detect the presence or absence of a Y-bearing cell line (PAR = pseudoautosomal region).

2.10.1. Detection of the three loci on the Y chromosome

The presence of Y-specific sequences at the SRY and AMEL-Y loci in the XXTH patients was detected by multiplex PCR and the presence of the 49a locus by a separate PCR reaction. Amplification of the SRY gene, which is located on the short arm of the Y chromosome, results in a product of 600bp. The amelogenin gene is found on the X chromosome and the amelogenin-like sequence is situated on the short arm of the Y chromosome. Although these two loci are about 90% homologous to each other, the X chromosome sequence has an additional 177bp insert (Nakahori *et al*, 1991). Thus, two fragments (925bp and 748bp) indicate a male while the amplification of the 925bp DNA product alone is indicative of a female. Amplification of the X chromosome sequence for the amelogenin locus, therefore, serves as a control for amplification of samples. The 49a system results in the amplification of an 800bp fragment when Y material is present. A control primer, $^{G}\gamma$-globin, which amplifies a 323bp fragment of the α-globin gene was also included in the 49a PCR reaction. This fragment serves as a control for amplification of samples.

The primer sequences used in the three Y chromosome assays were:

Amelogenin primers:

AMXY-1F	5' CTG ATG GTT GGC CTC AAG CCT GTG 3'	(24 bp)
AMXY-2R	5' TAA AGA GAT TCA TTA ACT TGA CTG 3'	(24bp)

(Nakahori *et al*, 1991)

SRY primers:

XES7'	5' CGA CAA TGC AAT CAT ATG CTT CTG 3'	(24bp)
XES2'	5' CTG TAG CGG TCC CGT TGC TGC 3'	(21bp)

(Spurdle *et al*, 1995)

49a primers:

49aa	5'
49am	5'

$^{G}\gamma$-globin primers:

$^{G}\gamma$	5' AGT GCT GCA AGA AGA ACA ACT ACC 3'	(24bp)
$^{G}\gamma$	5' CTC TGC ATC ATG GGC ACT GAG CTC 3'	(24bp)

(Kulozik *et al*, 1988)

The multiplex PCR reaction was carried out in a final volume of 25µl consisting of:

- 0.5-5ng DNA
- 1µl of a 10pmol stock of each of the forward and reverse primers for SRY and amelogenin
- 2.5µl of a 1.25mM stock of dNTPs
- 2.5µl of a 2.5mM stock of spermidine
- 2.5µl of 10x buffer
- 1U of *Taq* DNA polymerase

The samples were initially heated for seven minutes at 94°C after which the *Taq* DNA polymerase was added. This was followed by 30 cycles of denaturation at 94°C for one minute, reannealing at 66°C for two minutes and extension at 72°C for two minutes. A final extension was carried out at 72°C for 12 minutes.

The 49a reaction mix included dNTPs, spermidine, buffer and *Taq* DNA polymerase as for the SRY/amelogenin multiplex PCR, and 12.5pmoles of the 49aa and 49am primer and 1.4pmoles of the forward and reverse $^{G}\gamma$-globin control primers.

The 49a reaction mix was covered with mineral oil to prevent evaporation and amplified for 30 cycles (denaturation at 94°C for one minute, annealing at 65°C for one minute and extension at 72°C for one minute) with a final extension at 72°C for ten minutes.

After amplification, a 20µl aliquot of the PCR product was mixed with Ficoll dye and loaded on a 3% agarose gel. A 1kb ladder was run in the outside lanes to correctly size the PCR products. The gel was run for approximately one hour at 110V. Once the PCR product had run far enough, the gel was viewed on a UV transilluminator and a photograph taken with a Polaroid camera. Each individual was scored for the presence

or absence of the SRY (600bp), amelogenin (925bp and 748bp) and 49a (800bp) fragment (Figure 2.8).

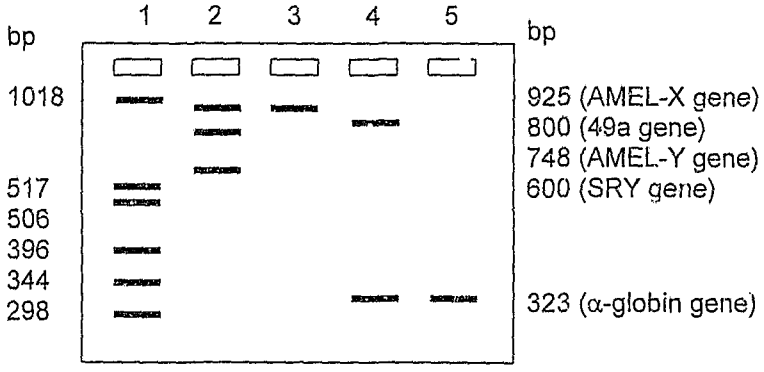


Figure 2.8. Diagrammatic representation of the banding pattern observed after amplification of the SRY, 49a and amelogenin loci in the presence and absence of any Y-specific sequence. Lane 1: 1kb molecular weight marker, Lane 2+3: Fragment sizes representative of amplification of the SRY/amelogenin loci in the presence or absence of Y material, respectively, Lane 4+5: Fragment sizes representative of amplification of the 49a loci in the presence or absence of Y material, respectively.

CHAPTER 3

3. RESULTS

3.1. Patients used in this study

In this study, DNA was available from 30 of the 41 black patients with suspected or confirmed hermaphroditism. These samples had been collected in the department over a number of years. Of the 41 patients, 34 were histologically demonstrated to be confirmed true hermaphrodites. Of the 30 cases on whom the genetic analyses were performed, 23 were histologically confirmed true hermaphrodites. Table 3.1 gives a breakdown of the histological findings and cytogenetic results (where available) of the 41 patients.

Table 3.1: Histological and cytogenetic data on the 41 patients from the study

XXTH	RIGHT GONAD	LEFT GONAD	AGE DIAGNOSED	SEX OF REARING	KARYOTYPE
XXTH 1†			12 years	Female	
XXTH 4†			3 years	Female	
XXTH 5†			9 months	Female	
XXTH 7	Ovotestis	Testis	4 years	Male	
XXTH 8†				Male	
XXTH 9#	Ovary	Ovotestis	17 months	Female	46,XX
XXTH 10	Ovotestis	Ovotestis	1 year	Male	46,XX
XXTH 11			15 years	Female	
XXTH 12	Ovotestis	Ovotestis	2 months	Male	46,XX
XXTH 13#	Ovotestis	Ovary	1 year	Female	46,XX
XXTH 14	Testis	Ovotestis	8 years	Male	46,XX
XXTH 15				Male	
XXTH 16	Testis	Ovary		Female	46,XX
XXTH 17				Unknown	

XXTH	RIGHT GONAD	LEFT GONAD	AGE DIAGNOSED	SEX OF REARING	KARYOTYPE
XXTH 20	Ovotestis	Ovary	5 years	Female	46,XX
XXTH 21				Female	
XXTH 24	Ovotestis	Ovary	1 year	Female	46,XX
XXTH 25	Ovary	Testis		Male	45,X/46,XY
XXTH 26#	Testis	Ovary	18 months	Male	46,XX
XXTH 27				Female	
XXTH 29				Male	
XXTH 30#	Testis	Ovary	3 months	Male	46,XX
XXTH 31	Ovotestis	Ovotestis	3 months	Male	46,XX
XXTH 32				Female	
XXTH 33	Ovary	Ovotestis	9 months	Male	46,XX
XXTH 34	Ovotestis	Ovotestis	11 months	Female	46,XX
XXTH 35	Ovotestis	Ovary	4 months	Female	46,XX
XXTH 36	Ovotestis		2 years	Female	46,XX
XXTH 37	Ovotestis	Ovary	4 months	Unknown	46,XX
XXTH 81*			1950	Female	
XXTH 82	Ovary	Ovotestis	8 years	Male	46,XX
XXTH 83*			1963	Male	
XXTH 84	Ovotestis	Ovotestis	14 years	Female	
XXTH 85*			1977	Female	
XXTH 86*			1970	Female	
XXTH 87	Ovotestis	Ovotestis		Male	46,XX
XXTH 88	Ovotestis		1970	Female	
XXTH 89	Ovotestis	Ovotestis	19 years	Male	46,XX
XXTH 90*			1968	Male	
XXTH 91*			1979	Female	
XXTH 92	Ovotestis	Ovary	3 months	Female	

† Confirmed XXTHs (Spurdle *et al*, 1995); * Confirmed XXTHs (Ramsay *et al*, 1988); # DNA extracted from paraffin-embedded tissue was available for these patients; DNA (extracted from blood) was available for XXTHs shown in blue

3.1.1. Karyotype analysis

The most common karyotype observed in our patients was the 46,XX chromosomal complement that accounts for 95.0% (19/20) of the above cases where the karyotype was known. The remaining case was a patient, XXTH 25, who had a mosaic karyotype: 45,X/46,XY. These findings are in keeping with other studies on the chromosomal profile of cohorts of hermaphrodites (van Niekerk & Retief, 1981b; Ramsay *et al*, 1988; Hadjiathanasiou *et al*, 1994).

3.1.2. The sex of rearing

Patients were assigned a male or female gender based on their sex of rearing. This information was obtained from questionnaires given to the patients, or their mothers, in a study by Ramsay *et al* (1988), or on whether their first names were male or female. In two of the 41 cases the names were ambiguous and therefore not included. From the remaining 39 cases, a male to female sex assignment ratio of 1: 1.3 (17:22) was obtained. If one were to only look at the 34 confirmed true hermaphrodites (gender identified in 33 of the 34 patients), a similar ratio of 1:1.2 (15:18) is obtained. The latter ratio is similar to a survey by Krob *et al* (1994) where the male to female assignment ratio was found to be close to one, independent of karyotype or presence of Y chromosome sequence. Similarly, when comparing our data to a study of 104 true hermaphrodites by van Niekerk and Retief (1981a), no significant difference ($p = 0.153$) in the ratio of male to female patients (1:0.67) was observed. Any differences that are seen are likely to be due to sampling error.

3.1.3. Gonadal distribution

When the gonadal distribution was looked at in the 24 true hermaphrodites on whom histopathology results were available, two groups were found to account for 75% of the true hermaphrodites:

i. The unilateral true hermaphrodite with an ovary/testis on one side and an ovotestis on the opposite side (45.83%).
ii. The bilateral true hermaphrodite with an ovotestis on both sides of the body (29.17%) (refer to Table 3.2).

Histopathology reports were only available for 15 of the 24 confirmed true hermaphrodites used in this study. The data from these patients are included in Table 3.2. We unfortunately did not have DNA from the remaining nine patients on whom histopathology reports were available and they were, therefore, not included in the molecular investigations.

Table 3.2: Gonadal distribution observed in 24 true hermaphrodites

TYPE OF DISTRIBUTION*	NUMBER OF PATIENTS	PERCENTAGE (%)
Ovary-Testis	4	16.67
Ovotestis-Ovary	9	37.50
Ovotestis-Ovotestis	7	29.17
Ovotestis-Testis	2	8.33
Ovotestis-?	2	8.33
TOTAL	24	100

* Laterality not taken into account

The 46 gonads that were examined in the 24 hermaphrodites were then arranged according to the side of the body on which they were located (see Table 3.3).

Table 3.3: The incidence of gonads occurring on the left or right side of the body in 24 true hermaphrodites

GONAD*	LEFT (%)	RIGHT (%)	TOTAL (%)
Ovary	9 (40.91)	4 (16.67)	13 (28.26)
Testis	2 (9.09)	4 (16.67)	6 (13.04)
Ovotestis	11 (50.00)	16 (66.66)	27 (58.70)
TOTAL	22	24	46

* Data extrapolated from Table 3.2

The ovary was found more often on the left side of the body (69.23%) while the testis and ovotestis occurred more frequently on the right side (66.67% and 59.26%,

respectively). It is also evident from Table 3.3 that the ovotestis is the most common gonad in the true hermaphrodite, accounting for 58.70% of the gonads. The ovary (28.26%) is the second most common gonad with the testis being present in only 13.04% of cases. These results are consistent with other studies on gonadal frequency reported in the literature (van Niekerk, 1976; Krob *et al*, 1994).

3.1.4. Histopathology of the gonads of XX true hermaphrodites

The ovarian tissue of true hermaphrodites has been described as being of normal histological appearance with many primordial follicles situated in the stroma. In contrast, the testicular tissue generally appears to be immature and in most cases no spermatogonia are observed. These results are consistent, regardless of whether or not the tissue is from a separate gonad (ovary or testis) or an ovotestis. The histologic appearances of the gonads of three XX true hermaphrodites from this study are shown in Figures 3.1 to 3.6.



Figure 3.1. Photomicrograph of the right gonad removed from XXTH 12, at two months of age, showing the ovotestis with the testicular portion (A) situated top left and the ovarian portion (B) bottom right. OS = ovarian stroma (Haematoxylin-eosin stain, x100).

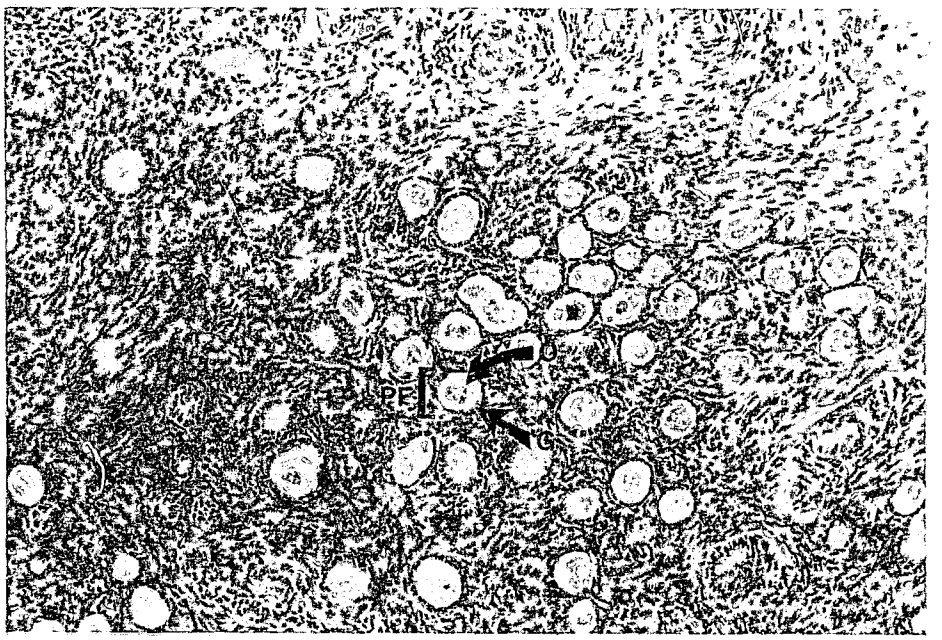


Figure 3.2. Photomicrograph of the ovarian portion of the ovotestis (left gonad) from XXTH 9. at 17 months of age, showing numerous primordial follicles (PF) lying within the ovarian stroma. Each oocyte (O) is surrounded by a single layer of flattened inactive granulosa cells (G) (Haematoxylin-eosin stain, x100).

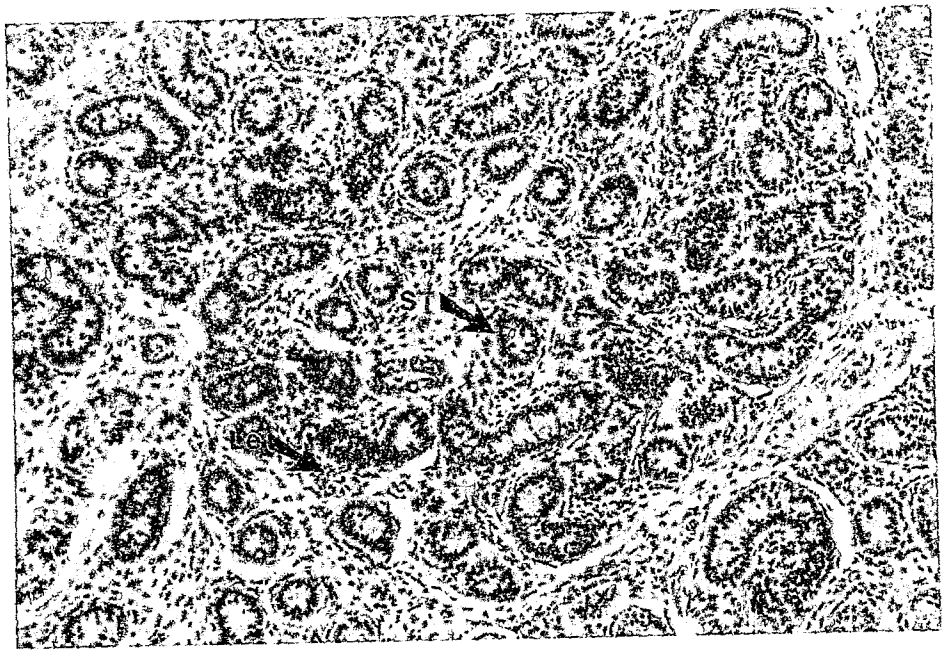


Figure 3.3. Photomicrograph of the testicular component of the ovotestis of XXTH 9, at 17 months of age, showing numerous solid, immature seminiferous tubules (ST) containing Sertoli cells. Germ cells are not apparent in this photograph. Leydig cells (Le) may be seen within the stroma. These cells should be referred to as interstitial cells and not Leydig cells because they are inert and not producing testosterone (Haematoxylin-eosin stain, x100).

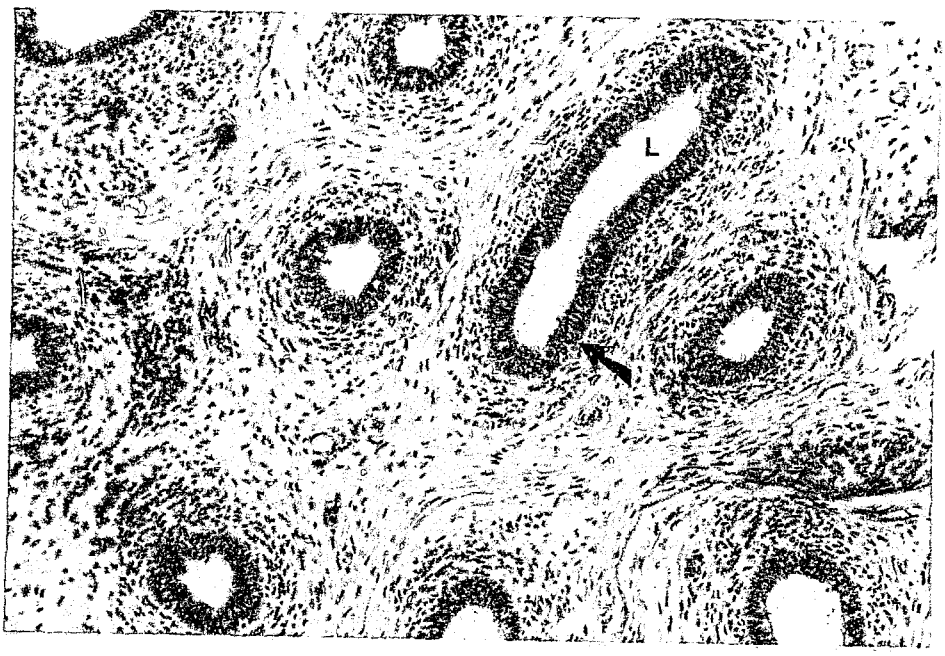


Figure 3.4. Photomicrograph showing the epididymis from the ovotestis of XXTH 9, at 17 months of age, indicating tubules lined by tall columnar ciliated epithelial cells (arrow), L = lumen of seminiferous tubules (Haematoxylin-eosin stain, x100).

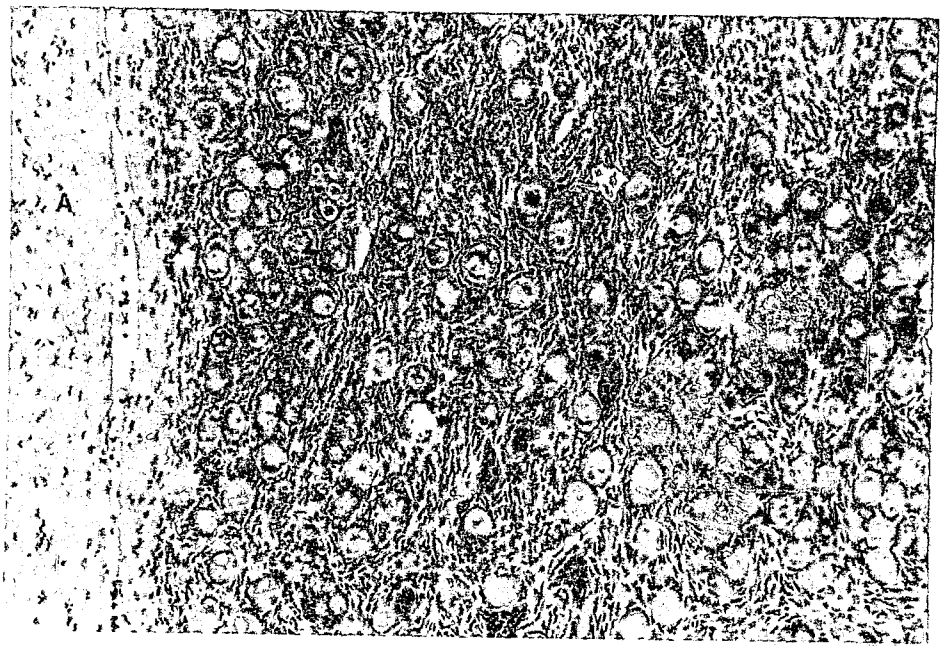


Figure 3.5. Photomicrograph of the left gonad from XXTH 30, at three months of age, showing it to be composed entirely of primordial follicles lying in the ovarian stroma. The ovarian cortex (A) is noted to the left (Haematoxylin-eosin stain, x100).

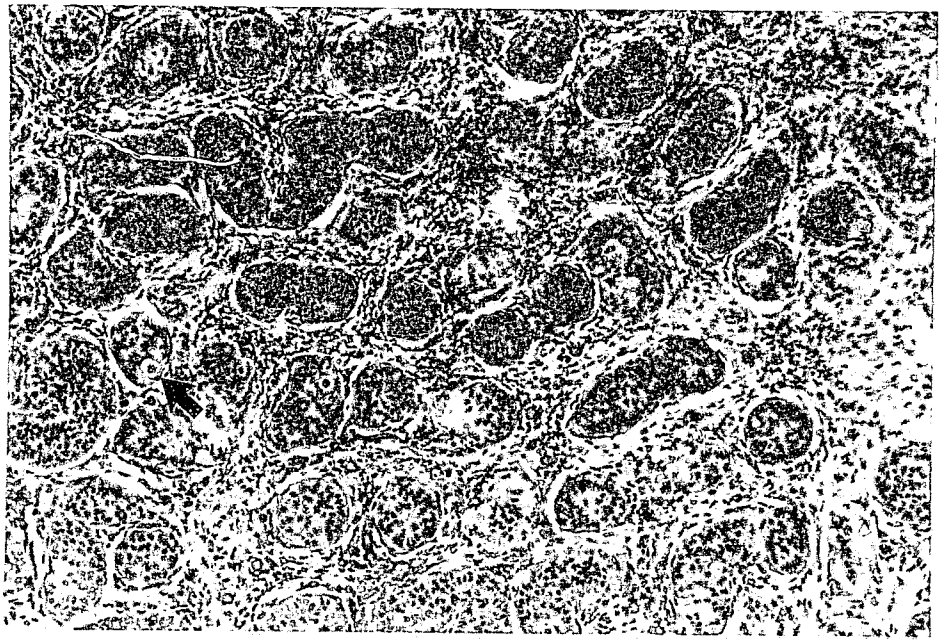


Figure 3.6. Photomicrograph of the right gonad of XXTH 30, at age three months, showing this to be composed entirely of solid seminiferous tubules. The arrow on the left of the picture indicates spermatogonia (germ cells) (Haematoxylin-eosin stain, x100).

As can be seen from the above photomicrographs, the histologic appearance of the gonads of these hermaphrodites is in keeping with those described in the literature.

3.2. An estimated incidence of XX true hermaphroditism in southern African black individuals

Two sources of data were used to determine a minimum estimate of the incidence of XX true hermaphroditism in the South African black population:

i. The Chris Hani-Baragwanath hospital birth defects database
ii. Cytogenetic analyses of individuals with sexual anomalies (data obtained from the Cytogenetics Laboratory at the SAIMR)

3.2.1. The Chris Hani-Baragwanath hospital birth defects database

Chris Hani-Baragwanath hospital has a birth defects database on which all babies born with a congenital abnormality, ranging from a cardiac defect to an extra digit, are entered. From this database we were able to obtain information on individuals born at Chris Hani-Baragwanath hospital, or transferred there within 24 hours of birth, that had some form of sexual anomaly. This included hypospadias, cliteromegaly, ambiguous genitalia and various anomalies of the external genitalia or sexual development. Over a period of ten years (1989 to 1998), 31 813 babies born with a congenital abnormality were entered onto the database. A search of the database for patients with signs associated with intersex disorders produced 92 patients.

The babies with a sexual anomaly were divided into two groups:

i. Babies born at Chris Hani-Baragwanath hospital (group 1)
ii. Babies born at other hospitals and clinics who were referred to Chris Hani-Baragwanath hospital within 24 hours of birth (group 2)

A total of 64 individuals fell into group 1 and 28 individuals into group 2. Table 3.4 gives a breakdown of the number of individuals recorded each year for a particular sexual anomaly.

Table 3.4: The number of black babies born with a sexual anomaly at Chris Hani-Baragwanath and other hospitals over a ten year period (1989 to 1998)

Chris Hani-Baragwanath births											
DOB	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	**TOT**
AG			1		2[b]	2[c]			2		7
AG+		2[a]		2	1			2		1	8
Hyp						1	1	5	4	13	24
Hyp+	1							1[d]	2	2	6
Cl				1	1			1			3
Other				1				1	9	5	16
S.Tot	1	2	1	4	4	3	1	10	17	21	**64**
Other hospitals											
DOB	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	**TOT**
AG		1			3[e]		1[f]			1	6
AG+		1		1		1	1	1		2	7
Hyp								1	2	2	5
Hyp+								1	2	1	4
Cl											0
Other								2	3	1	6
S.Tot	0	2	0	1	3	1	2	5	7	7	**28**
TOT	**1**	**4**	**1**	**5**	**7**	**4**	**3**	**15**	**24**	**28**	**92**

AG = Ambiguous genitalia; AG+ = Ambiguous genitalia in addition to hypospadias, cliteromegaly, and/or other symptoms associated with intersex; Hyp = Hypospadias; Hyp+ = Hypospadias together with other symptoms associated with intersex; Cl = Cliteromegaly; Other = Additional symptoms associated with intersex; S.Tot = Sub-total; TOT = Total; a - f = Confirmed XX true hermaphrodites (one case in each category with the exception of the two individuals with ambiguous genitalia in 1993, from Chris-Hani Baragwanath hospital, who were both confirmed hermaphrodites)

Histopathology and cytogenetic reports were retrieved for as many of the 92 patients as possible based on their laboratory numbers and/or first names to confirm or refute a

diagnosis of hermaphroditism. Seven true hermaphrodites were identified in this manner, five from group one and two from group two (Table 3.5).

Table 3.5: Individuals from the birth defects database that were identified as histologically confirmed true hermaphrodites

INDIVIDUAL*	SEXUAL ANOMALY	YEAR DIAGNOSED	AGE DIAGNOSED	KARYOTYPE
XXTH 9	Ambiguous genitalia	1993	17 months	46,XX
XXTH 10	Ambiguous genitalia	1993	1 year	46,XX
XXTH 13	Ambiguous genitalia	1993	1 year	46,XX
XXTH 24	Ambiguous genitalia	1995	1 month	46,XX
XXTH 31	Hypospadias, urethral meatus-scrotum and penis	1996	3 months	46,XX
XXTH 33	Ambiguous genitalia	1994	9 months	46,XX
XXTH 34	Ambiguous genitalia, CAH	1990	11 months	46,XX

* The XXTH numbers listed here correspond to those in Table 3.1; CAH = Congenital adrenal hyperplasia

From the results obtained in Table 3.4, it was calculated that 1 in 12.8 (5/64: 7.8%) black babies born with a sexual anomaly at Chris Hani-Baragwanath hospital and 1 in 14.0 (2/28: 7.1%) babies born at other hospitals and clinics were confirmed true hermaphrodites. There is no significant statistical difference between the observed percentage of hermaphrodites in the two groups ($p = 0.519$). A minimum estimate of 1 in 40 070 (95% CI: 1 in 39 921 to 1 in 40 219) was then calculated for the incidence of true hermaphroditism in the South African black population (Table 3.6). This figure was based on the number of confirmed cases from the database and the total number of births at the respective hospitals and clinics over the ten year period studied.

Birth rate figures were not available for 1996 to 1998 for group 2 (i.e., other hospitals and clinics). Data of the number of births for these three years were, therefore,

statistically calculated based on the trend observed for the period of 1989 to 1995 to enable us to determine the minimum incidence of hermaphroditism.

Table 3.6: A minimum incidence for true hermaphroditism in the South African black population

HOSPITAL	# OF CONFIRMED TH	# OF INDIVIDUALS WITH A SA	% TH	# OF BIRTHS	INCIDENCE OF HERMA-PHRODITISM
CHB	5	64	7.8	193 973	1 in 38 795
OTHER	2	28	7.1	86 513	1 in 43 258
TOTAL	**7**	**92**	**7.6**	**280 486**	**1 in 40 070**

TH = true hermaphrodites; CHB = Chris Hani-Baragwanath hospital (group 1); Other = other hospitals and clinics (group 2); SA = sexual anomaly; # = number; % = percentage

Using birth rate figures and population size estimates from 1994 and 1995 (Health trends in South Africa, 1994; Statistics in brief: RSA, 1996), respectively, the total number of babies expected to be born with XX true hermaphroditism was calculated (Table 3.7).

Table 3.7: The number of black babies expected to be born annually with XX true hermaphroditism in South Africa

INCIDENCE	BIRTH RATE*	POPULATION SIZE†	BIRTHS/YEAR	EXPECTED # OF XXTH BIRTHS
1 in 40 070	34 per 1 000	31 461 000	1 069 674	27

* 1994 statistics; † 1995 statistics; # = number

The percentages of individuals with a sexual anomaly were calculated with respect to the total number of births at the relevant hospitals/clinics (Table 3.8) and the number of individuals with a congenital abnormality entered on the birth defects database (Table 3.9).

Table 3.8: The total percentage of black babies born each year with a sexual anomaly

YEAR	NUMBER OF BIRTHS		TOTAL	% WITH SA (# of SA cases))
	BARAGWANATH (# of SA cases)	CLINICS (# of SA cases)		
1989	22 141 (1)	12 879 (0)	35 020	0.003 (1)
1990	23 972 (2)	12 197 (2)	36 169	0.011 (4)
1991	23 618 (1)	11 243 (0)	34 861	0.003 (1)
1992	20 486 (4)	9 823 (1)	30 309	0.016 (5)
1993	18 495 (4)	8 944 (3)	27 439	0.028 (7)
1994	17 680 (3)	7 719 (1)	25 399	0.016 (4)
1995	17 180 (1)	7 380 (2)	24 560	0.012 (3)
1996	16 350 (10)	6 268 (5)	22 618	0.066 (15)
1997	18 162 (17)	5 431 (7)	23 593	0.102 (24)
1998	15 889 (21)	4 629 (7)	20 518	0.136 (28)

= Number; % = Percentage; SA = Sexual anomaly

As can be seen from Table 3.8, there is a 45-fold increase in the percentage of black babies being born with a sexual anomaly from 1989 to 1998. A similar trend (50-fold increase) was observed when comparing the percentage of cases with a sexual anomaly to the total number of individuals entered onto the birth defects database for each year (Table 3.9).

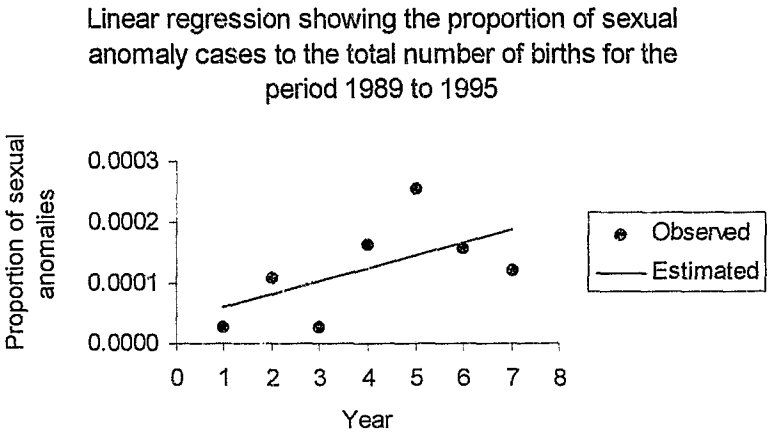
Table 3.9: The percentage of individuals entered on the Chris Hani-Baragwanath birth defects database that have a sexual anomaly

YEAR	# OF ENTRIES ON THE DATABASE	# OF INDIVIDUALS WITH SA	PERCENTAGE WITH SA
1989	4 812	1	0.021
1990	3 538	4	0.113
1991	3 232	1	0.031
1992	3 083	5	0.162
1993	3 029	7	0.231
1994	2 964	4	0.135
1995	2 683	3	0.112
1996	2 801	15	0.536
1997	2 941	24	0.816
1998	2 730	28	1.026

= Number; SA = Sexual anomaly

The data, for the first seven years (1989 to 1995) in the above two tables, was initially considered and a linear regression line fitted. As can be seen in Figure 3.7, the slopes of the lines (0.00002 and 0.00018, respectively) were not significantly different from zero. That is, the proportion of individuals diagnosed with a sexual anomaly from 1989 to 1995 was relatively constant with no significant statistical difference ($p = 0.41$ and $p = 0.36$ for Table 3.8 and 3.9, respectively). If this trend continued, we could expect the model to also fit the data for the following year, 1996. However, including the 1996 data point results in the null hypothesis (i.e., no change in the proportion of individuals with a sexual anomaly from year to year) (H_0: the slope of the regression line = 0) being rejected at the 5% level. This provides evidence for a significantly increasing trend of sexual anomaly cases recorded in the black population. This trend is further highlighted by the fact that when a logistic regression was fitted to the data, it clearly showed an exponential increase in the proportion of black babies diagnosed with a sexual anomaly over the ten year period studied (Figure 3.8).

A



B

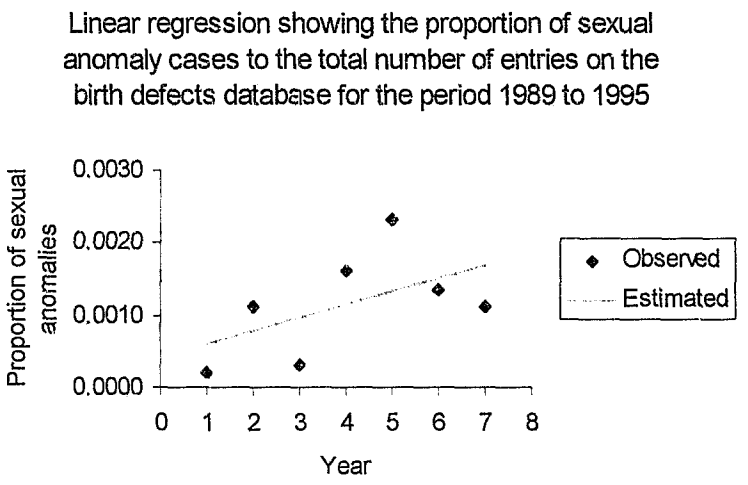
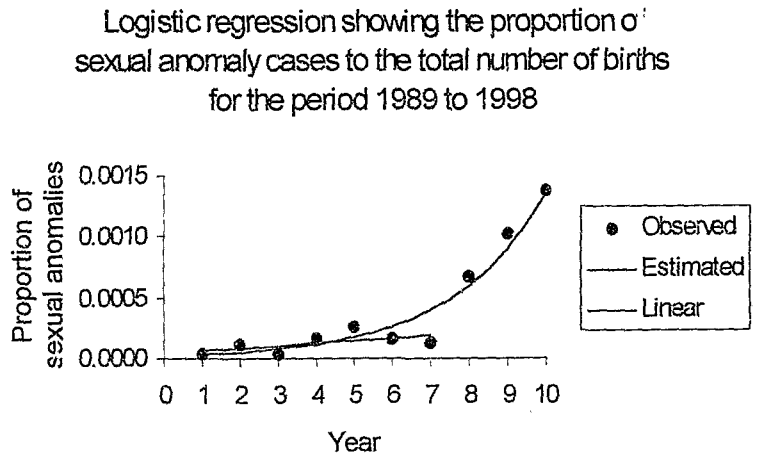


Figure 3.7. Linear regression showing the relatively constant number of black babies being born with a sexual anomaly over a seven year period (1989 to 1995) when looking at:

A. The total proportion of births at Chris Hani-Baragwanath hospital and other clinics.

B. The total proportion of entries on the Chris Hani-Baragwanath birth defects database.

A



B

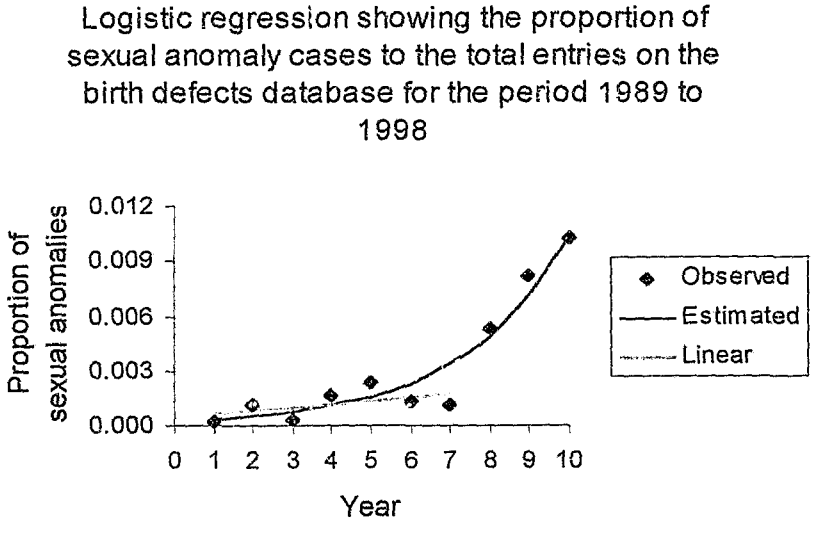


Figure 3.8 Logistic regression showing the exponential increase observed in the number of black babies detected with a sexual anomaly over a ten year period (1989 to 1998) when looking at:

A. The proportion of sexual anomalies compared to the total number of births at Chris Hani-Baragwanath hospital and other clinics.

B. The proportion of sexual anomalies compared to the total number of entries on the Chris-Hani Baragwanath hospital birth defects database.

From Tables 3.8 and 3.9 and Figure 3.8, it would appear that over the last three years there was a significant increase in the proportion of black babies being detected with a sexual anomaly ($p = 0.00020$ and $p = 0.00044$, respectively) when comparing the figures obtained in 1995 to those in 1996. Although the number of births and entries on the database has been decreasing over the last ten years, it appears that the proportion of cases with a sexual anomaly has increased.

The ratio of total births to the number of entries on the birth defects database was then calculated (Table 3.10) to determine whether or not the trend of increasing cases of sexual anomalies observed could be attributed to the birth rate and/or number of babies entered on the database with a congenital abnormality or if it is due to an external (i.e., genetic or environmental) factor.

Table 3.10: An odds ratio of the percentage of individuals with birth defects entered onto the database and the percentage of individuals born at the Chris Hani-Baragwanath and other hospitals from 1989 to 1998

YEAR	TOTAL NUMBER OF BIRTHS	TOTAL NUMBER OF ENTRIES ON THE DATABASE	RATIO OF BIRTHS TO ENTRIES ON THE DATABASE
1989	35 020	4 812	0.137
1990	36 169	3 538	0.098
1991	34 861	3 232	0.093
1992	30 309	3 083	0.102
1993	27 439	3 029	0.110
1994	25 399	2 964	0.117
1995	24 560	2 683	0.109
1996	22 618	2 801	0.124
1997	23 593	2 941	0.125
1998	20 518	2 730	0.133

From the above table, it is evident that the observed trend of increasing cases with sexual anomalies, cannot be attributed to a proportional increase in the total births or

number of birth defects entered on the database as these have remained constant over the ten year period (0.115). There must, therefore, be some other contributing factor that is resulting in the increased proportion of sexual anomaly cases detected.

3.2.2. The Cytogenetics database

From our cytogenetics records, we were able to retrieve karyotypes from 149 individuals that had been submitted for chromosomal analysis due to ambiguous genitalia, intersex or query hermaphroditism over a period spanning four years (1995 to 1998). The subjects were divided into two categories:

i. Individuals referred from Chris Hani-Baragwanath hospital (30.9% of cases)
ii. Those referred from elsewhere (69.1% of cases).

The patients, based on the indication for chromosomal analysis and the results of the karyotype, were then further sub-divided into four categories (Table 3.11 and 3.12):

i. Ambiguous genitalia
ii. Ambiguous genitalia and other sexual abnormalities including hypospadias, undescended testes etc.
iii. Intersex
iv. Hermaphroditism

Table 3.11: Karyotypes obtained from black individuals (born at Chris Hani-Baragwanath hospital) referred to our Cytogenetics laboratory for testing

	1995			1996		1997		1998		
	XX	XY	M	XX	XY	XX	XY	XX	XY	M
AG	2	1	1	10	4	5	2	3	2	1
AG+							1			1
IS	1			3	1	2	2	1		
H	1			1					1	
S.Tot	4	1	1	14	5	7	5	4	3	2
TOT	6			19		12		9		
FREQ	0.666	0.167	0.167	0.737	0.263	0.583	0.417	0.444	0.333	0.223

M = Mosaic/translocation; S.Tot = Sub-total; TOT = Total; FREQ = Frequency; AG = Ambiguous genitalia; AG+ = Ambiguous genitalia and other symptoms associated with intersex; IS = Intersex; H = Hermaphrodite

Table 3.12: Karyotypes obtained from black individuals (born at clinics and hospitals other than Chris Hani-Baragwanath) referred to our Cytogenetics laboratory for testing

	1995		1996		1997		1998		
	XX	XY	XX	XY	XX	XY	XX	XY	M
AG	4	3	7	4	10	11	12	10	1
AG+	1		1	1		2	1	2	
IS			4	3	3	4	2	3	
H		1	2	1	3	2	5		
S.Tot	5	4	14	9	16	19	20	15	1
TOT	9		23		35		36		
FREQ	0.556	0.444	0.609	0.391	0.457	0.543	0.556	0.417	0.027

M = Mosaic/translocation; S.Tot = Sub-total; TOT = Total; FREQ = Frequency; AG = Ambiguous genitalia; AG+ = Ambiguous genitalia and other symptoms associated with intersex; IS = Intersex; H = Hermaphrodite

Although the sample sizes are very small, the majority of patients referred for chromosomal analysis had the predominant karyotype found in true hermaphrodites, 46,XX. Four patients had mosaic karyotypes. They are listed in Table 3.13.

Table 3.13: Individuals with a mosaic or chromosome translocation karyotype

NAME	INDICATION	KARYOTYPE	DIAGNOSIS
XXTH 25	Ambiguous genitalia	45,X/46,XY	XXTH
XXTH 93	Ambiguous genitalia	45,X/46,X,+marker	Unknown
XXTH 94	Ambiguous genitalia/ truncus arteriosus/ CATCH22*	46,XY, add (9)(qter(p24::?)	Unknown
XXTH 95	Ambiguous genitalia	45,XX, -13, -14, t(13;14)	Unknown

* CATCH22 = This is an acronym for Cardiac defect, Abnormal face, Thymic hypoplasia, Cleft palate, Hypocalcemia, microdeletion 22q11 (Digilio *et al*, 1996)

The presence of testicular tissue in XXTH 25 and XXTH 93, could be explained by the sex chromosome abnormalities and the presence of Y chromosome in a cell line. In XXTH 94, the chromosomal abnormality may influence the sex determining gene on chromosome 9 causing ambiguous genitalia. In XXTH 95, the chromosome abnormality appears to be a balanced Robertsonian translocation. This would not be expected to influence phenotype in any way.

To determine if there was a statistical difference between the number of patients referred to the Cytogenetics laboratory for karyotyping before and after one and five years of age, respectively, a χ^2 test was performed. The null hypothesis tested was that the proportion of patients detected before a certain age is a half. When the number of individuals referred before and after one year of age were analysed, no significant statistical difference was found ($p = 0.799$) i.e., we fail to reject the null hypothesis. However, when examining patients referred before and after the age of five years, a statistical difference was found ($p = 0.006$) (Table 3.14).

Table 3.14: The age at referral of black babies born with a sexual anomaly at Chris Hani-Baragwanath hospital and other clinics and hospitals

	Chris Hani-Baragwanath					Other hospitals and clinics					
	'95	'96	'97	'98	S.Tot	'95	'96	'97	'98	S.Tot	TOT
0-1 mnth	2	5	3	5	15	3	7	9	8	27	**42**
1-6 mnth	2	2	2	1	7		2	7	9	18	**25**
6-12 mnth		1	2		3		5	5	1	11	**14**
1-5 years	1	4	5		10	1	2	3	6	12	**22**
5-10 years			1		1	2	2		4	8	**9**
> 10 years		2	1	3	6	8	4	10	7	29	**35**
U age	1	5	2	1	9		3	6	8	17	**26**
TOT	**6**	**19**	**16**	**10**	**51**	**14**	**25**	**40**	**43**	**122**	**173**

U = Unknown; S.Tot = Sub-total; TOT = Total; mnth = Month

3.3. Analysis of the SOX9 gene

Genomic DNA from affected individuals and normal controls were digested separately with the restriction enzymes *Apa*I, *EcoR*I, and *Sma*I. The samples were electrophoresed overnight, denatured *in situ* prior to Southern blotting and hybridised to the SOX9 probe p26.2. The film was developed after three day's exposure at -70°C (Figure 3.9). No information could be retrieved from the analysis of samples digested with *Sma*I (lanes 14 to 19) as only faint bands could be seen in three of the lanes. From the large and intense bands seen at the top of the lanes, it would seem that the digestion was incomplete. From the *Apa*I and *EcoR*I digests, however, it appeared as if

there may be a dosage effect occurring in some of the patients. In an attempt to determine whether or not the different band intensities observed on the SOX9 blot were due to a dosage effect, we tried to calculate the initial concentration of DNA that was used for the digest to see if this correlated to the band intensities on the blot. It was not, however, possible to accurately quantitate the concentration of DNA from the Polaroid photograph. We were, thus, unable to determine if there was a dosage effect or if it was an artefact of the gel. The blot was, therefore, stripped and hybridised with a cystic fibrosis probe, KM19 (results not shown), to see whether this was real or an artefact of DNA quantitation. Autoradiography of the KM19 blot (results not shown) was uninformative with respect to dosage since the fragments were very large for the *ApaI* and *SmaI* digests and because as many as seven bands were seen for the samples digested with *EcoRI*. Unfortunately, due to time constraints, the blot was not hybridised with another probe.

3.4. Investigation of the human androgen receptor element (ARE)

Samples were digested with the methylation sensitive enzyme *HpaII* prior to PCR. This restriction endonuclease is a rare cutter and one does not, therefore, expect to see an even smear in the lane (following digestion) as one would for other restriction enzymes. The active, unmethylated X chromosome is cut by the enzyme and as a result is not amplified in subsequent PCR reactions. The inactive, methylated X chromosome is not digested and is, therefore, amplified. However, because the process of X chromosome inactivation is a random event with, on average, 50% of the maternal and 50% of the paternal X chromosomes being inactivated, one would expect to see fragments representative of both maternally and paternally derived X chromosomes. The two X chromosomes are distinguished from one another on the basis of size as the region being amplified extends over a highly polymorphic trinucleotide repeat. If X-inactivation is not random, then preferential amplification of one of the X chromosomes will be observed. Following two rounds of amplification, radioactive (Figure 3.10) or fluorescent (Figure 3.11 to Figure 3.13) samples were run on polyacrylamide gels.

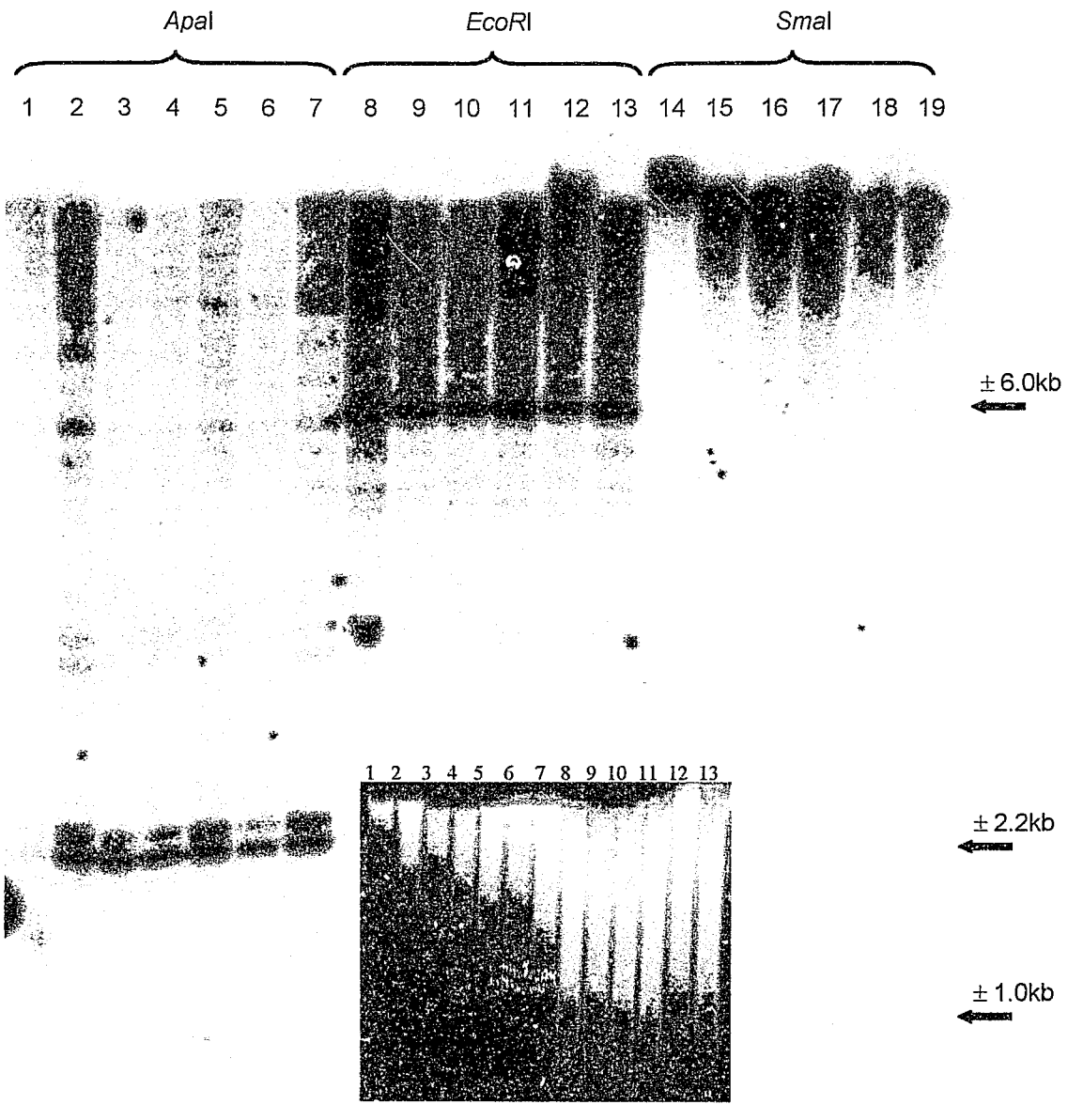


Figure 3.9. Autoradiograph of a blot hybridised with the SOX9 probe p26.2. Samples were digested separately with three enzymes: *Apa*I (lanes 1 to 7), *Eco*RI (lanes 8 to 13) and *Sma*I (lanes 14 to 19).

Lane 1 = XXTH 9; Lanes 2, 8, 14 = XXTH 1; Lanes 3, 9, 15 = XXTH 13; Lanes 4, 10, 16 = XXTH 15; Lanes 5, 11, 17 = XXTH 31; Lanes 6, 12, 18 = XXTH 88; Lanes 7, 13, 19 = female control.

Insert: Polaroid taken of the XXTH and a female control DNA digested with *Apa*I and *Eco*RI. Lanes 1 to 13 on the photo correlate with lanes 1 to 13 of the blot.

The following results were obtained. Firstly, DNA from 18 control females were amplified and the PCR products run on a 6% polyacrylamide gel to demonstrate the highly polymorphic nature of this locus (Figure 3. 10 and Table 3.15).

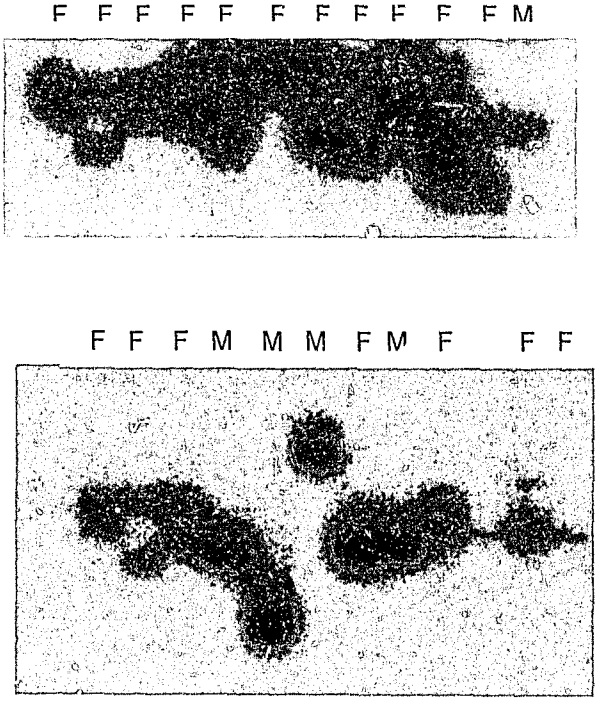


Figure 3.10. Autoradiograph of HUMARA PCR products of random black samples electrophoresed on a 6% polyacrylamide gel to illustrate the highly polymorphic trinucleotide repeat at this locus. Two bands corresponding to the different sized alleles on the two X chromosomes can be seen in most lanes. Individuals homozygous at this locus have a single band. In males who have a single X chromosome, only one band is seen.
F = Female control, M = Male control

Table 3.15: The heterozygosity frequency for the human androgen receptor gene

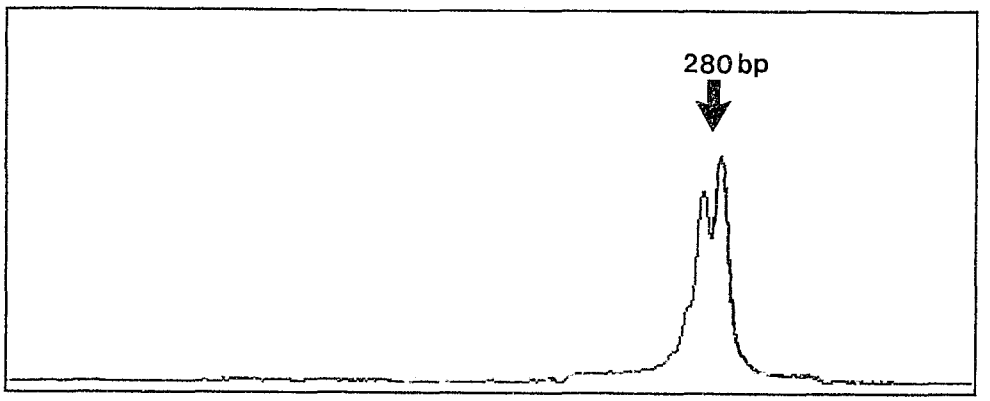
# OF FEMALE CONTROLS SCREENED*	# OF HETERO-ZYGOTES	# OF HOMO-ZYGOTES	% HETEROZYGOSITY
18	16	2	88.9

* = Data taken from results shown in Figure 3.11; # = Number; % = Percentage

When DNA from a control male individual was amplified prior to *Hpa*II digestion, a single peak was observed (Figure 3.11). PCR amplification, however, of the same sample after it has been digested with the methylation sensitive enzyme, *Hpa*II, resulted in no amplification occurring (i.e., no peak on the electropherogram). This is expected because males only have one active unmethylated X chromosome which is cut by *Hpa*II resulting in no amplification of the ± 270bp ARE fragment (Figure 3.11). In a control female exhibiting random X-inactivation, one or two peaks (depending on whether the individual is homozygous or heterozygous at the triplet repeat) are seen irrespective of whether the DNA was digested before or after PCR amplification of the ARE locus. Because this region is highly polymorphic, homozygotes are rare. Figure 3.12 illustrates the randomness of X chromosome inactivation in a normal female. Each allele (267bp and 282bp, respectively) of the ARE locus is represented by a single peak on the electropherogram. If X chromosome inactivation were not random, one allele would be preferentially amplified following digestion with *Hpa*II.

Despite numerous attempts, it was extremely difficult to get this assay to work on the DNA extracted from paraffin-embedded tissue samples. Amplification, prior to *Hpa*II digestion, was only observed for one of these samples (Figure 3.14). The peak area was, however, extremely small. After digestion, no amplification was observed for either X chromosome. Thus, no deductions can be made regarding the X-inactivation hypothesis and its role in the aetiology of XX true hermaphroditism.

A.



B.

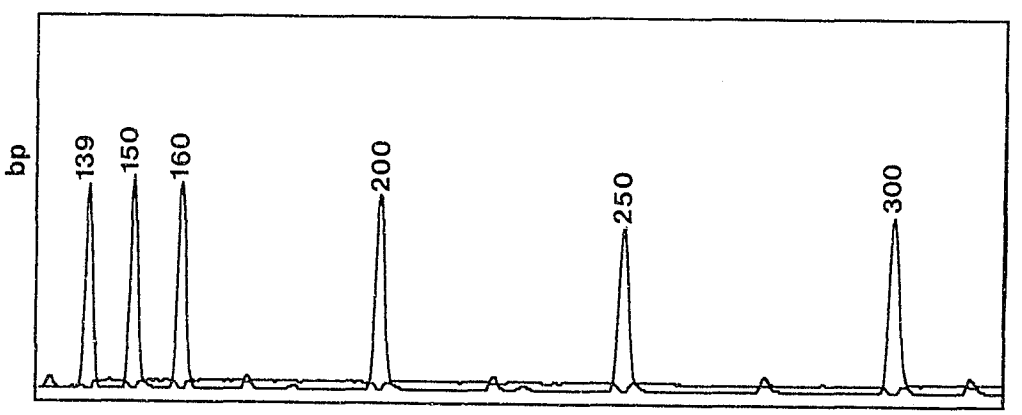
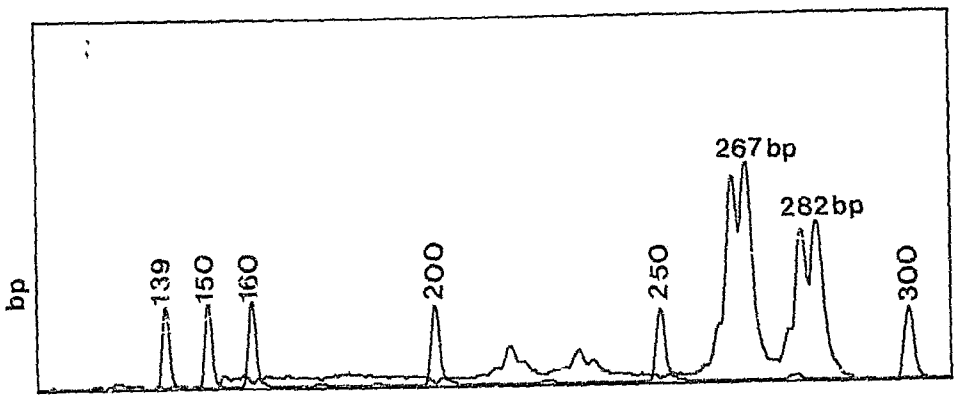


Figure 3.11. Electropherogram of a PCR fragment of the human androgen receptor gene amplified from a control male sample:

A. A single peak of 280bp is seen when the DNA sample is amplified prior to digestion with the methylation sensitive restriction enzyme *Hpa*II

B. PCR following restriction enzyme digestion results in the absence of any peaks as the single active (unmethylated) X chromosome has been cut. The red peaks on the electropherogram represent the ROX size standard that run with every sample.

A.



B.

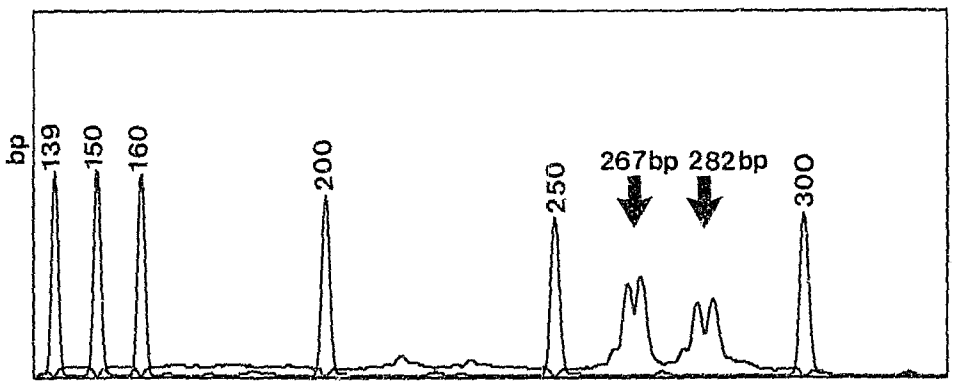
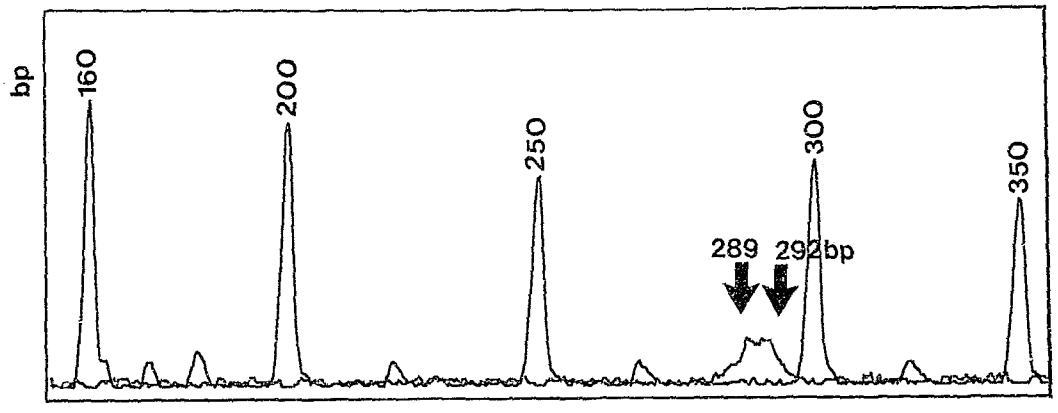


Figure 3.12. Electropherogram illustrating random X chromosome inactivation in a normal female DNA sample amplified with the ARE primers (the red peaks represent the internal size standard):

A. Two peaks representative of the two differently sized ARE alleles, 267bp and 282bp, are seen prior to *Hpa*II digestion of DNA from a female control

B. Following digestion of the DNA, two peaks, of equal proportion to those obtained before digestion, can be seen. This illustrates the random inactivation of the maternal and paternal X chromosomes.

A.



B. No amplification was observed

C. No amplification was observed

Figure 3.13. Electropherograms illustrating DNA amplification of the human androgen receptor gene after extraction from formalin fixed paraffin-embedded gonadal tissue of three different patients:

A. Amplification of DNA extracted from testicular tissue

B. Amplification of DNA extracted from ovarian tissue

C. Amplification of DNA extracted from the testicular portion of an ovotestis.

3.5. Molecular investigation of the DAX1 gene by means of SSCP analysis and sequencing

A total of 37 black individuals, including 23 confirmed true hermaphrodites, 6 suspected hermaphrodites and 8 normal female controls, were screened for mutations in the DAX1 gene. SSCP analysis of PCR generated products (labelled with [α-^{32}P] dCTP) was carried out under two sets of conditions, namely: MDE gels with 10% glycerol and those without glycerol. Any variants detected by this method were sequenced to identify the change responsible for the mobility shift. Table 3.16 lists the results obtained for the six regions of the DAX1 gene screened by SSCP analysis. DAX1.1 to 1.5 represent overlapping fragments of exon 1 and DAX 2.1 includes the entire coding sequence of exon 2.

If a single band was observed, individuals were scored as 1/1 homozygotes. If two bands were present on the autoradiograph, they were scored as 1/2 heterozygotes. Individuals with only the lower band were scored as 2/2 homozygotes. When four bands were present, two bands were scored as 1 and two bands as 2 i.e., four bands in one lane represent a heterozygous individual, 1/2 (see figure 3.14). The appearance of the bands in the homozygotes would guide the discussion of which bands represented each allele.

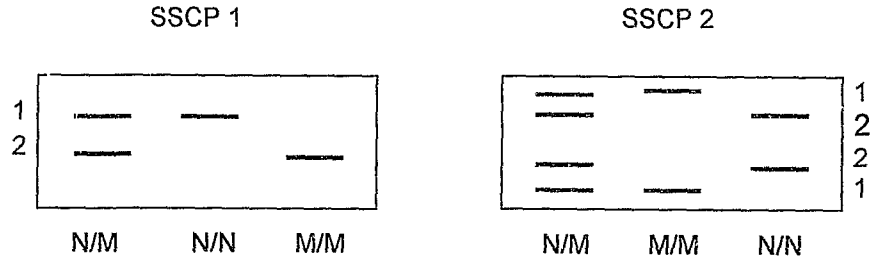


Figure 3.14. Diagram to illustrate how genotypes, for individuals on SSCP gels were scored (N = normal; M = mutant band)

SSCP analysis of the DAX1 gene showed mobility shifts in two of the six regions of the gene (Table 3.16). These were DAX1.1 (Figure 3.15) and DAX1.3 (Figure 3.16). No

variation was detected on autoradiography of the SSCP gels performed with the DAX1.2, DAX1.4, DAX1.5, and DAX2.1 primer pairs (Figure 3.17). A control individual and one patient exhibiting a shift were sequenced in the forward and reverse directions. Table 3.17 lists the results obtained upon sequencing of the relevant individuals.

Table 3.16: Results obtained from SSCP analysis of samples on MDE gels with and without glycerol*

INDIVIDUAL	PRIMERS IN THE DAX1 GENE**					
	DAX1.1	DAX1.2	DAX1.3	DAX1.4[†]	DAX1.5	DAX2.1
CONTROL 1	2/2		2/2	1	1/1	1/1
CONTROL 2	2/2	1/1	2/2	1	1/1	1/1
CONTROL 3	2/2	1/1		1	1/1	1/1
CONTROL 4		1/1			1/1	1/1
CONTROL 5	1/2	1/1		1		1/1
CONTROL 6				1		1/1
CONTROL 7			2/2			1/1
CONTROL 8			2/2			1/1
XXTH 1				1	1/1	
XXTH 4						
XXTH 5	2/2		1/1	1	1/1	
XXTH 7	2/2		2/2	1	1/1	
XXTH 9	2/2	1/1	1/2	1	1/1	
XXTH 11	2/2		2/2	1	1/1	
XXTH 12	2/2		1/2	1		
XXTH 13	2/2	1/1	1/1	1	1/1	
XXTH 14	2/2	1/1	1/1	1	1/1	
XXTH 15		1/1	2/2			
XXTH 17						
XXTH 20	2/2	1/1	1/1	1	1/1	
XXTH 21	2/2	1/1	1/1	1	1/1	1/1
XXTH 24	2/2	1/1		1		

INDIVIDUAL	PRIMERS IN THE DAX1 GENE**					
	DAX1.1	DAX1.2	DAX1.3	DAX1.4†	DAX1.5	DAX2.1
XXTH 25	2/2	1/1	1/1	1		1/1
XXTH 26						
XXTH 27#	2/2	1/1	1/1	1-	1/1	1/1
XXTH 29	2/2	1/1	1/1		1/1	
XXTH 30	2/2	1/1	1/1	1	1/1	1/1
XXTH 31	2/2	1/1	2/2	1		1/1
XXTH 32						
XXTH 81	2/2					1/1
XXTH 83	2/2	1/1	1/1		1/1	
XXTH 86	2/2	1/1	1/1	1	1/1	1/1
XXTH 87	2/2	1/1	1/1	1		
XXTH 88	2/2	1/1	1/1	1	1/1	1/1
XXTH 89	2/2	1/1	1/1	1	1/1	
XXTH 90	1/2		1/1	1	1/1	1/1
XXTH 91	2/2					
XXTH 92#	2/2		1/1	1-	1/1	

* The genotypes were the same irrespective of +/- glycerol MDE gels; ** Blank boxes indicate that no results were obtained; † Genotypes could not be assigned because of the pattern of bands obtained for this primer set; # XXTH 27 and XXTH 92 are denoted as 1- for DAX 1.4 as they were missing one band in their banding pattern which was, otherwise, identical to the pattern observed for all the other patients and controls studied.

Table 3.17: Sequencing results on controls and of representative individuals that showed mobility shifts in the DAX1 gene upon SSCP analysis

REGION OF DAX1 GENE	INDIVIDUAL	DNA VARIANT	AMINO ACID CHANGE
DAX1.1*	CONTROL 1(2/2)	Normal* (G)	Glutamic acid at amino acid four
	XXTH 90 (1/2)	Normal* (G)	Glutamic acid at amino acid four
DAX1.3	XXTH 30 (1/1)	TGT homozygote	Serine
	XXTH 9 (1/2)	TGC / TGT heterozygote	Cysteine / Serine
	CONTROL 2 (2/2)	TGC homozygote (normal)	Cysteine
DAX1.4	CONTROL 1 (1/2)	Normal	-
	XXTH 92 (2/2)	Normal	-

* At nucleotide 10 a G, and not a C (as given in GenBank), was found in all samples investigated. This was therefore taken to be the consensus sequence. After sequencing, no sequence differences were found between the assigned genotypes 2/2 and 1/2

The results obtained from SSCP analysis of DAX1.3 were potentially the most interesting and yielded the most variation (Figure 3.15 and Table 3.16). Although there are only two heterozygotes listed in the above table, four appear in Figure 3.15. This is because some parents of the hermaphrodites were included on the SSCP gel of DAX 1.3. Two of the parents were heterozygotes on the autoradiograph.

The allele and genotype frequencies were calculated from the results obtained for DAX1.3 and compared between normal and XXTH individuals. Allele 1 (p) occurred at a frequency of 0.773 in the XXTHs but was not observed in the four control subjects. The frequency of q was calculated to be 0.227 and 1 in the XXTHs and control individuals, respectively. Given the above allele frequencies for the XXTHs, it was then possible to calculate the expected genotype frequencies to determine if they were in Hardy-Weinberg equilibrium. Table 3.18 lists the observed and expected genotype frequencies for the XXTH patients. No significant statistical difference ($p = 0.456$) was found

between the observed and the expected genotypes. It thus appears that the hermaphrodite sample is in Hardy-Weinberg equilibrium.

Table 3.18: Genotype frequencies calculated for the XXTH patients based on allele frequencies obtained for DAX1.3

SAMPLE	GENOTYPE FREQUENCY		
	p^2 (n)	2pq (n)	q^2 (n)
OBSERVED	0.727 (16)	0.091 (2)	0.182 (4)
EXPECTED	0.598 (13)	0.351 (8)	0.051 (1)

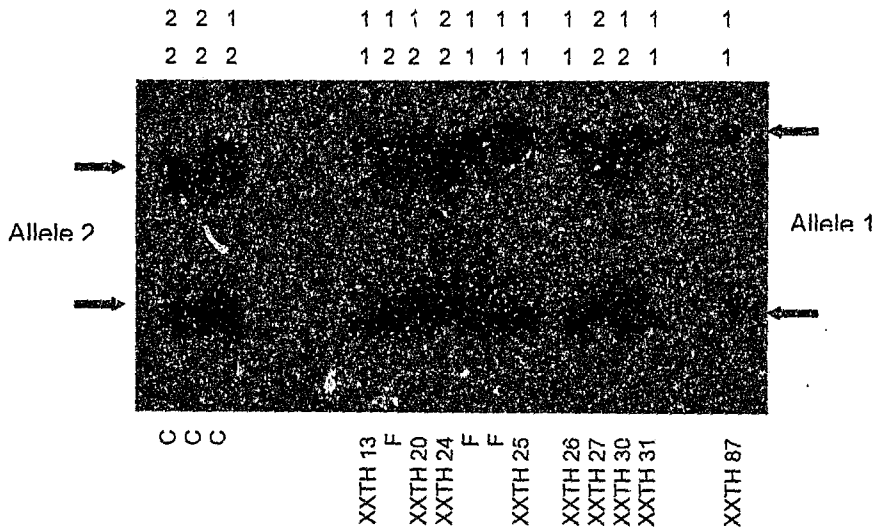
n = number of individuals

Sequencing of individuals representative of each genotype for the DAX1.3 region revealed a C to T transversion at nucleotide 660 of the DAX1 sequence. This results in an amino acid change: cysteine to serine. Cysteine and serine are both polar, uncharged amino acids. The molecular weight of cysteine is larger than that of serine and this could, therefore, result in a conformational change in the protein structure and hence to an altered function of the DAX1 protein. The sequencing results obtained for XXTH 9 (TGC/TGT heterozygote) corresponded with the genotype assigned to this patient from SSCP analysis i.e., 1/2. One would, therefore, expect the control patient (1/1) to be a TGC/TGC homozygote and the XXTH patients with the 2/2 genotype to be homozygous for the mutation. The opposite was observed in the control and XXTH patient that was sequenced. This finding merits further investigation, but unfortunately due to time constraints, could not be done for this study.

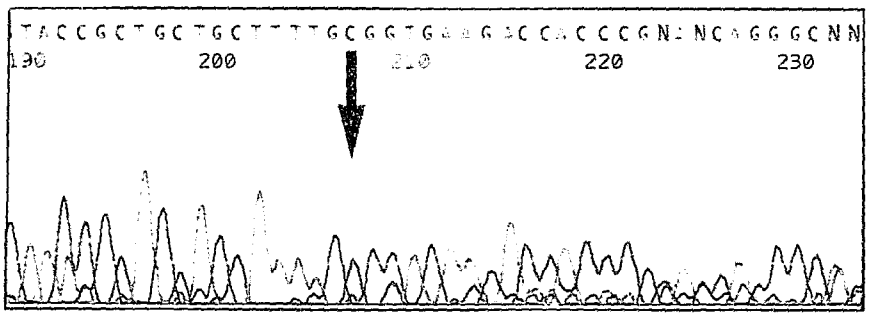
Although the SSCP shift observed in DAX1.1 was very clear, it is unlikely to be pathogenic because of its presence in one hermaphrodite and one control individual. Sequencing revealed no differences between the individuals with the shift and those without. All individuals tested were homozygous for a C to G transition at nucleotide 10 of the DAX1 gene sequence. Because the sequence reported by Zanaria *et al* (1994) also had a G at this position, and due to that fact that all individuals (XXTHs and controls) sequenced with the DAX1.1 primer pair had a G at nucleotide 10, this was taken to be the consensus sequence in our study.

controls) sequenced with the DAX1.1 primer pair had a G at nucleotide 10, this was taken to be the consensus sequence in our study.

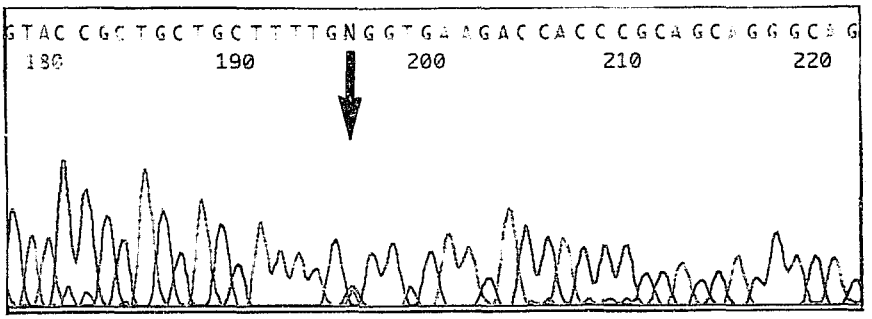
A.



B.i.



B. ii.



B.iii.

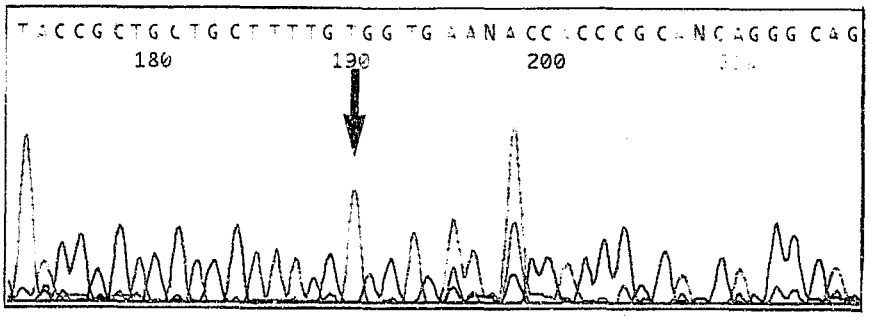


Figure 3.15. A. Autoradiograph of SSCP variants obtained with the DAX1.3 PCR system (hermaphrodite samples and some family members are shown) run on a MDE gel without glycerol. The arrows on the right indicate the band shifts that were detected. Individuals representative of each shift/genotype (1/2, 1/2, 2/2) were sequenced in the forward and reverse directions.

B. Electropherogram of sequencing results obtained for:

i. 1/1 homozygote

ii. 1/2 heterozygote

iii. 2/2 homozygote upon SSCP analysis of the DAX1.3 PCR fragment.

Results are shown for sequencing in the forward direction only. The arrows indicate the variant identified in the DNA sequence. It is a C to T transversion, resulting in a cysteine to serine amino acid change.

C = control, F = family member of XXTH

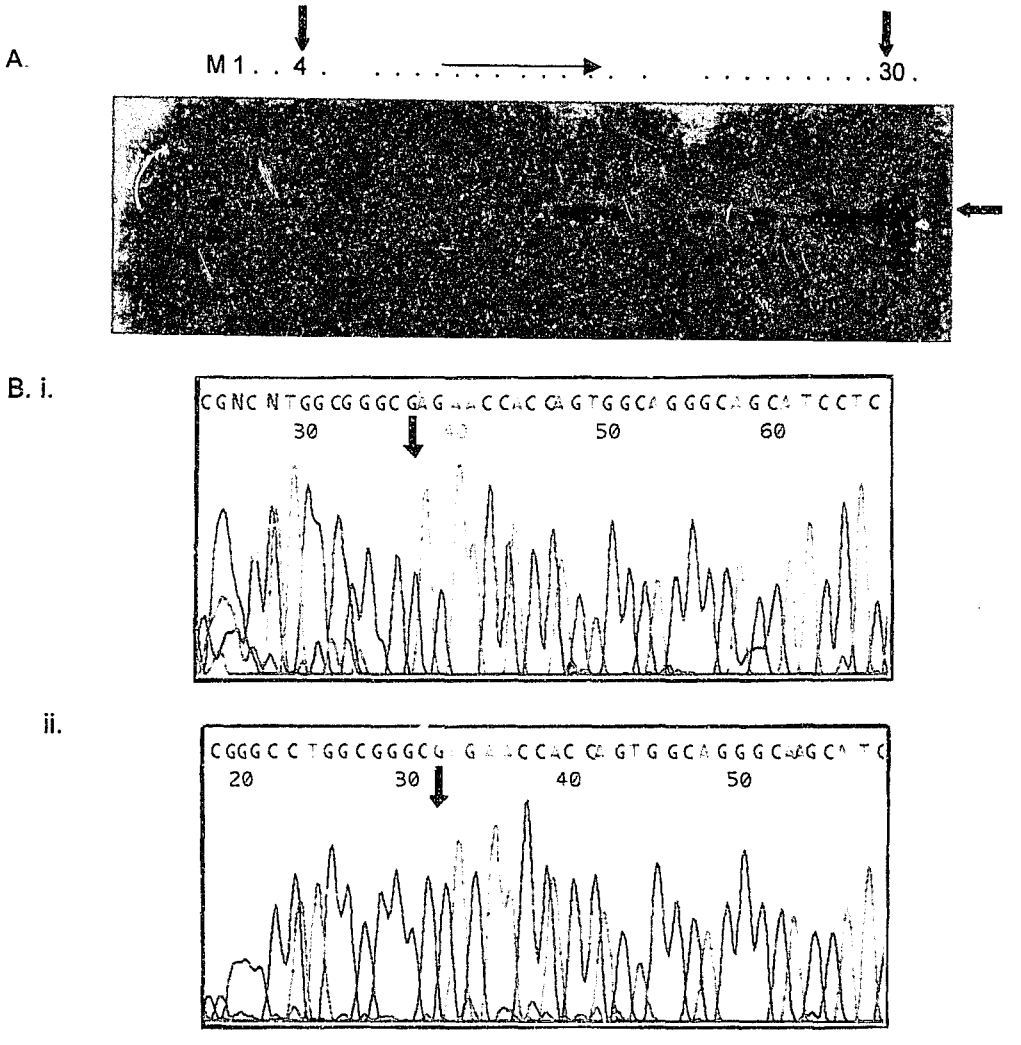


Figure 3.16. A. Autoradiograph of SSCP variants obtained with the DAX1.1 PCR system run on a 10% glycerol MDE gel. Lanes 1-4: controls, Lane 5-31: patients The arrow on the right indicates the band shift that was detected, and those above indicate the individuals with the shift. A normal control (lane 4) and one patient (XXTH 90) (lane 30) showing a shift were sequenced in the forward and reverse directions. B. Electropherogram of (i) a control sample and (ii) an XXTH sample that had a variant band on SSCP analysis of DAX1.1. The arrows indicate the position in the forward DNA sequence where it differed from the GenBank entry. The sequence downloaded from GenBank (accession number U31929) had a C at nucleotide 10 of the DAX1 gene. In the sequence reported by Zanaria *et al* (1994), however, a G was shown at this position. All individuals sequenced with the DAX1.1 primer pair had a G at nucleotide 10 of the DAX1 sequence. The G was, therefore, taken to represent the normal sequence in this study, and was not responsible for the band shift.

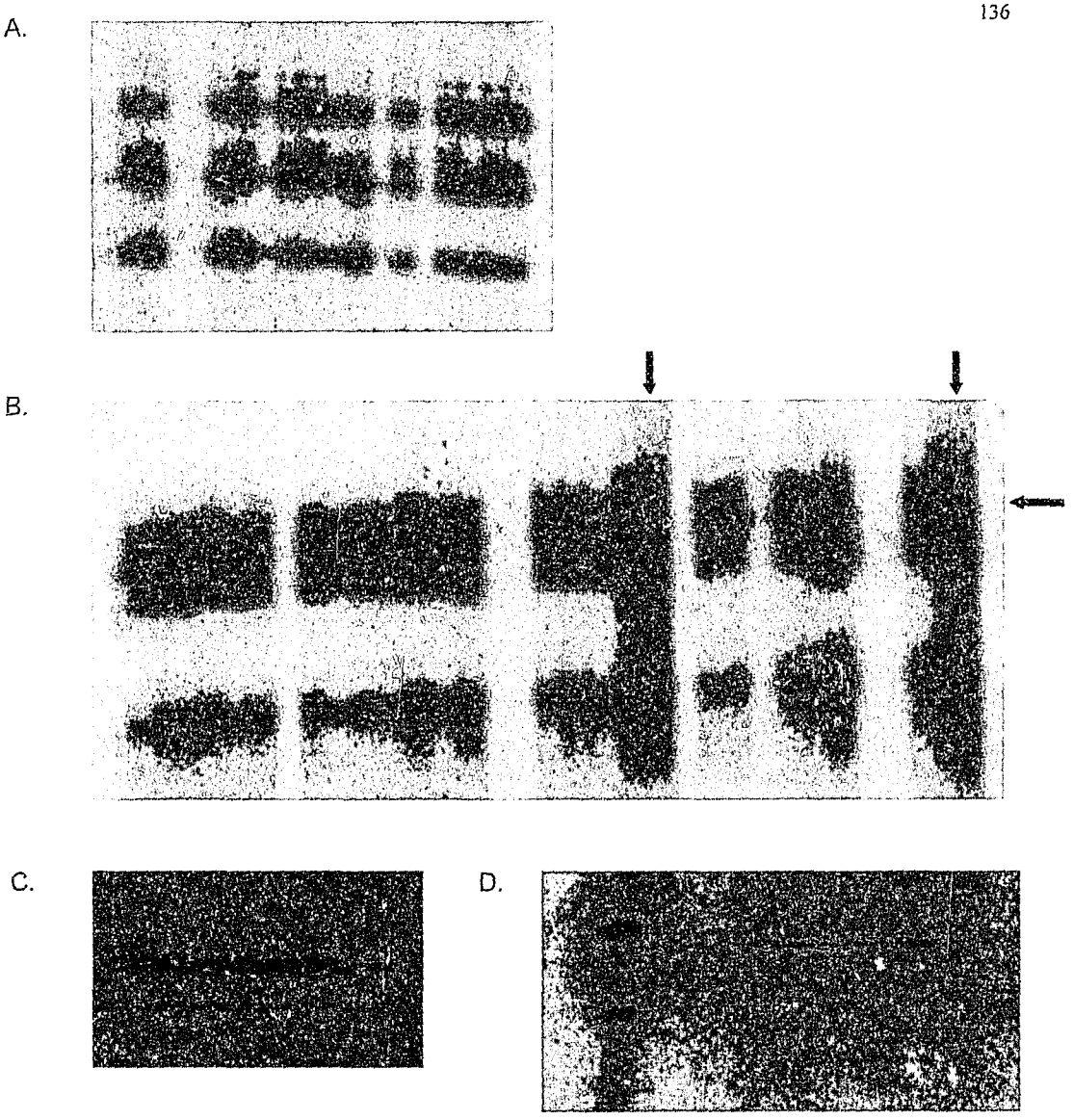


Figure 3.17. Autoradiograph of SSCP analysis using the following PCR primer sets:

A. DAX1.2

B. DAX1.4 (an arrow to the right of the autoradiograph indicates a possible absent band, and those above indicate the individuals detected with the alteration. Sequencing, however, did not reveal any differences to the normal sequence)

C. DAX1.5 and

D. DAX2.1.

No convincing mobility shifts were observed with these primers, although there was variation in the band intensities. This was not, however, reproducible/significant. Each gel contained 3 to 5 control samples that were no different to the XXTHs.

As seen in Figure 3.17B, a number of bands were present for all individuals amplified with the DAX1.4 primer pair and it could, therefore, not be properly scored. Variation was observed in two XXTH patients who are possibly missing a band (this was not very clear on the autoradiograph). No variation was found upon sequencing of one XXTH patient that looked as if a band was absent and a control individual. It is most likely that the absent band observed is due to an artefact of the gel.

If the DAX1 gene plays a role in the aetiology of XX true hermaphroditism, from SSCP and sequencing analysis, DAX1.3 would be the most likely region of the gene to be involved. This was the only region examined where a shift on the SSCP gel corresponded to a change in the DNA and protein sequences, and where the variant is associated with XXTH. No variation was detected in the other five regions of the DAX1 gene that were examined.

3.6. Gonadal mosaicism/chimerism in XX true hermaphrodites

The presence of three Y-specific loci: SRY, 49a and AMEL-Y, was investigated in our patients using DNA extracted from peripheral blood. In addition, gonadal tissue was microdissected and DNA extracted from the archival paraffin-embedded gonadal tissue of five confirmed XX true hermaphrodites. To determine how sensitive each assay was for detecting mosaicism or chimerism, dilutions of male DNA, relative to female DNA, were set up. The final concentrations of male DNA in the mixes were 0.1%, 1% and 5%. Following PCR amplification a 20μl aliquot was loaded on a 3% agarose gel (Figures 3.18). Individuals were scored for the presence or absence of Y material for the three loci examined (Table 3.19).

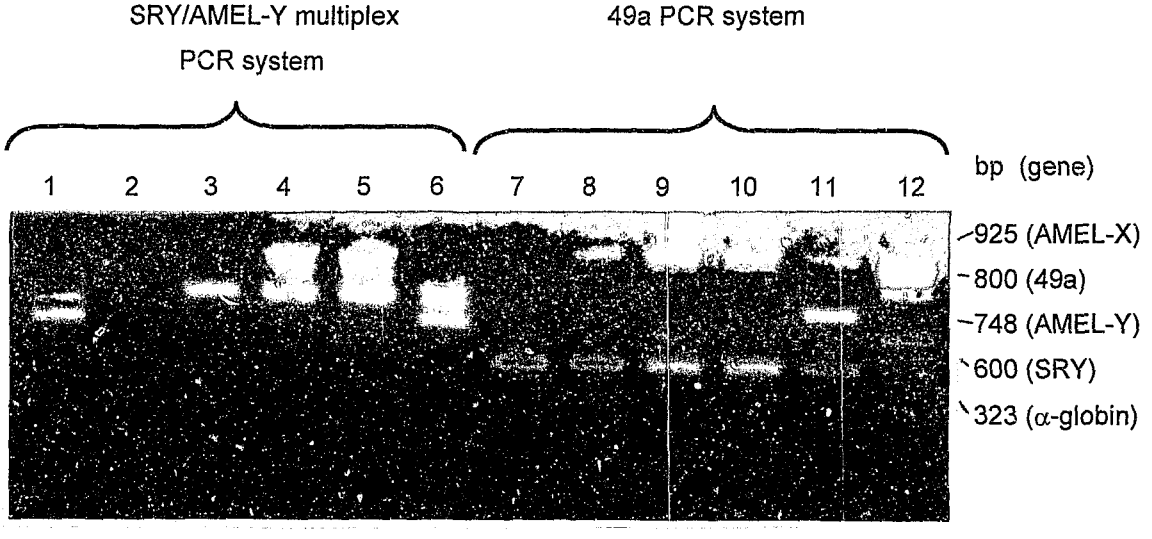


Figure 3.18. Photograph showing the results obtained on a 3% agarose gel of samples amplified for the three Y specific loci: SRY, AMEL-Y and 49a.
Lane 1: Male control, Lane 2: Blank, Lane 3: Female control, Lanes 4-6: XXTH patients, Lanes 7-9: XXTH patients, Lane 10: Female control, Lane 11: Male control, Lane 12: Molecular weight marker.

Table 3.19: Patients with Y chromosome material after investigation of three Y-specific loci on DNA extracted from lymphocytes and paraffin-embedded gonadal tissue

XXTH	KARYOTYPE (Blood lymphocyte)	HISTOPATHOLOGY		Y CHROMOSOME LOCI[1]			
		RIGHT GONAD	LEFT GONAD	SRY	AMELOGENIN[2]		49A
					X	Y	
0.1% MIX				-	-	-	
1% MIX				-	+	-	+
5% MIX				-	+	-	+
XXTH 15				+	+	+	+
XXTH 25	45,X/46,X+ marker	Ovary	Testis	+	+	+	-
XXTH 26	46,XX	Testis	Ovary	-	-	-	+
XXTH 27				+	+	+	+
XXTH 29				+	+	+	+
XXTH 83				-	+	-	+
XXTH 87	46,XX	Ovotestis	Ovotestis	-	+	-	+
XXTH 92		Ovotestis	Ovary	+	+	-	-
XXTH 9[3]	46,XX	Ovary	Ovotestis	-	-	-	+

1 = Blank boxes indicate that no results were obtained; 2 = The amelogenin gene is located on the X chromosome, and there is a homologous locus on the Y chromosome; 3 = results obtained using DNA extracted from archival formalin-fixed paraffin-embedded gonadal tissue

With the exception of XXTH 9, all the results listed in Table 3.19 were obtained from studies performed on DNA e4xtracted from peripheral blood.

The + signs in the above table refer to individuals who had a band present when their PCR products were analysed on a 3% agarose gel for a particular sexing system. The – signs in the table mean that no amplification was observed for the control band as well as the band for the particular sexing PCR system. The majority of the XXTHs showed amplification for the control band but not for the Y loci.

In total, 30 patients were examined for the presence of Y chromosome material. Six patients failed to amplify control or Y-specific sequences, 15 showed amplification but not for the Y chromosome and nine patients showed the presence of either one, two or all three Y specific loci. Three patients (XXTH 15, 27 and 29) were positive for all three loci on the Y chromosome that were investigated. Unfortunately no cytogenetic analyses had been performed on these patients and it is, therefore, not known whether they are 46,XY males with ambiguous genitalia or if they are 46,XX/XY chimeras/mosaics, both of which would explain the results obtained. One patient, XXTH 25, was positive for the two loci on the short arm of the Y chromosome (SRY and AMEL-Y) but was negative for the 49a locus which is found on the long arm of the Y chromosome. These results are consistent with the karyotype for this patient: 45,X/46,X,+ marker. The marker is more than likely the short arm of the Y chromosome. One patient, XXTH 92, was positive for only SRY. This could have arisen from a crossover event occurring during male meiosis in the patient's father. A further three patients (XXTH 26, XXTH 83 and XXTH87) were positive for the 49a locus alone. A possible explanation as to the sole amplification of the 49a locus in the three patients is that this assay is more sensitive than the multiplex PCR system for SRY and AMEL-Y. The evidence for this comes from the observation that bands were detected in the 1 and 5% mixes for the 49a PCR system but no amplification was observed for the same mixes when the multiplex PCR system was performed. The results may, therefore, suggest that these individuals are also positive for a low percentage of cells for SRY and AMEL-Y, but that the PCR was not sensitive enough to detect it. The experiments need to be repeated.

Another anomalous finding is the absence of product with AMEL-X in two individuals. Absence of an X chromosome would be incompatible with life and, therefore, these results are incorrect. They may cast doubt on the other results obtained for the multiplex experiments. One factor, however, that may explain this anomaly is that the SRY/AMEL-Y multiplex PCR system is less sensitive than the 49a PCR system. Thus, the DNA concentrations may have been insufficient in these two instances to result in amplification of the AMEL-X band. Also, as the AMEL-X band is the largest, it is possible that it would be the least efficiently amplified especially from poor quality DNA.

CHAPTER 4

4. DISCUSSION

The primary objective of this project was to determine the molecular aetiology of XX true hermaphroditism in the South African black population. The candidate gene approach was used to search for important factors leading to testicular development. The genes tested included SOX9 on chromosome 17 and DAX1 on the X chromosome, both of which have been shown to play a role in sex determination in humans. The randomness of X chromosome inactivation was also examined to determine whether or not this phenomenon, together with mutations on the X chromosome, plays a role in testicular differentiation and may explain the occurrence of ovotestes in XX true hermaphrodites. Lastly, subjects were screened for mosaicism/chimerism, including an XY cell line, using three loci on the Y chromosome, as a possible explanation for testicular development in the gonads of XXTH individuals.

In the past individuals were diagnosed with hermaphroditism or an intersex disorder when they reached puberty and began to develop secondary sexual characteristics that were not in accordance with the assigned sex of rearing e.g. breast development in males, menstruation or the lack thereof in males and females respectively. Today, however, with the increased awareness and knowledge of the importance of early detection and treatment of patients with ambiguous genitalia, hermaphrodites (and other intersex conditions) are being diagnosed at a much earlier age. This is imperative so that corrective surgery can be performed as soon as possible to minimise the psychological trauma the child may experience having to change from one gender to another. The diagnosis of true hermaphroditism is based solely on the presence of ovarian and testicular tissue in the gonads.

Based on information from referrals to our cytogenetic laboratory, early diagnosis of intersex individuals is slowly starting to occur in South Africa. No significant statistical difference was found between the number of individuals diagnosed with a sexual anomaly before and after one year of age ($p = 0.799$) over the four year period

investigated (1995 to 1998). A significant difference was observed when comparing the number of patients detected before and after five years of age ($p = 0.006$).

Perhaps as doctors, clinicians, nurses and other healthcare workers are informed as to the relatively high incidence of XX true hermaphroditism in the South African black population and the importance of diagnosing such patients early, more patients will be detected at, or soon after, birth. Whether the failure to diagnose patients early is due to the low attendance of black patients at hospitals because of their poor socio-economic status, the lack of adequate facilities and follow up examinations, or whether it is due to the belief that intersex disorders are rare, is not known.

4.1. Gonadal distribution in true hermaphrodites

In this study the unilateral (ovotestis on one side and ovary or testis on the other side of the body) and bilateral (an ovotestis on both sides of the body) hermaphrodites accounted for roughly 75% of the cases. These results correlate with the gonadal distribution observed in other South African studies by Ramsay *et al* (1988) and van Niekerk (1974, 1976) who found the unilateral and bilateral hermaphrodites to account for 78.38% and 81.48% of their cases, respectively. When comparing the gonadal distribution observed in South African black XX true hermaphrodites (van Niekerk, 1974; van Niekerk, 1976; Ramsay *et al*, 1988; present study) to that from studies performed in other parts of the world, a different distribution pattern is seen, with a lower percentage of individuals with ovotestes (refer to Table 4.1).

In the South African patients 59.76% of cases are accounted for by the unilateral distribution (ovotestis – ovary/testis) whereas in the rest of the world only 42.36% of cases of true hermaphroditism have this distribution. There are also differences in the percentages of bilateral (ovotestis-ovotestis) and lateral (ovary-testis) hermaphrodites (both approximately 10% more frequent in other areas than in southern Africa). These differences observed in the gonadal distribution are statistically significant ($p = 0.001$). With the exception of three subjects, all of the southern African hermaphrodites were black. In contrast, from the data obtained for the rest of the world, only 91/635 (14.33%) were black or of black mixed origin (85 were from Africa but unfortunately these were

not classified separately). The difference in gonadal distribution and the fact that 95% of true hermaphrodites in the South African black population have a 46,XX karyotype, as compared to about 70% in the rest of the world, may suggest a different aetiology for true hermaphroditism in this population.

Table 4.1: Gonadal distribution in hermaphrodites in southern Africa, compared to the rest of the world

POPULATION (Total sample size)	UNILATERAL		S.Tot (%)	BILATERAL (%)	TOTAL* (%)
	OT-O	OT-T			
Southern Africa I[1] (37)	19	3	***22*** ***(59.46)***	7 (18.92)	**29** **(78.38)**
Southern Africa II[2] (27)	14	4	***18*** ***(66.67)***	4 (14.81)	**22** **(81.48)**
Southern Africa III[3] (18)	7	2	***9*** ***(50.00)***	4 (22.22)	**13** **(72.22)**
S.Tot ***(82)***	***40***	***9***	***49*** ***(59.76)***	***15*** ***(18.29)***	***64*** ***(78.05)***
Other[4] (635)	198	71	***269*** ***(42.36)***	168 (26.46)	**437** **(68.82)**
TOTAL (717)	**238**	**80**	***318*** ***(44.35)***	**183** **(25.52)**	**501** **(69.87)**

1 = Ramsay *et al*, 1988; 2 = van Niekerk (1974, 1976); 3 = present study; 4 = De Marchi *et al*, 1976; van Niekerk & Retief, 1981b; Berkovitz *et al*, 1992; Green *et al*, 1994; Hadjiathanasiou *et al*, 1994; Krob *et al*, 1994; Ramos *et al*, 1996; Giltay *et al*, 1998; Slaney *et al*, 1998; * = The remaining individuals either had a lateral distribution (ovary-testis) or the gonad was only known on the one side and not the other

The ovotestis is the most common gonad in the hermaphrodite followed by the ovary and then the testis which is the least common gonad. This was also true for our patients in which the ovotestis accounted for 58.70% of the gonads, the ovary 28.26% and the testis 13.04%.

In the literature the ovary is predominantly situated on the left side of the body with the testis and ovotestis occurring more commonly on the right. A similar distribution pattern is seen in our patients with the ovary situated on the left side in 69.23% of cases and the testis and ovotestis occurring on the right side of the body in 66.67% and 59.26% of patients, respectively.

The unilateral (ovotestis-ovary) gonadal distribution occurs approximately three times more often than the unilateral ovotestis-testis combination. The ovotestis is, therefore, located more often on the right side of the body.

The asymmetrical distribution of ovaries and testes in human hermaphrodites can be explained by the observed asymmetry of gonadal growth in human foetuses: ovaries are smaller and do not grow as fast as testes; left gonads are smaller and grow slower than right gonads. Thus one would expect left gonads to have a tendency to become ovaries and right gonads to differentiate into testes.

4.1.1. Histology of gonadal tissue

Hermaphrodites are not usually fertile. A few descriptions of hermaphrodites giving birth have appeared in the literature (Narita *et al*, 1975; Kim *et al*, 1979). The infertility is the result of dysgenic and immature gonads. A comparison between normal and hermaphrodite tissue is given.

4.1.1.1. The normal testis vs. the true hermaphrodite testis

During foetal development the testis descends from the posterior wall of the peritoneal cavity to the scrotum. In the scrotum the testis is almost completely surrounded by a serous membrane, known as the tunica vaginalis. This cavity, which is an extension of the peritoneum, protects the testis by allowing it to move freely in the scrotal sac. The testis is encapsulated by a thick, fibrous, connective tissue layer, the tunica albuginea which is continuous with septa that divide the testis into 200 to 300 pyramidal lobules. Each lobule contains groups of about four highly convoluted seminiferous tubules. The seminiferous tubules are lined by a stratified epithelium which consists of two distinct populations of cells:

i. Cells in various stages of spermiogenesis
ii. Non-spermatogenic cells, called Sertoli cells, which support and nourish the developing spermatozoa.

Spermatozoa are produced in the seminiferous tubules and pass into the rete testis (a plexiform arrangement of spaces) which is connected to the epididymis via the ductuli efferentes. These small ducts conduct the spermatozoa to the epididymis (Ross & Reith, 1985; Wheater & Burkitt, 1987). In the interstitial spaces between the tubules the interstitial, or Leydig, cells are found. These are endocrine cells which are responsible for secretion of the male sex hormones.

The testis is the least common gonad found in true hermaphrodites and is predominantly found on the right side of the body. In van Niekerk's (1974) experience, the majority (5/6) of the testes occurred in the scrotum, while one was found in the inguinal region. Similar results were reported in the literature with most testes occurring in the scrotum, 22% in the normal ovarian position and 14% in the inguinal region. In 67% of cases of true hermaphrodites with testes, the testes were found to be of a smaller size than normal. The overall picture is that of an immature and histologically abnormal testis (Krob *et al*, 1994). Interstitial and peritubular fibrosis, hyalinisation of the base membranes of the tubules, atrophy of tubules and hyperplasia of Sertoli and Leydig cells has been described.

As shown in the photomicrographs in section 3.1.4., the testes were histologically immature with numerous solid seminiferous tubules containing Sertoli cells. Interstitial cells (termed such, and not Leydig cells, as they do not produce testosterone) were present in the interstitial spaces. Spermatogonia were seen in XXTH 30 who was three months old at the time of biopsy. Van Niekerk (1974) observed no undifferentiated germ cells or evidence of spermatogenesis upon examination of his patients. Krob *et al* (1994), however, reports the finding of normal spermatogonia in young patients. Dysgenetic tissue without spermatogonia, however, predominated in patients older than eight years.

4.1.1.2. The normal ovary vs. the true hermaphrodite ovary

The ovaries, paired organs lying in the pelvic cavity, are flattened, oval organs encapsulated by a single layer of low cuboidal or squamous cells. This layer, known as the germinal epithelium, is continuous with the mesothelium that covers the mesovarium. A fibrous connective tissue layer, the tunica albuginea, lies between the germinal epithelium and the cortex. The central portion of the ovary consists of spindle-

shaped cells, reticular fibres and ground substance, which together constitute the ovarian stroma. Ovarian follicles, each containing an oocyte in various stages of development, are embedded in the peripheral zone of the ovary, the cortex. In addition, there may also be post-ovulatory follicles of various kinds, i.e., corpora lutea or degenerative follicles i.e., corpora albicantes and atretic follicles.

In the mature ovary, undeveloped follicles exist as primordial follicles that are composed of a primary oocyte surrounded by a single layer of flattened follicular cells. When stimulated to develop, the primordial follicle enlarges to form a primary follicle in which the oocyte has greatly enlarged and the follicular cells have become cuboidal. A homogenous glycoprotein layer, the zona pellucida, develops between the oocyte and the surrounding follicular cells. During this stage of follicular maturation the surrounding connective tissue stroma begins to form an organised layer around the follicle called the theca folliculi. With further development, the primary follicle continues to enlarge and the follicular cells proliferate to form a layer several cells thick called the zona granulosa. The external connective tissue layer, the theca, begins to differentiate into two layers, the theca interna (which secretes oestrogen hormones) and theca externa. Primary follicles continue to develop until the stage at which they become known as secondary follicles.

Approaching maturity, further growth of the oocyte ceases and the first meiotic division is completed just before ovulation. At this stage the oocyte becomes known as the secondary oocyte and commences the second meiotic division. The first polar body remains inconspicuously within the zona pellucida. At ovulation, the mature follicle (the Graafian follicle) ruptures and the ovum, comprising the secondary oocyte, zona pellucida and corona radiata (cells surrounding the oocyte), is expelled into the peritoneal cavity near the entrance to the uterine tube (Ross & Reith, 1985; Wheater & Burkitt, 1987).

The ovary is the second most common gonad found in hermaphrodites and is most frequently found on the left side of the body. The ovary is usually situated in its normal position and is normal in size and shape. A small percentage of ovaries reported in the literature have been described as being smaller/larger than normal or cystic. In all of van Niekerk's (1974) patients over the age of fifteen years, a corpus luteum or corpus

albicans was present. Ova were seen in three of his patients who had ovaries. This is substantiated by the cases described in the literature of pregnancies in true hermaphrodites.

In the true hermaphrodites examined in this study, the ovarian tissue was histologically normal with many primordial follicles in the ovarian stroma.

4.1.1.3. The ovotestis

The ovotestis is the most common gonad found in true hermaphrodites and is generally situated on the right side of the body. Histologic examination of the ovarian portion of the ovotestis proved to be normal in 92% of cases with numerous follicles and a normal stroma (van Niekerk, 1974). In one patient there was evidence of endometriosis and in a second patient, a few maldeveloped follicles were seen among the ovarian stroma. In a review of the literature, the ovarian portion was judged normal in 69% of patients. The predominant abnormality reported was a reduction in the amount of primordial follicles in the ovotestis. Although the ovarian tissue may be normal, some hermaphrodites have a poorly developed or rudimental uterus which may act as an impediment against viable pregnancies. However, Krob *et al* (1994) found that the uterus was often of normal size and shape in 129 true hermaphrodites reported with a uterus.

The testicular portion of the ovotestis, on the other hand, presented a typical picture of an infantile testis. Fewer seminiferous tubules were present than normal and no spermatogonia were observed. Only 31% of van Niekerk's (1974) patients showed normal testicular histology. A high incidence of sclerosis was observed in the testicular portions of the ovotestis of older patients. There is often an abundance of Sertoli cells in the lumen of the seminiferous tubules. This is reminiscent of an infantile testis.

In our patients the ovarian and testicular tissue of the ovotestis had the same histological appearance as that seen in the separate ovarian and testicular gonads.

4.2. A minimum incidence for true hermaphroditism in the South African black population

Upon examination of the data gathered from the Chris Hani-Baragwanath hospital birth defects database, 7 of the 92 (7.61%) patients with a sexual anomaly were identified as histologically confirmed true hermaphrodites based on the appearance of their gonads. From these data and the total number of births at the respective hospitals and clinics for 1989 to 1998 (193 973 at Chris Hani-Baragwanath hospital and 86 513 at other hospitals), a minimum incidence for true hermaphroditism of 1 in 40 070 was calculated. This is an underestimate as many of the cases with ambiguous genitalia did not have gonadal biopsies and would thus remain undiagnosed.

However, given a minimum incidence for hermaphroditism of 1 in 40 070 in the South African black population and a birth rate of 34 per 1 000, one would expect to see 27 black babies being born each year in South Africa with true hermaphroditism (see Table 3.7). This figure is far from the number of individuals that are being detected/diagnosed with this disorder.

Possible reasons for the low detection rate of true hermaphrodites in the black population may be attributed to cultural and economic (extreme poverty) factors:

i. sexual anomalies are considered taboo in their society and such individuals may, therefore, be ostracised from the community and as a result their parent(s) would hide their disorder and they would grow up to do likewise
ii. such individuals are not aware of any anomaly until they reach puberty and develop contradictory secondary sexual characteristics e.g. breast development in males and failure to menstruate in females. These individuals would thus only seek medical attention after puberty
iii. they may live in rural areas and are not able to receive medical advice from qualified practitioners and therefore remain undiagnosed
iv. they may not be able to afford the costs incurred for transport and doctors' consultation fees and therefore remain undetected by the medical profession.
v. they are not ill and there is, therfore, no need to consult a doctor.

From the birth defects database it was also evident that there was a significant ($p = 0.00020$ and $p = 0.00044$) increase in the number of individuals detected with a sexual anomaly from 1996 onwards as compared to the numbers detected prior to 1996 (i.e., 1989 to 1995). This dramatic increase may be due to:

i. increased awareness as to the importance of early diagnosis and treatment of intersexed individuals
ii. the presence of a specialist or a specialist clinic at the hospital receiving referrals fro a large area
iii. human error – the lack of, or incorrect, entering of information on the database prior to 1995
iv. the cost of advanced technologies/techniques (such as gonadal biopsies) that are essential to make a diagnosis of sexual anomalies, may have changed over the years.
v. the criteria of what defines a sexual anomaly may have changed over the years resulting in more individuals being diagnosed in the later years
vi. an environmental agent(s) may be becoming more prevalent and could cause individuals with a predisposition to develop sexual anomalies

The first four points mentioned above may not be the entire reason for the observed increase in the number of cases of sexual anomalies detected from 1996 to 1998; the increase may be real. The last point has received considerable attention over the last few years and is a possible explanation/cause for the increased occurrence of individuals with a sexual anomaly, but is difficult to prove scientifically.

4.3. Environmental effects on sexual development in humans

During recent years, a new body of knowledge has gradually developed showing that a growing number of chemicals in food and water disrupt the growth and sexual development of living organisms like fish, birds, and mammals. Such chemicals have been found to possess either weak estrogenic, anti-androgenic or other hormonal activities and can mimic hormones. These are often referred to as endocrine disrupters. In some cases, the effects on wildlife have been dramatic. For example, development

of female sex organs in male herring gulls exposed to DDT and other organochlorine compounds (The wingspread statement, 1991).

Hormones are produced by the endocrine system – a bodily system consisting of highly specialised cells, tissues, and organs that create and secrete highly active organic chemicals called hormones. These chemical messengers are secreted into the bloodstream where they regulate other kinds of cells/organs in the body that contain specific receptors for these particular hormones. A small amount of a hormone attaches to a receptor site and the hormone-receptor pair then initiates a cascade of chemical changes, often with major and far reaching consequences (The wingspread statement, 1991).

The endocrine and nervous system are the two physiological regulatory systems of the body. The nervous system adjusts the body's response to a changing external environment on an immediate basis (shivering when cold), whereas the endocrine system copes with these environmental changes on a sustaining basis (the start of the female menstrual cycle each month). Examples of endocrine glands in humans include the adrenal gland, pancreas, ovaries and testes (The wingspread statement, 1991).

Male sexual differentiation is dependent on normal testicular function, including secretion of testosterone from the Leydig cells, and Müllerian inhibiting substance from the Sertoli cells. External factors, such as anti-androgens and estrogens, that disturb endocrine balance cause demasculinising and feminising effects in the developing male fetus. Estrogens also cause adverse effects in female fetuses, whereas anti-androgens have little influence.

The emerging adverse trends in human reproductive health, such as increased incidences of cryptorchidism, hypospadias and testicular cancer, and the ubiquitous presence of endocrine disrupters in the environment, support the hypothesis that disturbed sexual differentiation could in some cases be caused by increased exposure to environmental endocrine disrupters (Toppari & Skakkebaek, 1998). This is supported by the observations in humans and rodents exposed to hormonally active chemicals during sexual differentiation that can result in morphological pseudohermaphroditism (reviewed by Gray & Kelce, 1996).

4.4. Molecular investigations into the aetiology of XX true hermaphroditism

In an attempt to determine the molecular aetiology of XX true hermaphroditism, 23 confirmed and 6 query black hermaphrodite patients were investigated. This study included a screen of the SOX9 gene, analysis of the X-inactivation pattern in the gonadal tissue of subjects, SSCP analysis of the DAX1 gene and a search for three Y chromosome sequences in DNA isolated from archival formalin-fixed, paraffin-embedded gonadal tissue and peripheral lymphocyte tissue. The gonadal tissue was microdissected into testicular and ovarian components for the purpose of the X-inactivation study.

4.4.1. A possible role for SOX9 in the aetiology of XX true hermaphroditism

It is well established and known that haploinsufficiency of SOX9 leads to 46,XY sex reversal in approximately 70% of patients with CD (da Silva *et al*, 1996). This involvement of SOX9 in sex determination and the fact that previous studies (personal communication, Dr N. Madolo [SAIMR]) implicated a role for exon 3 of the gene in the aetiology of XX true hermaphroditism, made it an interesting candidate for investigation. The likelihood that SOX9 could be involved in the aetiology of XX true hermaphroditism was further strengthened by a very recent publication where it was found that duplication of part of chromosome 17, including SOX9, resulted in XX sex reversal (Huang *et al*, 1999). This report increases the probability of the association of SOX9 in the cause of hermaphroditism.

Southern blot analysis of the SOX9 gene revealed possible dosage differences in XXTHs. To investigate this further, the membrane was rehybridised with a cystic fibrosis gene probe, KM19. No convincing variation in the intensities of the bands was observed.

The Polaroid photograph of the digested DNA samples used for the SOX9 blot were examined and from this it was evident that lanes with greater intensity bands on the blot had a higher initial starting concentration of DNA.

Although these results did not show a convincing association between SOX9 and the aetiology of XX true hermaphroditism, it cannot be ruled out. Two reasons for the genes involvement in the aetiology of XX true hermaphroditism are:

1. A relatively crude method was used to assess the association between SOX9 and XX true hermaphroditism. Thus a more sensitive technique such as PCR may show a role for SOX9 in this disease
2. It is still possible that the regions of this gene that were not investigated in this study (e.g., the promotor region) may harbour mutations or rearrangements that result in the development of testicular tissue in the presence of a 46,XX karyotype.

4.4.2. The non-random X-inactivation hypothesis

The different cell types of the gonads arise from a number of different embryonic cell lineages, each starting with several cells. These cells are probably randomly inactivated. The gonads will therefore consist of groups of cells with the maternal X chromosome inactivated and groups with the paternal X chromosome inactivated. This fact could possibly explain the occurrence of ovotestes in true hermaphrodites. Cells from different lineages may have different X chromosomes (maternal or paternal) inactivated. When a mutation is present on the active X chromosome, it may result in testicular differentiation. When, however, the mutation is present on the inactive X chromosome, female differentiation proceeds. Thus, a gonad consisting of randomly distributed ovarian and testicular tissue arises. Depending on the position of the variously inactivated cells in the gonad, ovotestes with varying distributions of the two gonadal tissues could arise in hermaphrodites.

The human androgen receptor assay was optimised to detect the X-inactivation pattern in human DNA samples. Unfortunately, however, when this technique was applied to DNA extracted from archival paraffin-embedded gonadal tissue of the XX true hermaphrodite patients, no amplification was observed. In one sample, extremely small peaks were seen. The peaks were, however, too small to draw any conclusions regarding the randomness, or lack thereof, of X chromosome inactivation and the role of a mutated gene on the X chromosome in the aetiology of XX true hermaphroditism in the South African black population.

The extraction of high quality DNA from paraffin-embedded samples is problematic. The quality/quantity of DNA extracted depends on the fixative used and the method of fixation. Precipitive fixatives, such as ethanol and acetone, have been shown to produce DNA with better PCR amplification than cross-linking fixatives such as formalin (Goldsworthy *et al*, 1999).

The paraffin-embedded samples used in this study were archival formalin fixed gonadal tissue biopsies. These two factors, age and method of tissue fixation, may have contributed to the low concentration, poor quality, degraded DNA that was obtained from extraction. This may have been the cause for the lack of amplification observed for the HUMARA assay when using DNA extracted from these tissues.

The non-random X chromosome inactivation hypothesis remains one of considerable interest and further studies should be pursued. However, the role of X-inactivation in the aetiology of XX true hermaphroditism remains elusive.

4.4.3. Dosage sensitive sex determination (DAX1)

The DAX1 gene occurs in the dosage sensitive sex reversal (DSS) region of the X chromosome and may a play a role in sex reversal in XXTH individuals. SSCP analysis of the DAX1 gene identified variation in two regions of exon 1 of the gene (DAX1.1 and DAX1.3).

SSCP analysis of DAX1.1 revealed one patient, XXTH 90, and one control individual with a mobility shift. Sequencing, in the forward and reverse direction, of the DAX1.1 PCR product for XXTH 90, and a normal control, showed an apparent homozygous substitution of a C to G at nucleotide 10, creating a missense mutation at codon 4 (CAG → GAG) in all the cases studied. This apparent transition leads to an amino acid change: glutamine to glutamic acid. A discrepancy, however, arose when comparing the DAX1 sequence obtained from GenBank (accession number U31929) to a consensus sequence obtained from Zanaria *et al*, 1994. The sequences differed to one another at nucleotide 10 (C to G) in the coding region of the gene. From the sequencing results obtained for DAX1.1 of the gene, all samples in this study were found to have a G (and not a C as in the GenBank sequence) at nucleotide 10 of the

coding region. Since the G was present in all sequences, it was taken to be part of the consensus sequence in this study. Since the SSCP variant was also identified in one of the control samples it is unlikely to be disease causing, but more likely to be a polymorphism. No sequence variation was identified in the two individuals that showed a mobility shift on the SSCP gel for DAX1.1. The shift may have been an artefact.

DAX1.3 showed the most variation on SSCP analysis. Sequencing of XXTH 9 (genotype 1/2 on SSCP gel) identified a heterozygous mutation. This was a C to T transversion at nucleotide 660 of the gene. This mutation results in a cysteine to serine amino acid change (C220S). Both of these amino acids are polar and uncharged. Serine has a lower molecular weight than cysteine and this may alter the protein conformation and thus the interaction of DAX1 with other proteins. Sequencing results did not, however, correlate with those obtained from SSCP analysis. A normal control individual, genotype 2/2 on SSCP analysis, was homozygous for the C to T transversion. In contrast, an XXTH patient that was a 1/1 homozygote and, whom we would have expected to have been homozygous for the C220S mutation was in fact homozygous for the normal sequence Because the incidence of XX true hermaphroditism in the South African black population is relatively high, one would expect a common gene mutation to be involved in the aetiology. That is, we would expect most of the hermaphrodites to have the same mutation which is not what was observed for DAX1.3 as two of the patients were identified as being heterozygous for the C220S mutation and the remaining patients were homozygous normal. For many diseases expected to be due to a founder mutation, more than one common mutation has been found. This could well be the situation here, with one very common mutation and one or more rarer ones.

Although the coding region of DAX1 was extensively investigated, this gene cannot be ruled out completely as the intronic sequence and promoter region were not examined in this study. In addition, the fact that SSCP analysis does not detect 100% of mutations means that mutations in the coding region of the DAX1 gene may still be involved in the aetiology of XX true hermaphroditism in the South African black population. Further work is needed to confirm or refute a role for DAX1 in XXTH.

4.4.4. The presence of a Y-bearing cell line in XX true hermaphrodites

Sarafoylou *et al* (1998) tested the gonads of six XX true hermaphrodites with the marker sY279 to determine if the genetic aetiology of hermaphroditism resulted from the presence of an intact Y chromosome (chimerism or mosaicism) or from a Y chromosomal translocation. Their results confirmed the presence of a Y-bearing cell line in the ovotestes of true hermaphrodites (7/8 ovotestes showed moderate amplification and one showed weak amplification with Y specific primers). They concluded that 'the presence of areas of Y chromosome other than SRY support the hypothesis of gonadal mosaicism or chimerism as the likely genetic mechanism for true hermaphroditism'.

In 1990, after a thirty year long search, the gene responsible for inducing testicular development was discovered. This testis determining factor was called SRY for sex determining region of the Y chromosome (Sinclair *et al*, 1990). SRY has been considered as a candidate for the gene(s) causing development of testicular tissue in XXTHs. Only a small proportion of XXTHs, however, have been found to have Y chromosome DNA (Berkovitz *et al*, 1992; McElreavey *et al*, 1992), but most studies have been done on peripheral blood and would not detect a low level of XX/XY mosaicism in the gonads.

In this study, three loci on the Y chromosome were analysed and were found to be present in a total of nine individuals. Investigations on DNA extracted from lymphocytes will be discussed first followed by a discussion on the results obtained on DNA extracted from paraffin-embedded gonadal tissue.

i. Investigations performed on DNA extracted from peripheral blood lymphocytes:
The presence of all three Y chromosome loci were identified in three of the patients studied. However, none of these patients were histologically confirmed true hermaphrodites and no cytogenetic information was available regarding their chromosomal make-up. Thus it is not known whether they are in fact hermaphrodites or if they are 46,XY males with ambiguous genitalia. If they were 46,XX/XY mosaics, this would explain the presence of the three Y-specific loci. Five XXTH patients had either one or two of loci investigated on the Y chromosome. The two loci on the short arm of

the Y chromosome, SRY and AMEL-Y, were identified in XXTH 25. The 49a locus on the long arm was not present. Chromosome analysis revealed that this patient had a 45,X/46,X,+ marker karyotype. It is, therefore, more than likely that this marker chromosome is the short arm of the Y chromosome. One XXTH patient was positive for only the SRY band. A faint band representative of one of the three loci (49a) was seen in an additional three XXTH DNA samples amplified from blood. One might expect this to occur if there were a different cell line with a marker Y chromosome i.e., only part of the Y chromosome is present in a proportion of the cells. These results obtained for the 49a locus were, however, not reproducible.

ii. Investigations performed on DNA extracted from formalin fixed paraffin-embedded gonadal tissue:
Data from the investigation of gonadal biopsies of five XXTH individuals (one positive for the 49a locus) were not sufficient to exclude gonadal mosaicism/chimerism involving Y-specific sequences in the aetiology of XX true hermaphroditism as the tests could not be repeated due to lack of DNA. The possibility, therefore, still exists that mosaicism or chimerism with a Y-bearing cell line may be involved in the aetiology of this disorder in southern African blacks.

Dilutions of control male and female DNA samples were included in each PCR run to determine the sensitivity of the assays. The final concentration of male DNA in the mixes was 0.1%, 1% and 5%. Neither the SRY nor the AMEL-Y product was amplified from any of the mixes. For the 49a PCR system, however, amplification was achieved for the 1 and 5% mixes. Thus the 49a system is far more sensitive than the SRY/AMEL-Y multiplex PCR system. The absence of the other markers may be entirely technical, rather than due to only part of the Y chromosome being present. Gonadal mosaicism/chimerism can, therefore, not be entirely ruled out as a possible cause of XX true hermaphroditism.

The origin of Sarafoylou *et al* (1998) patients, where 7/8 of the ovotestes were positive for Y chromosome sequence, was not stated in the abstract and the work has not been published in full. But, because this study was performed by a group of Americans it is possible that the patients were white Americans. The presence of Y material in 9/30 of our South African black hermaphrodites needs to be confirmed and cannot, therefore,

support the findings of Sarafoylou *et al* (1998). It may, however, suggest that the aetiology of XX true hermaphroditism is different between white and black populations.

4.5. Future studies

Future work on the investigation of the aetiology of XX true hermaphroditism in the South African black population will first focus on getting definitive answers for the hypotheses proposed and investigated in this study. The two potential positive findings of this study, namely the DAX1.3 variant and the suggestion of the presence of Y material in XXTHs, will be the starting point for further work. Once conclusive answers have been reached regarding the involvement of DAX1.3 and Y sequence in the aetiology of XX true hermaphroditism, the next step will be to look at other chromosomes and genes that have been implicated in sex reversal and/or sex determination. These would include genes on chromosome 9, chromosome 10 and the recently identified locus on chromosome 22. If none of these loci should prove to be informative, the next best approach would be to do a genome screen to identify chromosomal regions with associations of sex determination/reversal and then to search for suitable candidate genes in these regions. The positional candidate genes would then be examined to investigate their possible role in XX true hermaphroditism.

CHAPTER 5

5. CONCLUSION

It can be regarded as fact that in all vertebrates, ourselves included, sexual development proceeds from potentially hermaphrodite rudiments. Although hermaphroditism has been recognised since pre-scientific times, man has failed to explain the occurrence of both testicular and ovarian tissue in a single individual. Today, the aetiology of XX true hermaphroditism still remains elusive despite the advanced technologies at our disposal and the ground breaking discoveries made regarding the plethora of genes involved in the sex determining pathway and their interaction with one another. Before present day efforts to explain the process in terms of molecular genetics can succeed, it will be necessary to be able to account for the facts of true hermaphroditism in man. It was after all the founder of British genetics who admonished the students of Cambridge University with these words: 'Treasure your exceptions! Keep them always uncovered and in sight. Exceptions are like the rough brickwork of a growing building which tells that there is more to come and shows where the next construction is to be' (reviewed by Mittwoch, 1986).

The gene(s) involved in the aetiology of XX true hermaphroditism have not yet been identified. A previous study on South African Bantu-speaking black XXTHs failed to implicate major environmental factors in the disorder (Ramsay *et al*, 1988). A simple single-gene explanation is unlikely if one considers that most cases are sporadic (Ramsay *et al*, 1988; Spurdle *et al*, 1995), although incomplete penetrance and variable expressivity cannot be excluded.

One easy way to explain hermaphroditism is to assume that the ovarian and testicular tissue arise from different cell lineages in the individual i.e., chimerism. This results when two eggs are fertilized by two separate spermatozoa, each with a different sex chromosome. Such an individual would, therefore, have a proportion of its cells with an XX chromosome complement and the remaining cells with an XY chromosome complement. The presence of the Y chromosome in a proportion of the cells would be

sufficient to initiate testicular development. In this study, it seems unlikely that mosaicism or chimerism with a Y bearing cell line is responsible for the phenotype observed in the majority of true hermaphrodites.

Another explanation as to the cause of hermaphroditism is that of non-random X chromosome inactivation together with a gene mutation on the active X chromosome. One could postulate that when a mutation is present on the active X chromosome, it may result in testicular differentiation in the absence of a Y chromosome. When, however, the mutation is present on the inactive X chromosome, female differentiation proceeds as the mutated gene is no longer transcribed and can, therefore, not interact with or activate/repress other genes that are involved in the initiation of testicular differentiation.

It is also not possible to rule out the option that hermaphroditism may be a multifactorial disorder with a number of genes at different loci interacting with one another and the environment.

There are a number of questions which still remain unanswered: Why is there a different pattern of gonadal distribution in the South African black XXTH population? Why is there a strikingly higher frequency of XX true hermaphroditism in the Bantu-speaking blacks? Does a genetic predisposition to the disorder exists in this population?

Until questions such as these can be answered, the presence of testicular tissue in individuals with a 46,XX karyotype will remain an enigma.

REFERENCES

ABBAS NE, TOUBLANC JE, BOUCEKKINE C et al (1990) A possible common origin of "Y-negative" human XX males and XX true hermaphrodites. *Hum Genet*, **84:** 356-360.

ABE S, NAKAE J, YASOSHIMA K, et al (1999) Novel missense mutation (Leu466Arg) of the *DAX1* gene in a patient with X-linked congenital adrenal hypoplasia. *Am J Med Genet*, **84:** 87-89.

ACHERMANN JC, ITO M, ITO M et al (1999) A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans. *Nature Genet*, **22(2):** 125-126.

ALECK KA, ARGUESO L, STONE J et al (1999) True hermaphroditism with partial duplication of chromosome 22 and without *SRY*. *Am J Med Genet*, **85:** 2-4.

ALLEN RC, ZOGHBI HY, MOSELEY AB et al (1992) Methylation of *Hpa*II and *Hha*I sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. *Am J Hum Genet*, **51:** 1229.

ALTERMAN LA, de ALWIS M, GENET S et al (1993) Carrier determination for X-linked agammaglobulinemia using X inactivation analysis of purified B cells. *J Immunol Methods*, **166:** 111-116.

ANON (1988) The Y chromosome and sex determination. *Lancet*, **1:** 973-974.

BARDONI B, ZANARIA E, GUIOLI S et al (1994) A dosage sensitive locus at chromosome Xp21 is involved in male to female sex reversal. *Nature Genet*, **7(4):** 497-501.

BEHRINGER RR, FINEGOLD MJ, CATE RL (1994) Müllerian-inhibiting substance function during mammalian sexual development. *Cell*, **79:** 415-425.

BENNETT CP, DOCHERTY Z, ROBB SA et al (1993) Deletion 9p and sex reversal. *J Med Genet*, **30:** 518-520.

BERKOVITZ GD, FECHNER PY, MARCANTONIO SM, et al (1992) The role of the sex-determining region of the Y chromosome (SRY) in the etiology of 46,XX true hermaphroditism. *Hum Genet*, **88:** 411-416.

BLYTH B, DUCKETT JW Jr (1991) Gonadal differentiation: a review of the physiological process and influencing factors based on recent experimental evidence. *J Urol*, **145(4):** 689-94.

BOGAN JS, PAGE DC (1994) Ovary? Testis? – A mammalian dilemma. *Cell*, 76: 603-607.

BROWN CJ, GOSS SJ, LUBAHN DB et al (1989) Androgen receptor locus on the human X chromosome: regional localization to Xq11-12 and description of a DNA polymorphism. *Am J Hum Genet*, **44:** 264-269.

BUSQUE L, ZHU J, De HART D et al (1994) An expression based clonality assay at the human androgen receptor locus (HUMARA) on chromosome X. *Nucleic Acids Res*, **22(4):** 697-698.

CAPEL B (1998) Sex in the 90s: SRY and the switch to the male pathway. *Annu Rev Physiol*, **60:** 497-523.

CATE RL, MATTALIANO RJ, HESSION C et al (1986) Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. *Cell*, **45(5):** 685-698.

CHERFAS J (1991) Sex and the single gene. *Science*, **252:** 782.

DAMIANI D, FELLOUS M, McELREAVEY K et al (1997) True hermaphroditism: clinical aspects and molecular studies in 16 cases. *Eur J Endocrinol*, **136(2):** 201-204.

DA SILVA SM, HACKER A, HARLEY V et al (1996) *Sox9* expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. *Nature Genet*, **14:** 62-68.

de la CHAPELLE A, HORTLING H, NIEMI M et al (1964) XX sex chromosomes in a human male: first case. *J Acta Med Scand*, **412:** 25S-38S.

DIAMOND M, SIGMUNDSON HK (1997) Sex reassignment at birth: Long-term review and clinical implications. *Arch Pediatr Adolesc Med*, **151:** 298-304.

DIGILIO MC, MARINO B, GRAZIOLI et al (1996) Comparison of occurrence of genetic syndromes in ventricular septal defect with pulmonic stenosis (classic tetralogy of Fallot) versus ventricular septal defect with pulmonic atresia. *Am J Cardiol*, **77(15):** 1375-1376.

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(3):** 293-301.

DREGER AD (1998) "Ambiguous sex" – or ambivalent medicine? Ethical issues in the treatment of intersexuality. *Hastings Center Report*, **28(3):** 24-35.

EHRHARDT AA, MEYER-BAHLBURG HFL (1981) Effects of prenatal sex hormones on gender-related behavior. *Science*, **211:** 1312-1318.

FEDERMAN DD, DONAHOE PK (1995) Ambiguous genitalia – etiology, and therapy. *Adv Endocrinol Metab*, **6:** 91-116.

FERGUSON-SMITH MA (1966) X-Y chromosomal interchange in the aetiology of true hermaphroditism and of XX Klinefelter's syndrome. *Lancet*, **2:** 475-476.

FLEJTER WL, FERGESTAD J, GORSKI J et al (1998) A gene involved in XY sex reversal is located on chromosome 9, distal to marker D9S1779. *Am J Hum Genet*, **63(3):** 794-802.

FORD CE (1970) Genetic activity of sex chromosomes in germinal cells. *Phil Trans R Soc Lond B Biol Sci*, **259:** 53-55.

FOSTER JW, DOMINGUEZ-STEGLICH MA, GUIOLI S et al (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature*, **372:** 525-530.

FRACCARO M, TIEPOLO L, ZUFFARDI O (1979) Familial XX true hermaphroditism and the H-Y antigen. *Hum Genet*, **48(1):** 45-52.

GAHMBERG CG, TOLVANEN M (1996) Why mammalian cell surface proteins are glycoproteins. *Trends Biochem Sci*, **21(8):** 308-311.

GALE RE, WHEADON H, GOLDSTONE AH et al (1996) Frequency of clonal remission in acute myeloid leukaemia. *Lancet*, **341:** 138-142.

GIESE K, COX J, GROSSCHEDL R (1992) The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. *Cell*, **69(1):** 195-195.

GINSBURG M, SNOW MH, MCLAREN A (1990) Primordial germ cells in the mouse embryo during gastrulation. *Development*, **110(2):** 521-528.

GOLDSWORTHY SM, STOCKTON PS, TREMPUS CS et al (1999) Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. *Mol Carcinog*, **25(2):** 86-91.

GOTO T, CHRISTIANS E, MONK M (1998) Expression of an Xist promoter-luciferase construct during spermatogenesis and in preimplantation embryos: regulation by DNA methylation. *Mol Reprod Dev*, **49(4):** 356-367.

GREY LE, KELCE WR (1996) Latent effects of pesticides and toxic substances on sexual differentiation of rodents. *Toxicol Ind Health*, **12:** 515-531.

GUBBAY J, COLLIGNON J, KOOPMAN P et al (1990) A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature*, **346:** 245-250.

GUBBAY J, VIVIAN N, ECONOMOU A et al (1992) Inverted repeat structure of the Sry locus in mice. *Proc Natl Acad Sci USA*, **89:** 7953-7957.

GUSTAFSON ML, DONAHOE PK (1994) Male sex determination: Current concepts of male sexual differentiation. *Annu Rev Med.* **45:** 505-524.

HACKER A, CAPEL B, GOODFELLOW P et al (1995) Expression of *Sry*, the mouse sex determining gene. *Development*, **121:** 1603-1614.

HADJIATHANASIOU CG, BRAUNER R, LORTAT-JACOB S et al (1994) True hermaphroditism: Genetic variants and clinical management. *J Pediatr*, **125(5):** 738-744.

HAQQ CM, DONAHOE PK (1998) Regulation of sexual dimorphism in mammals. *Physiol Rev*, **78(1):** 1-33.

HARLEY VR, JACKSON DI, HEXTALL PJ et al (1992) DNA binding activity of recombinant SRY from normal males and XY females. *Science*, **255:** 453-456.

HARLEY VR, LOVELL-BADGE R, GOODFELLOW PN (1994) Definition of a consensus DNA binding site for SRY. *Nucleic Acids Res*, **22(8):** 1500-1501.

HAWKINS JR, TAYLOR A, BERTA P et al (1992) Mutational analysis of SRY: nonsense and missense mutations in XY sex reversal. *Hum Genet*, **88:** 471-474.

HAWKINS R (1994) Sex determination. *Hum Mol Genet*, **3:** 1463-1467.

HAYASHI K (1991) PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods and Applications*, **1:** 34-38.

HAYASHI K, YANDELL DW (1993) How sensitive is PCR-SSCP? *Hum Mutation*, **2:** 338-346.

HINMAN F: Principles and practice of urology. Philadelphia, Saunders, 1935, p256.

HODGKIN J (1990) Sex determination compared in *Drosophila* and *Caenorhabditis*. *Nature*, **344:** 721-728.

HUANG B, WANG S, NING Y et al (1999) Autosomal XX sex reversal caused by duplication of SOX9. *Am J Hum Genet*, **65(4):** A6

HUNTER RHF (1995) *Sex determination, differentiation and intersexuality in placental mammals*. First edition, Cambridge University Press, Great Britain.

IKEDA Y, SHEN WH, INGRAHAM HA et al (1994) Developmental expression of mouse steroidogenic factor 1, an essential regulator of the steroid hydroxylases. *Mol Endocrinol*, **8:** 654-662.

JACOBS PA (1969) The chromosome basis of some types of intersexuality in man. *J Rep Fertil*, **7:** 73S-78S.

JIMÉNEZ R, SANCHEZ A, BURGOS M et al (1996) Puzzling out the genetics of mammalian sex determination. *Trends Genet*, **12(5):** 164-166.

JIMÉNEZ R, BURGOS M (1998) Mammalian sex determination: joining pieces of the genetic puzzle. *BioEssays*, **20(9):** 696-699.

JONES HW Jr, SCOTT WW: Hermaphroditism, genital anomalies and related endocrine disorders. Baltimore, Williams and Wilkins, 1958, p45.

JONES KW, SINGH L (1981) Conserved repeated DNA sequences in vertebrate sex chromosomes. *Hum Genet*, **58:** 46-53.

KIM HJ, HSU LYF, GOLDSMITH LC et al (1977) Familial translocation with partial trisomy of 13 and 22: evidence that specific regions of chromosome 13 and 22 are responsible for the phenotype of each trisomy. *J Med Genet*, **14:** 114-119.

KIM MH, GUMPEL JA, GRAFF P (1979) Pregnancy in a true hermaphrodite. *Obstet Gynecol*, **53(3):** 40S-42S.

KLUG WS, CUMMINGS MR (1991) *Concepts of genetics*. Third edition, Macmillan Publishing Company, USA.

KNEBELMANN B, BOUSSIN L, GUERRIER D et al (1991) Anti-Müllerian hormone Bruxelles: A nonsense mutation associated with the persistent Müllerian duct syndrome. *Proc Natl Acad Sci USA*, **88:** 3767-3771.

KOOPMAN P, MÜNSTERBERG A, CAPEL B et al (1990) Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature*, **348:** 450-452.

KOOPMAN P, GUBBAY J, VIVIAN N et al (1991) Male development of chromosomally female mice transgenic for *Sry*. *Nature*, **351:** 117-121.

KREIDBERG JA, SARIOLA H, LORING JM et al (1993) WT-1 is required for early kidney development. *Cell*, **74:** 679-691.

KROB G, BRAUN A, KUHNLE U (1994) True hermaphroditism: geographical distribution, clinical findings, chromosomes and gonadal histology. *Eur J Pediatr*, **153:** 2-10.

KUHNLE U, SCHWARZ HP, LÖHRS U et al (1993) Familial true hermaphroditism: paternal and maternal transmission of true hermaphroditism (46,XX) and XX maleness in the absence of Y-chromosomal sequences. *Hum Genet*, **92(6):** 571-576.

KULOZIK AE, LYONS J, KOHNE E et al (1988) Rapid and non-radioactive prenatal diagnosis of β thalassaemia and sickle cell disease: application of the polymerase chain reaction (PCR). *Br J Haematol*, **70:** 455-458.

KWOK C, TYLER-SMITH C, MENDONCA BB et al (1996) Mutation analysis of the 2kb 5' to SRY in XY females and XY intersex subjects. *J Med Genet*, **33:** 465-468.

LAU YF, YANG-FENG TL, ELDER B et al (1992) Unusual distribution of Zfy and Zfx sequences on the sex chromosomes of the wood lemming, a species exhibiting XY sex reversal. *Cytogenet Cell Genet*, **60(1):** 48-54.

LEHNINGER AL, NELSON DL, COX MM (1993) *Principles of biochemistry*. Second edition, Worth Publishers, New York.

LOVELL-BADGE R, HACKER A (1995) The molecular genetics of *Sry* and its role in mammalian sex determination. *Phil Trans R Soc Lond B Biol Sci*, **350:** 205-214.

LOVELL-BADGE R, ROBERTSON E (1990) XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy. *Development*, **109(3):** 635-645.

LUKS FI, HANSBROUGH F, KLOTZ DH Jr et al (1988) Early gender assignment in true hermaphroditism. *J Pediatr* Surg, **23(12):** 1122-1126

LUO X, IKEDA Y, PARKER KL (1994) A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell*, **77:** 481-490.

MARSHALL GRAVES JA (1998) Interactions between *SRY* and *SOX* genes in mammalian sex determination. *Bioessays*, **20(3):** 264-269.

MARX J (1995) Snaring the genes that divide the sexes for mammals. *Science*, **269:** 1824-1825.

MAXAM AM, GILBERT W (1977) A new method of sequencing DNA. *Proc Natl Acad Sci USA*, **74:** 560-564.

MAYOU BJ, ARMON P, LINDENBAUM RH (1978) Pregnancy and childbirth in a true hermaphrodite following reconstructive surgery. *Br J Obstet and Gynaecol*, **85:** 314-316.

McELREAVEY K, RAPPAPORT R, VILAIN E et al (1992) A minority of 46,XX true hermaphrodites are positive for the Y-DNA sequence including SRY. *Hum Genet*, **90:** 121-125.

McELREAVEY K, VILAIN E, ABBAS N et al (1993) A regulatory cascade hypothesis for mammalian sex determination: SRY represses a negative regulator of male development. *Proc Natl Acad Sci USA*, **90:** 3368-3372.

McLAREN A (1991) The making of male mice. *Nature*, **351:** 96.

MIGEON BR (1994) X-chromosome inactivation: molecular mechanisms and genetic consequences. *Trends Genet*, **10(7):** 230-235.

MILLER SA, DYK DD, PELESKY HF (1988) A simple salting-out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*, **16(3):** 1215.

MIRO R, CABALLIN MR, MARINA S et al (1978) Mosaicism in XX males. *Hum Genet*, **45(1):** 103-106.

MITTWOCH U (1936) Males, females and hermaphrodites. *Ann Hum Genet*, **50:** 103-121.

MITTWOCH U (1992) Sex determination and sex reversal: genotype, phenotype, dogma and semantics. *Hum Genet*, **89(5):** 467-479.

MITTWOCH U, KIRK D (1975) Superior growth of the right gonad in human foetuses. *Nature*, **257:** 791-792.

MOHANDAS T, SPARKES RS, SHAPIRO LJ (1981) Reactivation of an inactive human X chromosome: Evidence for X inactivation by DNA methylation. *Science*, **211:** 393-396.

MÜNSTERBERG A, LOVELL-BADGE R (1991) Expression of the mouse anti-Müllerian hormone gene suggests a role in both male and female sexual differentiation. *Development*, **113:** 613-624.

MUSCATELLI F, STROM TM, WALKER AP et al (1994) Mutations in the *DAX-1* gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. *Nature*, **372:** 672-676.

NAKAHORI Y, HAMANO K, IWAYA M et al (1991) Sex identification by polymerase chain reaction using X-Y homologous primer. *Am J Med Genet*, **39:** 472-473.

NARITA O, MANBA S, NAKANISHI T et al (1975) Pregnancy and childbirth in a true hermaphrodite. *Obstet Gynecol*, **45(5):** 593-595.

NASRIN N, BUGGS C, KONG XF et al (1991) DNA-binding properties of the product of the testis-determining gene and a related protein. *Nature*, **354:** 317-320.

NEW MI, KITZINGER ES (1993) Pope Joan: A recognizable syndrome. *J Clin Endocrinol Metab*, **76(1):** 3-13.

NICHOLL RM, GRIMSLEY L, BUTLER L et al (1994) Trisomy 22 and intersex. *Arch Dis Child*, **71:** F57-F58.

NORDQVIST K, LOVELL-BADGE R (1994) Setbacks on the road to sexual fulfilment. *Nature Genet*, **7:** 7-9.

ORITA M, SUZUKI Y, SEKIYA T et al (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics*, **5:** 874-879.

PAGE DC, MOSHER R, SIMPSON EM (1987) The sex-determining region of the human Y chromosome encodes a finger protein. *Cell*, **51:** 1091-1104.

PALMER MS, SINCLAIR AH, BERTA P et al (1989) Genetic evidence that ZFY is not the testis-determining factor. *Nature*, **342:** 937-939.

PALMER MS (1990) Mammalian sex determination. *Eur J Cancer*, **26(9):** 992-993.

PARADIS V, LAURENT A, FLEJOU J et al (1997) Evidence for the polyclonal nature of focal nodular hyperplasia of the liver by the study of X-chromosome inactivation. *Hepatology*, **26(4):** 891-895.

PARKER KL, SCHEDL A, SCHIMMER BP (1999) Gene interactions in gonadal development. *Annu Rev Physiol*, **61:** 417-433.

PARKHURST SM, MENEELY PM (1994) Sex determination and dosage compensation: Lessons from flies and worms. *Science*, **264:** 924-932.

PELLETIER J, BRUENING W, KASHTAN CE et al (1991) Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. *Cell*, **67:** 437-447.

PETIT C, de la CHAPELLE A, LEVILLIERS J et al (1987) An abnormal terminal X-Y interchange accounts for most but not all cases of human XX maleness. *Cell*, **49:** 595-602.

POULAT F, DESCLOZEAUX M, TUFFERY S et al (1998) Mutation in the 5' noncoding region of the SRY gene in an XY sex-reversed patient. *Hum Mutat*, **1:** S192-S194.

PROBER JM, TRAINOR GL, DAM RJ et al (1987) A System for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science*, **238:** 336-341.

RAINE J, ROBERTSON ME, MALCOLM S et al (1989) Absence of Y specific DNA sequences in two siblings with 46XX hermaphroditism. *Arch Dis Child*, **64(8):** 1185-1187.

RAMKISSOON Y, GOODFELLOW P (1996) Early steps in mammalian sex determination. *Curr Opin Genet Dev*, **6:** 316-321.

RAMOS ES, MOREIRA-FILHO CA, VICENTE YAMVA et al (1996) SRY-negative true hermaphrodites and an XX male in two generations of the same family. *Hum Genet*, **97:** 596-598.

RAMSAY M, BERNSTEIN R, ZWANE E et al (1988) XX True hermaphroditism in southern African blacks: An enigma of primary sexual differentiation. *Am J Hum Genet*, **43:** 4-13.

RAYMOND CS, PARKER ED, KETTLEWELL JR et al (1999) A region of human chromosome 9p required for testis development contains two genes related to known sexual regulators. *Hum Molec Genet*, **8(6):** 989-996.

REINER WG (1997) Sex assignment in the neonate with intersex or inadequate genitalia. *Arch Pediatr Adolesc Med*, **151:** 1044-1045.

ROBERTS L (1988) Zeroing in on the sex switch. *Science*, **239:** 21-23.

RÖSLER A, SILVERSTEIN S, ABELIOVICH D (1996) A (R80Q) mutation in 17β-hydroxysteroid dehydrogenase Type 3 gene among Arabs of Israel is associated with pseudohermaphroditism in males and normal asymptomatic females. *J Clin Endocrinol Metab*, **81(5):** 1827-1831.

ROSS MH, REITH EJ (1985) *Histology: A text and atlas*. Second edition, Harper & Row, Publishers, JB Lippincot Company, New York.

ROTHWELL NV (1993) *Understanding Genetics: A molecular approach*. First edition, 91-126, A John Wiley and Sons, Inc., New York.

SAMBROOK J, FRITSCH EF, MANIATIS T (1989) *Molecular cloning: A laboratoty manual*. Second edition, Cold Spring Harbour Laboratory Press.

SANGER F, NICKLEN S, COULSON AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA*, **74:** 5463-5467.

SARAFOGLOU K, ORTENBERG J, GUILLEN-NAVARRO E et al (1998) Genetic mechanisms of true hermaphroditism.

SASAKI M, MAKINO S (1960) *Texas Rep Biol Med*, **18:** 493.

SCHERER G, HELD M, ERDEL M et al (1998) Three novel SRY mutations in XY gonadal dysgenesis and the enigma of XY gonadal dysgenesis cases without SRY mutations. *Cytogenet Cell Genet*, **80:** 188-192.

SCHMIDT, DU SART (1992) Functional disomies of the X chromosome influence the cell selection and hence the X inactivation pattern in females with balanced X-autosome translocations: a review of 122 cases. *Am J Med Genet*, **42(2):** 161-169.

SCHMITT-NEY M, THIELE H, KALTWABER P et al (1995) Two novel SRY missense mutations reducing DNA binding identified in XY females and their mosaic fathers. *Am J Hum Genet*, **56:** 862-869.

SEAVER LH, GRIMES J, ERICKSON RP (1994) Female pseudohermaphroditism with multiple caudal anomalies: absence of Y-specific sequences as pathogenetic factors. *Am J Med Genet*, **51(1):** 16-21.

SHEFFIELD VC, BECK JS, KWITEK AE et al (1993) The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics*, **16:** 325-332.

SHEN W, MOORE CCD, IKEDA Y et al (1994) Nuclear receptor steroidogenic factor 1 regulates the Müllerian inhibiting substance gene: A link to the sex determination cascade. *Cell*, **77:** 651-661.

SINCLAIR AH, BERTA P, PALMER MS et al (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 et al (1987) Familial 46,XX males coexisting with familial 46,XX true hermaphrodites in same pedigree. *J Pediatr*, **110(2):** 244-248.

SLANEY SF, CHALMERS IJ, AFFARA NA et al (1998) An autosomal or X linked mutation results in true hermaphrodites and 46,XX males in the same family. *J Med Genet*, **35:** 17-22.

SMITH LM, SANDERS JZ, KAISER RJ et al (1986) Fluorescence detection in automated DNA sequence analysis. *Nature*, **321:** 674-679.

SMITH SA, EASTON DF, EVANS DGR et al (1992) Allele losses in the region 17q12-21 in familial breast and ovarian cancer involve the wild-type chromosome. *Nature Genet*, **2:** 128-131.

SMITH MJ (1994) Turning on sex. *Current Biology*, **4(11):** 1003-1005.

SOUTHERN E (1975) Gel electrophoresis of restriction fragments. *Methods in Enzymol*, **68:** 152-176.

SPURDLE AB, SHANKMAN S, RAMSAY M (1995) XX True hermaphroditism in southern African blacks: Exclusion of SRY sequences and uniparental disomy of the X chromosome. *Am J Med Genet*, **55:** 53-56.

SWAIN A, ZANARIA E, HACKER A et al (1996) Mouse *Dax1* expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function. *Nat Genet*, **12(4):** 404-409.

SWAIN A, NARVAEZ V, BURGOYNE P et al (1998) *Dax1* antagonizes *Sry* action in mammalian sex determination. *Nature*, **391:** 761-767.

TALERMAN A, VERP MS, SENEKJIAN E et al (1990) True hermaphrodite with bilateral ovotestes, bilateral gonadoblastomas and dysgerminomas, 46, XX/46, XY karyotype, and a successful pregnancy. *Cancer*, **66(12):** 2668-2672.

TAR A, SÓLYOM J, GYÖRVÁRI B et al (1995) Testicular development in an SRY-negative 46,XX individual harboring a distal Xp deletion. *Hum Genet*, **96:** 464-468.

TOPPARI J, SKAKKEBAEK NE (1998) Sexual differentiation and environmental endocrine disrupters. *Baillieres Clin Endocrinol Metab*, **12(1):** 143-156.

VAN NIEKERK WA (1974) *True hermaphroditism: Clinical, morphologic and cytogenetic aspects.* First edition, Harper & Row, Publishers, Inc., New York.

VAN NIEKERK WA (1976) True hermaphroditism: An analytic review with a report of 3 new cases. *Am J Obstet Gynecol*, **126(7):** 890-907.

VAN NIEKERK WA, RETIEF AE (1981a) Nuwe kennis met betrekking tot die etiologie en patogenese van ware hermafrodiete. *S Afr Med J*, **60(5):** 195-198.

VAN NIEKERK WA, RETIEF AE (1981b) The gonads of human true hermaphrodites. *Hum Genet*, **58:** 117-122.

VAUGH J, GONZALEZ-ANGULO A (1961) True hermaphrodite with ovarian tumour. *J Urol*, **86(6):** 776-781.

VEITIA R, ION A, BARBAUX S et al (1997) Mutations and sequence variants in the testis-determining region of the Y chromosome in individuals with a 46,XY female phenotype. *Hum Genet*, **99:** 648-652.

VERGNAUD G, PAGE DC, SIMMLER MC et al (1986) A deletion map of the Y chromosome based on DNA hybridisation. *Am J Hum Genet*, **38:** 109-124.

VERP MS, SIMPSON JL (1987) Abnormal sexual differentiation and neoplasia. *Cancer Genet Cytogenet*, **25(2):** 191-218.

WACHTEL SS, OHNO S, KOO GC et al (1975) Possible role for H-Y antigen in the primary determination of sex. *Nature*, **257:**235-236.

WACHTEL SS, TIERSCH TR (1994) *Molecular genetics of sex determination.* First edition, Academic Press, New York.

WACHTEL SS (1998) X-linked sex-reversing genes. *Cytogenet Cell Genet*, **80:** 222-225.

WERNER MH, HUTH JR, GRONENBORN AM et al (1996) Molecular determinants of mammalian sex. *Tibs*, **21:** 302-308.

WHEATER PR, BURKITT HG (1987) *Functional histology: A text and colour atlas.* Second edition, Churchill Livingstone, Edinburgh.

WILKIE AOM, CAMPBELL FM, DAUBENEY P et al (1993) Complete and partial XY sex reversal associated with terminal deletion of 10q: Report of 2 cases and literature review. *Am J Med Genet*, **46:** 597-600.

Kits and manufacturer's protocols

- ABI PRISM™ 377 DNA sequencer user's manual, 1995
- Basic PCR: *Protocols and optimization strategies*
- Comparative PCR sequencing: A guide to sequencing-based mutation detection, 1995
- dRhodamine terminator cycle sequencing ready reaction kit protocol – Perkin Elmer
- MDE protocol – FMC
- The wingspread statement – Part 1: Chemicals in environment affect sexual development in wildlife. and humans?, 1991.
- Health Trends in South Africa (1994) Department of Health: 10.
- Statistics in brief: RSA (1996) Pre Rand Printers, Central Statistics (CSS).

APPENDIX 1

1.1. List of reagents and suppliers

REAGENT	SUPPLIER
Absolute ethanol	BDH Chemicals
40%(w/v) Acrylamide/Bis solution (19:1)	Gibco BRL
Agar	Difco Laboratories
Agarose D1 LE	Whitehead Scientific
Ammonium persulfate	Amresco
BioMax X-ray film	Kodak
Boric acid	Merck
Bovine Serum Albumin	NEB
Bromophenol blue sodium salt	Merck
Centrisep spin columns	PE Applied Biosystems
Chloroform	SMM Chemicals
Dextran	BDH Chemicals
DNA Sequencing Kit: dRhodamine terminator cycle sequencing ready reaction kit	PE Applied Biosystems
DMSO	Fluka
dNTPs	Boehringer Mannheim
EDTA	BDH Chemicals
Ethidium bromide	Sigma
Ficoll	Sigma
Filter paper	Whatman
Formamide	Fluka
Gel slick	FMC BioProducts
Genescan^{R} - 350 (ROX) Size Standard	PE Applied Biosystems
Glucose	Fine Chemicals
Glycerol	BDH Chemicals

Hybond-N hybridisation transfer membrane	Amersham
Hydrochloric acid 32%	Sky Chem
Isoamylalcohol	Merck
Isopropanol	Merck
KCl	Merck
$MgCl_2$	Merck
$MgSO_4$	BDH Chemicals
MDE gel solution	FMC BioProducts
Molecular weight marker	Gibco BRL
Mineral oil	Sigma
Na-acetate	BDH Chemicals
NaCl	Sky Chem
NaOH	SMM Chemicals
Nonidet P-40	Sigma
NuSieve SGT agarose	FMC BioProducts
PCR product pre-sequencing kit Exonuclease I Shrimp alkaline phosphatase	 Amersham
Phenol	Biosolve
Polyethylenglycol	Merck
Polyoxyethylenesorbitan monolaurate (Tween 20)	Sigma
Polyvinylpyrrolidone	Sigma
PRISM™ dUTP Set	Applied Biosystems
Proteinase K	Boehringer Mannheim
Restriction enzymes and supplied buffers: *Apa*I *EcoR*I *Hpa*II *Hpa*II *Sma*I	 Boehringer Mannheim Boehringer Mannheim Boehringer Mannheim New England Biolabs Boehringer Mannheim
SDS	BDH Chemicals
Sephadex G-50	Sigma

Spermidine	Sigma
Sterivex – GS 0.22µm filter unit	Millipore
Sucrose	Saarchem
Taq DNA polymerase and supplied buffer	Boehringer Mannheim
Temed	Stratagene
Tris	Saarchem
Triton-X 100	Sigma
Tryptone	Difco Laboratories
Urea	Research Organics
Wizard™ Plus Maxipreps DNA purification system	Promega
Wizard™ Plus Minipreps DNA purification system	Promega
Xylene	SMM Chemicals
Xylene cyanol FF	BDH Laboratory reagent
Yeast extract	Oxoid

1.2. Solutions

4.3% Acrylamide solution

10.6ml	40% (w/v) Acrylamide/Bis solution
10ml	10 x TBE
36g	Urea

Make up to 100ml with dH_2O.
Filter sterilise.

Agarose gels

Weigh out agarose and dissolve in 1 x TBE buffer (300ml/gel).
Boil until clear.
Add 3µl of ethidium bromide (10mg/ml) per 100ml of gel.

10% Ammonium persulphate

Dissolve 0.5g APS in 5ml of dH_2O.
Wrap in tinfoil and store at 4°C.

Ampicillin

Dissolve 50mg/ml stock in dH_2O.
Sterilise by filtration through a 0.22µm filter.
Store at -20°C.

Bromophenol blue dye

0.1%	Bromophenol blue sodium salt
0.1M	EDTA
50%	Glycerol

Add dH_2O to make up to volume.

Chloroform – Isoamyalcohol (24:1)

960ml	Chloroform
40ml	Isoamylalcohol

Make up in fume hood.

Denaturing solution

1.5M NaCl
0.5M NaOH

Make up to 1l with dH_2O.

100x Denhardt's solution

1g Ficoll
1g PVP
1g BSA

Make up to 50ml with dH_2O.
Store at -20°C.

dNTP mix

125µl 10mM dATP
125µl 10mM dCTP
125µl 10mM dGTP
125µl 10mM dTTP

Make up to 1ml with dH_2O.
This gives a final concentration of 125mM.
Store at -20°C.

0.2M EDTA pH 8.0

37.24g EDTA

Make up to 500ml with dH_2O.
Adjust to the correct pH with NaOH pellets.

0.5M EDTA pH 8.0

93.1g	EDTA

Make up to 500ml with dH_2O.
Adjust to the correct pH with NaOH pellets.
Autoclave.

Ficoll loading dye

50%	Sucrose
50mM	EDTA (pH 7.0)
0.1%	Bromophenol blue dye
10%	Ficoll

Make up to volume with dH_2O.
Store at 4°C.

Formamide loading dye

10ml	Formamide
10mg	Xylene cyanol FF
10mg	Bromophenol blue sodium salt
200µl	0.5M EDTA pH 8.0

1M Glucose

180.16g	Glucose

Make up to 1l with dH_2O.
Prepare fresh

80% Isopropanol

80ml	Isopropanol
20ml	dH_2O

1kb DNA ladder (Gibco BRL)

250µl	Ladder (250µg)
2.1ml	1 x TE
125µl	Ficoll loading dye

Luria agar

1%	Tryptone
0.5%	Yeast extract
0.5%	NaCl
1.5%	Agar

Make up to volume with dH_2O.
Autoclave. Allow to cool slightly before adding the antibiotic.

Luria broth

1g	Tryptone
0.5g	Yeast extract
1g	NaCl

Make up to 100ml with dH_2O.
Adjust the pH to 7.5 with NaOH.
Autoclave.

MDE polyacrylamide gels for SSCPs

With glycerol:

7.5ml	MDE
1.8ml	10 x TBE
3ml	glycerol
17.7ml	dH_2O

Without glycerol:

7.5ml	MDE
1.8ml	10 x TBE
20.7ml	dH_2O

1M $MgCl_2$

50.83g	$MgCl_2$

Make up to 250ml with dH_2O.
Autoclave.

1M $MgSO_4$

34.6 g	$MgSO_4$

Make up to 250ml with dH_2O.
Autoclave.

3M Na – acetate (pH 4.6)

Dissolve 40.81g sodium acetate in 50ml dH_2O.
Adjust the pH to 4.6 with glacial acetic acid.
Make up to 100ml with dH_2O.
Autoclave.

Neutralising solution

1.5M	NaCl
0.5M	Tris base
0.001M	EDTA
42ml	Concentrated HCl acid

Make up to 1l with dH_2O.
If necessary, adjust the pH to 7.2 with more HCl acid.

Special neutralising solution

3M	NaCl
0.5M	Tris

Make up to volume with dH_2O.
pH to 7.0 with HCl.

Nusieve gels

Weigh out agarose and dissolve in 1 x TAE buffer (300ml/gel).
Boil until clear.
Add 3µl of ethidium bromide (10mg/ml) per 100ml of gel.
Pour and allow gel to set at 4°C.

Paraffin-embedded DNA extraction buffer

10mM	Tris pH 8.3
50mM	KCl
1.5mM	$MgCl_2$
100µg/ml	BSA
0.45%	Nonidet P-40

0.45%	Polyoxyethlenesorbitan monlaurate (Tween 20)
100µg/ml	Proteinase K

Make up to 10ml with dH_2O.
Filter sterilise.

6% Polyacrylamide gel solution

7.5ml	40% (w/v) Acrylamide/Bis solution
5ml	10 x TBE
21g	Urea

Make up to 40ml with dH_2O.

Prehybridisation solution

50ml 20x SSC
10ml 100x Denhardts solution
10ml 10%SDS

Make up to 100ml with dH_2O.
Store at -20°C.

Proteinase K solution (10mg/ml)

Weigh out powder.
Add dH_2O to give a concentration of 10mg/ml.
Aliquot and freeze.

Proteinase K mix (for salting out procedure)

1%	SDS
2mM	EDTA
2mg/ml	Proteinase K
350µl	dH_2O

Salmon sperm DNA

Dissolve 5mg/ml ssDNA in dH_2O.
Make up to 20ml.
Allow to dissolve overnight.
Shear by sonication (9 x 2 minutes).
Freeze at -20°C in 1ml aliquots.

Saturated NaCl

Add NaCl to dH_2O until it no longer dissolves.

SSCP gel marker

There are two components:

1. Buffer:	2µl	1M Tris pH7.4
	2µl	1M $MgCl_2$
	12µl	0.1M DTT
	2µl	10mM dATP
	2µl	10mM dGTP
	2µl	10mM dTTP
	178µl	dH_2O

Store at -20°C.

2. Marker:	1µl	pBR322 DNA (259ng/µl)	
	1µl	*Hpa*II	
	1µl	10 x *Hpa*II buffer	
	7µl	dH_2O	

Incubate for 1 hour at 37°C.

Make up to 150µl with TE buffer.

Store at -20°C.

To make up the marker:	20µl	Buffer
	2µl	Klenow (2U/µl)
	1µl	^{32}PdCTP
	25µl	Marker

Incubate at 30°C for 10 minutes.

Add 8µl of formamide dye.

Store in a lead peg at -20°C.

Load approximately 3µl on a gel.

10%SDS

10g/100ml

Weigh in a fume hood. Autoclave the dH_2O before adding the SDS powder.

20x SSC

3M	NaCl
0.3M	Na Citrate

Make up to volume with dH_2O.

1M Tris-HCl pH 8.0

121.1g Tris

pH to 8.0 with HCl and make up to volume with dH_2O.

50x TAE buffer

1M Tris base
28.55ml Glacial acetic acid
50ml 0.5M EDTA pH 8.0
Make up to 500ml with dH_2O.
Autoclave.

10x TBE buffer

108g Tris base
55g Boric acid
7.4g EDTA

Make up to 1l with dH_2O.
Autoclave.

1x TE buffer pH8.0

10mM Tris base
1mM EDTA

Make up to 1l with dH_2O.
If necessary pH with HCl.

T20E5

20mM	Tris pH8.0
5mM	EDTA

Make up to volume with dH_2O.

Autoclave.

Triton-X lysing buffer

10ml	1M Tris-HCl pH8.0
5ml	1M $MgCl_2$
10ml	Triton-X

Make up to 1l with dH_2O.

Autoclave. Just prior to use, add 109.5g sucrose.

TSB (make fresh)

1g	10% PEG
0.5ml	1M DMSO
0.1ml	1M $MgCl_2$
0.1ml	1M $MgSO_4$
0.2ml	1M Glucose

Make up to 10ml with Luria broth

All solutions were stored at room temperature unless otherwise stated.

APPENDIX 2

2.1. DAX1 primer design

There is a large region in the DAX-1 gene that contains repetitive sequences. It was, therefore, extremely important not to have primers located in this region as this would result in non-specific amplification.

2.1.1. Primer design method

The DAX-1 gene sequence was downloaded from GenBank on the Internet. The sequence was then edited using editseq of DNAstar so that it was compatible for use in the primer design programme, primerselect, of DNAstar. Because SSCP analysis was to be performed on this gene, fragments between 250bp and 350bp were desirable so that restriction enzyme digestion of amplified PCR products would not be necessary. This meant that five primer pairs were required to span the entire coding region of exon 1 and that one primer pair was necessary to cover the whole coding sequence of exon 2 of the DAX-1 gene.

The regions in the gene where one would like the forward and reverse primer to be situated are highlighted and the programme then diagrammatically represents the positions on the DNA sequence of the possible primers. One can then select a primer pair and examine parameters such as GC content, primer melting temperatures and primer annealing to select the best primer pair option.

APPENDIX 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Ramsay

CLEARANCE CERTIFICATE PROTOCOL NUMBER M 960915

PROJECT	Molecular and Psychosocial studies on XX True Hermaphroditism in Southern African Blacks
INVESTIGATORS	Dr M Ramsay
DEPARTMENT	Human Genetics, SAIMR
DATE CONSIDERED	960927
DECISION OF THE COMMITTEE	Approved unconditionally
DATE	961009

CHAIRMAN. ..... (Professor P E Cleaton-Jones)

c c Supervisor: Dr M Ramsay
Dept of Human Genetics, SAIMR

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE....14-10-96.....SIGNATURE .....M. Ramsay.....

Author Padoa C J
Name of thesis The Aetiology Of Xx Tru Hermaphroditism In The Southern African Black Population Padoa C J 1999

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