MOLECULAR GENETICS OF HUMAN 3ß - HYDROXYSTEROID DEHYDROGENASE DEFICIENCY

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by

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This thesis is dedicated to my mother and father,

for all their love, support, and encouragement.

DECLARATION

The research reported in this thesis is my own original work except where otherwise stated and has not been submitted for any other degree.

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SUMMARY

3ß-hydroxysteroid dehydrogenase (3ß-HSD) catalyses a series of obligatory biosynthetic steps in the synthesis of mineralocorticoids, glucocorticoids and sex steroids. Specifically, it converts the Δ^5 3ß-hydroxysteroids pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone, and androstene 3ß,17ß-diol into the respective Δ^4 3-ketosteroids: progesterone, 17alpha-hydroxyprogesterone, androstenedione and testosterone. In rodents at least three genes for 3ß-HSD have been found to be expressed. When analysed on southern blots, human DNA shows evidence of six or more sequences with homology to 3ß-HSD probes. So far, two highly homologous but distinct human genes have been cloned, encoding 3ß-HSD type I expressed principally in the placenta, and 3ß-HSD type II expressed in adrenal, ovary and testis. Though the type I and II enzymes differ in their kinetics in detail, they are both capable of converting the same Δ^5 substrates.

It has long been recognised that inherited defects of 3ß-HSD can result in congenital adrenal hyperplasia (CAH) and nonsense and frameshift mutations in the gene for 3ß-HSD type II have recently been described in classical cases of severe CAH affected with salt-losing crises, deficiencies in glucocorticoid metabolism and incomplete masculinisation. A range of less severe phenotypes have also been ascribed to deficiencies of 3ß-HSD, including hypospadias, male pseudohermaphroditism, and late onset virilisation in women. In some cases these are associated with a degree of salt loss, in others salt handling is normal. It is not clear to what extent these different clinical phenotypes are due to different mutations in the same gene, or to mutations in other 3ß-HSD genes encoding enzymes with somewhat different functions.

This thesis describes the investigation of three families (Ran, Sta, and Pfe) affected with putative 3ß-HSD deficiency. The aim of this thesis was to identify the molecular

genetic basis of 3ß-HSD deficiency in each of the three families. I visited the laboratory of Professor D. A. Hopkinson and Dr. P. H. Johnson, MRC Human Genetics, Galton Laboratories, London to compare the use of several screening techniques for detecting mutations: chemical cleavage, temperature gradient gel electrophoresis (TGGE), and denaturing gradient gel electrophoresis (DGGE). DGGE was judged to be the most useful of these techniques and was subsequently used to search for mutations in the type I and type II genes for 3ß-HSD. More recently, automated DNA sequencing of the 3ß-HSD type II gene was carried out in a further attempt to detect mutations.

In family Ran the propositus, a 5 year old XY pseudohermaphrodite, was found to have a defect of steroid biosynthesis consistent with a partial deficiency of 3ß-HSD. There was no evidence of salt loss and no detectable abnormality of glucocorticoid metabolism. A mutation in the gene for 3ß-HSD type II was observed at codon 173 (CTA->CGA), leading in the affected patient to the homozygous substitution of $173^{leu->arg}$. The propositus' 2 year old XX sister was also $173^{arg/arg}$; she showed the biochemical signs of 3ß-HSD deficiency but was without clinical symptoms. The mutation segregated as an autosomal recessive. A number of family members heterozygous for the codon 173 substitution exhibited biochemical and clinical abnormalities though to a lesser degree than the two homozygous children.

Family Sta was characterised by two brothers both affected with hypospadias. Urinary steroid analysis indicated a partial deficiency of 3ß-HSD. One of the brothers also showed evidence of mild salt loss and demonstrated an impaired cortisol response to synacthen. No other members of the family were affected indicating an autosomal recessive condition. DGGE and subsequent DNA sequencing revealed a single point mutation in the gene for 3ß-HSD type II. A missense mutation at codon 100 (AAT->AGT) resulted in the substitution of Asn for Ser. The mother and both of the brothers were heterozygous for this mutation. To date no evidence of mutation has

been detected in either the 3ß-HSD type I or 3ß-HSD type II genes of the father in family Sta.

Family Pfe contained four female members affected with varying degrees of dysmenorrhea and hirsutism. Despite a diagnosis of partial 3ß-HSD deficiency it was conclusively shown by use of polymorphic markers in 3ß-HSD types I and II that the disease phenotype did not co-segregate with either of the known 3ß-HSD genes. This finding has important implications for the future diagnosis of 3ß-HSD deficiency.

Future work on families Ran and Sta will involve the expression of the mutant alleles *in vitro* to determine the effect of each substitution on the activity of 3ß-HSD type II. As the hirsutism in family Pfe appears not to be caused by a deficiency in 3ß-HSD, future work may include the typing of family Pfe for a panel of polymorphic markers in candidate genes such as aromatase in an attempt to establish linkage.

During the course of this work a number of polymorphic markers and mutations were identified in both the type I and type II genes. These markers were used to determine the segregation of genes within each family. The markers were also used to show that the genes for 3ß-HSD types I and II are in close genetic linkage on chromosome 1p13. In addition to polymorphisms and mutations in 3ß-HSD types I and II, a number of sequences homologous to 3ß-HSD were isolated by the use of DGGE. The possibility of a tightly clustered gene family raises the possibility that in some cases of 3ß-HSD deficiency, mutations may be due to unequal crossing over or to gene conversion, as has been observed in several other gene clusters. It is hoped that the use of somatic cell hybrids will allow the mapping of 3ß-HSD homologues that have been detected by DGGE, to chromosome 1p1-2 and hence to determine their linkage relationships (if any) to 3ß-HSD types I and II.

ABBREVIATIONS

å - alpha

ß - beta

Δ - delta

ø - phi

3ß-HSD - 3 beta-hydroxysteroid dehydrogenase

ACTH - adrenocorticotropic hormone

ATP - adenosine triphosphate

CAH - congenital adrenal hyperplasia

DNA - deoxyribonucleic acid

cAMP - cyclic adenosine-3', 5'-monophosphate

cDNA - complementary DNA

CMO - corticosterone methyl oxidase

dATP - deoxyadenosine triphosphate

dCTP - deoxycytidine triphosphate

dGTP - deoxyguanosine triphosphate

dTTP - deoxythymidine triphosphate

DF - deoxycortisol

DGGE - denaturing gel electrophoresis

DHEA - dehydroepiandrosterone

DHEA-S - dehydroepiandrosterone sulphate

DOC - deoxycorticosterone

DTT - dithiotheitol

EDTA - ethylenediaminetetra-acetic acid (disodium salt)

FSH - follicle-stimulating hormone

hCG - human chorionic gonadotropin

HLA - human lymphocyte antigens

LB - Luria-Bertani

LOD - $\log_{10}$ of the odds

MPH - male pseudohermaphroditism

MRF - Mullerian regression factor

mRNA - messenger RNA

nt - nucleotide

OHP - hydroxyprogesterone

ORF - open reading frame

PCR - polymerase chain reaction

PRA - plasma renin activity

PRL - prolactin

RFLP - restriction fragment length polymorphism

RNA - ribonucleic acid

RNase A - ribonuclease A

SD - standard deviation

SDS - sodium dodecylsulphate

SSC - sodium chloride, sodium citrate

SSCP - single strand conformation polymorphism

TAE - Tris-acetate, EDTA

TBE - Tris-borate, EDTA

TE - Tris, EDTA

TEMED - N, N, N', N'-Tetra methyl ethylenediamine

TGGE - temperature gradient gel electrophoresis

THDOC - tetrahydrodeoxycorticosterone

THS - tetrahydro-11-deoxycortisol

Tm - melting temperature

Tris - tris 9 hydroxymethyl - amino ethane

W.H.O. - World Health Organisation

X-gal - 5-bromo-4-chloro-3-indolyl-ß-galactoside

Measurements

l - litre

dl - decilitre

ml - millilitre (10^{-3} l)

ul - microlitre (10^{-6} l)

M - Molar (moles per litre)

mM - millimolar

mmol - millimole (10^{-3} mole)

pmol - picomole (10^{-12} mole)

kg - kilogram (10^{3} g)

g - gramme

ug - microgram (10^{-6} g)

ng - nanogram (10^{-9} g)

cm - centimetre (10^{-2} m)

mm - millimetre (10^{-3} m)

bp - base pair

kb - kilo base pair

pH - acidity [negative $\log_{10}$ (Molar concentration H^{+} ions)]

^{0}C - degrees centigrade

rpm - revolutions per minute

V - volts

mA - milliamps (10^{-3} A)

Ci - Curies

dpm - disintegrations per minute

secs - seconds

(Tk) - time constant

kDa - kilo Dalton

INTRODUCTION

Hormones are widely distributed being found in almost all living cells. Hormones exert wide ranging effects on the activity of the body in response to changes in both the internal and external environment. Some hormones act locally i.e. they are secreted and exert their effect within individual tissues, for example, growth factors. Other hormones have a long range effect, reaching their target tissues by diffusing into the circulation. From the chemical standpoint there are three groups of hormones. Firstly, there are derivatives of the amino acid tyrosine, secondly, peptide and protein hormones and thirdly, steroid hormones. Cholesterol is the natural precursor of all steroid hormones. The biochemical pathway for steroid biosynthesis starts from acetate, via mevalonate and squalene to cholesterol. Though steroid hormones are present in almost all living cells, organs such as the adrenals, testes, ovaries and placenta are highly specialised in the metabolism of cholesterol and the synthesis of steroid hormones. According to current concepts, the biosynthetic pathways in all endocrine organs that form steroid hormones are similar, the organs differing from one another only in the enzyme systems they express. For example, some tissues are capable of metabolising cholesterol whereas other tissues require to be supplied with steroid intermediates which they can then metabolise.

Adrenal steroidogenesis

The adrenals are situated at the superior pole of each kidney and consist of two endocrine organs, one surrounding the other (Fig 1a, b, c). The inner adrenal medulla secretes the catecholamines adrenaline, noradrenaline, and dopamine, which are involved in preparing the individual to deal with emergencies. The adrenal cortex secretes steroid hormones. The adrenal cortex is divided into three zones. The outer zona glomerulosa is made up of whorls of cells which often appear discontinuously

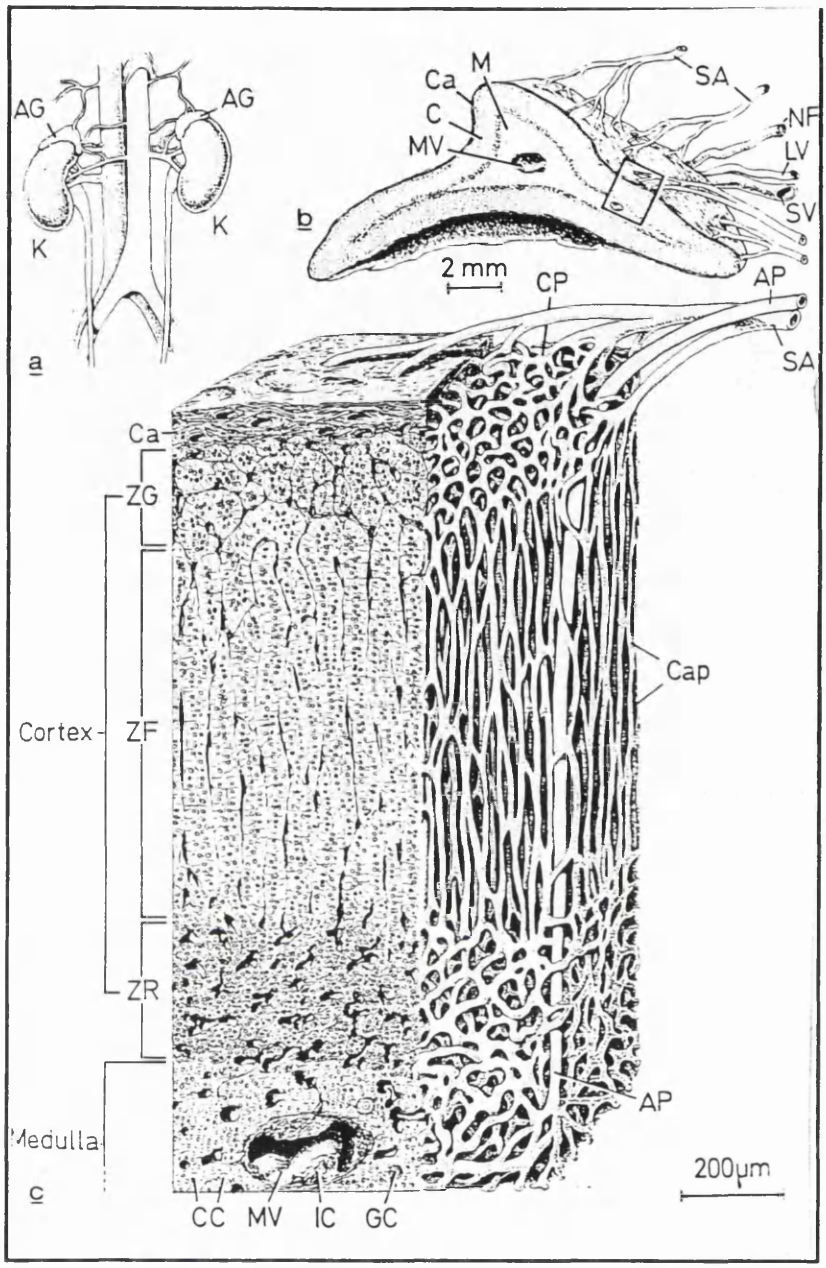


Figure 1: The adrenal glands (adapted from R.V.Krsic 1991)

KEY: kidneys - K; suprarenal glands - AG; capsule - CA; cortex - C; medulla - M; medullary veins - MV; suprarenal vein - SV; suprarenal arteries - SA; nerve fascicles - NF; lymphatic vessels - LV; zona glomerulosa - ZG; zona fasciculata - ZF; zona reticularis - ZR; chromaffin cells - CC; ganglion cells - GC; subendothelial cushions - IC; capsular plexus - CP; capillaries - Cap; arteriolae perforantes - AP.

under the glandular capsule. The zona fasciculata is a wide zone consisting of columns of cells separated by venous sinuses. The cells of the zona fasciculata have abundant reserves of lipid. The inner portion of the zona fasciculata merges into the more compact zona reticularis. The blood flow in the adrenal cortex is large, as it is in most endocrine glands, and it is thought that pooling and reflux of blood carrying secretory products may be significant for control of steroidogenic activity in different locations. Arterial blood reaches the adrenal from many small branches of the phrenic and renal arteries and the aorta. The blood then proceeds from the subcapsular region to the central portal vein (Dobbie et al. 1968).

Three classes of steroids are produced in the adrenal cortex: mineralocorticoids, glucocorticoids and sex steroids. The mineralocorticoids are essential to the maintenance of sodium balance and extracellular fluid volume. The production of the major mineralocorticoid aldosterone is limited to the zona glomerulosa. Glucocorticoids have widespread effects on the metabolism of carbohydrate and protein. Cortisol, the principal glucocortcoid, is produced in the zona fasciculata (Nussdorfer 1986). The sex steroids, androgens and oestrogens, are involved in the differentiation of the genital ducts during gestation and also in the development and maintenance of secondary sex characteristics at puberty. The zona reticularis produces the androgenic C19 steroids.

Although numerous steroids have been isolated from adrenal tissue, the only steroids normally secreted in physiologically significant amounts are aldosterone, cortisol, corticosterone, dehydroepiandrosterone (DHEA), and androstenedione. Other steroids that are secreted in small amounts are 18-hydroxycorticosterone, pregnenolone, progesterone, testosterone, and oestrogens.

Adrenocortical secretion is controlled primarily by adrenocorticotropic hormone (ACTH) from the anterior pituitary, but mineralocorticoid secretion is also subject to

control by the renin-angiotensin system. ACTH binds to high affinity receptors on the plasma membrane of adrenocortical cells and activates adenylate cyclase. The resultant increase in cAMP activates phosphoprotein kinases which in turn increase the activity of a number of cell processes. Activated cholesterol esterase produces free cholesterol from cholesterol esters stored in the cytoplasm. The free cholesterol is transported to the mitochondria where it is converted to the common steroid hormone precursor, pregnenolone, at the inner mitochondrial membrane by the action of P450scc (cholesterol 20, 22-desmolase)(Lambeth and Stevens 1985). Continued elevation of ACTH increases mRNA transcription and protein translation rates for each of the enzymes in the steroidogenic pathway (Boggaram et al. 1985; Voutilainen and Miller 1987).

An outline of steroid biosynthesis in the adrenal cortex is given in Figure 2. The location of each enzyme in the adrenal cortex is indicated.

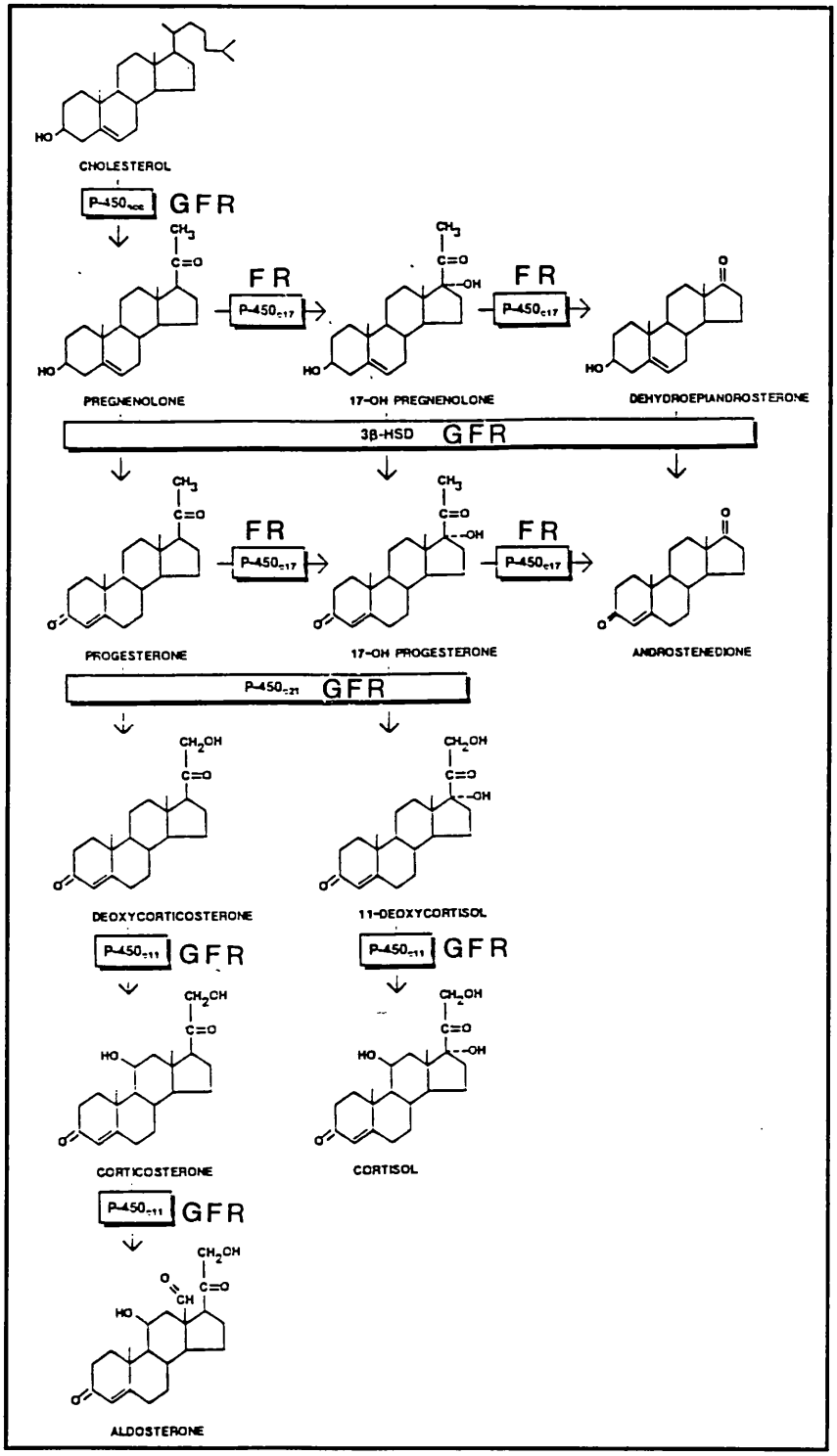


Figure 2: Simplified scheme for adrenal steroidogenesis.

The location of each enzyme in the adrenal cortex is indicated.

G - zona glomerulosa; F - zona fasciculata; R - zona reticularis.

Testicular steroidogenesis

The testes are the major site of androgen production in the male. Androgens are essential for the development and maintenance of the male genitalia and secondary sex characteristics.

The testes are made up mainly of loops of convoluted seminiferous tubules, along the walls of which spermatozoa are formed from primitive germ cells. Between the seminiferous tubules are nests of cells known as Leydig cells which are responsible for the bulk of androgen production. Small amounts of androgen are also produced by the seminiferous tubules (Bell et al. 1971; Dufau, et al. 1971). As Leydig cells do not contain many of the enzymes found in the adrenal cortex e.g. 11- and 21-hydroxylases, no glucocorticoids or mineralocorticoids are synthesised.

Although testosterone is the principal androgen secreted by the testes, in many target tissues it is converted to the more potent 5 alpha dihydrotestosterone by the enzyme 5 alpha reductase. In plasma, 97% of testosterone is bound to protein: 40% to sex hormone binding globulin (SHBG), 40% to albumin, and 17% to other proteins (Doe and Seal 1963; Burke and Anderson 1972). Only the testosterone which is not bound to the SHBG is available for binding to the androgen receptor (Lasnitzki and Franklin 1972). In addition to testosterone, testosterone precursors such as androstenedione and DHEA also enter the circulation. Figure 3 illustrates the biosynthesis of testosterone in the testes. There are two major pathways for testosterone biosynthesis: the Δ5 pathway involving the 5-ene-3ß-hydroxysteroids pregnenolone, 17 hydroxypregnenolone and DHEA, and the Δ4 pathway involving the 4-ene-3-oxosteroids, progesterone, 17 hydroxyprogesterone, and 4-androstenedione (Dorfman and Ungar 1965). Various workers have attempted to determine the relative importance of the Δ4 and Δ5 pathways. In rodents the Δ4 pathway is favoured (Shikita et al. 1964; Slaunwhite and Burgett 1965), whereas in humans the Δ5 pathway is the most important (Gower and Bicknell 1972). Small amounts of

oestrogens are also produced by the testes. 70% of oestradiol in the plasma of adult men is formed by aromatization of circulating testosterone and androstenedione and most of the remaining 30% is secreted directly by the testes (Longcope et al. 1972).

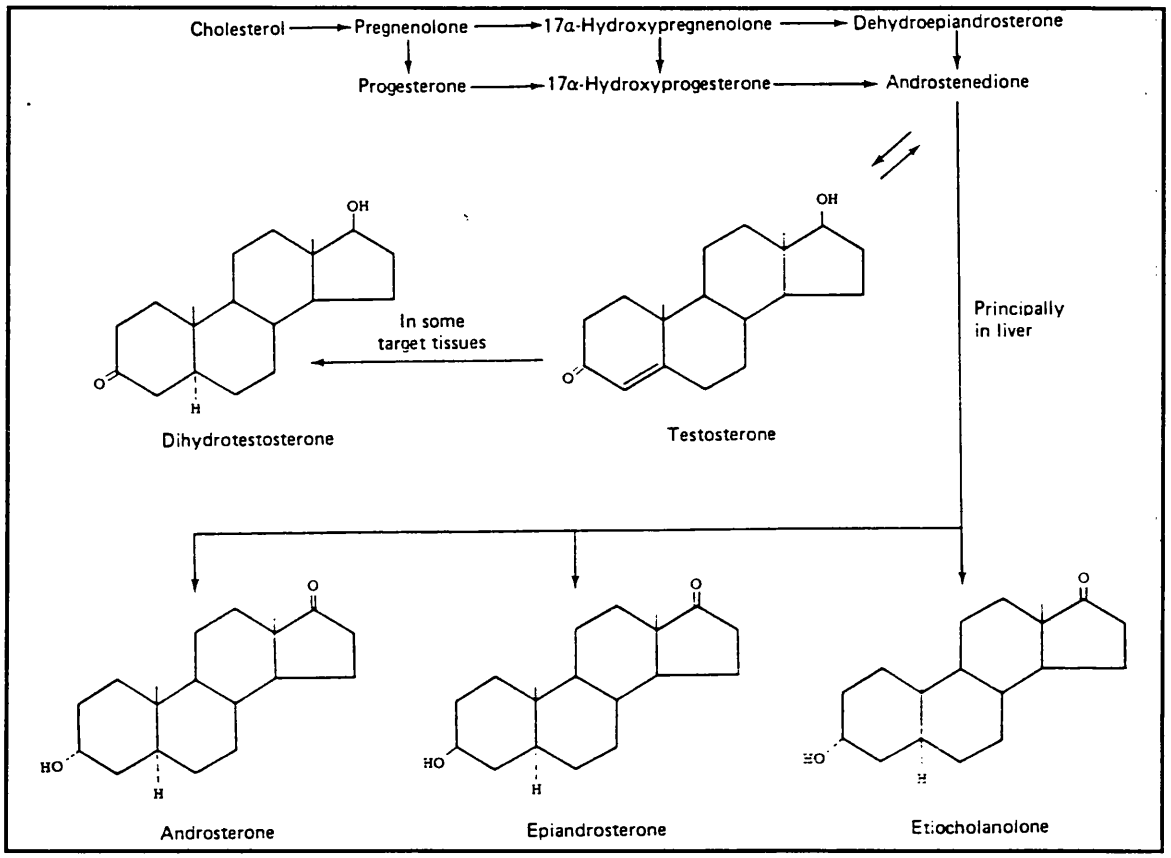


Figure 3: Biosynthesis and metabolism of testosterone

Although the main secretory product of the Leydig cells is testosterone, some of the precursors also enter the circulation.

The production of testosterone is controlled by pituitary luteinising hormone (LH). LH stimulates the formation of cholesterol from cholesterol esters and the conversion of cholesterol to pregnenolone via the activation of protein kinases. Testosterone and other androgens exert an inhibitory feedback effect on pituitary LH.

Ovarian steroidogenesis

The ovaries are situated in the pelvis on either side of the uterus. Each ovary is attached to the broad ligament of the uterus by a fold of peritoneum. The centre of each ovary is known as the medulla or stroma and consists mainly of connective tissue containing many collagen fibres. Surrounding the medulla is the cortex which consists of a layer of epithelium (germinal epithelium) and a number of cystic spaces of various sizes, the Graafian follicles, each of which contains an immature ovum.

At the start of each menstrual cycle, several of these follicles enlarge, and a cavity forms around the ovum. In humans, one of the follicles in one ovary continues to develop while the others regress. The structure of the maturing follicle is shown in Fig 4. The theca interna and granulosa cells of the follicle are the respective sites of progesterone and oestrogen biosynthesis in the ovary. After ovulation the cells of the corpus luteum continue to secrete large amounts of progesterone. If pregnancy occurs the corpus luteum persists and there are usually no more periods until after delivery. If there is no pregnancy the corpus luteum degenerates (Guraya 1971; Fuchs and Klopper 1977).

In addition to oestrogens and progesterone, the ovaries also secrete 17-hydroxyprogesterone, pregnenolone, 17-hydroxypregnenolone, and a small amount of androgens; testosterone, androstenedione, and DHEA. The oestrogens synthesised are responsible for the development of the female sex organs and the secondary sex characteristics. They are involved in preparing the female reproductive tract in order to receive the fertilised ovum. During pregnancy, oestrogens act synergistically with the other ovarian hormone progesterone to maintain gestation (Guraya 1971; Fuchs and Klopper 1977). They also effect the development of the breasts, the typical female body shape, texture of the skin, and hair growth. 17ß-oestradiol, the major secreted oestrogen and the most potent, is in equilibrium in the circulation with oestrone. Oestrone is further metabolised to oestriol, the least potent oestrogen,

mainly in the liver. Approximately 3% of the circulating oestradiol is free and the remainder is bound to protein: 60% to albumin and 37% to SHBG (Burke and Anderson 1972).

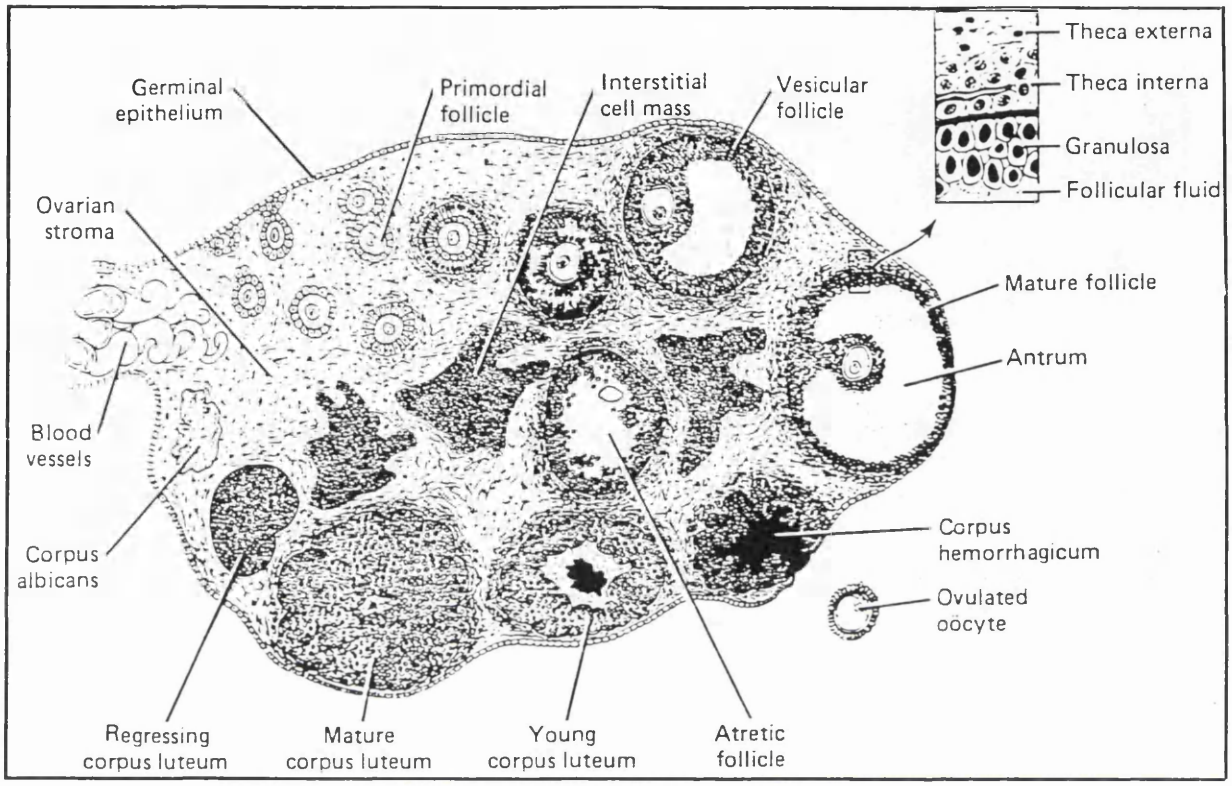


Figure 4: Ovarian cycle(Gorbman and Bern 1962)

Diagram of a mammalian ovary, showing the sequential development of a follicle, formation of corpus luteum, and, in the centre, follicular atresia. A section of the wall of a mature follicle is enlarged at the upper right.

Progesterone is responsible for the progestational changes in the endometrium and the cyclic changes in the vagina and cervix. It decreases the number of oestrogen receptors in the endometrium and increases the rate of conversion of 17ß-oestradiol to less active oestrogens, in breast it stimulates the development of lobules and alveoli. During the luteal phase of the cycle progesterone production by cells of the corpus luteum increases approximately 20 fold, mainly via the Δ4 pathway (Guraya 1971; Fuchs and Klopper 1977).

The granulosa cells of the follicle are the major site of oestrogen production.However they can only do so when provided with androgens from the theca interna cells. Theca interna cells have many LH receptors, and LH acts via cyclic AMP to increase conversion of cholesterol to androstenedione mainly by increasing the flux of Δ5 steroids to DHEA. Androstenedione synthesised in the thecal cells is secreted directly into the circulation. Some androstenedione also enters adjacent granulosa cells. Aromatase, an enzyme expressed in granulosa cells, converts the androstenediol to oestradiol (Fig 5). The same enzyme also catalyses the biosynthesis of oestrone by aromatization of androstenedione. The oestradiol synthesised by the granulosa cells is discharged into the circulation and some is also secreted into the follicular fluid. As the granulosa cell layer is relatively avascular it is not clear whether the newly synthesised oestrogens pass directly into the circulation or whether they have to pass through the theca interna cells to reach the capillary network (Hillier 1991). The possibility exists that steroids secreted by the granulosa cells may be altered by the thecal cells before they reach the circulation.

Granulosa cells have many follicle stimulating hormone (FSH) receptors, and FSH facilitates their secretion of 17ß-oestradiol by acting via cyclic AMP to increase aromatase activity in the cells. FSH also induces LH receptors coupled to steroid biosynthesis in granulosa cells; aromatase is therefore under direct control of both FSH and LH in the preovulatory follicle (Hillier 1991)(Fig 6).

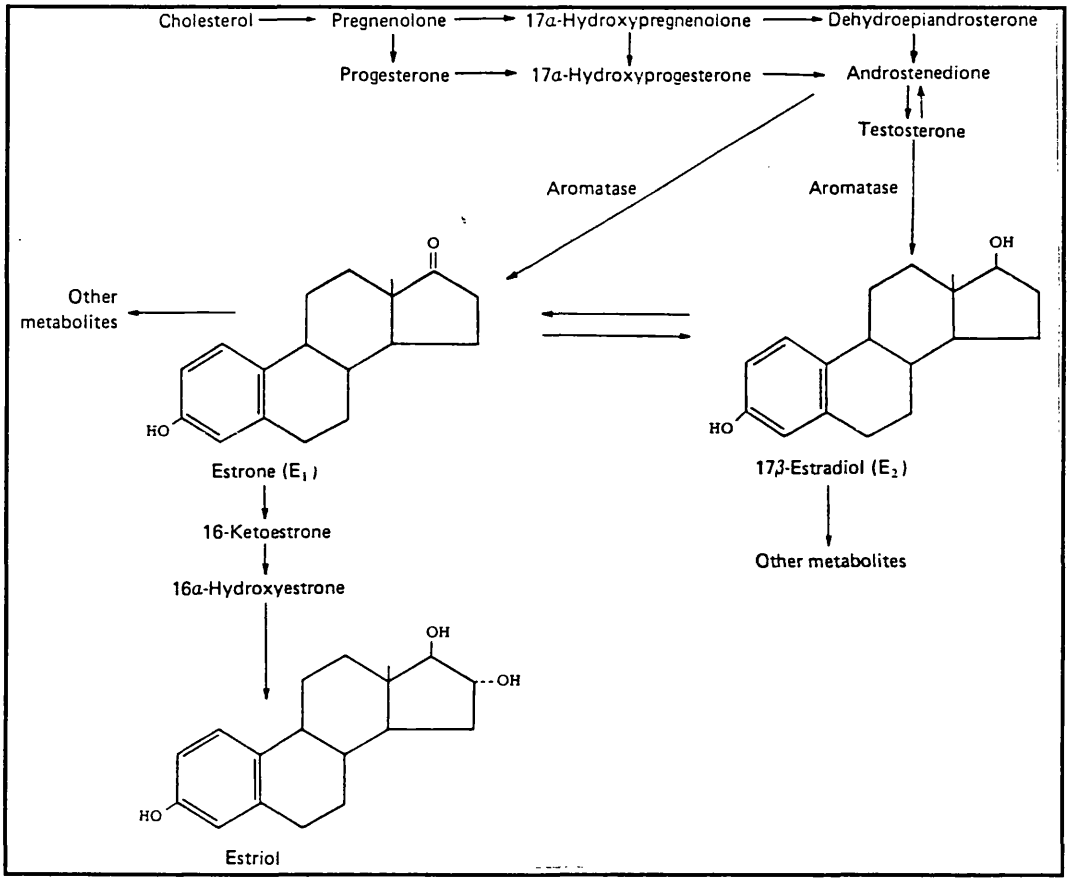


Figure 5: Biosynthesis and metabolism of oestrogens.

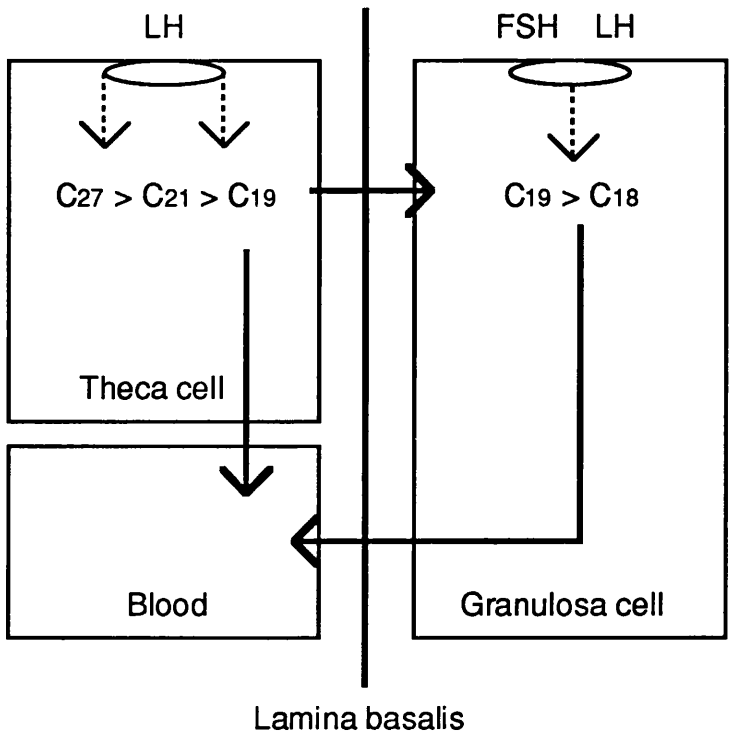


Figure 6: Regulation of oestrogen biosynthesis in the ovary.

(adapted from Hillier 1991)

Androstenedione synthesised in thecal cells enters the efferent follicular vasculator to be secreted as the major follicular androgen. Androstenedione also traverses the lamina basalis and enters adjacent granulosa cells where it is aromatized to oestradiol. Oestradiol formed in granulosa cells is discharged into the ovarian vein.

Male pseudohermaphroditism (MPH)

Testosterone and Mullerian regression factor (MRF), a glycoprotein secreted by the Sertoli cells of the testes, are essential in the sexual development of the male fetus. MRF acts alone to inhibit the development of the Mullerian ducts; MRF and testosterone act together to develop the epididymides, vas deferens, seminal vesicles, and ejaculatory ducts from the Wolffian ducts (Jost 1970). Testosterone is also required for suppression of the breast anlage (Wilson et al. 1980) and indirectly for the normal formation of the male external genitalia.

If the embryonic testes are defective MPH results. A pseudohermaphrodite is an individual with the genetic constitution and gonads of one sex and the genitalia of the opposite sex. Failure of the testes to produce testosterone and MRF results in genetic males with both external and internal female genitalia. However, if the problem is limited to a defect in production of testosterone or of 5 alpha dihydrotestosterone (the active form of testosterone in some tissues) then the female genital ducts do not form due to the production of MRF. Individuals with this syndrome exhibit micropenis, hypospadias, and gynaecomastia (Jost 1970; Wilkins 1975; Wilson et al. 1980).

MPH can result from a deficiency in any one of the enzymes involved in the biosynthesis of testosterone and 5 alpha dihydrotestosterone. In other forms of MPH, androgen receptors on target tissues may be absent or abnormal. MPH may also occur in the presence of normal androgen receptors and testosterone production when there are congenital defects in the molecular events that occur after receptor binding (Morris 1953; Goldstein and Wilson 1974; Wilson and Goldstein 1975; Griffin and Wilson 1980).

Androgens in the female

The four primary circulating androgenic steroids in women are DHEA, DHEA-S, androstenedione and testosterone (in some tissues testosterone is converted to 5 alpha dihydrotestosterone by 5 alpha reductase). Only testosterone and 5 alpha dihydrotestosterone are capable of binding to the intracellular androgen receptor, DHEA, DHEA-S and androstenedione do not bind to the androgen receptor and are classed as androgen pre-hormones. The androgenic activity of these latter steroids depends solely on the extent to which they are converted to testosterone or 5 alpha dihydrotestosterone in peripheral tissues.

In normal women, approximately 50-70% of circulating testosterone is derived from peripheral conversion of androstenedione (Horton and Tait 1966; Bardin and Lipsett 1967), the androstenedione being synthesised in the ovary and the adrenals. Direct ovarian production of testosterone is estimated to be between 5 and 20% of total circulating testosterone. The adrenal contribution is estimated between 0 and 30% (Kirschner and Bardin 1972; Moltz et al. 1983). Metabolism of DHEA and Δ5 androstenediol accounts for approximately 10-15% of plasma testosterone (Kirschner et al. 1973; Clark 1986). In non-pregnant women, 75-80% of circulating testosterone is bound to gonadal steroid-binding globulin, 20-25% is loosely bound to albumin (Vermuelen et al. 1971) and about 1% circulates freely. Only the testosterone that is not bound to SHBG is available for binding to the androgen receptor. SHBG does not bind androstenedione, DHEA, or DHEA-S.

The levels of circulating testosterone fluctuates very little in normal women, however DHEA, DHEA-S, and androstenedione levels demonstrate significant diurnal fluctuations. Circulating DHEA and DHEA-S are almost exclusively of adrenal origin (80% and 90% respectively)(Horton and Tait 1966). By contrast, during the follicular phase of the menstrual cycle, androstenedione production is evenly divided

between the ovary and the adrenals. At midcycle, however, the ovarian contribution increases to two thirds of the total (Abraham 1974).

Neither ovarian nor adrenal androgen secretion in women is directly regulated. Although LH and ACTH do stimulate adrenal and ovarian androgen secretion respectively, the secreted androgens do not feed back in an autoregulatory manner at the level of the hypothalamus-pituitary (Kirschner and Bardin 1972).

The physiological role of androgens in men is well defined, however, in the female the physiological significance of androgens is largely unknown. Androgens are known to serve as oestrogen precursors and may be involved in the regulation of ovarian function at gonadal level. Growth and development of pubic and axillary hair are also androgen dependent. An excess of androgen production during gestation results in female pseudohermaphroditism. Individuals with this syndrome have normal internal female genitalia, however varying degrees of labial fusion and clitoromegaly occur depending on the levels of androgen and the duration of exposure during gestation. In prepuberal and adult females an excess of androgen production leads to the adrenogenital syndrome. Symptoms include clitoromegaly , oligomenorrhea or amenorrhea, hirsutism, obesity, acne, male muscle mass, and in some cases polycystic ovaries. Excessive androgen production in females is commonly the result of congenital enzyme deficiencies in 21-hydroxylase, 11ß-hydroxylase, or 3ß-hydroxysteroid dehydrogenase. Excess androgens can also occur as a result of Cushings disease, where excess ACTH secretion results in stimulation of adrenal androgen production in addition to hypercortisolism. Less commonly, excess levels of androgen can occur as a result of virilizing tumours of the ovary or adrenal.

Congenital adrenal hyperplasia (CAH)

CAH affects about 1 in 5000 births. It results from a deficiency in one or another of the enzymes involved in cortisol biosynthesis. Deficient cortisol synthesis leads to a rise in ACTH production which in turn leads to hyperplasia of the adrenal cortex. The degree of hyperplasia depends on the extent to which enzyme activity is reduced.

The initial diagnosis of CAH is based on clinical symptoms and both serum and urinary steroid analysis. Enzyme assays can only be carried out either post-mortem in severe cases or occasionally when tissue is removed therapeutically in some milder cases. Steroid analysis can be enhanced by the use of synacthen (synthetic ACTH). Artificial stimulation of the cortisol biosynthetic pathway by synacthen leads to an overproduction and accumulation of cortisol precursors proximal to the block. Each enzyme deficiency produces characteristically abnormal levels of adrenal hormones and precursors (Finkelstein and Schaeffer 1979). Defects in the following enzymes have been associated with CAH (Table 1). Only 21, 11ß, and 3ß deficiencies are associated with virilisation.

Table 1

Deficiency	Enzyme	Frequency
Cholesterol desmolase	P450scc	Rare
3ß-hydroxysteroid dehydrogenase	3ß-hydroxysteroid dehydrogenase	Frequent?
17alpha-hydroxylase	P450c17	Rare
17,20-lyase	P450c17	Rare
21-hydroxylase	P450c21	1:12,000
11-hydroxylase	P450c11	1:100,000
Corticosterone methyl oxidase type II	P450c11	Rare

21-hydroxylase deficiency

This enzyme deficiency is the most common cause of CAH accounting for >90% of all cases. 21-hydroxylase converts progesterone to 11-deoxycorticosterone, and 17-hydroxyprogesterone to 11-deoxycortisol. Deficiency in 21-hydroxylase is generally characterised by raised levels of 17-hydroxyprogesterone in blood and large quantities of metabolites such as pregnenetriol and 17-hydroxypregnenolone in the urine. As 17-hydroxyprogesterone is a precursor of androstenedione and testosterone, the secretion of both of these androgens is also increased. Excessive androgen production results in virilisation of both males and females. In female newborns, the external genitalia are masculinized; gonads and internal genitalia are normal. If untreated postnatally, both males and females may manifest rapid growth, penile or clitoral enlargement, precocious adrenarche, and ultimately early epiphyseal closure and short stature.

There are four recognised clinical forms of 21-hydroxylase deficiency: salt losing, simple virilising, late onset (non-classical), and cryptic, all of which are inherited in an autosomal recessive manner. The salt wasting form accounts for approximately half of the reported cases. Inadequate production of salt retaining steroids, especially aldosterone, in the zona glomerulosa results in the inability to retain urinary sodium. Loss of sodium can result in shock or death during the neonatal period. The simple virilising form is due to an isolated defect in the cortisol pathway i.e. only the enzyme activity in the zona fasciculala-zona reticularis is affected. Aldosterone synthesis and conservation of urinary sodium are normal. The cryptic and late-onset forms of 21-hydroxylase deficiency are relatively mild in comparison to the classical forms. Usually patients exhibit a mild deficiency in cortisol synthesis and either remain asymptomatic (cryptic form) or become virilised in late childhood or at puberty (late-onset form)(New, White et al. 1989).

There are two genes encoding 21-hydroxylase, CYP21A and CYP21B, both of which are closely linked to the HLA major histocompatibility complex on the short arm of

chromosome 6 (White et al. 1983; Carrol et al. 1985; White et al. 1985). In addition there are specific associations between HLA haplotypes and different forms of 21-hydroxylase deficiency (O'Neill, et al. 1982). DNA sequence analysis has confirmed that the CYP21B gene encodes 21-hydroxylase, whereas the CYP21A gene is a pseudogene. Analysis of the 21-hydroxylase genes in deficient families has revealed a variety of gene defects including point mutations, gene deletions, gene conversions, and unequal crossovers of genetic material between the inactive gene A and the active gene B (Raum et al. 1984; Carrol et al. 1985; Donohoue et al. 1986; Werkmeister et al. 1986; Globerman et al. 1987; Amor et al. 1988; White et al. 1988). It has been suggested that 21-hydroxylase contains two active sites: one active on progesterone and the other specific for 17 alpha hydroxyprogesterone. In the salt losing variety both sites are defective and in the simple virilizing form only the second site is defective (Orta-Flores et al. 1976). This hypothesis may be investigated in the future by site directed mutagenesis of the CYP21B gene.

11ß-hydroxylase deficiency

This enzyme deficiency accounts for approximately 5-8% of reported cases of CAH (Wilkins 1965; Bongiovanni 1978; Werder et al. 1980). The deficiency results in elevated levels of 11-deoxycortisol (compound S) and deoxycorticosterone (DOC) in the serum. An elevation of the corresponding tetrahydro metabolites, tetrahydro-11-deoxycortisol (THS) and tetrahydrodeoxycorticosterone (THDOC) can be observed in urine (Eberlain and Bongiovanni 1956). The cortisol precursors that accumulate proximal to the block are shunted into the androgen biosynthetic pathway. Excessive foetal androgen production in females results in ambiguous genitalia with normal internal female reproductive organs. In both males and females, penile and clitoral enlargement, precocious adrenarche, and early epiphyseal closure can result if untreated. Mild, late-onset, and even cryptic forms of 11ß-hydroxylase deficiency have also been reported (Gabrilove et al. 1965; Newmark et al. 1977; Cathelineau et

al. 1980; Birmbaum and Rose 1984; Rosler and Leibermann 1984; Hurwitz et al. 1985). In many cases of 11ß-hydroxylase deficiency, hypokalaemia and hypertension are thought to be caused by increased levels of DOC and other precursors in the mineralocorticoid pathway but this is not certain.

Initial work by Hall et al (1985) indicated that the enzymatic activities of 11ß-hydroxylase and corticosterone methyl oxidase type I (CMOI:18 hydroxylase) and type II (CMOII:18 dehydrogenase)(Yanagibashi et al. 1986) resided in the one protein. Further work has shown that two distinct proteins exist (Chua 1987) and the cDNAs encoding for two putative human cyt P-450$_{11ß/18}$ genes have recently been cloned. The two genes CYP11B1 and CYP11B2, which are 95% identical, have been assigned to chromosome 8q22. The physical distance between these genes is not yet known, however, they are closely linked and thought to lie in tandem on chromosome 8q22, CYP11B1 upstream of CYP11B2 (Mornet et al. 1989). It is thought that CYP11B1 encodes cyt P-450$_{11ß}$ (11ß-hydroxylase) and CYP11B2 encodes a seperate enzyme cyt P-450 aldosterone synthase. A variety of mutations have been found in both CYP11B1 and CYP11B2 (White et al. 1991; Yanase et al. 1991; Helmberg et al. 1992; Pascoe et al. 1992).

A chimaeric 11ß-hydroxylase / aldosterone synthase gene has recently been identified in a family characterised by glucocorticoid - remediable aldosteronism and hypertension. A non-homologous crossover fusing the 5' regulatory region of 11ß-hydroxylase (expressed in both adrenal fasciculata and glomerulosa) to the coding sequences of aldosterone synthase (expressed only in adrenal glomerulosa) is thought to be responsible for the physiological abnormalities observed in this family (Lifton et al. 1992).

17 alpha hydroxylase deficiency

17 alpha hydroxylase deficiency is a rare defect in which the conversion of pregnenolone and progesterone to 17-hydroxypregnenolone and 17-hydroxyprogesterone is reduced. Consequently the production of glucocorticoids and sex steroids is reduced, and plasma levels of mineralocorticoids, especially corticosterone, are increased.

Affected females present as sexually infantile phenotypic females; males exhibit pseudohermaphroditism including incomplete Wolffian duct development (Mullerian structures are absent due to normal testicular production of MRF), and gynaecomastia. Both males and females exhibit low renin hypertension and hyperkalaemia which is probably due to elevated levels of mineralocorticoids (Grumbach and Conte 1985). 17,20-lyase activity resides in the same protein as 17 alpha hydroxylase (Kominami et al. 1982). Deficiency of 17,20-lyase causes an isolated defect in the synthesis of the sex steroids (Zachmann et al. 1972).

It is probable that at least four allelic variants of 17 alpha hydroxylase deficiency may exist. Bigliere et al (1966) reported males and females with complete absence of enzyme activity. New et al (1970) and Jones et al (1982) reported an incomplete block in 17 alpha hydroxylase-lyase activity, and cases of 17 alpha hydroxylase deficiency with intact 17,20-lyase activity have also been reported (Bradshaw, et al. 1987).

The 17 alpha hydroxylase/17,20-lyase protein is encoded by a single gene which has been localised to chromosome 10. To date a variety of point mutations, duplications, and deletions have been found in cases of combined 17 alpha hydroxylase/17,20-lyase deficiency (Kagimoto et al. 1988). Mutations which affect only one function of the protein have yet to be found.

Cholesterol desmolase deficiency

Cholesterol desmolase deficiency is an extremely rare defect also known as lipoid adrenal hyperplasia or Prader's syndrome (Prader and Gurtner 1955; Prader and Siebenman 1957). The affected patients have severely impaired synthesis of all steroids characterised by gonadal hypogenesis or agenesis, severe fluid and electrolyte disturbances, and Addisonian pigmentation. In most cases, death occurs during infancy due to an acute salt losing crisis.

The gene that encodes P-450scc has been allocated to chromosome 15 (Chung et al. 1986). As yet no gross deletions or rearrangements of the gene have been reported, and linkage of the disease to polymorphisms of this gene remains to be demonstrated.

3ß-hydroxysteroid dehydrogenase (3ß-HSD) deficiency

3ß-HSD catalyses a series of obligatory biosynthetic steps in the synthesis of mineralocorticoids, glucocorticoids, and sex steroids. 3ß-HSD is expressed in adrenal, ovary, testes, placenta, and possibly skin sebaceous glands and hair follicles (Luu-The et al. 1989; Lorence et al. 1990a). Specifically it converts the Δ5 3ß-hydroxysteroids pregnenolone, 17-hydroxypregnenolone, DHEA, and androstene 3ß, 17 diol into the respective Δ4 3-ketosteroids; progesterone, 17 alpha hydroxyprogesterone, androstenedione, and testosterone (Bongiovanni and Kellenbenz 1962; Miller 1988; Thomas et al. 1989). The conversion involves oxidation of the steroid A ring at the C3 position accompanied by a shift of the C-5,6 double bond on the steroid B ring to C4,5 on the A ring. A single protein catalyses both steps (Lachance et al. 1990; Lorence et al. 1990a).

3ß-HSD deficiency results in a high ratio of Δ5 to Δ4 steroids, characterised specifically by elevated serum levels of the Δ5 steroids, pregnenolone, 17-hydroxypregnenolone, and DHEA, and increased excretion of the Δ5 metabolites

pregnenetriol and 16-pregnenetriol in the urine. Baseline serum hormonal concentration measurements are insufficient for diagnosis of 3ß-HSD deficiency, instead, ACTH stimulation or the use of urinary 24 hour hormonal concentrations is required.

Evidence based on the assay of steroid hormones and their precursors indicates that there are a variety of inherited deficiencies of 3ß-HSD including salt wasting, non-salt wasting, classical, and late-onset. The classical form of 3ß-HSD deficiency is usually associated with salt wasting caused by insufficient mineralocorticoid production though in some cases the ability to conserve sodium has been intact. In males, defective testosterone biosynthesis results in pseudohermaphroditism including micropenis, hypospadias, and in later life, gynaecomastia (Bongiovanni and Kellenbenz 1962; Mendonca et al. 1987). In contrast, affected newborn females exhibit normal sexual differentiation or partial virilisation including clitoral enlargement and variable degrees of labial fusion. In later life, females exhibit hirsutism, primary or secondary amenorrhea, or irregular menses (Pang et al. 1985). The virilisation in females is a result of high levels of circulating DHEA which is almost exclusively adrenal in origin. It is likely that conversion of DHEA to potent androgens such as testosterone and 5 alpha dihydrotestosterone takes place in peripheral tissues such as skin sebaceous glands and hair follicles.

In the past decade there has been an increase in the number of reports of non-classical (late-onset) deficiencies of 3ß-HSD which are not associated with aldosterone synthesis or salt wasting. Children with non-classical 3ß-HSD deficiency may exhibit early appearance of pubic and axillary hair and accelerated growth (Temeck et al. 1987; Oberfield and Levine 1988). Adult females may exhibit hirsutism, acne, temporal balding, irregular menses, and infertility (Axelrod et al. 1965; Lobo and Goebelsmann 1981; Bongiovanni 1983; Pang et al. 1985; Bongiovanni 1987;

Redmond et al. 1988; Schram and Zerah 1990). Little is known about non-classical 3ß-HSD deficiency in adult men.

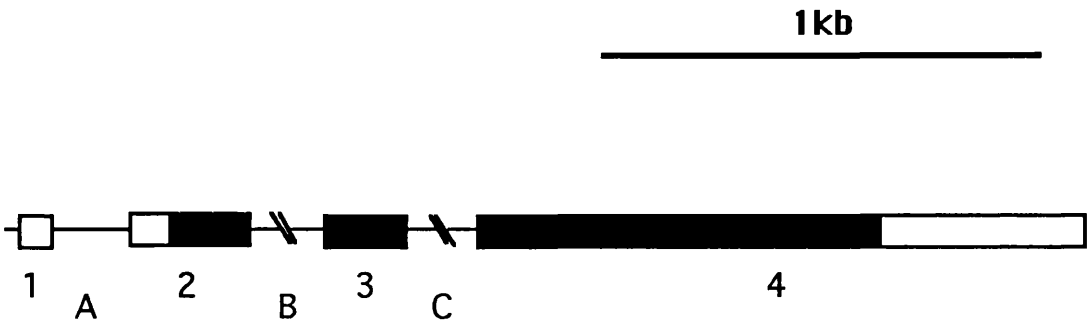
Molecular biology of 3ß-HSD

Following purification of human placental 3ß-HSD and the development of antibodies in rabbits (Luu-The et al. 1988; Luu-The et al. 1990), the cDNA encoding 3ß-HSD type I (Luu-The et al. 1989; Lorence et al. 1990a) and its corresponding gene (Lachance et al. 1990; Lorence et al. 1990b) were isolated by the Medical Research Council Group in Molecular Endocrinology (C.H.U.L. Research Centre and Laval University, Quebec). A full length cDNA encoding 3ß-HSD type I was also cloned by Nickson et al (1990)(Glasgow University) as part of a project sponsored by the W.H.O. Task Force on Vaccines for Fertility Regulation. This project involved the isolation and eukaryotic expression of cDNAs for placental specific surface proteins from a lambda gt11 placental library using monoclonal antibodies raised against placental trophoblast (Mueller et al. 1987). More recently, the cDNA encoding 3ß-HSD type II (Rheaume et al. 1991) and its corresponding gene (Lachance et al. 1991) were isolated by the Quebec group. To date, only two human genes encoding 3ß-HSD have been isolated, however, in rodents at least three genes for 3ß-HSD have been found to be expressed (Zhao et al. 1990; Bain et al. 1991; Zhao et al. 1991) and when analysed on southern blots, human DNA shows evidence of six or more sequences with homology to 3ß-HSD probes (Fig 13 and Lachance et al 1990). Ribonuclease protection assays have shown 3ß-HSD type I expression principally in the placenta and skin, and 3ß-HSD type II expression in adrenals, ovary, and testis (Rheaume et al. 1991).

3ß-HSD type I (Fig 7) contains a 5' untranslated exon of 53bp and three successive translated exons of 232, 165, and 1218bp, respectively, separated by introns of 129, 3883, and 2162bp. A putative TATA box (ATAAA) is situated 28 nucleotides

upstream from the transcription start site and a putative CAAT box is located 57 nucleotides upstream from the TATA box (Lachance et al. 1990; Lorence, et al. 1990b).

The type II gene (Fig 7) contains four exons of 57, 231, 165, and 1214 bp, respectively, separated by introns of 128, 3383, and 2162 bp. Two putative TATA boxes are situated 28 and 140 nucleotides upstream from the transcription start site and two putative CAAT boxes are located 57 and 38 nucleotides upstream from the TATA boxes respectively. The nucleotide sequence of the exons of the type II 3ß-HSD gene shows 77.4, 91.8, 94, and 91% similarity with the corresponding exons of the type I gene and 84, 83.1, and 73.9% similarity for the introns (Lachance et al. 1991).



TYPE I		TYPE II	
EXONS	INTRONS	EXONS	INTRONS
1. 53 bp	A. 129 bp	1. 57 bp	A. 128 bp
2. 232 bp	B. 3883 bp	2. 231 bp	B. 3383 bp
3. 165 bp	C. 2162 bp	3.165 bp	C. 2162
4. 1218 bp		4.1214 bp	

CHROMOSOMAL LOCATION 1p13.1

Figure 7: Gene structure of 3ß-HSD type I and type II.

The 3ß-HSD type I gene encodes a single 42 kDa protein composed of 372 amino acid residues (Luu-The et al. 1989; Lorence et al. 1990a). The type II gene encodes a 41.921 kDa protein of 371 residues (Rheaume et al. 1991). When compared, the two protein sequences show 93.5% homology. Both the type I and type II genes contain a putative nucleotide cofactor binding site of the ß-å-ß motif at residues Ser5>Asp36 and Ser4>Asp35 respectively. The central portion of this 3ß-HSD sequence is GXGGXXG (residues 10-16 type I, residues 9-15 type II), which resembles the consensus GXGXXG (Wierenga et al. 1986).

Though the enzymes are both capable of converting the same Δ5 substrates they differ in their kinetics in detail. The type I 3ß-HSD protein possesses a significantly higher affinity (approximately 5 fold greater) for pregnenolone, DHEA, and dihydrotestosterone than the type II protein. The type I 3ß-HSD protein possesses equivalent affinity for C21 (pregnenolone) and C19 (DHEA and dihydrotestosterone) steroids, and this is also true for the 3ß-HSD type II protein (Lorence et al. 1990a; Rheaume et al. 1991). Like most steroidogenic enzymes, 3ß-HSD types I and II may be able to catalyse many steps other than their principle action in the pathway in which they reside. Though it has been shown that the 3ß-HSD type I and type II proteins have similar substrate specificity for their principle substrates, significant differences may exist in their affinities for less common substrates.

The 3ß-HSD type I gene was assigned to chromosome 1p13.1 by Berube et al. (1989) and Morrison et al. (1990). No evidence was produced to suggest the presence of 3ß-HSD homologues elsewhere in the genome. The chromosomal location of 3ß-HSD type II has not yet been determined though it is likely that it also is situated on chromosome 1p13.1. I will present evidence which suggests close linkage of the 3ß-HSD type I and type II genes on chromosome 1.

The central role of the adrenals and gonads in steroid biosynthesis leads to the expectation that mutations in the gene for 3ß-HSD type II will be responsible for most of the inherited syndromes of 3ß-HSD deficiency. Indeed, stop codons and another mutation in 3ß-HSD type II have recently been reported in patients with severe CAH due to 3ß-HSD deficiency (Rheaume et al. 1992). These patients have profound deficiencies in the synthesis of mineralocorticoids and glucocorticoids and males are affected with pseudohermaphroditism due to a lack of testosterone. This severe autosomal recessive condition is now best understood as a failure to synthesize steroid precursors in the adrenals and gonads (Rheaume et al. 1992). The three families (Ran, Sta, and Pfe) to be presented in this thesis are affected by a less severe form of the deficiency. In each family the deficiency is limited to the production of the sex steroids. With the possible exception of family Sta, glucocorticoid and mineralocorticoid synthesis remains normal.

Detection of mutations

A variety of methods now exist for the detection of new mutations. Methods such as denaturing gradient gel electrophoresis (DGGE)(Myers et al. 1987), temperature gradient gel electrophoresis (TGGE)(DIAGEN GmbH), single strand conformation polymorphism (SSCP)(Orita et al. 1989), hydrolink gels (Keen et al. 1991), RNase A cleavage (Myers et al. 1985), and chemical cleavage (Cotton et al. 1989) provide screening systems that allow a mutation to be located to an exon of a specific gene, or even to a specific isoform of a gene family, and so focus the requirement for direct sequencing. The ready detection of homozygotes and heterozygotes allows rapid tests of the genetic co-segregation of the disease and the putative underlying mutation. DGGE has proved a powerful tool in this respect and several methodological aspects have been encountered in this study. Denaturing gradient gel electrophoresis allows the separation of DNA molecules which differ by as little as a single base substitution. The method involves the electrophoresis of double stranded DNA

fragments through a standard acrylamide gel containing a linear gradient, increasing from top to bottom, of DNA denaturants, such as urea and formamide. The increasing concentration of denaturants in the gel is equivalent to a proportional increase in temperature. DNA molecules differing by as little as one base pair will have a slightly different melting temperature and will be separated on the gel. A full description of DGGE is given by Myers et al (1987).

The DGGE method does require to be tuned for specific gene segments, and mutations that can barely be distinguished from controls can often be convincingly and reproducibly resolved by small changes in DGGE conditions. Some important factors to be taken into account when running DGGE are (1) the % denaturant to use (2) the size of pores in the gel i.e. the acrylamide concentration (3) the length of run (4) the voltage to use. The MELT programmes of Lerman and Silverstein (1987) were used to optimise DGGE for the screening of each exon of 3ß HSD types I and II. The computer readouts were open to interpretation and were therefore only treated as a rough guide. As suggested by Lerman, different gradients and run times were attempted for each fragment of DNA.

I have used DGGE to analyse the coding sequence of the 3ß-HSD type I and type II genes in each of the affected patients. In addition, automated sequencing of PCR products was used to screen the coding sequence of 3ß-HSD type II. Initially, only the affected patients were available for analysis. The collection of samples from other family members frequently proved difficult and time consuming as some came from outside Scotland. For this reason, some results are not presented in chronological order.

MATERIALS AND METHODS

Steroid assays: Serum steroids were measured by standard radioimmunoassay procedures (Forest et al. 1980). Urinary steroid profile analysis was performed by capillary column chromatography of steroid methyloxime-trimethylsilyl ether derivatives by a method similar to that developed by Shackleton and Honour (1976).

Isolation of genomic DNA: 10ml of peripheral blood was mixed with 75ml of 0.32M sucrose, 10mM Tris-HCl (pH 7.5), 5mM MgCl, and 1% Triton X-100 at 4^0C. White cells were collected by centrifugation at 10,000 rpm for 10 minutes at 4^0C. White cells were resuspended in 4.5ml of 0.075M NaCl, 0.024M EDTA using a pasteur pipette. SDS and proteinase K were added to a final concentration of 0.1% and 200ug/ml, respectively, and the suspensions were incubated at 37^0C overnight. DNA was extracted by phenol/chloroform, and precipitated by adding 0.5ml 3M Sodium acetate and 11ml ethanol (100%). DNA was dissolved slowly over 2 days at 4^0C in 1ml TE buffer (10mM Tris pH 7.6, 1mM EDTA). A 1 in 50 dilution of the sample was examined spectrophotometrically at 260nm and 280nm to determine the quality and purity of the DNA.

Agarose gel electrophoresis: Unless otherwise indicated, all agarose gels were run in TBE buffer (89mM Tris, 89mM boric acid, 2mM EDTA). Ethidium bromide was added to each gel at a concentration of 0.5ug/ml. The sample loading buffer used consisted of 0.25% bromophenol blue, 40% (w/v) sucrose in water (6x buffer).

Southern blot analysis: Human genomic DNA was isolated from peripheral blood as described. 5ug of genomic DNA was digested with EcoRI and XbaI restriction endonucleases. Each reaction mixture (final volume 30ul) consisted of 5ug human genomic DNA, EcoRI reaction buffer (5mM Tris (pH8.0), 1mM $MgCl_2$, 10mM NaCl) or XbaI reaction buffer (5mM Tris (pH8.0), 1mM $MgCl_2$, 5mM NaCl), spermidine

100mM, and 30 units of enzyme. The reaction mixture was incubated at 37^0C overnight. The DNA was electrophoresed through a 0.8% agarose gel at 3V/cm of gel length overnight. HindIII/EcoRI-digested lambda DNA fragments were used as molecular size markers. The DNA was transferred to nylon membranes (PALL) as described (Southern 1975). Filters were prehybridised for 4 hours at 65^0C in 6xSSC, 5xDenhardts, 0.5% SDS, and 100ug/ml denatured herring sperm DNA. The filters were then probed with 3ß-HSD type I exon specific DNA (Fig 12). Probes 1/2, 3, and 4 were amplified from human genomic DNA by PCR using primers a&b, c&d, and e&h (Fig 23), respectively, under the conditions specified in Table 8. PCR products were electrophoresed through a 1% agarose gel at 8V/cm gel length for 1-2 hours. Bands were visualised using long wavelength ultraviolet light and relevant bands were excised using a scalpel. DNA was purified from the gel slice using a Magic Prep column (Promega). The probes were labelled with [alpha-^{32}P]dCTP (3000Ci/mmol; Amersham) by the random primer method to a specific activity of 10^9dpm/ug. Probes were heated at 100^0C for 2 minutes. 5x10^6dpm of probe was added to 10ml of fresh prehybridisation buffer. The filters were hybridised at 65^0C overnight. The filters were washed twice (15 minutes each) in 2xSSC with 0.5% SDS at 22^0C and twice (60 minutes each) at 65^0C in 2xSSC with 0.5% SDS. Autoradiography was performed at room temperature using Kodak X-omat S films for 2 days.

Random priming of DNA: 20ng of DNA was labelled with [alpha-^{32}P]dCTP (3000Ci/mmol; Amersham) by the random primer method, using a Ready-To-Go DNA labelling kit (Pharmacia), to a specific activity of 10^9dpm/ug. The reaction mixture (final volume 50ul) consisted of 20ng DNA in 45ul water, 5ul [alpha-^{32}P]dCTP (10uCi/ul), and Reaction Mix (dATP, dGTP, dTTP, FPLC *pure*^{R} Klenow fragment (4-8 units)), and random oligodeoxyribonucleotides, primarily 9-mers.). DNA was heated at 100^0C for 2 minutes before adding to the above mix. The reaction was incubated at room temperature overnight then the reaction was stopped using 2ul of 0.5M EDTA. After adjusting the volume to 100ul with TE the labelled DNA was separated from the

unincorporated [alpha-^{32}P]dCTP by chromatography on a Sephadex G-50 column, using a 2cm x 1cm bed (height x diameter).

Polymerase chain reaction (PCR): Segments of the genes for 3ß-HSD types I and II were amplified from lymphocyte DNA by PCR. PCRs were performed in a Hybaid "Omnigene" for 40 cycles of: [1] 91^{0}C for 30sec, [2] annealing temperature for 30sec, [3] 72^{0}C for 30sec. A final cycle of 95^{0}C for 10 minutes was included to aid the formation of heteroduplexes. The annealing temperature of each pair of primers is shown in Table 8.(p 61) The reaction mixture (final volume 50ul) consisted of amplification buffer (50mM KCl, 10mM Tris-HCl (pH9.0), 0.1% Triton X-100, 15mM MgCl), 0.2mM of each dNTP, 1uM of each oligonucleotide primer, 0.5-1 unit of Taq DNA polymerase (Promega), and 10-50ng of DNA template.

BglII RFLP in 3ß-HSD type I exon 4: The PCR amplified product of 3ß-HSD type I exon 4 was precipitated and then digested with 10 units of BglII (Promega) at 37^{0}C for 2 hours. The restriction fragments were then separated on a 1% agarose gel at 8V/cm gel length for 1-2 hours, or analysed by DGGE for haplotype analysis.

Denaturing gradient gel electrophoresis: The DGGE system including materials, reagents, gel preparation, and electrophoresis has been described in detail by Myers et al (1987). Segments of the genes for 3ß-HSD types I and II were amplified from lymphocyte DNA by PCR, using the primers, GC clamps and conditions shown in Table 8. In each case the sequence of the GC clamp at the 5' end of the PCR primer was that defined by Sheffield et al (1989). PCR fragments, with GC clamps in either orientation, were screened for mutations using DGGE. DGGE was carried out in 6.5% polyacrylamide gels (37.5:1, acrylamide:bis acrylamide) in a buffer tank containing 9 l of TAE buffer (40mM Tris, 20mM sodium acetate, 1mM EDTA, pH 7.4) (Fig 8). Denaturing gradient gels (16 x 18 x 0.1 cm) were poured using a Dual Slab Electrophoresis SE 600 gel former (Hoeffer). A Grant Immersion Thermostat (

TA 0-150) was used to circulate the electrophoresis buffer at a constant temperature of 60^{0}C. The power pack used was a Kikusui PAB (250-0.25). It was necessary to remove the small metal bar connecting the positive and negative electrodes before using for DGGE. Failure to do so resulted in a loss of current. Appropriate denaturant and acrylamide concentrations were achieved in each gel by the mixing of two stock solutions, 0% denaturant stock (6.5% acrylamide (37.5:1, acrylamide:bis acrylamide) in TAE), and 100% denaturant stock (6.5% acrylamide (37.5:1, acrylamide:bis acrylamide), 7M urea, 40% formamide in TAE). Stock solutions were filtered twice through Whatman No.1 paper and stored in brown bottles at 4^{0}C. 1/200 gel volume of 20% ammonium persulphate and 1/2000 gel volume of TEMED (N, N, N', N'- Tetra methyl ethylenediamine) were used as polymerisation catalysts. Approximate denaturant gradients, electrophoresis voltages and times were selected for each amplified segment of genomic DNA with the aid of the MELT programme (Lerman and Silverstein 1987) and were refined by trial and error. The conditions for best resolution of each gene segment are shown in Table 8. All DGGE gels were run at 80V, 100mA for 20 hours. Best resolution was achieved in the centre and lower two thirds of the gel matrix. Parameters including the concentration of acrylamide and denaturants in the DGGE gel and the voltage and duration of electrophoresis were varied to allow the DNA sample to resolve at approximately half way down the gel. DGGE gels were stained by immersion in 280 ug/ul ethidium bromide for 30 minutes.

Samples which exhibited altered mobility on DGGE were originally analysed by excising the band of interest from the gel, reamplifying the DNA fragment, then sequencing the resulting PCR product directly. This method was rendered obsolete by the advent of PCR cloning vector technology.

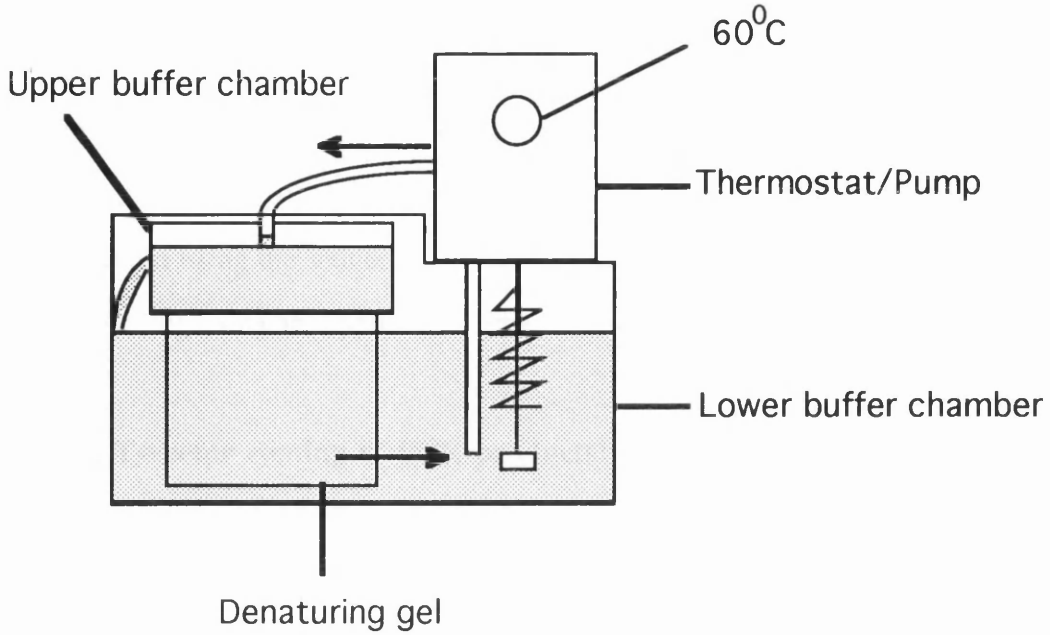


Figure 8: Denaturing gradient gel electrophoresis

Figure 8 illustrates the equipment used in the running of denaturant gradient gels. The direction of buffer flow is indicated by arrows.

Isolation of DNA fragments from DGGE gels: DGGE gels were examined under long wavelength ultraviolet light and bands of interest were excised using a scalpel. A fresh scalpel blade was used for each band. The gel slice was transferred to a microfuge tube and crushed against the wall of the tube using a disposable pipette tip. 200ul of elution buffer (0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA (pH 8.0), 0.1% SDS) was added to the tube. The sample was incubated at 37^{0}C on a rotating wheel for 12-16 hours. The sample was centrifuged at 12,000g for 1 minute and the supernatant transferred to a fresh microfuge tube. The DNA was precipitated then resuspended in 100ul water. 10ul was used as template for PCR amplification of the fragment. PCR products were run on suitable denaturing gels alongside controls (the original PCR product) to confirm successful isolation and reamplification.

Direct sequencing of PCR products: PCR products were electrophoresed through a 1% agarose gel at 8V/cm gel length for 1-2 hours. Bands were visualised using long wavelength ultraviolet light and relevant bands were excised using a scalpel. DNA was purified from the gel slice using a Magic Prep column (Promega). Sequencing was carried out using the sequenase version 2.0 DNA sequencing kit (U.S.B.) or by cycle sequencing using an Applied Biosystems automatic sequencer.

pT7 Blue T-vector cloning of PCR products: 50ng (0.03pmol) of pT7 Blue T-vector was ligated with 10-50ng (0.04-0.2pmol) of PCR product. PCR products were not band purified before cloning into pT7 Blue T-vector. The reaction mixture (final volume 10ul) consisted of T4 DNA ligase buffer (30mM Tris-HCl (pH7.8), 10mM $MgCl_2$ 10mM DTT), 10mM ATP, 50ng pT7 Blue T-vector, 10-50ng PCR product, and 2-3 Weiss units T4 DNA ligase (B.R.L.). The reaction mixture was incubated at 15^0C overnight. 1ul of ligation reaction was added to 20ul of Nova Blue competent cells (Invitrogen) and placed on ice for 30 minutes. The cells were heated for 40 seconds in a 42^0C water bath and then placed on ice for 2 minutes. 80ul of SOC medium (20g bacto-tryptone, 5g bacto-yeast extract, 0.5g NaCl, 20ml of 1M glucose, 950ml H_2O) was added and the cells incubated at 37^0C for 1 hour. 300ul more SOC medium was added and 100ul portions were spread on LB (Luria-Bertani) agar plates (10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl, 15g bacto-agar, 950ml H_2O) containing 50ug/ml ampicillin plus 15ug/ml tetracycline. For blue/white screening of recombinants 35ul of 50mg/ml X-gal in dimethylformamide and 20ul 100mM IPTG in water were spread on the plates and allowed to soak in for about 30 minutes prior to plating. Plates were incubated at 37^0C overnight. Putative clones were identified by blue/white selection.

Confirmation of putative clones by PCR: Individual white colonies were selected and grown up in 2ml LB broth (10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl, 950ml H_2O) incubated at 37^0C overnight. 10ul of culture was added to 40ul water

and heated in a boiling water bath for 5 minutes. 10ul of the boiled culture was used as template in the appropriate PCR reaction (Table 8). PCR products were screened for artefactual PCR mutations by resolution on the appropriate DGGE gel alongside controls (the original PCR product). Only independent clones which resolved indistinguishably from the original genomic template were selected for sequencing. Large scale plasmid preparation of DNA for sequencing was performed using Qiagen columns. Sequencing was carried out using the sequenase version 2.0 DNA sequencing kit (U.S.B.).

RESULTS

Patient profiles

The following families were selected for analysis on the basis of clinical and biochemical data suggestive of 3ß-HSD deficiency.

Family Sta (Fisher et al. 1988)(Fig 9)

Sta III-1 presented at birth with normal testes and perineal hypospadias. From early childhood onwards, several episodes of hyponatraemia were diagnosed. Urinary steroid analysis showed high basal levels of pregnenolone, 16 alpha hydroxypregnenolone and 16 alpha hydroxyDHEA with normal cortisol metabolites, establishing the diagnosis of partial 3ß-HSD deficiency (Table 2). The raised basal plasma renin activity (PRA) suggested a mild salt loss. The fall in urine sodium while on a low sodium diet was surprising since plasma aldosterone levels declined. Fisher suggests that the presence of a dexamethasone suppressible aldosterone antagonist may be responsible.

Sta III-2 presented at birth with normal testes, micropenis, and perineal hypospadias. No episodes of hyponatraemia occured. In later life (8-10 years), premature adrenarche was diagnosed. Urinary steroid analysis confirmed 3ß-HSD deficiency with higher levels of steroid metabolites than his brother (Table 2).

Both parents, StaII-2 and Sta II-3, were unaffected.

Fisher suggests that the 3ß-HSD enzyme deficiency in cases Sta III-1 and Sta III-2 is present in the testes and adrenals, as poor testosterone response to hCG stimulation was observed in both cases and Sta III-2 also demonstrated an impaired cortisol

response to synacthen. Most of the circulating testosterone was adrenal in origin as levels were suppressed by dexamethasone.

Family Pfe (Fig 10)

Patients Pfe III-2, Pfe IV-1, Pfe IV-2, and Pfe IV-5 were diagnosed as having mild deficiency of 3ß-HSD mainly on the basis of having elevated levels of plasma 17-hydroxypregnenolone after ACTH stimulation. Patients presented with varying degrees of hirsutism and oligomenorrhoea. The mode of inheritance appeared to be autosomal dominant, however, X linked inheritance cannot be ruled out. Table 3 gives a summary of steroid gonadotropin and prolactin values of family Pfe. Table 4 gives information on the clinical findings for each affected member of the family.

Family Ran (Fig 11)

Family Ran is characterised by both parents and the two children having marked levels of 16 alpha metabolites of 17-hydroxypregnenolone and DHEA suggestive of non-salt losing 3ß-HSD deficiency (Table 5). The propositus (Ran III-3) presented at age five with a history of clitoral enlargement. No other signs of excess androgens were present. Her karyotype was 46 XY indicating that she was in fact a male pseudohermaphrodite. Her sister (Ran III-4) had no detectable clinical abnormality and had a normal female karyotype (46 XX). Biochemical data on Ran III-4 indicates that she has the same defect which is likely to manifest itself later in females. The mother (Ran II-7) is affected with severe oligomenorrhea (1-2 periods per year lasting 2-7 days) but is not virilised. The propositus was conceived spontaneously while her sister was conceived with the help of clomiphene therapy. An increase in the 16 alpha hydroxylation of DHEA (an androgen precursor) to 16 alpha hydroxy-DHEA (an oestrogen precursor) may account for the mother's lack of virilisation. The father (Ran II-8), though having an abnormal steroid profile had no

detectable clinical abnormality. Neither Ran III-3, Ran III-4, Ran II-7, nor Ran II-8 have disturbances of mineralocorticoid or glucocorticoid metabolism. Comparison of steroid profiles indicates that both parents are possibly heterozygotes for the deficiency whereas both sibs, who are much more severely affected, are probably homozygotes.

FAMILY *STA* : male pseudohermaphroditism with mild salt loss

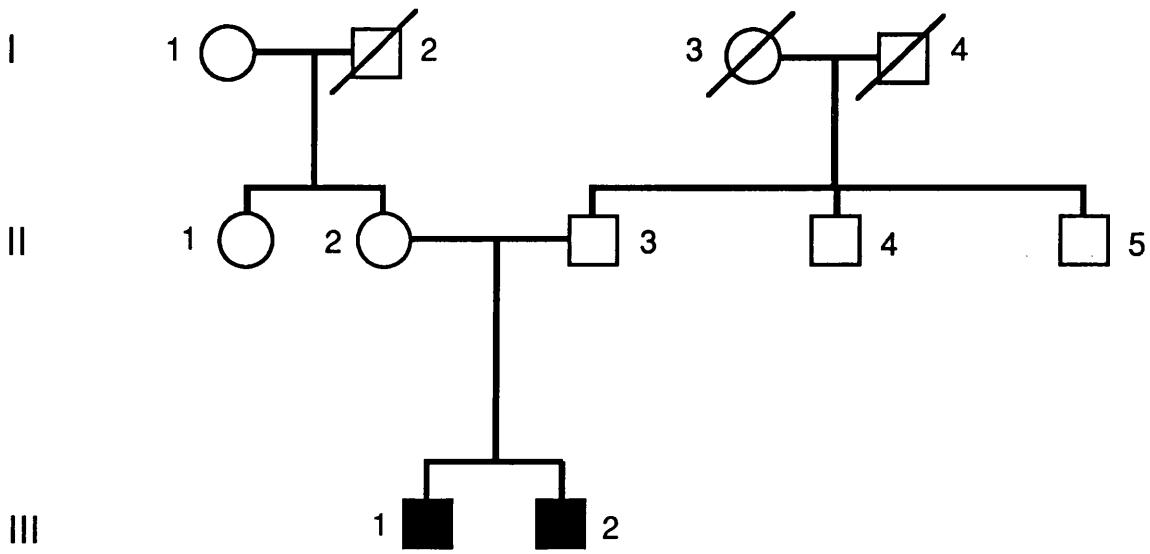


Figure 9

Table 2 (Family Sta)

		Basal	1h post ACTH	Low sodium diet*	Pre-HCG**	Post-HCG***
Cortisol 09.00, 220-775 nmol/l	III-1 III-2 control	361 537 451	587 554 881		<20 21 40	<20 <20 26
17alpha-OHP < 5 nmol/l	III-1 III-2 control	15.7 21.2 1.3	29.9 36.7 10.0		1.2 1.4 <1.0	1.3 1.3 1.5
Testosterone 0.8-1.5 nmol/l	III-1 III-2 control	0.2 1.4 1.4	0.2 1.4 1.3		<0.2 <0.2 <0.2	0.6 3.3 8.8
DHEA-S 0.3-1.8 umol/l	III-1 III-2 control	6.4 20 1.4	6.3 21.6 1.4		5.6 13.2 0.3	3.8 8.4 1.1
Plasma aldosterone, recumbent, 100-500 pmol/l	III-1 III-2 control	410 350 120		220 230 145		
PRA, recumbent, 1.1-2.7 pmol/ml/h	III-1 III-2 control	6.8 2.1 2.6		9.9 5.2 3.0		
Urine aldosterone 10-50 nmol/24h	III-1 III-2 control	16 18 <10		16.5 23 46		

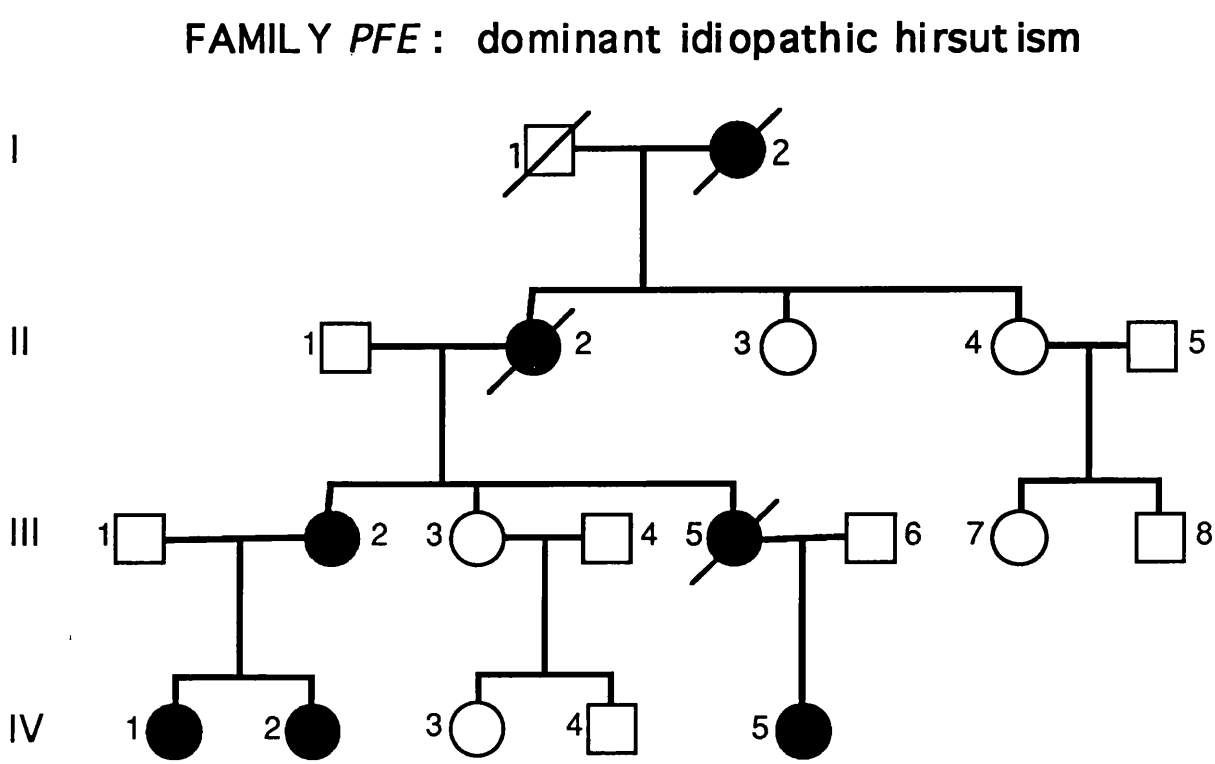

FAMILY *PFE* : dominant idiopathic hirsutism
I
II
III
IV
1
2
1
2
3
4
5
1
2
3
4
5
6
7
8
1
2
3
4
5

Figure 10

Table 3 (Family Pfe)

Steroid (reference values +/- SD basal/stimulated with ACTH)	III-2 basal	III-2 60'	IV-1 basal	IV-1 60'	IV-2 basal	IV-2 60'	IV-5 basal	IV-5 60'
17OH Preg. ng/dl (433 +/- 390/828 +/- 344)	174	1006	480	2829	259	3062	509	846
DHEA ng/dl (868 +/- 553/1102 +/- 329)	810	1838	1503	2905	306	2512	1673	5286
DHEA-S ug/dl (286 +/- 120)	259	-	288	-	183	-	486	-
17OH Prog. ng/dl (149 +/- 98/255 +/- 145)	119	233	115	421	110	216	132	135
21 DF ng/dl (16 +/- 9/37 +/- 12)	11	69	16	62	12	41	27	72
Testosterone ng/dl (31 +/- 14)	39	-	43	-	31	-	56	-
Androstenedione ng/dl (242 +/- 60/270 +/- 99)	102	29	423	615	86	159	207	330
Cortisol ng/ml (50-250)	53	478	157	340	97	326	68	230
LH (1.8-13 ng/ml)	2.0	-	7.2	-	3.4	-	1.9	-
FSH 1-14 U/l	0.8	-	4.9	-	2.0	-	2.9	-
PRL (2.3-20 ng/ml)	4.3	-	9	-	5.6	-	5.4	-

Table 4 (Family Pfe)

	III-2	IV-1	IV-2	IV-5
Hirsutism	+++	++++	+	+
Menarche (years of age)	10	10	12	13
Eumenorrhoea	-	-	+	+
Oligomenorrhoea	+	+	-	-
Amenorrhoea	-	-	-	-
Polycystic ovaries	n.i.	No	No	n.i.
Height (cm)	157	162	167	163
Weight (kg)	65	73	69	53
Age (years)	46	22	24	19

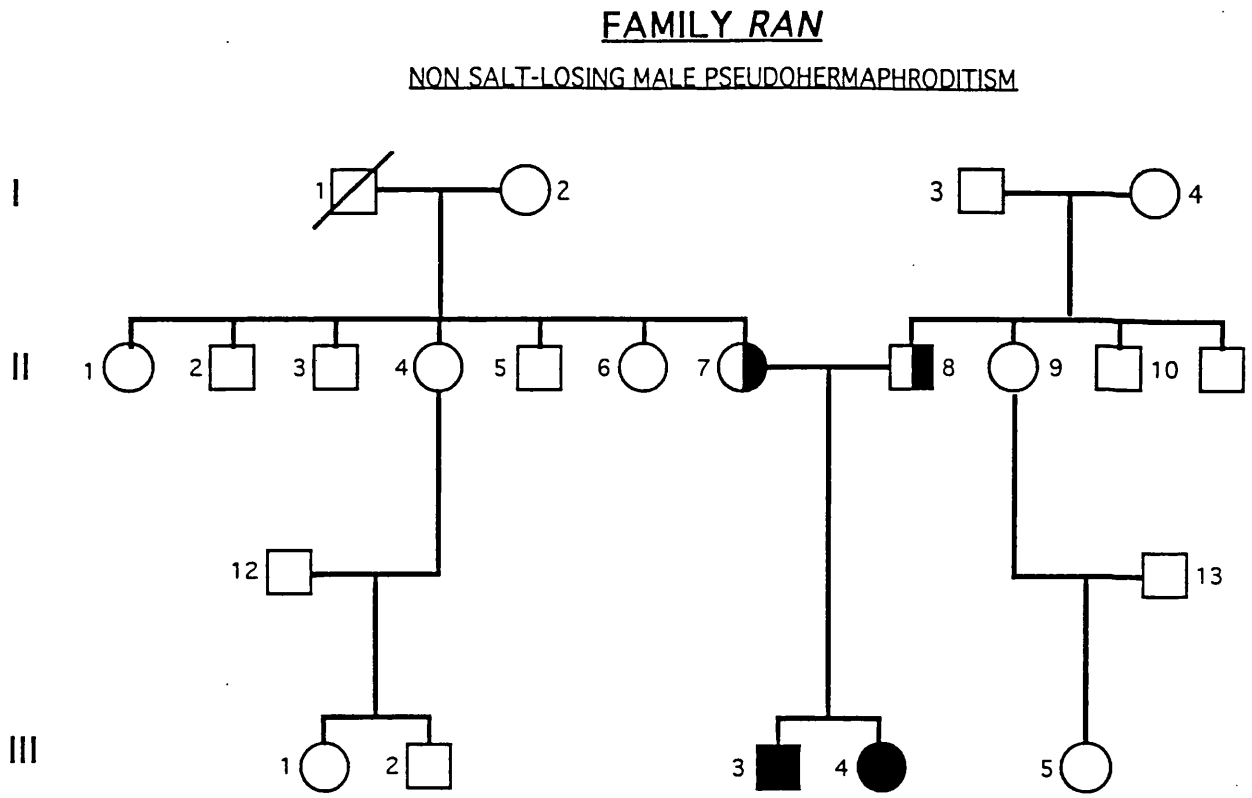

FAMILY RAN
NON SALT-LOSING MALE PSEUDOHERMAPHRODITISM
I
II
III
1
2
3
4
5
6
7
8
9
10
11
12
13

Figure 11

Table 5 (Family Ran)

URINARY STEROID METABOLITES	III-3	III-4	Normal child upper limit	II-7	Normal female adult upper limit	II-8	Normal male male upper limit
delta-5 metabolites:							
Dehydroepiandrosterone	93	ud	ud	940	350	180	2090
16a OH dehydroepiandrosterone	504	150	ud	2040	1360	700	1820
5-androstene-3ß-16a-17ß triol	255	110	ud	700	1300	770	1700
5-pregnene-3ß-17a-20a triol	645	170	ud	890	300	480	450
delta-4 metabolites:							
Androsterone	78	30	140	670	1940	1350	4500
Aetiocholanolone	60	20	130	810	2400	1140	3700
11-oxo-aetiocholanolone	59	30	130	280	500	160	640
11ß OH androsterone/17OH pregnanolone	297	110	210	750	1250	850	2600
11ß OH aetiocholanolone	120	40	170	440	780	210	820
5ß-pregnane-3a-20a-diol	ud	ud	ud	860	1480	ud	740
Pregnanetriol	363	350	ud	1300	850	910	1600
Tetrahydrocortisone	1177	590	2050	3250	5100	3710	8200
Tetrahydro-11-dehydrocorticosterone	199	ud	130	350	530	170	570
Tetrahydrocorticosterone	ud	ud	120	280	460	220	730
Allo-tetrahydrocorticosterone	ud	ud	300	ud	1100	ud	1790
Tetrahydrocortisol	525	180	650	1580	2700	1920	4510
Allo-tetrahydrocortisol	409	380	940	1060	1990	1700	5220
a-cortolone	340	200	180	1340	1500	1120	1950
ß-cortolone plus ß-cortol	107	300	440	1310	1700	1370	2360
a-cortol	510	100	130	330	640	300	790
Reference group			children, n=19		females, n=21		males, n=22
Age range			prepubertal		20-45		20-45

Southern blot analysis of DNA from patients and controls

Introduction

The aim of this experiment was to determine how many sequences homologous to 3ß-HSD type I were present in the human genome. It was also hoped that any gross rearrangements of DNA in affected families would be detected by this method.

Genomic DNA from control individuals and from patients were restricted with XbaI or EcoRI and resolved on Southern blots probed with 3ß-HSD type I exon specific probes (Fig 12). At the time of this experiment only 3ß-HSD type I had been identified. The restriction enzymes EcoRI and XbaI were chosen because neither cut within its coding regions and this was subsequently found to be true for 3ß-HSD type II.

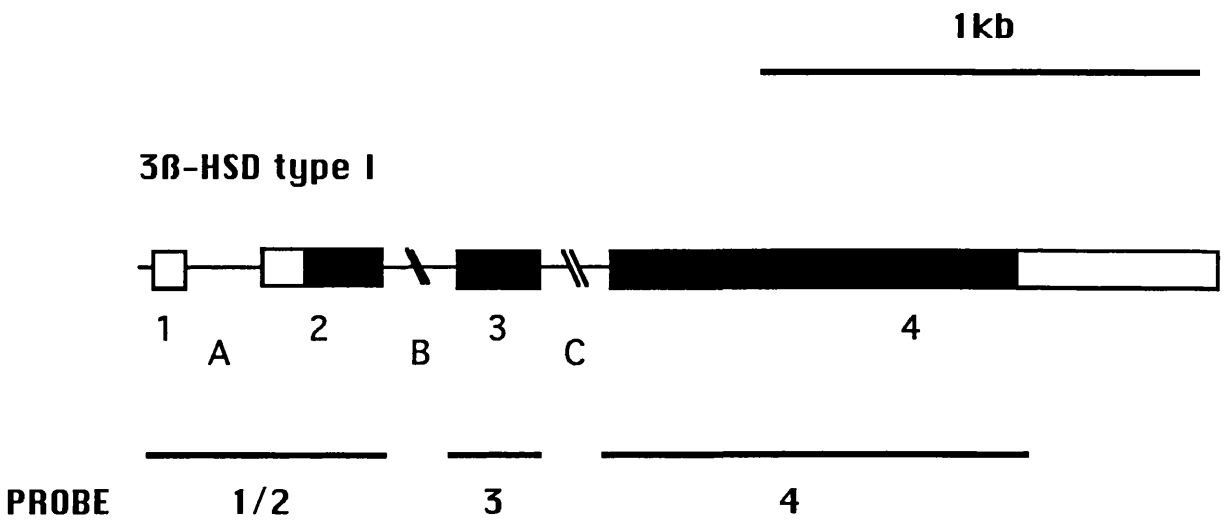


Figure 12: Details of hybridisation probes.

Figure 12 shows the extent of the DNA probes used for southern blots.

Results and Discussion

The probes hybridised to several restriction fragments ranging from 0.7 kb to 30 kb (Fig 13). For the purposes of presentation, only one example of each enzyme digest is shown for each probe. Identical band patterns were observed for each of the patients,

Sta III-1, Ran III-3, and Pfe IV-1, and for controls. This indicated that there were no detectable gross rearrangements of 3ß-HSD sequences in these families.

In all cases, fragments of predictable size were observed as intense bands however other bands of varying size and intensity were also present. Although some of these bands could be pseudogenes the results emphasise the possibility that further functional genes for 3ß-HSD exist in the human genome.

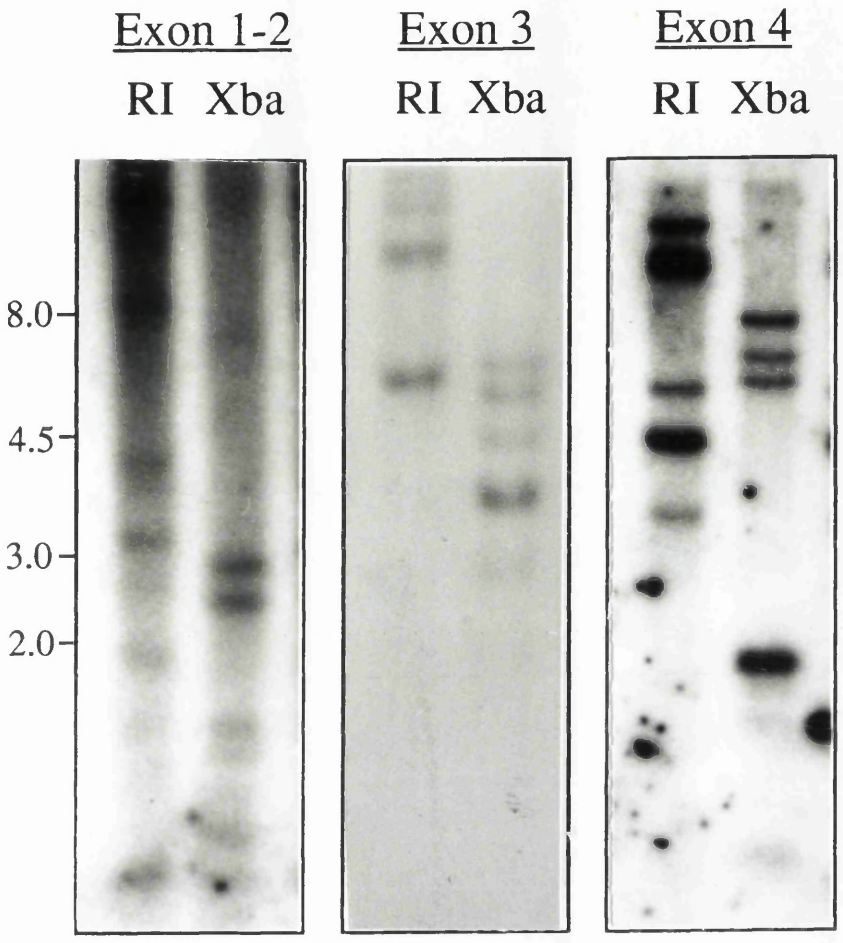


Figure 13: Southern blot analysis.

3ß-HSD type I exon specific probes hybridised to several restriction fragments ranging from 0.7kb to 30kb. Intense bands were observed which corresponded to the sizes predicted by the restriction map of the known 3ß-HSD genes.

Synonymous and non-synonymous polymorphisms in type I 3ß-HSD exon 4 (Fig 14)

Introduction

The full length cDNA of 3ß-HSD type I was cloned from a lambda gt11 placental library by Nickson et al 1990. Sequencing of independent clones revealed two polymorphisms both in exon 4 (Russell et al. 1991a; Russell, et al. 1991b). The first polymorphism identified was a RFLP at base 8715 corresponding to codon 338 in 3ß-HSD type I. The substitution of a C for a T resulted in the loss of a BglII site but no change in the amino acid specified. The polymorphism was typed in individual samples of DNA by amplifying exon 4 by PCR (primers (e) and (h) Fig 23) and then digesting the product with BglII. The 0.8% agarose gel in Fig 15 shows the 927 bp product of exon 4 being reduced in length to 757 bp after digestion with BglII. Homozygote BglII +/+, homozygote BglII -/-, and a heterozygote BglII +/- are shown. The second polymorphism was a non-synonymous polymorphism. The substitution of an A for a C at base 8803 resulted in the change of amino acid residue from Asn to Thr at codon 367 in 3ß-HSD type I. Table 6 shows the allele frequencies of each polymorphic marker. It is not known at present what effect this amino acid substitution has on the activity of the enzyme. Initially this polymorphism was more difficult and time consuming to detect in individuals as it could only be detected by the direct sequencing of PCR products. Figure 16 shows sequence from a homozygote Asn, a homozygote Thr, and a heterozygote Asn/Thr. It was later found that DGGE could be used as a much more rapid and efficient method for detecting the polymorphism at codon 367. The coding sequence of exon 4 was first amplified with a 5' GC clamp (primers (e) and (h) Fig 23). When run on DGGE the Thr allele migrated to a lower level in the gel than the Asn allele. Fig 17 shows a homozygote Asn, a homozygote Thr, and a heterozygote Asn/Thr. The BglII digestion of exon 4 PCR products followed by DGGE analysis allowed the haplotypes of each individual to be determined. In the case of double heterozygotes i.e. BglII +- / 367 Asn/Thr this

proved particularly useful. Fig 18 shows the result of three double heterozygotes who all proved to be (BglII+/Asn) (BglII-/Thr).

Together the BglII and 367 polymorphisms were used to determine the segregation of 3ß-HSD type I alleles in the three families (Ran, Sta, and Pfe) (Figs 20, 21, & 22)).

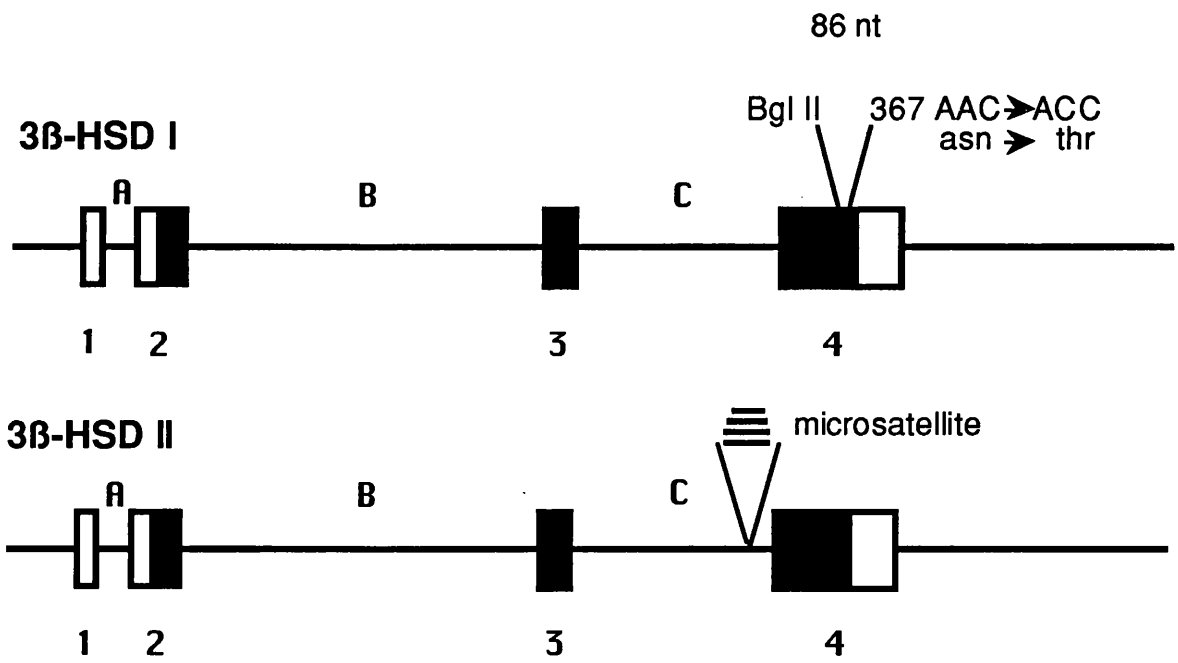


Figure 14: Polymorphisms in the genes for 3ß-HSD types I & II

Figure 14 shows the relative positions of the polymorphisms in 3ß-HSD type I and 3ß-HSD type II.

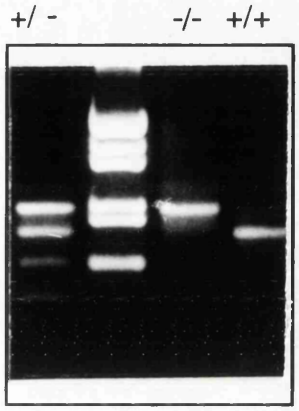


Figure 15: Detection of BglII RFLP in 3ß-HSD type I exon 4.

The substitution of a C for a T at base 8715 resulted in the loss of a BglII site. Figure 15 shows the 927 bp product of exon 4 being reduced in length to 757 bp after digestion with BglII. Homozygote BglII +/+, homozygote BglII -/-, and a heterozygote BglII +/- are shown.

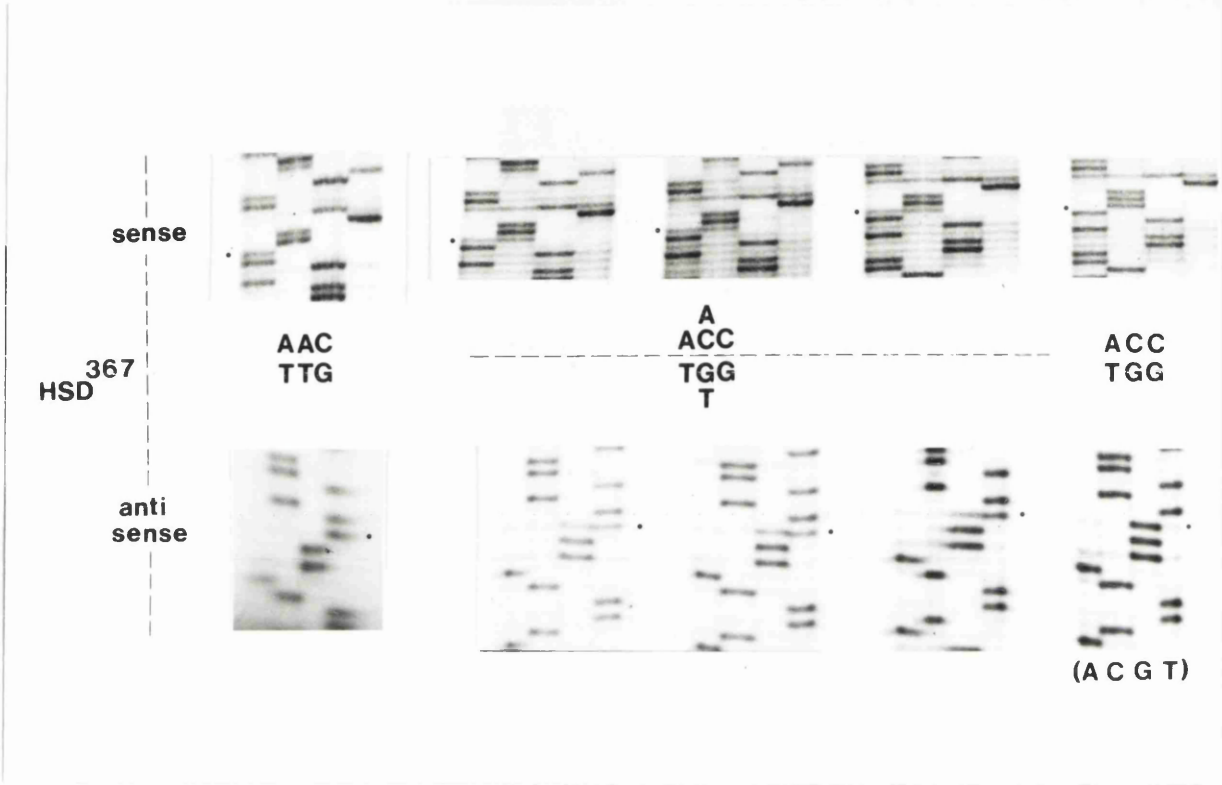


Figure 16: Detection of non-synonymous substitution at codon 367 3ß-HSD type I by direct sequencing of PCR products.

The PCR product was purified on an agarose gel then sequenced using Sequenase Version 2.0 (USB) to sequence either the sense strand (top row) or anti-sense strand (bottom row) of the DNA. Each sequence track is marked with a dot at base 1100. The replacement of A (allele 1) by C (allele 2) results in codon 367 changing from Asn to Thr.

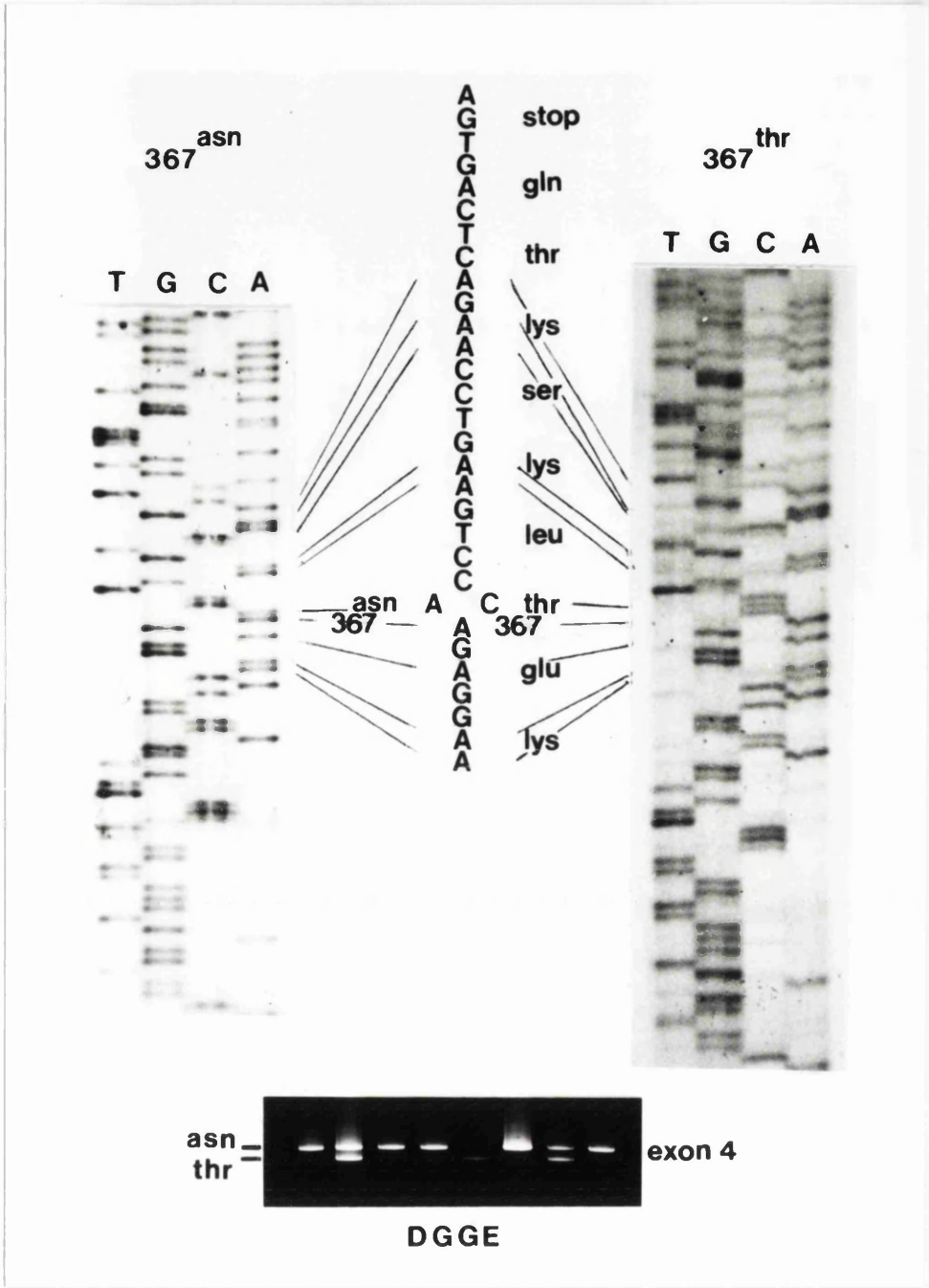


Figure 17: Detection of non-synonymous substitution at codon 367 3ß-HSD type I by DGGE.

Figure 17 shows sequence data outlining the codon substitution at residue 367. The bottom panel shows the separation of 3ß-HSD type I exon 4 alleles by DGGE. Homozygotes and heterozygotes are easily identified. The gradient of denaturants was 40-60%. The gel was run at 100mA, 80V for 20 hours.

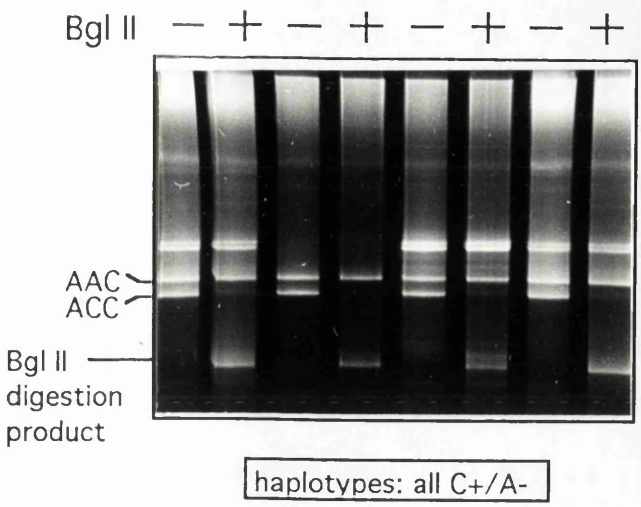


Figure 18: Haplotype analysis of 3ß-HSD type I

Table 6

Genotypes	frequency
Alleles:	
Bgl +	0.5
Bgl -	0.5
codon 367^{asn}	0.65
codon 367^{thr}	0.35
Haplotypes:	
codon 367^{asn} Bgl +	0.22
codon 367^{asn} Bgl -	0.46
codon 367^{thr} Bgl +	0.22
codon 367^{thr} Bgl -	0.07

Microsatellite marker of the 3ß-HSD type II gene

Introduction

A complex set of simple sequence repeats are located in the third intron of 3ß-HSD type II, just 5' to the start of exon 4 (Fig 14). The high number of sequence repeats within this small area led us to suspect that this might be a useful source of microsatellite variation. Primers (u) and (v) (Table 8) were designed to amplify a 424bp fragment (nucleotides 7330 - 7753) of 3ß-HSD type II intron C. PCR products of varying length were produced indicating considerable genetic variation within this region. The flanking sequence of these amplified segments has been determined confirming that amplification was from the expected segment of the 3ß-HSD type II gene, rather than from unrelated genomic segments.

Results

To date 4 alleles have been identified (Fig 19), with the following frequencies (20 unrelated males and females):

allele	frequency
1 (approx 385bp)	0.48
2 (approx 425bp)	0.12
3 (approx 450bp)	0.28
4 (approx 475bp)	0.12

Alleles were separated on 3% agarose gels. The identification of further alleles by use of polyacrylamide gels is expected. The microsatellite analysis of families Ran, Sta, and Pfe is shown in Figs 20, 21, and 22.

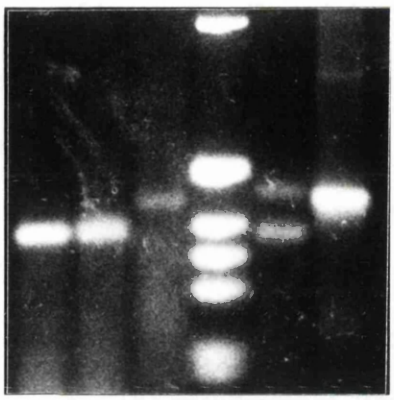


Figure 19: Analysis of 3ß-HSD type II intron C microsatellite.

Figure 19 shows the separation of PCR amplified microsatellite DNA on a 3% agarose gel. Only alleles 1, 3, and 4 are shown.

Track 1: 1/1 homozygote

Track 2: 1/1 homozygote

Track 3: 3/3 homozygote

Track 4: lambda 1kb ladder (Gibco)

Track 5: 1/4 heterozygote

Track 6: 3/3 homozygote

Results and Discussion: polymorphic markers in 3ß-HSD type I and type II

Ran family

Analysis of the BglII and codon 367 polymorphisms in 3ß-HSD type I and the microsatellite in intron C of 3ß-HSD type II indicated that both affected sibs, Ran III-3 and Ran III-4, inherited the same alleles from either parent i.e. both inherited a BglII+/Thr/3^{haplotype} from the father and a BglII+/Thr/3^{haplotype} from the mother. The BglII+/Thr/3^{haplotype} on the father's side of the family originated from the grandmother Ran I-4. No other individuals on the father's side of the family possessed the BglII+/Thr/3^{haplotype}. On this side of the family only the father, Ran II-8, exhibited an abnormal steroid profile (Table 5). The grandmother, Ran I-4, from whom the BglII allele originated was post menopausal and had reduced adrenal function, therefore no conclusions could be drawn from steroid analysis of this individual.

The BglII+/Thr/3^{haplotype} from the mother's side of the family was deduced to have originated from the grandfather Ran I-1. All available offspring on this side of the family possessed a BglII+/Thr/3^{haplotype}, however, only Ran II-6 and Ran II-7 presented abnormal steroid profiles (see pg101). This did not disprove the possibility of the BglII+/Thr/3^{haplotype} being linked to the abnormal phenotype. Our hypothesis is that Ran I-1 was a BglII+/Thr/3, BglII+/Thr/3 homozygote. One BglII+/Thr/3 was linked to a mutation in one of the 3ß-HSD genes and was passed to Ran II-6, Ran II-7, and subsequently to Ran III-3 and Ran III-4.

Sta family

Analysis of the polymorphic markers in 3ß-HSD type I and type II indicated that both affected sons inherited the same alleles from either parent i.e. both sons inherited a BglII+/Asn/2^{haplotype} from the father and a BglII+/Thr/1^{haplotype} from the mother. No other individuals on the father's side of the family possessed the BglII+/Asn/2^{haplotype}.

Analysis of the father's side of the family also revealed evidence of a possible crossover between the 3ß-HSD type I and 3ß-HSD type II genes. The BglII+/Thr/1 haplotype from the mother's side of the family may have originated from either grandparent and may also be present in Sta II-1 who is affected by hirsutism.

Pfe family

Analysis of the microsatellite in intron C of the type II gene indicated that the disease phenotype in family Pfe was not linked to 3ß-HSD type II though the possibility of linkage with the type I gene could not be ruled out. Affected individuals Pfe III-1, IV-1, and IV-2, all possessed a BglII+/Thr/4 haplotype, whereas IV-5, who was also affected, did not possess the BglII+/Thr/4 haplotype. Furthermore, the BglII+/Thr/4 haplotype was present in Pfe III-3 who was unaffected.

Figures 20, 21, and 22 show that the polymorphic markers in 3ß-HSD types I and II co-segregate. To date, only one recombination event has been observed between these genetic markers (family Sta, see Fig 21). The LOD scores for these markers were: 3.568 for Ran, 0.301 for Sta, and 0.301 for Pfe. When combined with families 1 and 2 from Rheaume et al.(1992), a total LOD score of 5.374 was obtained indicating tight genetic linkage between the two loci (Table 7).

	θ								
	0.000	0.001	0.002	0.003	0.004	0.005	0.010	0.020	0.050
Family *RAN*	3.568	3.561	3.554	3.548	3.541	3.534	3.499	3.429	3.216
Family *S*	0.301	0.300	0.299	0.298	0.298	0.297	0.292	0.283	0.258
Family *P*	0.301	0.300	0.299	0.298	0.298	0.297	0.292	0.283	0.258
Rheaume *1*	0.602	0.601	0.599	0.598	0.597	0.596	0.585	0.567	0.515
Rheaume *2*	0.602	0.601	0.599	0.598	0.597	0.596	0.585	0.567	0.515
TOTAL	5.374	5.363	5.350	5.340	5.331	5.320	5.253	5.129	4.762

Table 7: LOD scores for the linkage of the genes for 3ß-HSD types I and II.

LOD scores were calculated for values of ø of 0 - 0.05. Values for families 1 and 2 were computed from the pedigrees of Reaume et al (1992).

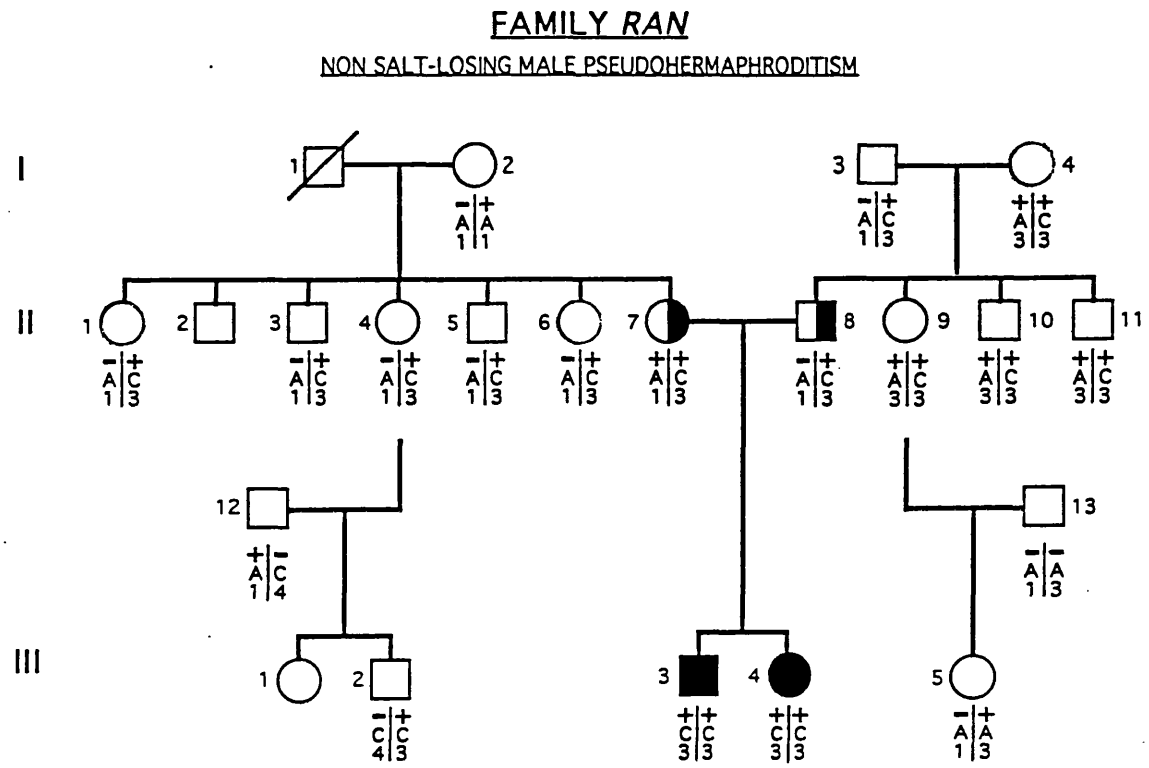
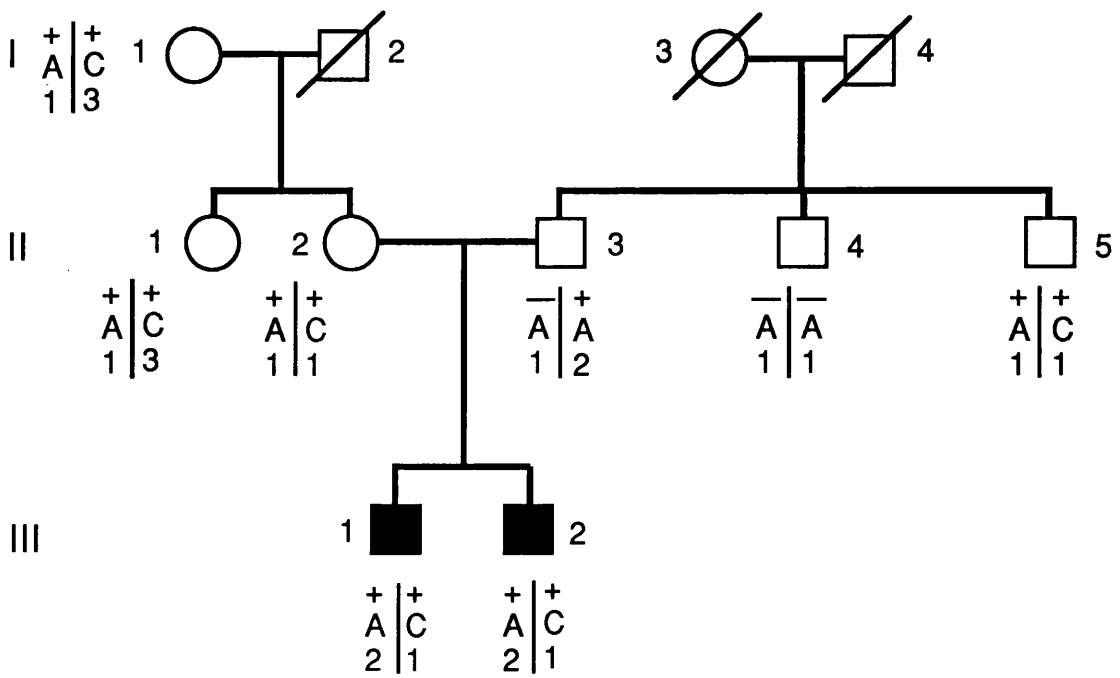

FAMILY RAN
NON SALT-LOSING MALE PSEUDOHERMAPHRODITISM
I
II
III
Haplotype key
3ß-HSD I – | + BglII
A | A codon 367
3ß-HSD II 1 | 1 intron C microsat

Figure 20

FAMILY *STA* : male pseudohermaphroditism with mild salt loss



Haplotype key

3ß-HSD I	—	+	BgI II
	A	A	codon 367
3ß-HSD II	1	1	intron C microsat

Figure 21

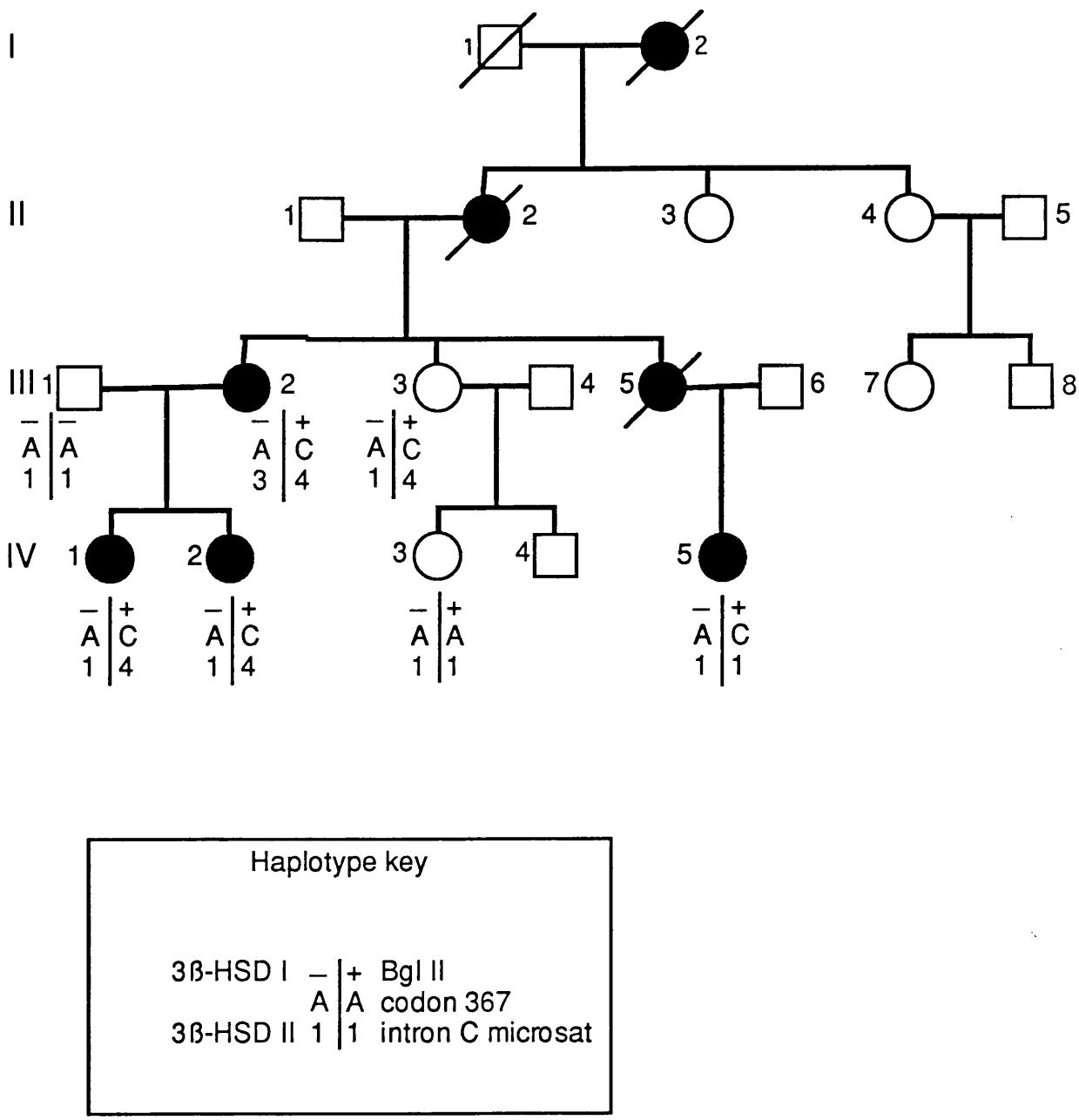

FAMILY PFE : dominant idiopathic hirsutism
I
II
III
IV
Haplotype key
3ß-HSD I
3ß-HSD II
Bgl II
codon 367
intron C microsat

Figure 22

DGGE screening of 3ß-HSD type I and type II

Introduction

The aim of the following experiments was to detect any sequence variation in the 3ß-HSD type I and II genes which may have accounted for the clinical and biochemical symptoms in our patients. PCR fragments from affected patients were compared to normal controls using DGGE. In most cases only the most severely affected member of each family was screened initially. The computer programmes of Lerman and Silverstein (1987) were used to predict the melting behaviour of each fragment. This allowed the design of primers (Fig 23) and the optimisation of DGGE for each of the fragments (Table 8). All relevant MELT maps are shown on pages A1-A19.

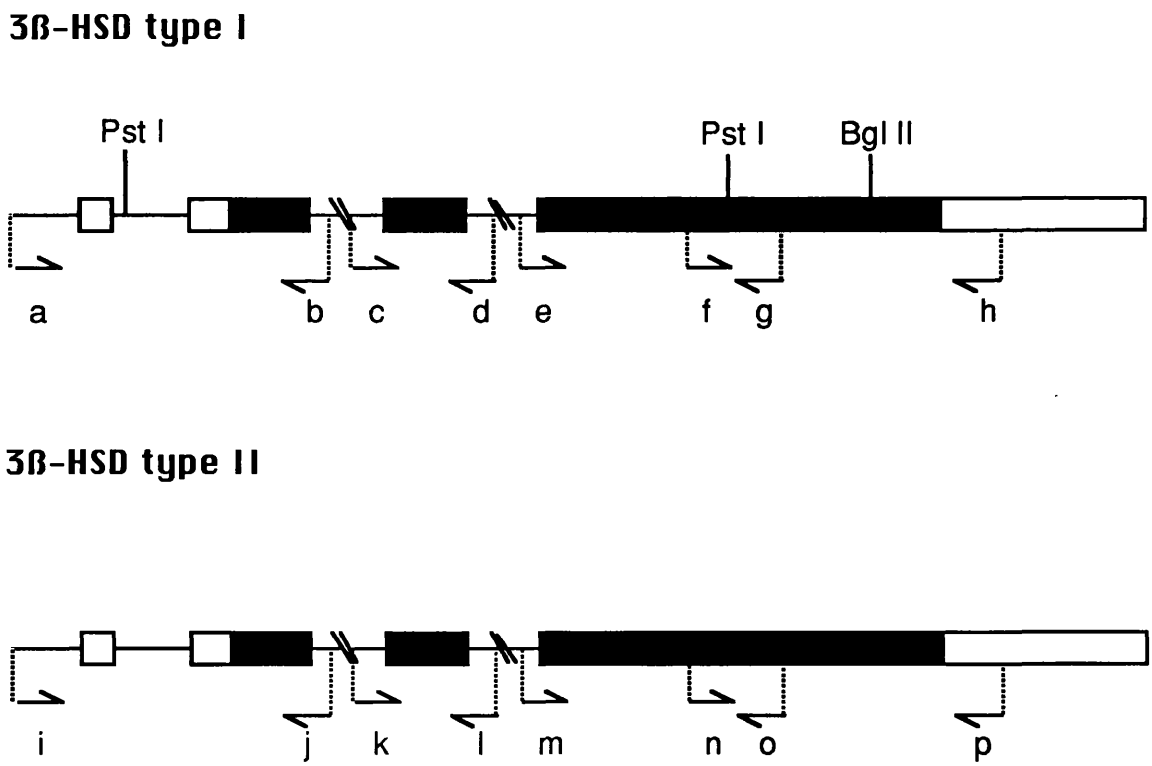


Figure23: PCR primers

Figure 23 shows the relative positions of PCR amplimers used in the screening of 3ß-HSD types I and II by DGGE. The position of relevant restriction endonuclease sites is also shown. Further details of PCR primers and conditions are given in Table 8.

Table 8

3ß-HSD type I	Primers	Annealing temp.	DGGE % denaturant
exon 1/2	(a) 1354 - AGTGCATAAAGCTTCAG - 1370 (b) 1855 - CCACATACATGCAGTGT - 1839	50°C	30 - 60%
exon 3	(c) 5655 - CCAATGACCTGACCTGT - 5671 (d) 5881 - CCACCTTGCTGCATCTC - 5865	52°C	55 - 85%
exon 4	(e) 7955 - CGTGGTTGGCACCTCTT - 7971 (h) 8881 - GGAGCTTGATGACATCT - 8865	52°C	35 - 65%
exon 4A	(e) 7955 - CGTGGTTGGCACCTCTT - 7971 (g) 8466 - AGTAGAACTGTCCTCGG - 8450	52°C	40 - 70%
exon 4B	(f) 8400 - CACATTCTGGCCTTGAG - 8416 (h) 8881 - GGAGCTTGATGACATCT - 8865	52°C	40 - 65%
3ß-HSD type II			
exon 1/2	(i) 1219 - AGAGCATAAAGCTCCAG - 1235 (j) 1720 - CCACACCCATGCAGAGT - 1704	50°C	30 - 60%
exon 3	(k) 5488 - CCAATGACCTGACCTGT - 5504 (l) 5714 - CCAACTTGTTTTATCTC - 5698	48°C	35 - 65%
exon 4A	(m) 7849 - TGTGGTTGCAGCTCCTT - 7865 (o) 8270 - AGACTGGGTTGACTGAT - 8254	50°C	45 - 75%
exon 4B	(n) 8181 - GCCCATTCCTTTCTGCC - 8197 (p) 8774 - GAGTTTGATGACATTTC - 8758	49°C	40 - 70%
ø specific	(q) 5662 - CCTGACCTGTGTTCTTG - 5678 (r) 5786 - AGGCGGTGCGGATGACT - 5770	52°C	
intron B > ø	(s) 5269 - CATGATGCTGGAGTCCC - 5285 (r) 5786 - AGGCGGTGCGGATGACT - 5770	52°C	
ø > intron C	(q) 5662 - CCTGACCTGTGTTCTTG - 5678 (t) 6268 - GTGACTACAGTGGCACC - 6252	52°C	
microsatellite	(u) 7330 - CTCAGAGTATAGCCTCC - 7346 (v) 7753 - ATGATACAGCCGTAGGA - 7737	52°C	

DGGE screening of 3ß-HSD type I exon 1/2

Exons 1 and 2 extend over only 414 bp and were therefore initially amplified with intron A as a single segment (nt 1354 - 1855). This PCR product was initially screened with a GC clamp at the 5' end and then at the 3' end. According to the melt map (A2) the 3' GC clamped product was predicted to comprehensively screen the lowest melting domain of the molecule (nt 1354 - 1567) although nucleotides 1568 to 1855 would not be screened as thoroughly. Digestion of the 3' GC clamped product with PstI resulted in the reduction in size of the lower melting domain from 214 bp to 91 bp. This increased the effectiveness of the screening of nucleotides 1568 to 1855 (A3).

Results

When exon 1/2 type I was amplified with a 5' GC clamp, Pfe, Ran, and Sta samples showed no difference in migration on DGGE. When the PCR product was cut with PstI all control and patient samples again yielded a band of similar mobility.

When amplified with a 3' GC clamp, Ran and Sta samples produced a single band on DGGE which corresponded to controls. All three available members of family Pfe (Pfe III-2, Pfe IV-1, Pfe IV-2) produced this same band (A) plus a second band (B) which resolved at a higher level in the gel i.e. a lower Tm (Fig 24).

PCR product from Pfe IV-1 containing both bands A and B was cloned into pT7 Blue T-vector and putative clones were identified by blue/white selection. The presence of insert in these samples was confirmed by PCR. PCR product from a number of positive clones were run on DGGE alongside control Pfe IV-1 DNA. A number of clones corresponding in migration to bands A and B were detected and in addition several clones were present whose migration did not correspond to bands A and B (

Fig 25). When digested with PstI, clones 5 and 6 (bands B and A respectively) migrated with the other cut samples (Fig 26). This indicated that the sequence variation between the two clones was most likely to be located in the segment which was deleted by the PstI digestion i.e. nt 1354 - 1476.

Sequencing of clones 5 (band B) and 6 (band A)

Each clone was sequenced over its entire length using primers (a) and (b) (Fig 23). Clone 6 (band A) showed no differences when compared to the published sequence. Clone 5 (band B) showed one base difference when compared to the published sequence, a C > A substitution at base 1426 (Fig 27) i.e. within the untranslated exon 1. No other sequence variations were found in this clone. The location of the substitution, between bases 1354 and 1476, was as predicted.

Screening of extended family Pfe

When screened for the C > A substitution in exon 1, Pfe III-1, Pfe IV-3, and Pfe IV-5 showed no difference in migration when compared to controls. Pfe III-3 produced both bands A and B indicating the presence of the C > A substitution in exon 1.

Discussion: type I exon 1/2

According to computer predictions (A2) the screening of full length exon 1/2 with a 3'GC clamp should have comprehensively screened the lower melting domain of the PCR product i.e. nt 1354 - 1567. This analysis resulted in the detection of a C > A substitution at nt 1426 in the three available members of the Pfe family (Pfe III-2, IV-1 & IV-2). As the substitution was present in the untranslated exon 1 it was unlikely to be the cause of the 3ß-HSD deficiency in this family, however as all three

affected members possessed the same substitution it was hoped that it may show linkage with the phenotype in other affected members of the same family. When an extended family was finally obtained it was found that Pfe IV-5, the other affected member of the family, did not contain the C > A substitution in exon 1. It was also shown that Pfe III-3, an unaffected member of the family, did contain the C > A substitution. The polymorphic markers in 3ß-HSD types I and II strongly suggest that neither 3ß-HSD type I nor type II is linked to the disease phenotype in family Pfe.

The methods used in the detection cloning and confirmation of clones proved effective. According to the DGGE pattern the sequence variation in Pfe samples was expected to reduce the Tm of the molecule. This prediction was confirmed by the eventual identification of the C > A substitution. The location of the substitution between nt 1354 and 1476 was accurately predicted initally by the melt maps (A2 & A3) and later by the PstI digestion of samples (Fig 26). The need to screen positive clones on DGGE before sequencing was clearly necessary as 6 out of 11 positive clones tested did not co-migrate with bands A and B (Fig 25). These spurious PCR products were most probably the result of Taq polymerase errors during the initial amplification from Pfe genomic DNA.

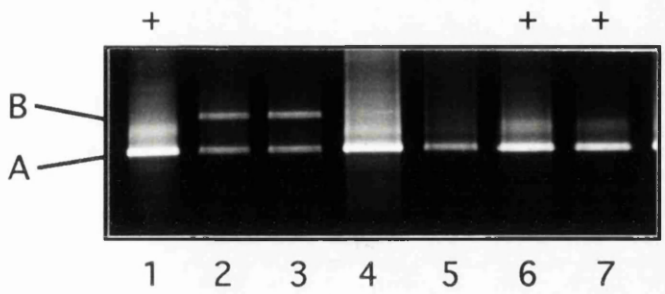


Figure 24: DGGE gel showing the migration of 3ß-HSD type I exon 1/2

(+) = control patient. Tracks 2-5 contain samples Pfe IV-1, Pfe IV-2, Ran III-3 and Sta III-1 respectively. Bands A and B are identified at left hand side.The gradient of denaturants was 30-60%. The gel was run at 100mA, 80V for 20 hours.

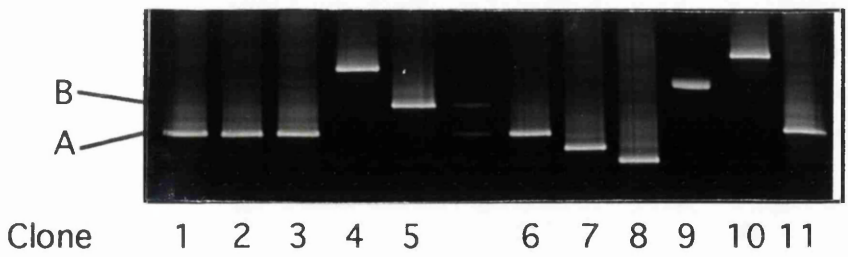


Figure 25: Selection of positive clones by DGGE (3ß-HSD type I exon 1/2)

Track 6 contains Pfe IV-1 exon 1/2 as a positive control. Bands A and B are identified at left hand side. The gradient of denaturants was 30-60%. The gel was run at 100mA, 80V for 20 hours.

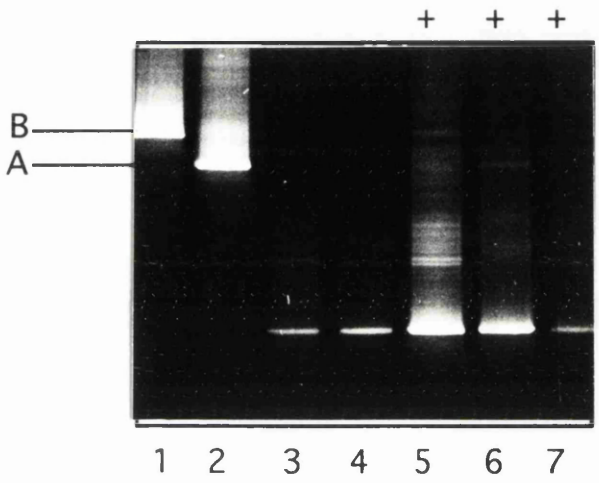


Figure 26: PstI restriction analysis of Pfe IV-1 clones (3ß-HSD type I exon 1/2)

Clones 5 (band B) and 6 (band A)(Fig 25) were re-run in tracks 1 and 2 respectively. Tracks 3 and 4 contain clones 5 and 6 both cut with Pst I. No difference in migration is observed after Pst I digestion. (+) = control sample digested with PstI. Bands A, and B are identified at left hand side. The gradient of denaturants was 30-60%. The gel was run at 100mA, 80V for 20 hours.

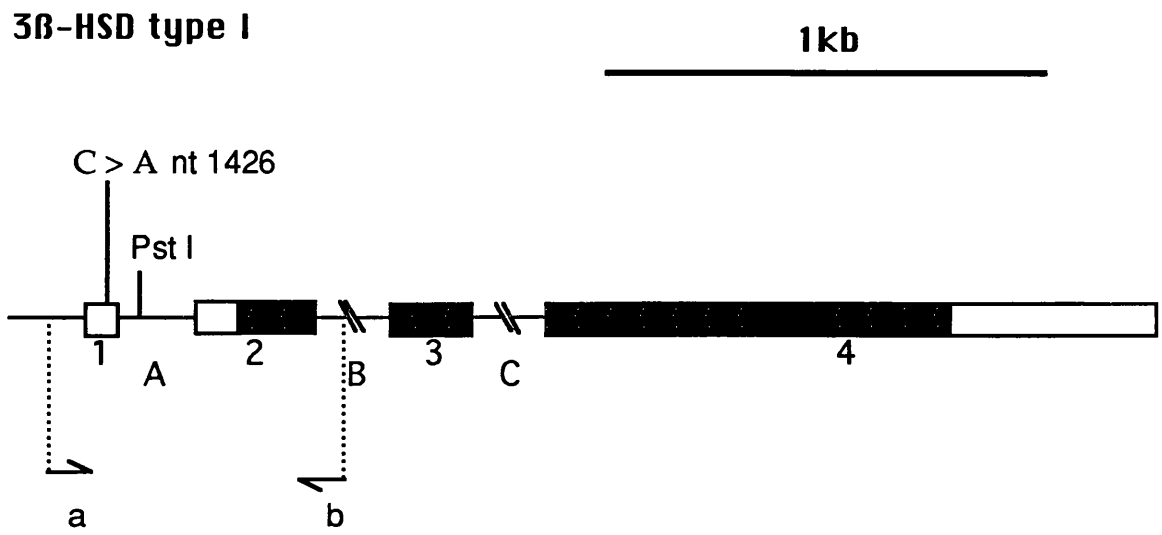


Figure 27: Nucleotide substitution in 3ß-HSD type I exon 1.

Figure 27 shows the relative position of the nucleotide substitution in 3ß-HSD type I exon 1. The substitution of an A for a C at base 1426 has to date only been detected in family Pfe. The position of the relevant PCR primers and of the Pst I restriction site are also shown.

DGGE screening of 3ß-HSD type I exon 3

The melting profiles of type I exon 3 (A4 & A5) indicated a single melting domain from nt 5655 - 5881. Screening first with a 5'GC clamp then with a 3'GC clamp should have been adequate for detecting >99% of all possible mutations.

Using a 3'GC clamp on the PCR product, all samples produced one band which migrated to the same depth in gel as controls.

With a 5'GC clamp, PCR products of exon 3 produced two bands å and ß. All three available Pfe samples (Pfe III-2, IV-1, and IV-2) also produced a third band ø (Fig 28). Bands å, ß, and ø were cut from the gel and reamplified by PCR. PCR products were run on an identical gel to confirm successful isolation. Each PCR product was then sequenced directly. The sequence obtained from å and ß was poor but both showed homology to 3ß-HSD. Sequence from band ø was of better quality and showed a number of sequence variations when compared to 3ß-HSD type I. Of 132 bp sequenced, 13 substitutions were detected, 3 in intron B and 10 in exon 3 (Fig 29).

Cloning and sequencing of 3ß-HSD type I exon 3 DGGE variants

To obtain more extensive sequence of å, ß, and ø, the bulk type I exon 3 5'GC PCR product from Pfe IV-1 (unseparated by DGGE) was cloned into pT7 Blue T-vector and putative clones were identified by blue/white selection. The presence of insert in these samples was confirmed by PCR. To date 4 independent clones have been identified. Clone 1 was found to be identical to the published 3ß-HSD sequence. Clone 2 was found to be identical to the previous sequence obtained from band ø (Fig 29). Complete sequence of ø revealed an open reading frame (ORF) of 165bp. Clones 3 and 4 were also shown to have ORFs of 165bp. Comparisons of 3ß-HSD type I and type II with clones 2 (ø), 3, and 4 are shown in Figures 30 and 31. Clone 2

(ø) differs from type I in 23 nucleotides and 9 amino acids and from type II in 24 nucleotides and 9 amino acids. Clone 3 differs from type I in 18 nucleotides and 9 amino acids and from type II in 26 nucleotides and 9 amino acids. 16 of the nucleotide substitutions and 8 of the amino acid substitutions observed were also present in clone 2 (ø). Clone 4 showed much more homology to 3ß-HSD type I than either clones 2 or 3. Only 9 nucleotides and 4 amino acid differences were observed when compared to 3ß-HSD type I. 19 nucleotide and 4 amino acid substitutions were observed when compared to type II.

Clones 1, 3, and 4 were run on DGGE alongside controls (amplified from Pfe IV-1 genomic DNA) to determine if any of them corresponded to bands å or ß (Fig 32). Clone 1 co-migrated with band å. No clone was isolated which corresponded to band ß although clone 3 migrated to a similar level in the gel. Clone 4 migrated to a lower level in the gel than either of bands å, ß, or ø. There were too many mutations present in clone 4 for it to have been the result of Taq polymerase errors, also its 3' end was not a direct copy of any of the other clones, therefore it was unlikely to have originated through "jumping PCR" (the small size of the product also makes "jumping PCR" an unlikely explanation). The most likely explanation is that clone 4 is a legitimate copy of a sequence that occurs in the genome and that it did not appear on the original DGGE gel simply because it was not amplified to a sufficient concentration.

Investigation of ø

All three available members of the Pfe family (Pfe III-2, IV-1, and IV-2) produced an extra band (ø) on DGGE and it was hoped that ø might account for the clinical and biochemical symptoms in family Pfe. The high number of base substitutions present in ø made it very unlikely that ø was the result of spontaneous mutation of either the 3ß-HSD type I or type II genes. It was our hypothesis that ø originated

elsewhere in the genome. To test our hypothesis on the origin of ø, primers were designed which would specifically amplify ø (Fig 33). The aim of this experiment was to find if ø could be amplified from unaffected individuals and not only from family Pfe. In a further experiment, PCR primers were designed to sit on intronic sequence flanking 3ß-HSD type I exon 3 (Fig 33). It was hoped that by using a combination of these primers and ø specific primers an insight may be gained as to whether ø was attached to the type I gene in family Pfe as a result of DNA rearrangement.

Results

The amplification of a putative ø band (125 bp) using ø specific primers is shown in Figure 34. It was shown that this fragment could be amplified from unaffected individuals and not only from family Pfe. The 125 bp PCR product was cloned into pT7 Blue T-vector and putative clones identified by blue/white selection. Positive clones were confirmed by PCR. Sequencing of clones revealed identical sequence to the ø band of family Pfe. Primers (s) and (r), and (q) and (t) were unsuccessful in amplifying a 3ß-HSD type I - ø hybrid product.

Discussion: type I exon 3

The use of ø specific primers showed that ø was present in the genome of unaffected individuals and not only family Pfe.

The appearance of ø in only one family (Pfe) when primers (c) and (d) were used could be explained by the following possibilities. It was possible that the ø sequence originated from a pseudogene or even from another functional 3ß-HSD gene. The

primers used would not normally have amplified ø , however, in family Pfe a point mutation at one of the intronic primer sites could have resulted in the amplification of ø. If this was the case then ø was unlikely to be the cause of the deficiency in family Pfe. Another possibility was that some sort of DNA rearrangement may have occurred in the Pfe family between either of the known genes and another functional 3ß-HSD gene or pseudogene. This hypothesis was unlikely as no evidence of gross rearrangement was observed on southern blots (Fig 13). If rearrangement had occurred, the type I gene would be the most likely candidate for rearrangement as ø was amplified using type I specific primers.

The attempt at linking ø to 3ß-HSD type I by using a combination of intronic primers and ø specific primers failed to produce a PCR product. This may mean that ø was not linked to 3ß-HSD type I although it is still possible that some form of DNA rearrangement may have occurred and that the boundaries of such a rearrangement were outwith the intronic primer sites.

The identification of four separate clones homologous to 3ß-HSD type I and type II supports the previous southern blot data indicating the existence of other 3ß-HSD sequences within the genome. It is not known if these clones have originated from pseudogenes or from functional genes, each one has an ORF and each clone extends into intronic sequence indicating that they have not originated from processed pseudogenes.

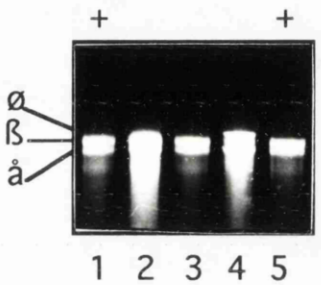


Figure 28: DGGE gel showing the migration of human 3ß-HSD type I exon 3.

(+) = control patient. Tracks 2, 3, and 4 contain samples Ran III-3, Sta III-1, and Pfe IV-1 respectively. Bands å, ß and ø are identified at the left hand side. The gradient of denaturants was 55-85%. The gel was run at 100mA, 80V for 20 hours.

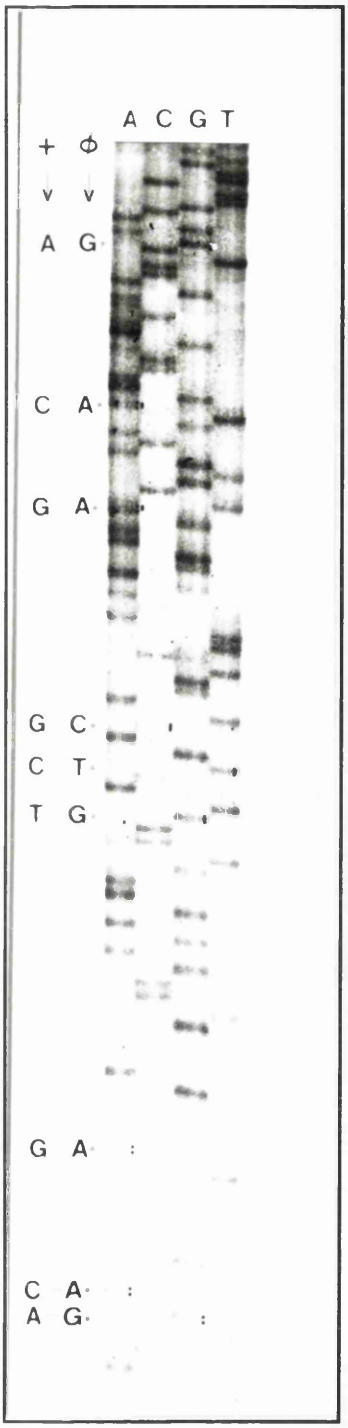


Figure 29: Direct PCR sequence of band ø.

Figure 29 shows the sequence of part of band ø obtained by direct sequencing of PCR product. Nucleotides that do not match with the known sequence of 3ß-HSD type I exon 3 are written in left margin (ø) with the corresponding wild type (+) nucleotide in the far left margin of the sequence.

```
          5674
3ß-HSD I     TCACACAGAA CTCCAGAACA AGACCAAGCT GACAGTGCTG GAAGGAGACA
3ß-HSD II    TCACACAGAG CTCCAGAACA GGACCAAGCT GACTGTACTT GAAGGAGACA
Clone 2(ø)   TCTTGCAGAG CTCCAGAACA AGACCAAGAT GACAGTGCTA GAAGGAGACA
Clone 3      TCACACAGAA CTCCAGAACA AGACCAAGCT GACAGTGCTG GAAGGAGACA
Clone 4      TCTTGCAGAG CTCCAGAACA AGACCAAGAT GACAGTGCTA GGAGGAGACA
ConSensus    TC...CAGA. CTCCAGAACA AGACCAAG.T GACAGTGCT. GaAGGAGACA

          5724
3ß-HSD I     TTCTGGATGA GCCATTCCTG AAGAGAGCCT GCCAGGACGT CTCGGTCATC
3ß-HSD II    TTCTGGATGA GCCATTCCTG AAAAGAGCCT GCCAGGACGT CTCGGTCGTC
Clone 2(ø)   TTCTGGATCA GTCATGCCTG AAGAGAGCCT GCCAGGACAT CTCGGTAGTC
Clone 3      TTCTGGATCA GTCATGCCTG AAGAGAGCCT GCCAGGACAT CTCGGTAGTC
Clone 4      TTCTGGATGA GCCATTCCTG AAGAGAGCCT GCCAGGACGT CTCGGTCGTC
ConSensus    TTCTGGAT.A G.CAT.CCTG AAGAGAGCCT GCCAGGAC.T CTCGGT.gTC

          5774
3ß-HSD I     ATCCACACCG CCTGTATCAT TGATGTCTTC GGTGTCACTC ACAGAGAGTC
3ß-HSD II    ATCCACACCG CCTGTATCAT TGATGTCTTT GGTGTCACTC ACAGAGAGTC
Clone 2(ø)   ATCCACACCG CCTCTATCAT TGACATCTTT GGTGTCACTC ACAGAGAGTC
Clone 3      ATCCACACCG CCTCTATCAT TGACATCTTC GATGTCACTC ACAGAGAGTC
Clone 4      ATCCGCACCG CCTGTATCAT TGATGTCTTC GGTGTCACTC ACAGAGAGTC
ConSensus    ATCCaCACCG CCT.TATCAT TGA..TCTTc GgTGTCACTC ACAGAGAGTC

          5824
3ß-HSD I     TATCATGAAT GTCAATGTGA AAGGTATGGT AGGCTGGGGA GGAGATGCAG
3ß-HSD II    CATCATGAAT GTCAATGTGA AAGGTACAGT AGCCTGGGGA GGAGATAAAA
Clone 2(ø)   TATCATGAAC TTCAACGTGA AAGGTACAGT AGCCTGAGGA GGAGATGCAG
Clone 3      TATCATGAAC TTCAACGTGA AAGGTACAGT AGCCTGAGGT GGAGATGCAG
Clone 4      TATCATGAAT GTCAATGTGA AAGGTATGGT AGGCTGGGGA GGAGATGCAG
ConSensus    TATCATGAA. .TCAA.GTGA AAGGTA..GT AG.CTG.GGa GGAGATGCAG

          5874
3ß-HSD I     CAAGGTG
3ß-HSD II    CAAGTTGG
Clone 2(ø)   CAAGGTGG
Clone 3      CAAGGTGG
Clone 4      CAAGGTG
ConSensus    CAAGGTGG
```

Figure 30: Nucleotide sequence comparison of clones 2-4 (Pfe IV-1: 3ß-HSD type I exon 3)

Figure 30 shows a comparison of clones 2-4 (Pfe IV-1: 3ß-HSD type I exon 3) with the nucleotide sequences of 3ß-HSD types I and II. Clone 1 was found to be identical to the published sequence of 3ß-HSD type I.

```
    codon 50
3ß-HSD I    LQNKTKLTVL EGDILDEPFL KRACQDVSVI IHTACIIDVF GVTHRESIMN
3ß-HSD II   LQNRTKLTVL EGDILDEPFL KRACQDVSVV IHTACIIDVF GVTHRESIMN
Clone 2(ø)  LQNKTKMTVL EGDILDQSCL KRACQDISVV IHTASIIDIF GVTHRESIMN
Clone 3     LQNKTKLTVL EGDILDQSCL KRACQDISVV IHTASIIDIF DVTHRESIMN
Clone 4     LQNKTKMTVL GGDILDEPFL KRACQDVSVV IRTACIIDVF GVTHRESIMN
Consensus   LQNKTK.TVL eGDILD...L KRACQD.SVv IhTA.IID.F gVTHRESIMN

   codon 100
3ß-HSD I    VNVK
3ß-HSD II   VNVK
Clone 2(ø)  FNVK
Clone 3     FNVK
Clone 4     VNVK
Consensus    NVK
```

Figure 31: Protein sequence comparison of clones 2-4

(Pfe IV-1: 3ß-HSD type I exon 3)

Figure 31 shows a comparison of clones 2-4 (Pfe IV-1: 3ß-HSD type I exon 3) with the protein sequence of 3ß-HSD types I and II.

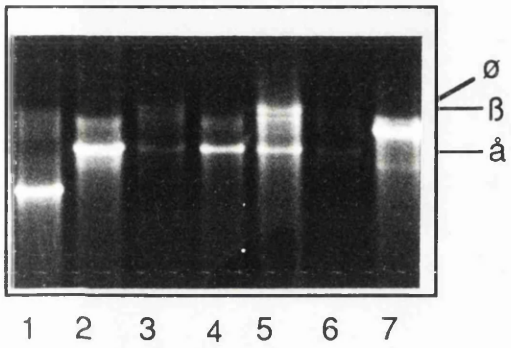


Figure 32: DGGE analysis of clones 1, 3, and 4 (Pfe IV-1: 3ß-HSD type I exon 3)

Tracks 3, 5, and 6 contain bands å, ß, and ø amplified from Pfe IV-1 genomic DNA.

Tracks 2 and 4 contain PCR product amplified from clone 1.

Track 1 contains PCR product amplified from clone 4.

Track 7 contains PCR product amplified from clone 3.

Bands å, ß and ø are identified at the right hand side. The gradient of denaturants was 55-85%. The gel was run at 100mA, 80V for 20 hours.

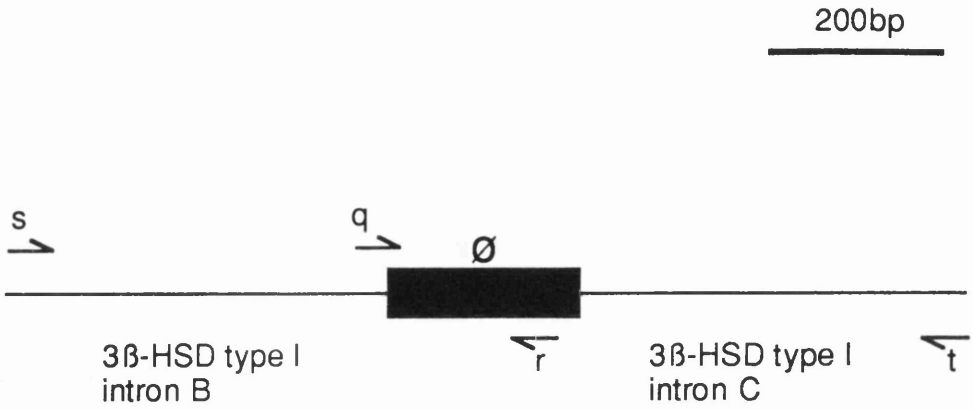


Figure 33: The relative position of primers used in the investigation of band ø.

Primers (q) and (r) are ø specific. Primers (s) and (t) are 3ß-HSD type I specific. Further details of PCR primers and conditions are given in Table 8.

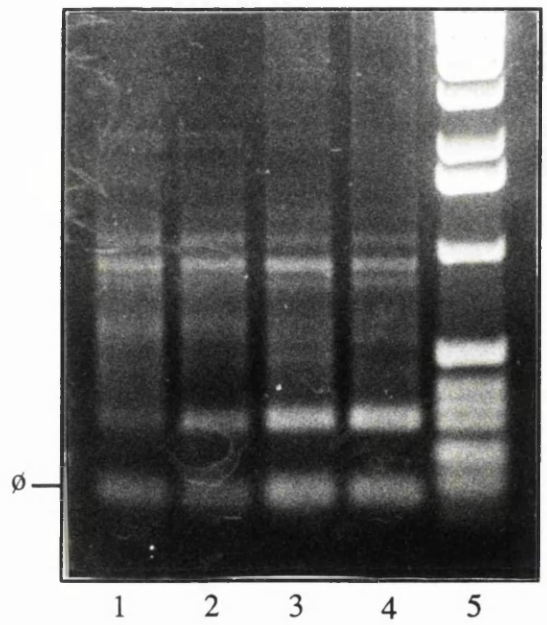


Figure 34: Investigation of band ø.

a) Amplification of the 125bp band ø from 3 unaffected individuals. Samples were chosen randomly from a panel of unrelated individuals. Track 4 contains band ø amplified from Pfe IV-2 as a positive control. Track 5 contains lambda 1kb size marker (Gibco).

DGGE screening of 3ß-HSD type I exon 4

In the following experiments part of the coding sequence of 3ß-HSD type I plus a small amount of flanking non-coding sequence was screened by DGGE. The majority of the 3' untranslated portion of the gene was not screened.

The melt maps of exon 4 indicated that a full length PCR product of 964 bp (primers (e) & (h) Fig 23) would be poorly screened by DGGE (A6 & A7). Therefore it was decided to use a combination of primers and enzyme digestions to screen exon 4 thoroughly (A8-11). The amplification of a 964 bp 5'GC clamped product would have resulted in the inadequate screening of bases 7955 - 8460 by DGGE. The same PCR product when digested with PstI resulted in the screening of bases 8000 - 8420 by DGGE (A8). The use of primers (e) and (g) to amplify a 3' GC clamped product of 549bp resulted in the screening of bases 7955 - 8420 by DGGE (A9). An attempt was made to screen bases 8400 - 8881 by DGGE using PCR primers (f) and (h) however this proved to be unsuccessful. In the following results nucleotides 7955 - 8466 are referred to as exon 4A. An overlapping fragment of nucleotides 8400 - 8881 is referred to as exon 4B.

Results

When the 5' GC clamped product of exon 4 was digested with PstI, all samples (Pfe, Ran, and Sta) produced one band which migrated to the same depth in gel as controls. Similarly, when either primers (e) and (g) (exon 4A 3'GC), or (f) and (h) (exon 4B 5'GC) were used, all samples (Pfe, Ran, and Sta) produced one band which migrated to the same depth in gel as controls.

Only when exon 4B was amplified with a 3'GC clamp was any difference in migration observed on denaturing gels (Fig 35). The Ran sample (Ran III-3) produced one band (A) which migrated to the same depth in gels as controls.
The Sta sample (Sta III-1) produced one band (A) which migrated to the same depth in gels as controls, plus an extra band (B) which resolved at a higher level in the gel i.e. a lower Tm. When other available members of the Sta family were run on a similar gel, samples Sta III-2 and Sta II-3 produced an identical pattern to Sta III-1. Sta II-2 produced one band (A) which migrated to the same depth in gels as controls. The PCR product of Sta III-1 was cloned into pT7 Blue T-vector and putative clones identified by blue white selection. The presence of insert in these clones was confirmed by PCR. PCR product from positive clones was run on DGGE alongside Sta control samples. Clones which co-migrated with bands A and B were selected for sequencing. PCR products which did not co-migrate with bands A or B were also observed.

The Pfe sample (Pfe III-2) produced a single band (C) which migrated to a lower depth in gel than controls i.e. a higher Tm. When other available members of the Pfe family were run on a similar gel, both Pfe IV-1 and Pfe IV-2 produced the same band as Pfe III-2 plus a second band (A) which migrated to the same depth in gel as controls. The PCR product of Pfe III-2 was cloned into pT7 Blue T-vector and putative clones identified by blue white selection. The presence of insert in these clones was confirmed by PCR. PCR product from positive clones was run on DGGE alongside Pfe control samples and clones which co-migrated with bands A and C were selected for sequencing . PCR products which did not co-migrate with bands A or C were also observed.

Sequencing of clones

The sequence obtained from clones is shown in figure 36. All of the clones contained numerous sequence variations when compared to 3ß-HSD type I exon 4. The corresponding protein sequence of each clone shows that stop codons were present in all cases (Fig 37).

Discussion: type I exon 4

No sequence variation in exon 4A of 3ß-HSD type I was found in any of the families but analysis of exon 4B gave an unexpected result. It appears that the primers used amplified a different 3ß-HSD sequence to the one expected. The DGGE results suggested that Sta III-1, III-2, and II-3 possessed one normal allele (band A) and one mutant allele (band B). Pfe IV-1 and IV2 appeared to possess one normal allele (band A) and one mutant allele (band C), and Pfe III-2 appeared to possess two mutant alleles (both band C). The Ran sample (Ran III-3) and the control samples only produced band A.

Sequencing revealed that none of the bands observed on DGGE were 3ß-HSD type I exon 4. All clones examined were homologous to 3ß-HSD type I however each had a series of point mutations present. Some of these point mutations would have resulted in the introduction of stop codons into the the coding sequence of 3ß-HSD. It is probable that these sequences were amplified from pseudogenes. The only bands to appear on DGGE were the bands which were cloned and none of these bands was the expected 3ß-HSD exon 4. There is no easy explanation as to why this happened as even if pseudogenes were amplified 3ß-HSD exon 4 should also have been amplified.

The 3' half of exon 4 (nt 8460 - 8881) has already been screened for each of the families during the analysis of the non-synonymous polymorphism at codon 367 (see

pg47). This analysis involved the screening of a full length exon 4 PCR product amplified with a 5'GC clamp. Although no mutations were detected in any of the families it would have been preferable to screen this region of exon 4 using a shorter PCR product.

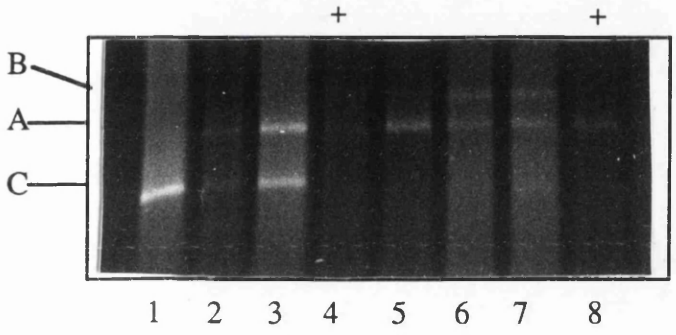


Figure 35: DGGE gel showing the migration of human 3ß-HSD type I exon 4B.

(+) = control patient. Tracks 1-8 contain samples Pfe III-2, Pfe IV-1, Pfe IV-2, (+), Ran III-3, Sta III-1, Sta III-2, and (+) respectively. The gradient of denaturants was 40-65%. The gel was run at 100mA, 80V for 20 hours. Bands A, B, and C are identified at the left hand side.

```
          8450
3ß-HSDI       CCGAGGACAG TTCTACTATA TCTCAGATGA CACGCCTCAC CAAAGCTATG
3ß-HSDII      CCGAGGTCAA TTCTATTACA TCTCAGATGA CACGCCTCAC CAAAGCTATG
Band A        CCTAGGACAG TTCTACTATA TCTCAGATGA CACGCCTCAC CAAAGTTATG
Band B        CCTAGGACAG TTCTACTATA TCTCAGATGA CACGCCTCAC CAAAGTTATG
Band C                                TCAGATGA CACGCCTCAC CAAAGTTATG
ConSensus     CC.AGGaCAg TTCTAcTAtA TCTCAGATGA CACGCCTCAC CAAAGtTATG

          8500
3ß-HSDI       ATAACCTTAA TTACACCCTG AGCAAAGAGT TCGGCCTCCG CCTTGATTCC
3ß-HSDII      ATAACCTTAA TTACATCCTG AGCAAAGAGT TTGGCCTCCG CCTTGATTCC
Band A        ATAACCTTAA TTACATCTTG AGCAAAGAGT TCGGCCCCTG CCTTGATTCC
Band B        ATAACCTTAA TTACATCCTG AGCAAAGAGT TCGGCTCCTG CCTTGATTCC
Band C        ATAACCTTAA TTACATCCTG AGCAAAGAGT TCGGCCCCTG CCTTGATTCC
ConSensus     ATAACCTTAA TTACAtCcTG AGCAAAGAGT TcGGCccCtG CCTTGATTCC

          8550
3ß-HSDI       AGATGGAGCT TTCCTTTATC CCTGATGTAT TGGATTGGCT TCCTGCTGGA
3ß-HSDII      AGATGGAGCC TTCCTTTAAC CCTGATGTAC TGGATTGGCT TCCTGCTGGA
Band A        AGATGAAGCC TTCTTTTATT CCTGATGTAC
Band B        AGATGAAGCC TTCTTTTATT CCTGATGTAC TGGATTGGCT TCCTGCTGGG
Band C        AGATGAAGCC TTCTTTTATT CCTGATGTAC TGGATTGGCT TCCTGCTGGG
ConSensus     AGATGaAGCc TTCtTTTAtt CCTGATGTAc TGGATTGGCT TCCTGCTGG.



          8697
3ß-HSDI       AAGAAGGCTC AGCGAGATTT GGCGTATAAG CCACTCTACA GCTGGGAGGA
3ß-HSDII      AAGAAGGCTC AGCGAGATCT GGCGTATAAG CCACTCTACA GCTGGGAGGA
Band A        AAGAAGGCTC AGCGAGATCT GGCATATAAG CCACTTTACA GCTGGGAGGA
Band B        AAGAAGGCTC AGCGAGATCT GGCATATAAG CCACTTTACA GCTGGGAGGA
Band C        AAGAAGGCTC AGCGAGATCT GGCATATAAG CCACTTTACA GCTGGGAGGA
ConSensus     AAGAAGGCTC AGCGAGATcT GGCaTATAAG CCACTtTACA GCTGGGAGGA

          8747
3ß-HSDI       AGCCAAGCAG AAAACGGTGG AGTGGGTTGG TTCCCTTGTG GACCGGCACA
3ß-HSDII      AGCCAAGCAG AAAACCGTGG AGTGGGTTGG TTCCCTTGTG GACCGGCACA
Band A        AGCCAAGCAG AAAACCATGG AGTGAGTTGG TTCCCTTGTG GACCGGCACA
Band B        AGCCAAGCAG AAAACCATGG AGTGAGTTGG TTCCCTTGTG GACCGGCACA
Band C        AGCCAAGCAG AAAACCATGG AGTGAGTTGG TTCCCTTGTG GACCGGCACA
ConSensus     AGCCAAGCAG AAAACcaTGG AGTGaGTTGG TTCCCTTGTG GACCGGCACA

          8797
3ß-HSDI       AGGAGAACCT GAAGTCCAAG ACTCAGTGAT TTAAGGATGA CAGAGATGTG
3ß-HSDII      AGGAGACCCT GAAGTCCAAG ACTCAGTGAT TTAAGGATGA CAGAGATGTG
Band A        AGGAGAACCT GAAGTCCAAG ACTCAGTGCT TTAAGGATGA CAGAGATGTG
Band B        AGGAAAACCT GAAGTCCAAG ACTCAGTGCT TTAAGGATGA CAGAGATGTG
Band C        AGGAGAACCT GAAGTCCAAG ACTCAGTGCT TTAAGGATGA CAGAGATGTG
ConSensus     AGGAgAaCCT GAAGTCCAAG ACTCAGTGcT TTAAGGATGA CAGAGATGTG
```

Figure 36: Nucleotide sequence of clones (3ß-HSD type I exon 4B)

Figure 36 shows the nucleotide sequence of clones detected during the analysis of 3ß-HSD type I exon 4B by DGGE. When compared to 3ß-HSD type I exon 4, all clones contained numerous sequence variations.

```
     codon 250
3ß-HSDI       RGQFYYISDD TPHQSYDNLN YTLSKEFGLR LDSRWSFPLS LMYWIGFLLE
3ß-HSDII      RGQFYYISDD TPHQSYDNLN YILSKEFGLR LDSRWSLPLT LMYWIGFLLE
Band A        LGQFYYISDD TPHQSYDNLN YILSKEFGPC LDSR*SLLLF LMY
Band B        LGQFYYISDD TPHQSYDNLN YILSKEFGSC LDSR*SLLLF LMYWIGFLL
Band C              SDD TPHQSYDNLN YILSKEFGPC LDSR*SLVLF LMYWIGFLLG
Consensus     .GQFYYISDD TPHQSYDNLN YiLSKEFG.c LDSR*Sl.Lf LMYWIGFLLe

     codon 332
3ß-HSDI       KKAQRDLAYK PLYSWEEAKQ KTVEWVGSLV DRHKENLKSK TQ*FKDDRDV
3ß-HSDII      KKAQRDLAYK PLYSWEEAKQ KTVEWVGSLV DRHKETLKSK TQ*FKDDRDV
Band A        KKAQRDLAYK PLYSWEEAKQ KTME*VGSLV DRHKENLKSK TQCFKDDRDV
Band B        KKAQRDLAYK PLYSWEEAKQ KTME*VGSLV DRHKENLKSK TQCFKDDRDV
Band C        KKAQRDLAYK PLYSWEEAKQ KTME*VGSLV DRHKENLKSK TQCFKDDRDV
Consensus     KKAQRDLAYK PLYSWEEAKQ KTME*VGSLV DRHKENLKSK TQCFKDDRDV
```

Figure 37: Protein sequence of clones (3ß-HSD type I exon 4B)

Figure 37 shows the protein sequence of clones detected during the analysis of 3ß-HSD type I exon 4B by DGGE. Stop codons were present in all clones.

DGGE screening of 3ß-HSD type II exon 1/2

Exons 1 and 2 extend over only 416bp and were therefore co-amplified with intron A as a single segment (nt 1219 - 1720). Using primers (i) and (j) a 5'GC clamped product was amplified. When run on DGGE this resulted in the screening of bases 1250 - 1480 and 1590 - 1720 (A12). The 3'GC clamped product resulted in the screening of bases 1219 - 1430 (A13).

In both cases all samples, Pfe, Ran, and Sta produced one band which migrated to the same depth in the gel as controls.

DGGE screening of 3ß-HSD type II exon 3

The melting profiles of type II exon 3 (A14 & A15) indicated a single melting domain from nt 5488 - 5714. Screening first with a 5'GC clamp then with a 3'GC clamp should have been adequate for detecting >99% of all possible mutations.

When amplified with a 5'GC clamp, samples Pfe, Ran, and controls produced a single band (A). The Sta sample (Sta III-1) produced the same band (A) plus three extra bands, band B which migrated to a lower depth in the gel i.e. a higher Tm than controls, and bands C and D which appeared as a doublet resolving at a higher level in the gel i.e. a lower Tm than controls (Fig 38).

Samples amplified with a 3'GC clamp produced a similar result. This experiment run on the same gel showed the 3'GC clamped products resolving at a lower point in the gel than the 5' GC clamped products. Once again Pfe and Ran samples migrated to the same depth in gel as controls. Sta III-1 produced the same four bands as before only this time the separation of bands C and D was more pronounced.

Screening of family Sta

When run on a similar gel 5'GC clamped samples of Sta III-2 and Sta II-2 produced the same four bands as Sta III-1. Sta I-1, II-1, II-3, II-4, and II-5 all produced a single band which migrated to the same depth in gel as band A.

PCR product from Sta III-1 was cloned into pT7 Blue T-vector and putative clones identified by blue/white selection. The presence of insert in these clones was confirmed by PCR. Clones which corresponded to bands A and B on DGGE (Fig 39) were selected for sequencing (clones 8 and 10, and 9 and 11 respectively). Other PCR products which did not correspond to either band A or B were observed. No clones were found that corresponded to bands C or D.

Sequencing of clones (Fig 40)

Clone 8 (corresponding to band A) was found to agree exactly with the published sequence of 3ß-HSD type II exon 3.

Clone 10 (corresponding to band A) was found to have two substitutions, an A→ G at base 5524 and a T→ C at base 5575.

Clone 9 (corresponding to band B) was found to have a single substitution, an A→ G at base 5671.

Clone 11 (corresponding to band B) was found to have two substitutions, an A→ G at base 5671 i.e. the same substitution found in clone 9, and an A→ G at base 5556.

The obvious discrepencies between clones necessitated the further analysis of each clone by DGGE. The selection of clones had been based on DGGE analysis using 5' GC clamped products. It was decided to further analyse clones 8, 10, 9, and 11 using a 3' GC clamp (Fig 41). The 5' GC clamped samples showed the same pattern as before i.e. clones 8 and 10 co-migrated with band A, and clones 9 and 11 co-migrated with band B. When tested with a 3' GC clamp only clone 8 co-migrated with band A. Clone 10 3' GC resolved at a much lower point in the gel. Only clone 9 co-migrated with band B as before. Clone 11 resolved at a much lower point in the gel.

Discussion: type II exon 3

The screening of type II exon 3 by DGGE showed no differences in Pfe and Ran samples when compared to controls. In family Sta both affected sons (Sta III-1 and III-2) and their mother (Sta II-2) produced four bands, A, B, C, and D. Two clones each of bands A and B were sequenced giving a different result in each case. The

clones had originally been selected for sequencing on the basis of DGGE analysis using 5'GC clamped products, however, further DGGE analysis using 3'GC clamped products revealed that clones 10 and 11 were the product of Taq polymerase errors. Only clones 8 and 9 co-migrated with bands A and B using both 5' and 3'GC clamped products and were therefore the only two legitimate clones.

Every sample tested produced band A (clone 8) which has been confirmed as wild type 3ß-HSD type II. Both affected sons and their mother had a second allele (clone 9) containing an A→ G point mutation at base 5671 (Fig 40). This point mutation resulted in the substitution of an Asn for a Ser at codon 100. The substitution at base 5671 has not been detected in over 30 genes selected from the normal population. It is not known at present what effect this amino acid substitution will have on the activity of the enzyme.

No clones of bands C or D were observed (Fig 39). Bands C and D were most probably heteroduplexes formed by the melting and reannealing of bands A and B during the PCR amplification. Although these heteroduplexes were probably successfully cloned into pT7 Blue T-vector, subsequent transformation and amplification in the host *Escherichia coli* may have resulted in the repair of the mismatch by the host *E. colis* repair mechanisms. This experiment emphasised the problems which may be encountered when sequencing cloned PCR products. Four separate clones of 227 bp produced four different results indicating that the error rate of Promega Taq polymerase may be much higher than expected.

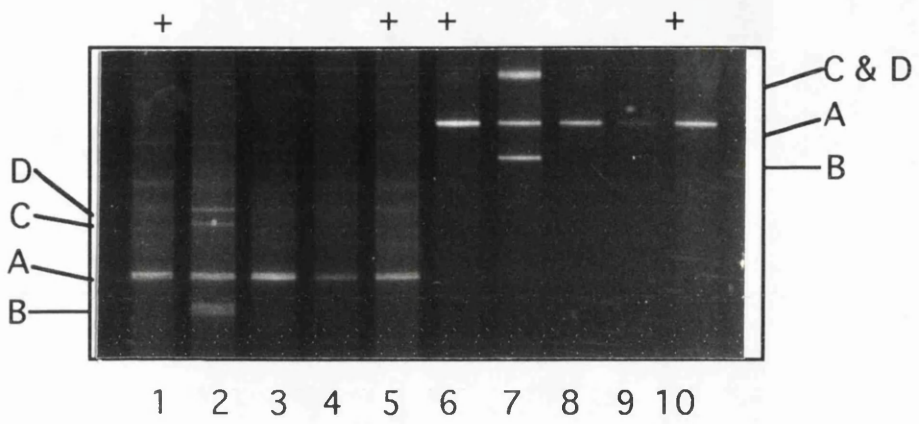


Figure 38: DGGE gel showing the migration of human 3ß-HSD type II exon 3.

(+) = control patient.

Tracks 1-5 contain samples amplified with a 3'GC clamp.

Tracks 6-10 contain samples amplified with a 5'GC clamp.

Tracks 2 & 7 = Sta III-1

Tracks 3 & 8 = Pfe IV-2

Tracks 4 & 9 = Ran III-3

Bands A, B, C, and D are identified at each side of the gel. The gradient of denaturants was 35-65%. The gel was run at 100mA, 80V for 20 hours.

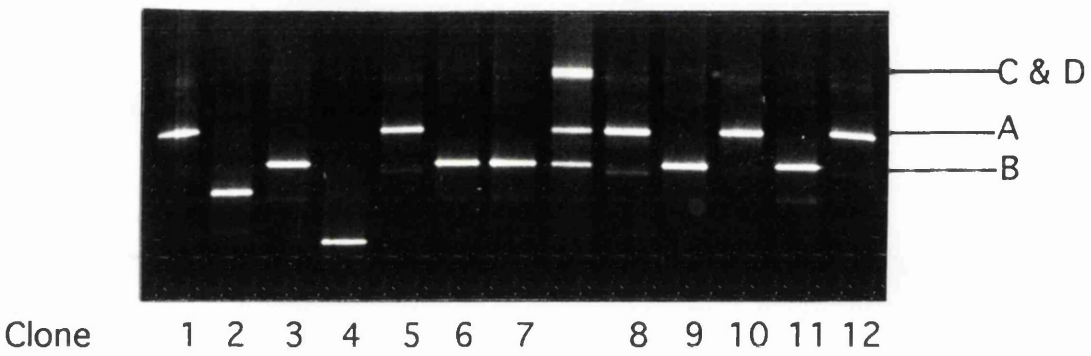


Figure 39: Selection of positive clones by DGGE (Sta III-1: 3β-HSD type II exon 3)

All samples were amplified using a 5'GC clamp. Track 8 contains Sta III-1 exon 3 as a positive control. Bands A, B, C, and D are identified at right hand side. The gradient of denaturants was 35-65%. The gel was run at 100mA, 80V for 20 hours.

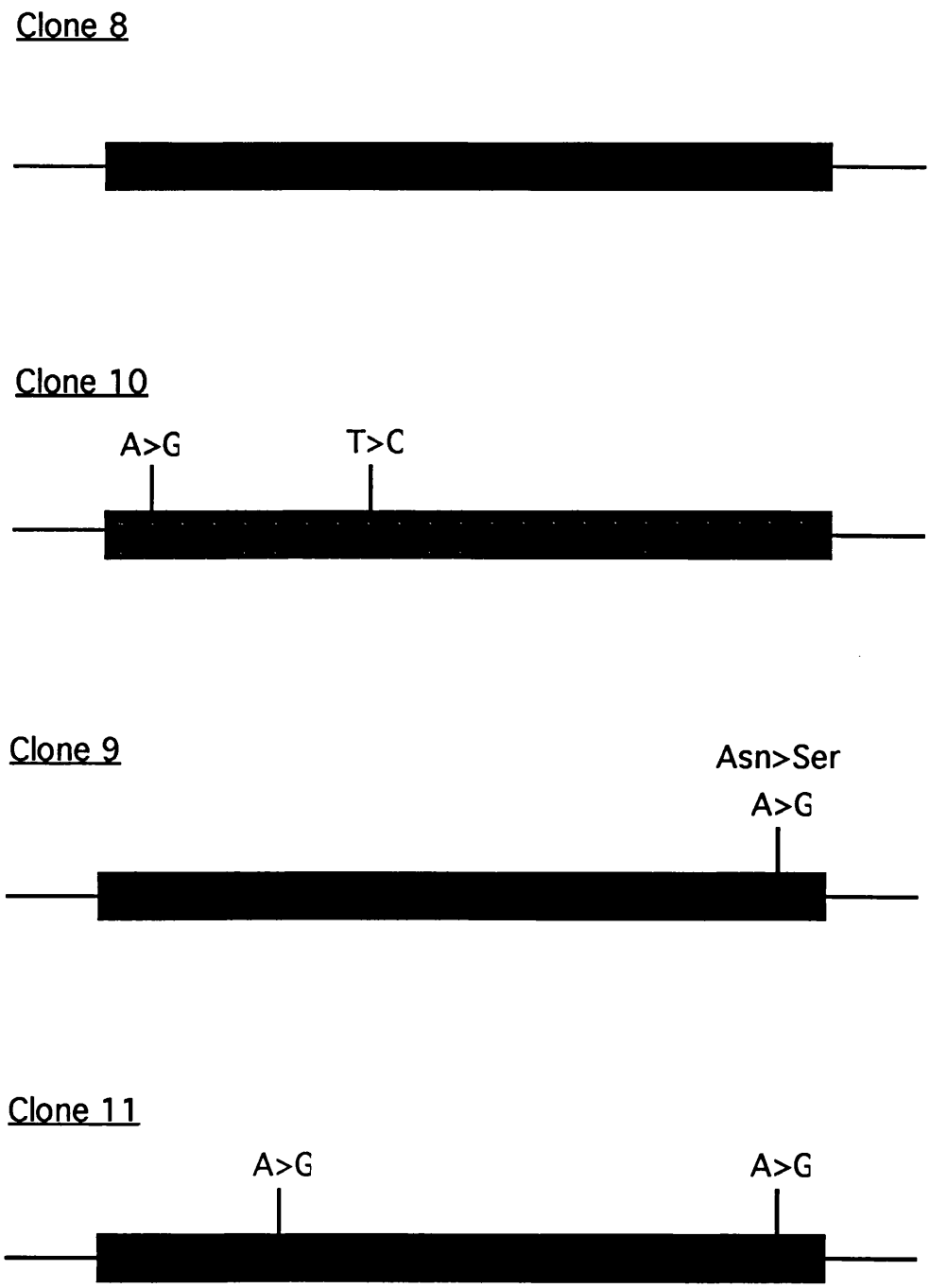


Figure 40: Clones 8, 10, 9, and 11(Sta III-1: 3ß-HSD type II exon 3)

Point mutations present in each clone are highlighted.

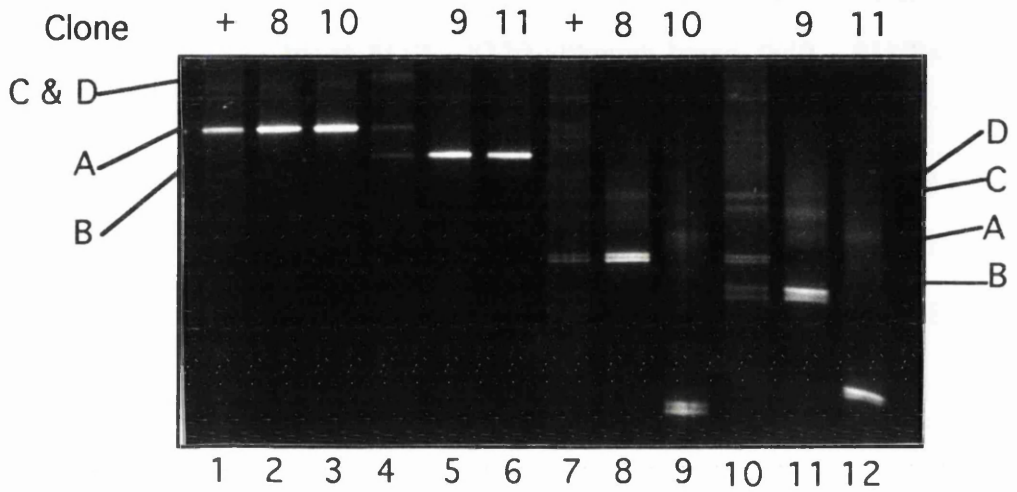


Figure 41: Comparison of clones using both 5' and 3' GC clamps (Sta III-1: 3ß-HSD type II exon 3).

(+) = control patient.

Tracks 1-6 contain samples amplified with a 5'GC clamp. Tracks 7-12 contain samples amplified with a 3'GC clamp. Tracks 4 and 10 contain Sta III-1 exon 3 as a positive control. Bands A, B, C, and D are identified at each side of the gel. The gradient of denaturants was 35-65%. The gel was run at 100mA, 80V for 20 hours.

DGGE screening of 3ß-HSD type II exon 4

In the following experiments a large portion of the coding sequence of exon 4 and a small amount of flanking non-coding sequence was screened by DGGE. The majority of the 3' untranslated sequence was not screened. According to the melt maps (A16-19), the use of primers (m) and (o) to amplify a 5'GC clamped fragment would result in the screening of bases 8110 - 8270 although bases 7849 - 8110 would remain unscreened (A16). The same PCR product when amplified with a 3'GC clamp would result in the screening of bases 7849 - 8270 (A17). By using primers (n) and (p) bases 8181 - 8775 were comprehensively screened (A18 & 19). In the following results nucleotides 7849 - 8270 are referred to as exon 4A. An overlapping fragment of nucleotides 8181 - 8775 is referred to as exon 4B.

Results

Using primers (n) and (p) no sequence variations were detected in any of the samples.

When exon 4A was amplified with a 5'GC clamp (primers (m) and (o)), Sta and Pfe samples produced a single band which migrated to the same depth in gel as controls. The Ran III-3 band, however, migrated to a slightly lower depth in the gel than controls (Fig 42). Screening of Ran III-4, Ran II-7, and Ran II-8 revealed that both parents (Ran II-7 and Ran II-8) produced two bands, one band which migrated to the same depth in gel as controls (band A) and one band which resolved at a slightly lower depth in the gel (band B). Both sibs, Ran III-3 and Ran III-4, only produced band B (Fig 43).

The remaining PCR products of Ran III-3, III-4, II-7, and II-8 were cloned separately into pT7 Blue T-vector and putative clones identified by blue/white selection. The presence of insert in these clones was confirmed by PCR. PCR products from positive

clones were run on DGGE alongside Ran controls. In the case of Ran II-7 and Ran II-8, clones which co-migrated with bands A and B were selected for sequencing. For Ran III-3 and Ran III-4, clones which co-migrated with band B were selected. Clones which did not co-migrate with the control bands were disregarded.

Sequencing of clones

All clones corresponding to band A showed no sequence variation when compared to the published sequence of 3ß-HSD type II exon 4.

All clones corresponding to band B revealed a single point mutation T → G at base 8118. This point mutation would lead to the substitution of Arg for the wild type Leu at codon 173 (Fig 44). No other sequence variations were found in these clones.

Screening of extended Ran family

All available members of the Ran family were screened for the point mutation at base 8118. Samples Ran I-4, II-1, II-6, II-7, and II-8, were all found to possess both bands A and B i.e. one normal allele and one mutant allele. Samples Ran I-2, I-3, II-2, II-3, II-4, II-5, II-9, II-10, II-11, II-12, II-13, III-1, III-2, and III-5 only produced the normal allele, band A. Samples Ran III-3 and Ran III-4 only produced band B, the mutant allele.

The mutation at base 8118 has not been detected in over 40 genes selected from the normal population.

Discussion: type II exon 4

No sequence variations were found in Sta and Pfe samples of 3ß-HSD type II exon 4.

A single point mutation T→ G at base 8118 was found in several members of the Ran family. The effect of the mutation on the melting properties of the PCR fragment was quite small. This was expected since the substitution occurs at a transition point between two melting domains in the PCR product (A16). Nevertheless, the effect of the mutation was sufficient to distinguish both homozygotes and heterozygotes.

The point mutation was found to be present on both sides of the family, originating from Ran I-1 and Ran I-4 (Fig 45). This raises the possibility of consanguinity at an earlier generation.

The T→ G substitution at base 8118 would lead to the substitution of Arg for the wild type Leu at codon 173. As Arg is a hydrophilic, basic amino acid, and Leu is a hydrophobic, non-polar amino acid it is probable that this substitution would have a marked effect on the activity of the enzyme. Our hypothesis is that this mutation is the underlying cause of the 3ß-HSD deficiency in this family.

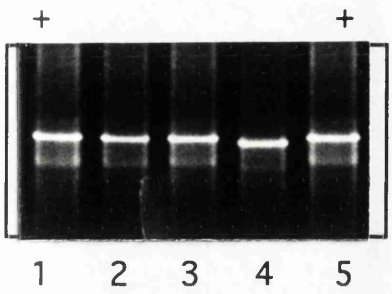


Figure 42: DGGE gel showing the migration of human 3ß-HSD type II exon 4A.

(+) = control patient. Tracks 2-4 contain samples Pfe IV-2, Sta III-1, and Ran III-3 respectively. Sample Ran III-3 migrated to a slightly lower point in the gel. The gradient of denaturants was 45-75%. The gel was run at 100mA, 80V for 20 hours.

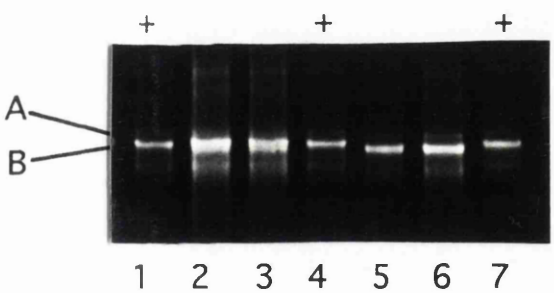


Figure 43: Analysis of family Ran 3ß-HSD type II exon 4A by DGGE.

(+) = control patient. Tracks 2 and 3 contain samples Ran II-7 and Ran II-8 respectively. Tracks 5 and 6 contain samples Ran III-3 and Ran III-4 respectively. Bands A and B are identified at the left hand side of the gel. The gradient of denaturants was 45-75%. The gel was run at 100mA, 80V for 20 hours.

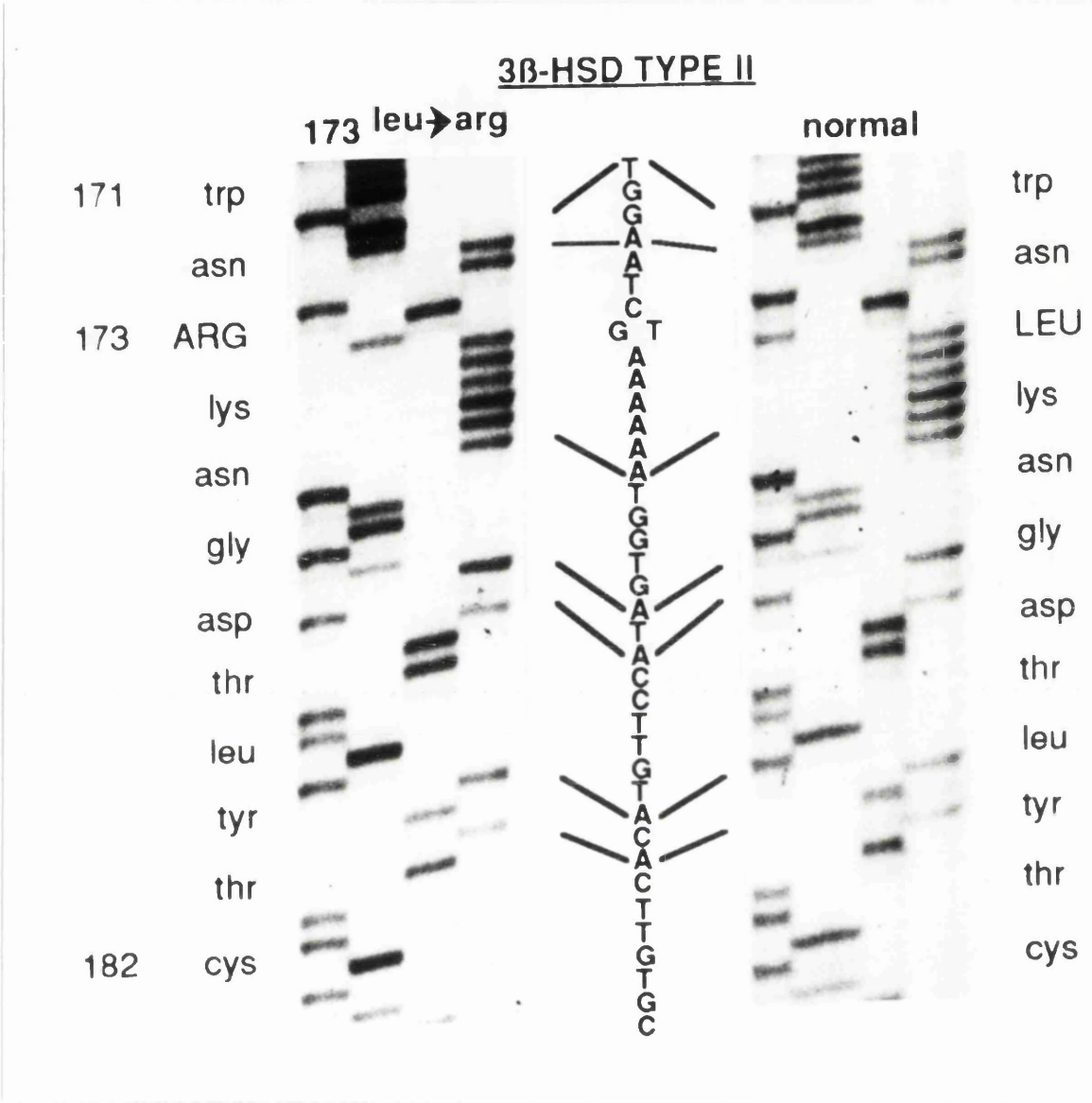


Figure 44: Non-synonymous substitution at codon 173 3ß-HSD type II.

Figure 44 shows sequence data outlining the codon substitution at residue 173. The substitution of a G for a T at base 8118 results in codon 173 changing from Leu to Arg.

Automated sequencing of PCR products

To ensure the detection of all possible mutations in the 3ß-HSD type II gene, automated sequencing of PCR products was carried out on affected members of all three families (Ran, Sta, and Pfe). Both the sense and anti-sense strands of each PCR product was sequenced. No evidence of mutation was found in any of the patients (data not shown).

DISCUSSION

Family Ran: A summary of clinical, biochemical, and genetic information on family Ran is given in Fig 45 and table 9. When typed for the two genetic polymorphisms in exon 4 of the type I gene (pg47) and the microsatellite in intron C of the type II gene, the sibs (Ran III-3 and Ran III-4) were found to have inherited the same polymorphic alleles from their parents.

Using a combination of DGGE and direct sequencing of PCR products, no evidence of mutation could be found in the type I gene, however, a mutation was detected in the proximal segment of exon 4 of the type II gene. Sequencing showed the mutation to be a single missense mutation of CTA > CGA in codon 173, leading to the substitution in the affected patient of arginine for the normal leucine. No other sequence variations were found in 3ß-HSD type II. The 173 Arg codon is not found in the gene for 3ß HSD type I and cannot therefore be the product of a gene conversion event between the type I and the type II genes. Figure 45 shows that the polymorphic markers in 3ß HSD type I and type II co-segregate, together with the mutation in codon 173 of the type II gene. No recombination events were observed between these markers in family Ran.

Insufficient is known at present of the protein structure to predict what effect this substitution may have on the activity of the enzyme, however as Arg is a positively charged hydrophilic amino acid and leu is a strongly hydrophobic amino acid it is probable that the substitution would have an effect on enzyme activity. Neither of the sibs homozygous for the codon 173 substitution show disturbances of mineralocorticoid or glucocorticoid metabolism, indicating that the main effect of the substitution is to reduce the conversion of C19 substrates to androstenedione and testosterone, leaving substantially unaffected the conversion of C21 substrates to progesterone and 17 alpha hydroxyprogesterone. The type II codon 173 mutation is

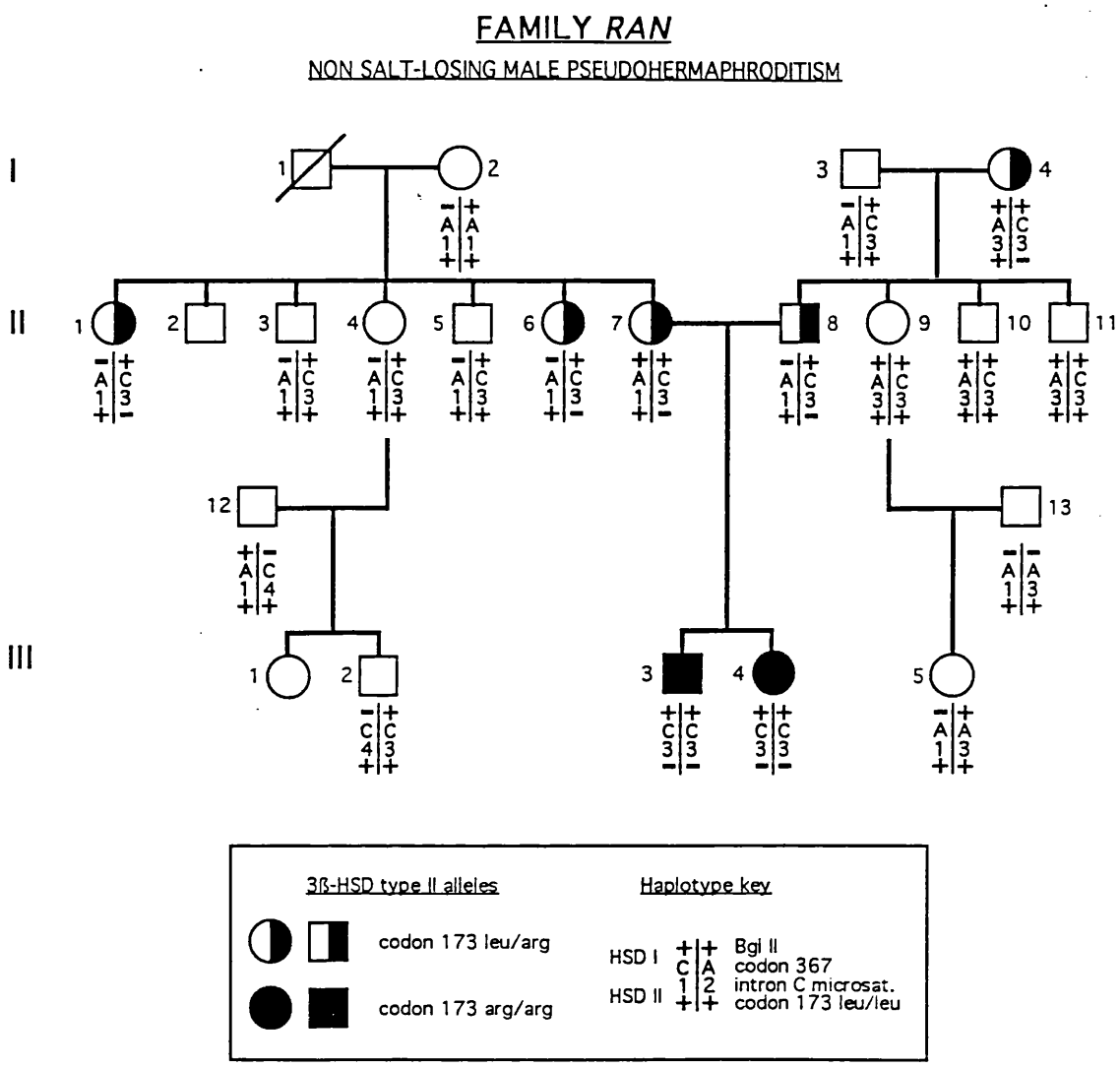

FAMILY RAN
NON SALT-LOSING MALE PSEUDOHERMAPHRODITISM
I
II
III
3ß-HSD type II alleles
codon 173 leu/arg
codon 173 arg/arg
Haplotype key
HSD I
HSD II
Bgl II
codon 367
intron C microsat.
codon 173 leu/leu

Figure 45

Table 9

ID No	CLINICAL	BIOCHEMISTRY UNDER BASAL CONDITIONS										GENETICS
		Urinary steroid metabolites					Serum steroids					
		DHA	16-OH DHA	Preg-3	A-3	P-3	OHP	A-4	T	17OH Preg	DHA	DHAS	codon 173 leu>arg
III-3	PROPOSITUS: Male pseudohermaphrodite (XY). Reared as a girl. Presented at age 5 with ambiguous genitalia.	H	H	H	H	H	n	n	n	H	H	H	173 arg/arg
III-4	SISTER OF PROPOSITUS: Female aged 2 years (XX karyotype). Phenotypically normal.	H	H	H	H	H	n	H	n	H	H	H	173 arg/arg
II-1	Female aged 38 years. History of menstrual irregularities. Mother to 3 children.	-	-	-	-	-	n	n	n	-	-	n	173 arg/leu
II-2	Male aged 36 years. Father to 3 children No further information available.	-	-	-	-	-	-	-	-	-	-	-	-
II-3	Male aged 34 years. Father to one daughter. No significant clinical abnormality.	H	n	?H	n	n	n	n	-	-	-	n	173 leu/leu
II-4	Female aged 33 years. Mother to 2 children.	n	n	n	n	n	n	n	-	-	-	n	173 leu/leu
II-5	Male aged 28 years. Unmarried. No relevant clinical history.	-	-	-	-	-	n	n	-	-	n	n	173 leu/leu
II-6	Female aged 22 years. Not virilised or hirsute. Overweight.	H	H	?H	n	n	n	H	-	-	H	n	173 arg/leu
II-7	MOTHER OF PROPOSITUS: 29 years. ;not virilised/hirsute. Delayed menarche, oligomenorrhea. Early menopause. Conceived younger daughter while on Clomid	H	n	?H	H	H	n	n	n	n	n	n	173 arg/leu
II-8	FATHER OF PROPOSITUS: Male aged 30 years. No relevant clinical details.	n	n	?H	n	n	H	H	-	H	H	H	173 arg/leu
II-9	Female aged 36 years. No relevant clinical history. One daughter.	n	n	n	n	n	n	n	-	-	n	n	173 leu/leu
II-10	Male aged 34 years. No relevant clinical details.	n	n	?H	n	n	n	n	-	-	n	n	173 leu/leu
II-11	Male aged 28 years. No relevant clinical details.	n	n	n	n	n	n	n	-	-	n	n	173 leu/leu
I-2	Female aged 68 years. No relevant clinical details.	n	n	n	n	n	n	n	-	-	n	n	173 leu/leu
I-3	Male aged 65 years. No relevant clinical details.	n	n	n	n	n	n	n	-	-	n	n	173 leu/leu
I-4	Female aged 64 years.	n	n	n	n	n	n	n	-	-	n	n	173 arg/leu

therefore most likely to result in a partial, substrate specific loss of enzyme activity. Whether the kinetic effects of the 173Leu→Arg mutation are limited to Δ5 substrates that are precursors to the sex steroid pathway, or if it also exerts an effect on mineralocorticoid or glucocorticoid synthesis, should emerge from work in progress on the expression of the allele *in vitro*.

It is clear that homozygosity of the mutant allele results in the grossly abnormal steroid profile exhibited by Ran III-3 and Ran III-4, however biochemical and clinical data suggest that the codon 173 mutation may also exert an affect on members of the family heterozygous for the substitution. The mother (Ran II-7) and father (Ran II-8) of the affected sibs exhibited abnormal levels of 3ß-HSD metabolites suggestive of 3ß-HSD deficiency. In addition, Ran II-7 exhibited particularly infrequent and irregular periods, on average 1-2 periods a year only lasting 2-7 days. Although Ran III-3 was conceived spontaneously, Ran III-4 was conceived with the help of clomiphene therapy. Ran II-7 has recently been diagnosed as menopausal at the age of 31 years and is currently on hormone replacement therapy. Steroid analysis of Ran II-1 showed no abnormality in the levels of Δ5 steroids, however, she had previously undergone a radical hysterectomy and was effectively post-menopausal. Prior to the hysterectomy she had exhibited menstrual irregularities similar to Ran II-7. Ran II-6 has no history of menstrual irregularities though steroid analysis revealed levels of Δ5 steroids indicative of a 3ß-HSD deficiency. The paternal grandmother (Ran I-4) of the affected sibs was post menopausal and had reduced adrenal function, therefore no conclusions about her status could be drawn from steroid analysis. Ran I-4 had no history of menstrual irregularities or fertility problems. The elevated levels in serum of the Δ4 steroids androstenedione in the mother (Ran II-7) and of progesterone, 17-hydroxyprogesterone and androstenedione in the propositus (Ran III-3), her sister (Ran III-4) and their father (Ran II-8) have been reported in other cases of biochemically-diagnosed 3ß-HSD deficiency. It is beleived that the increased levels

of Δ5 steroids entering the blood from the adrenals and ovaries leads to greater conversion of Δ5 to Δ4 steroids through the activity of 3ß-HSD in peripheral tissues.

As 3ß-HSD is a relatively rare condition, the identification of the same mutation originating from both sides of the family was an unexpected result. Family histories over 4 generations revealed no evidence of consanguinity.

Family Pfe: Screening of the 3ß-HSD type I and type II genes by DGGE and direct sequencing of PCR products showed no evidence of mutation in the coding sequence of either gene. The only point mutation observed was present in the non-coding exon 1 of 3ß-HSD type I. When this mutation was used in conjunction with the microsatellite in intron C of 3ß-HSD type II it was conclusively shown that the disease phenotype did not co-segregate with either the 3ß-HSD type I or type II genes (Fig 46).

Since the diagnosis of 3ß-HSD deficiency has been based on clinical and biochemical criteria, without access to tissue samples, it is possible that the hirsutism in family Pfe may be the result of mutations in an as yet undiscovered 3ß-HSD. 3ß-HSD enzyme activity is expressed in other mammalian tissues and distinct genes have been cloned from rat and mouse (Zhao et al. 1990; Bain et al. 1991; Zhao et al. 1991) that are expressed in liver and are members of the 3ß-HSD family. In the human, similar additional isoforms of the enzyme have yet to be defined. Southern blots of human genomic DNA (Fig 13 and Lachance et al. 1990) and DGGE (Figs 30 & 36) have provided evidence for the existence of several isoforms of 3ß-HSD in humans however the chromosomal location of these sequences is not yet known. SI protection studies on RNA expression (Rheaume et al. 1991) and the expression of 3ß-HSD epitopes detected with monoclonal antibodies (Nickson et al. 1990) have shown that the type I and type II enzymes are not significantly expressed in tissues other than trophoblast, adrenals, gonads, and skin. The existence of additional isoforms of 3ß-

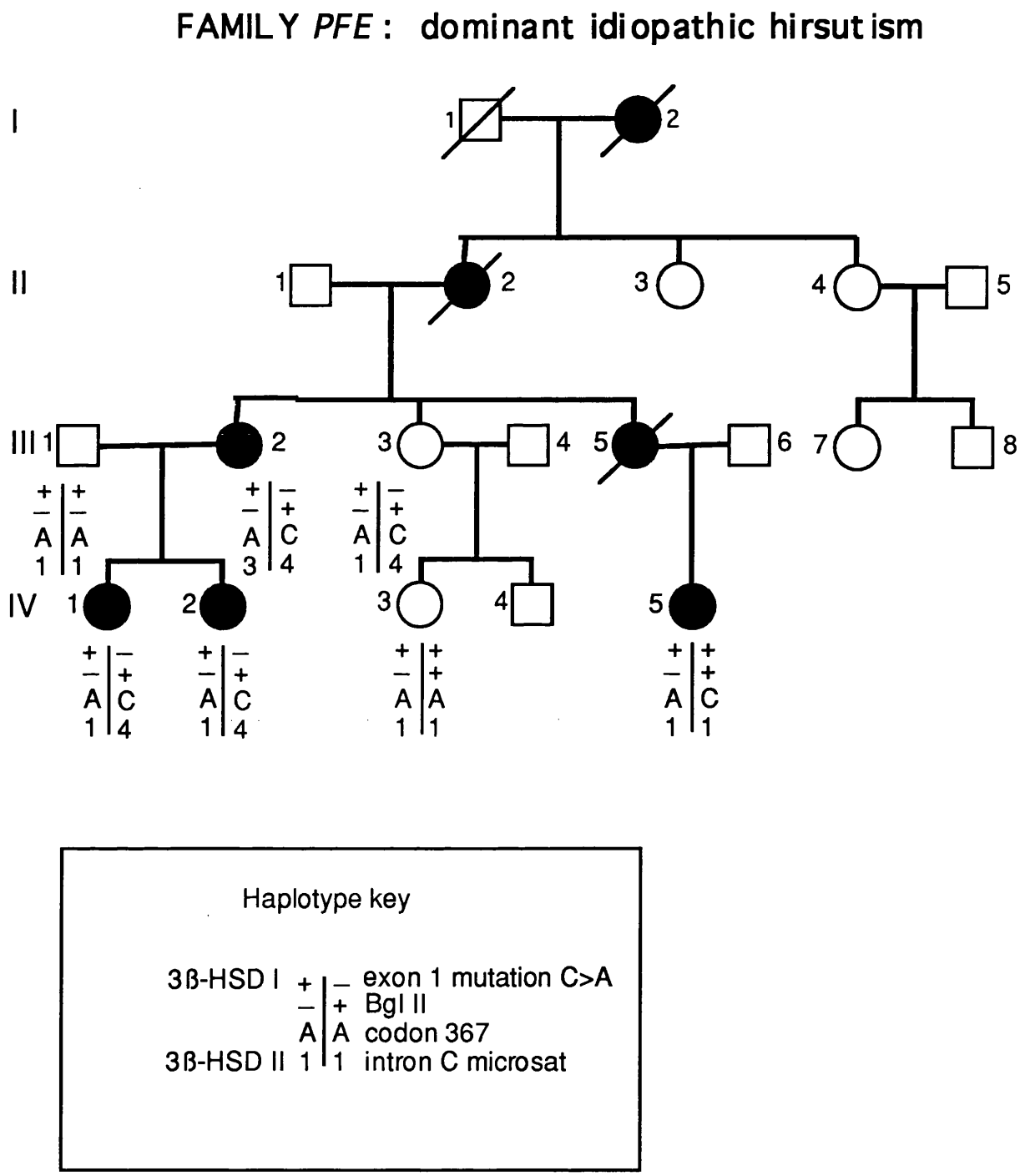

FAMILY PFE : dominant idiopathic hirsutism
I
II
III
IV
Haplotype key
3ß-HSD I
3ß-HSD II
exon 1 mutation C>A
Bgl II
codon 367
intron C microsat

Figure 46

HSD would explain why 3ß-HSD enzyme activity is expressed in other tissues such as the liver. It remains a possibility however that the 3ß-HSD activity detected in the liver is the result of another unrelated dehydrogenase enzyme.

The allocation of 3ß-HSD type I to chromosome 1p13.1 by in situ hybridisation (Berube et al. 1989; Morrison et al. 1990) showed no evidence to suggest the presence of 3ß-HSD homologues elsewhere in the genome. If there is a tightly clustered gene family of 3ß-HSD at 1p13 then the markers used in the typing of the type I and type II genes strongly suggest that the hirsutism in family Pfe is not the result of a 3ß-HSD deficiency.

When compared to the other families in this study (Ran and Sta) family Pfe differed in various aspects. The mode of inheritance in family Pfe appeared to be dominant, although sex linkage could not be ruled out. This may be tested by typing the family with a panel of X chromosome polymorphic markers. The mode of inheritance in families Ran and Sta was clearly autosomal recessive. In addition, affected members of family Pfe had normal basal levels of Δ5 steroids in serum. Marked elevation of Δ5 steroids and their precursors only occurred after ACTH stimulation. This was in marked contrast to the steroid profiles of family Ran and family Sta. In these families elevated basal levels of Δ5 steroids and their precursors was observed. The hirsutism in family Pfe and the exaggerated response to ACTH stimulation may be due to a variety of factors other than 3ß-HSD deficiency. It is possible that a deficiency exists in the catabolism of Δ5 steroids in family Pfe. Insufficient conversion of circulating androgens to oestrogens by the enzyme aromatase may also be a factor. Future work on family Pfe may include the use of genetic markers in candidate genes such as aromatase in an attempt to establish linkage.

The findings in family Pfe have important implications for the future diagnosis of 3ß-HSD deficiency. Reports on the prevalence of 3ß-HSD deficiency in women with

symptoms of androgen excess vary between 9 and 61% (Zerah et al. 1991). As many diagnoses have been based on steroid profiles similar to that found in family Pfe it is possible that 3ß-HSD deficiency is not as common as some may believe.

Family Sta: A summary of the clinical and genetic information in family Sta is given in figure 47. When typed for the two genetic polymorphisms in exon 4 of the type I gene (p47) and the microsatellite in intron C of the type II gene it was shown that both affected sons inherited the same polymorphic alleles from either parent. The same genetic markers also revealed evidence of a possible crossover between the 3ß-HSD type I and type II genes on the father's side of the family.

Screening of the type I gene by DGGE revealed no evidence of mutation. Screening of the type II gene by DGGE revealed a mutation in exon 3 of the type II gene. Sequencing showed the mutation to be a single missense mutation of AAT⇒AGT in codon 100 leading to the substitution of Ser for the normal Asn. No other sequence variations were found in 3ß-HSD type II. The Ser codon is not found in the gene for 3ß-HSD type I and cannot therefore be the product of a type I⇒type II gene conversion event. The substitution was detected in both affected sibs and also in their mother. No other members of the family possessed the substitution, including Sta II-1 who is affected with hirsutism. If the Asn⇒Ser substitution is involved in the manifestation of MPH in the two sibs then it would appear that Sta II-1 is a sporadic case.

Insufficient is known at present of the protein structure to predict what effect the substitution may have on the activity of the enzyme. Both Ser and Asn have a neutral charge though Ser is slightly more hydrophobic. Though the main defect in family Sta is in the conversion of C19 substrates to androstenedione and testosterone, it appears that the conversion of the C21 substrates to progesterone and 17 alpha hydroxyprogesterone is also affected as Sta III-2 demonstrated an impaired cortisol

FAMILY *STA* : male pseudohermaphroditism with mild salt loss

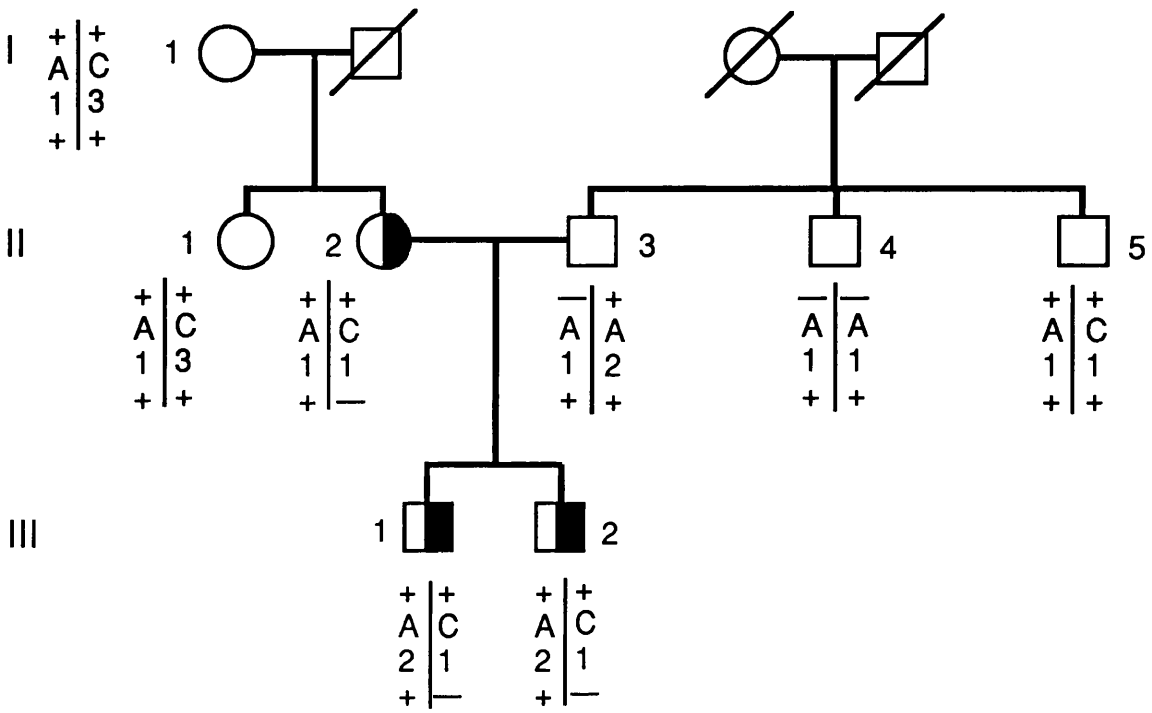


Figure 47

response to synacthen. The expression of the mutant allele is currently under test *in vitro*.

To date no evidence of mutation has been detected in either the 3ß-HSD type I or 3ß-HSD type II genes of the father in family Sta. As mentioned previously it remains a possibility that a deficiency exists in an as yet undiscovered 3ß-HSD gene though the results of both HCG and synacthen tests suggest that both the testes and the adrenals are affected. This means that 3ß-HSD type II is still the most likely source of a deficiency. The possibility that a mutation may still lie undetected in either the coding sequence or even in a 5' or 3' regulatory region of the fathers type II gene is currently under investigation.

The aim of this thesis was primarily to detect the underlying genetic factors which result in 3ß-HSD deficiency, however, the polymorphic markers discovered during the course of this work and the techniques which have been employed will prove useful in other areas. The polymorphic markers can be used to build up a more detailed genetic map in the region of chromosome 1p1-2. Analysis of polymorphic markers (Figs 45, 46, and 47) has demonstrated significant genetic linkage between the genes for 3ß-HSD types I and II. When the LOD scores for each of the families presented in this report were added to those of families 1 and 2 from Rheaume et al. 1992, a combined score of 5.374 was obtained, indicating a high probability of tight linkage. At present the physical linkage between the two loci has not been determined, nor have the linkage relationships with other members of the putative gene family been determined. It is hoped that the use of somatic cell hybrids may allow the mapping of 3ß-HSD homologues, detected by DGGE (Figs 30 & 36), to chromosome 1p1-2. The possibility of a tightly clustered gene family raises the possibility that in some cases of 3ß-HSD deficiency mutations may be due to unequal crossing over or to gene conversion, as has been observed for aldosterone synthase and 11ß-hydroxylase (Lifton et al. 1992) and in several other gene clusters including

21-hydroxylase (Donohoue et al. 1986; Garlepp et al. 1986; Harada et al. 1987). This hypothesis is unlikely to apply to the families presented in this thesis as only single point mutations were observed, however, as it is not known what other homologous elements may be clustered on chromosome 1, the possibility cannot be ruled out.

A study of the genetic basis of inherited breast cancer is currently underway which will also utilise the polymorphic markers in 3ß-HSD. The existence of families with raised incidences of breast cancer has long been recognised and is currently the focus of considerable work to find the genes responsible (Schildkraut 1989; Hall 1990; Lynch 1990; Ponder 1990; Claus 1991). Results from the breast cancer consortium indicate that about one third of families result from a predisposition due to a locus on chromosome 17q. These families have a mixture of breast and ovarian cancer. In other families, with breast cancer alone, the disease is clearly not linked to 17q. Current work to look for predisposing genes elsewhere in the genome focuses on segregation studies of DNA which map in the main to those parts of the genome where cytogenetic abnormalities have been detected or where oncogenes or anti-oncogenes are located. The chromosomal location of 3ß-HSD (1p13.1) is of interest as Nras maps to the same region. 1p13 is also one of the commonest sites for cytogenetic abnormalities in breast cancer. Mapping studies have not so far given a clear positive linkage to this region, studies are limited by a lack of good polymorphic markers and possible genetic heterogeneity. The use of the polymorphic markers presented in this thesis may prove to be a significant step in establishing linkage.

It is possible that 3ß-HSD may play a direct role in the incidence of breast cancer. Several lines of evidence indicate that sex hormones influence the management and incidence of breast cancer (Henderson 1981; Key 1988; Bulbrook 1989) and it has been suggested that genetic variation in sex hormone receptors or in the enzymes involved in steroid biosynthesis, e.g. 3ß-HSD, may contribute to the risk of breast cancer by changing effective local steroid hormone concentrations at particular stages

of development. Candidate genes and their known polymorphic markers are shown in Table 10. DGGE will prove useful in the detection of polymorphic markers in genes for which so far none have been reported.

Table 10

ENZYME NAME	MAP DATA	ALLELE SYSTEM
Cholesterol side chain cleavage (P450scc)	15q21.1	(CA)n repeat
Aromatase (P450Arom)	15q21.1	(CA)n repeat
17-alpha hydroxylase (P450 17c)	10	-
3ß hydroxysteroid dehydrogenase/isomerase (3ßHSD)	1p13.1	1 intragenic RFLP 1 intragenic substitution 1 microsatellite
17ß-hydroxysteroid dehydrogenase (17ß-HSD)	17q	-
Steroid sulphatase (STS)	Xp22.3	(CA)n repeat in GMGX9 within 600kb of STS
Oestrogen receptor (ESR)	6q24-27	3 RFLP 1 intragenic substitution
Androgen receptor (AR)	Xq12	(CAG)n intragenic repeat

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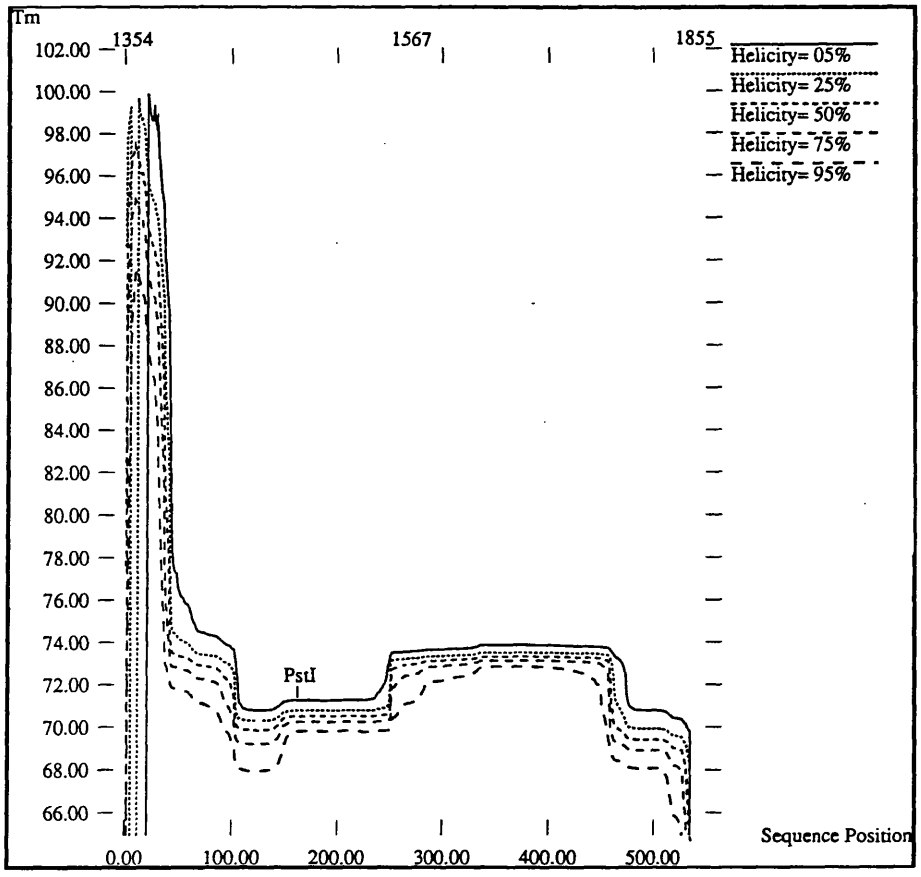
APPENDIX

Melt maps

The following pages contain the melting profiles of each region of the 3ß-HSD type I and type II genes calculated using the MELT programmes of Lerman and Silverstein (1987). The melting information gained from these graphs was used to determine the denaturing gradient conditions and electrophoresis times that would result in maximum resolution in the gel of DNA fragments differing by single base changes.
A detailed description of the use of melt maps and their application to DGGE is given by Lerman and Silverstein (1987).

A plot of Tm as a function of position along a DNA molecule is called a melting map. The computer analysis is based on the assumption that each base pair in a molecule of DNA can exist in only two possible states; either stacked , helical, and hydrogen bonded, or unstacked, disordered and without base pairing i.e. melted. The existence of fairly long regions, 30-300bp, termed domains, at which all bases melt at very nearly the same temperature is typical. The melting maps shown in the following pages show the positions and Tms of melting domains in each fragment of DNA based on its nucleotide sequence. Also shown are a series of plots which calculate the difference in migration rates through the denaturing gradient between the fully base paired helix and molecules with single mismatches as a function of time after starting electrophoresis. DGGE is most effective when detecting mutations in the lowest melting domain of the DNA molecule. The calculations are based on a gel temperature of 60^0C. Time (Tk) and displacement are given in arbitrary units, approximately equal to hours and millimetres respectively.

Melting contour plot across sequence 3ß-HSD type I exon 1/2 5'GC



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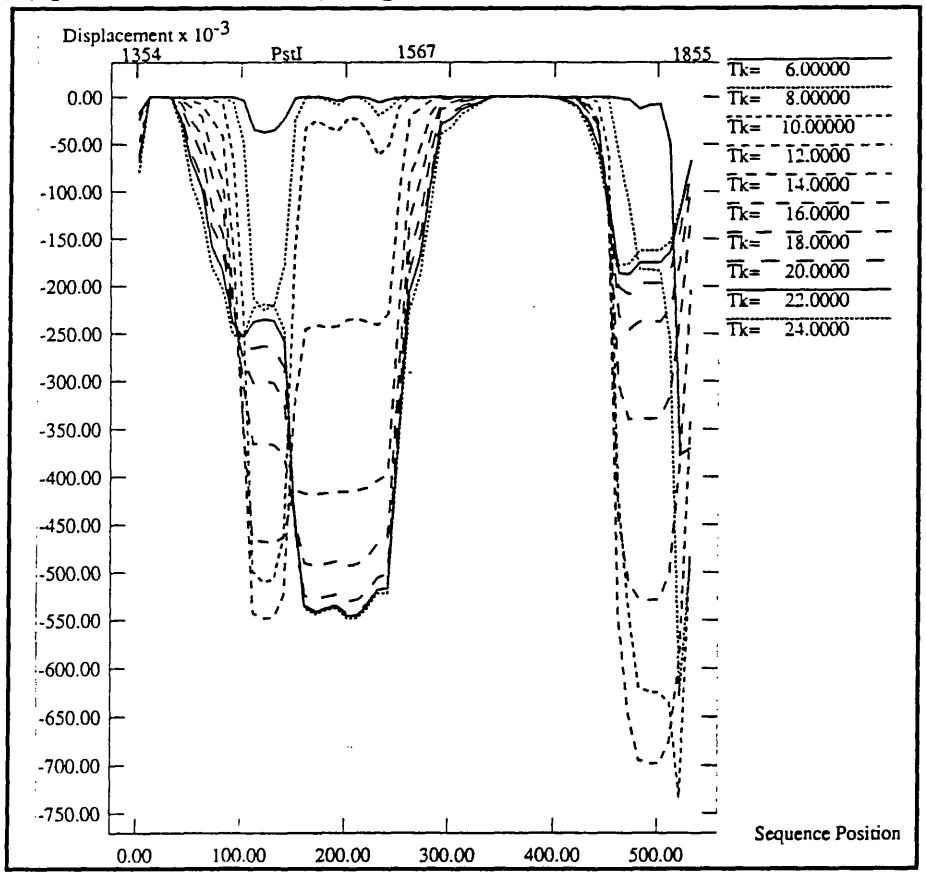

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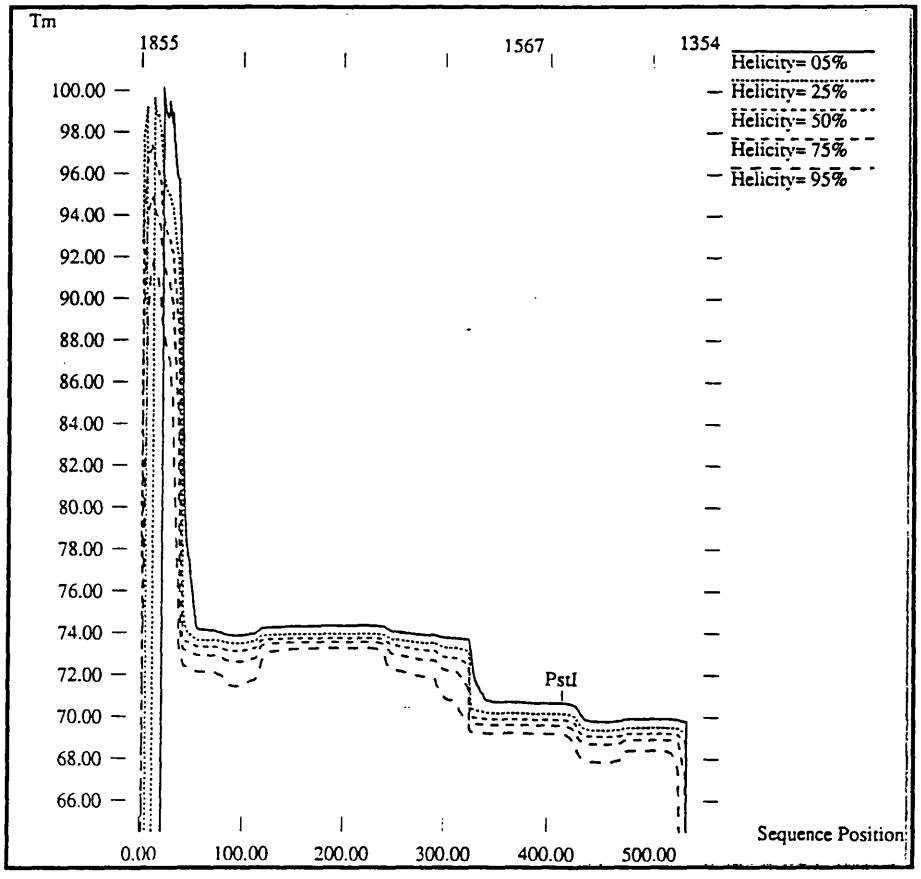

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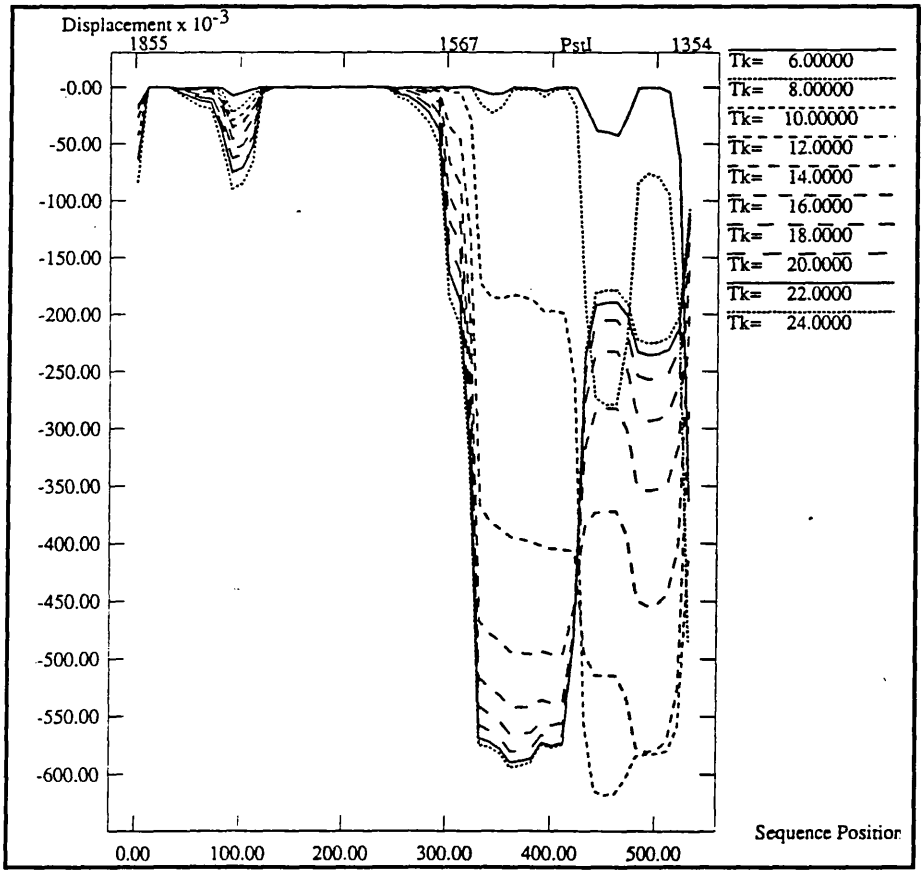

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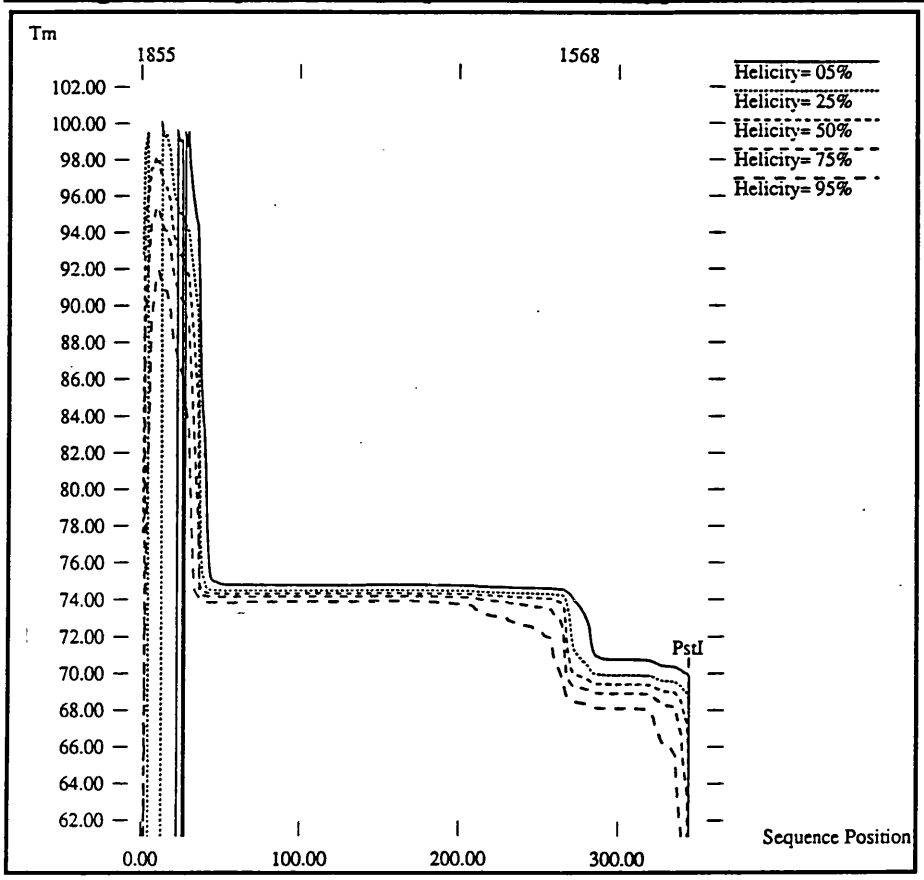

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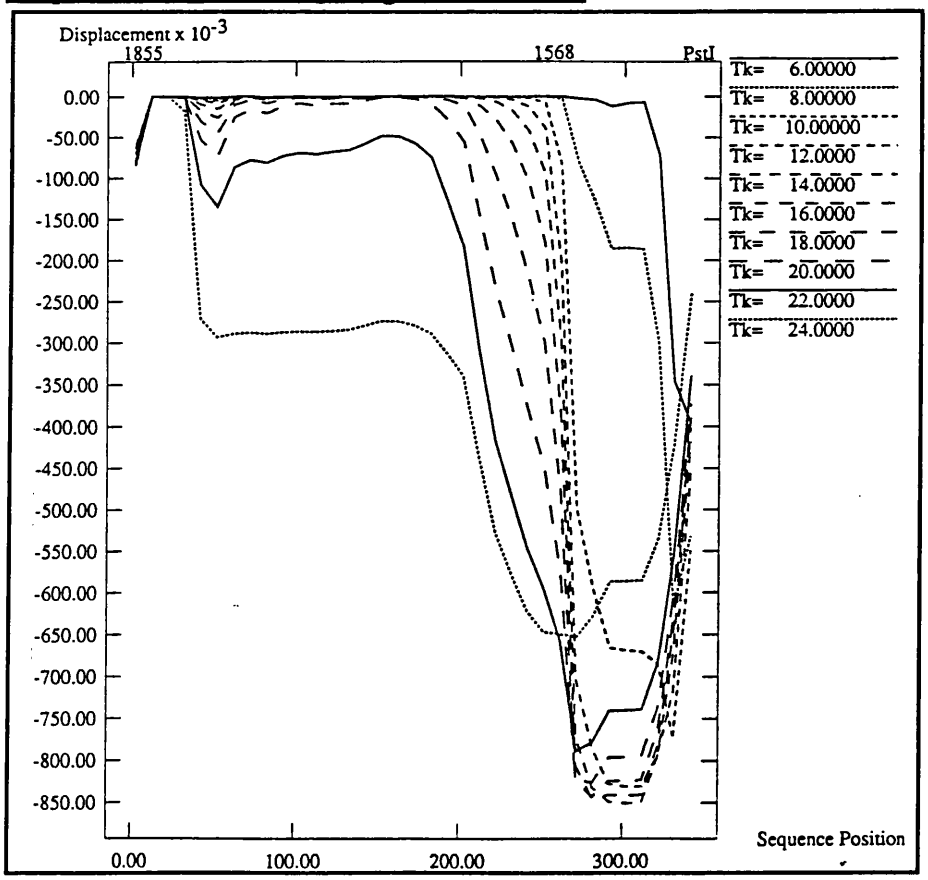

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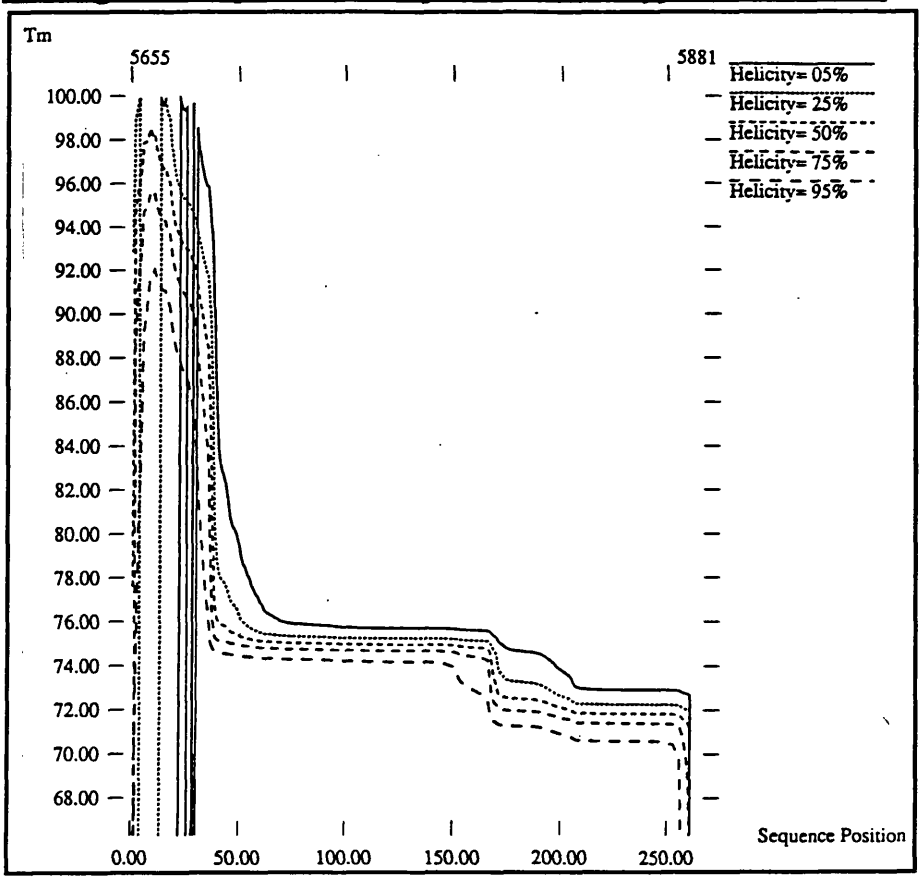

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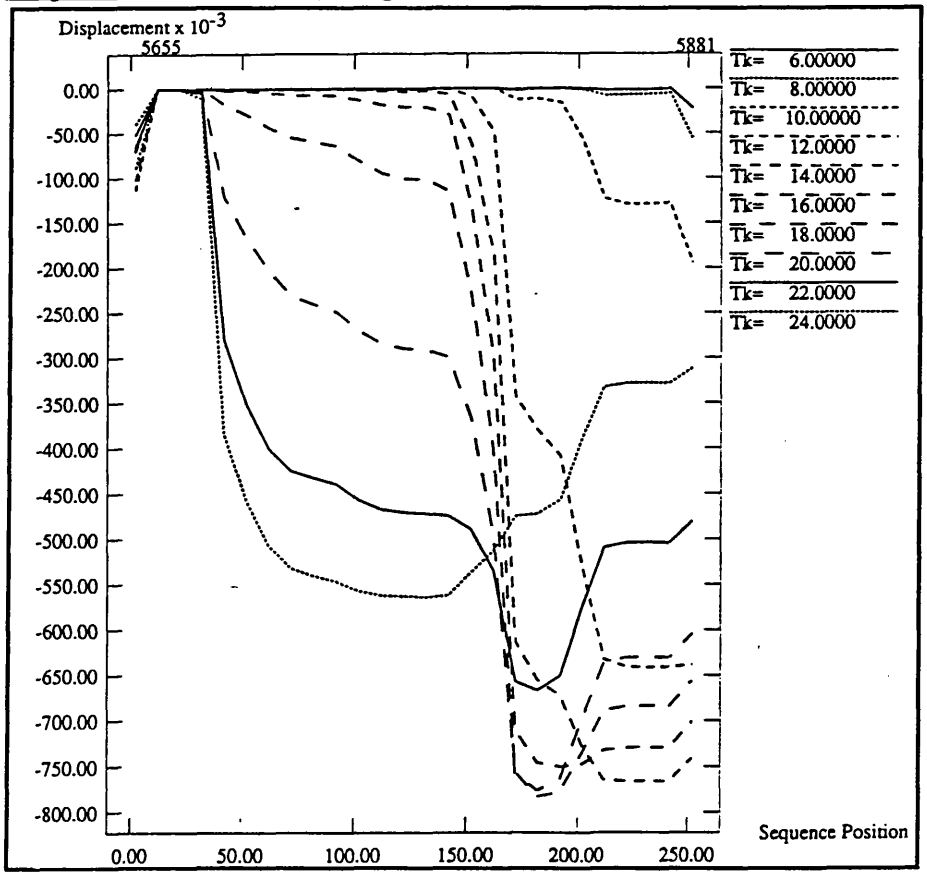

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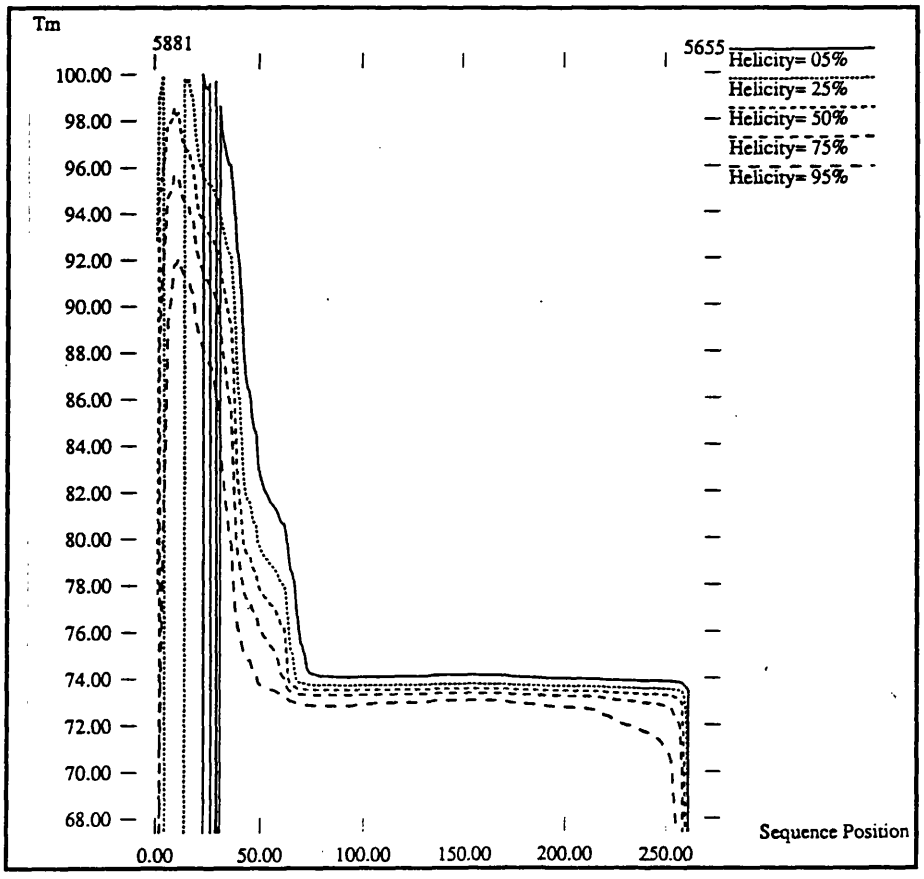

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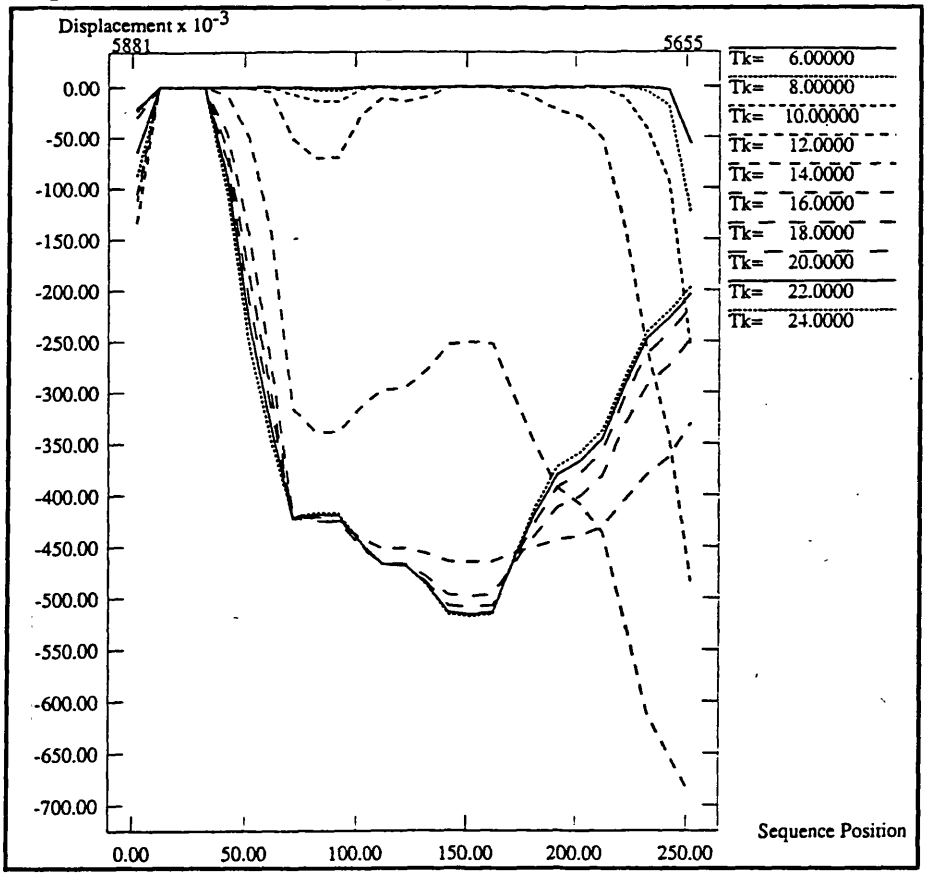

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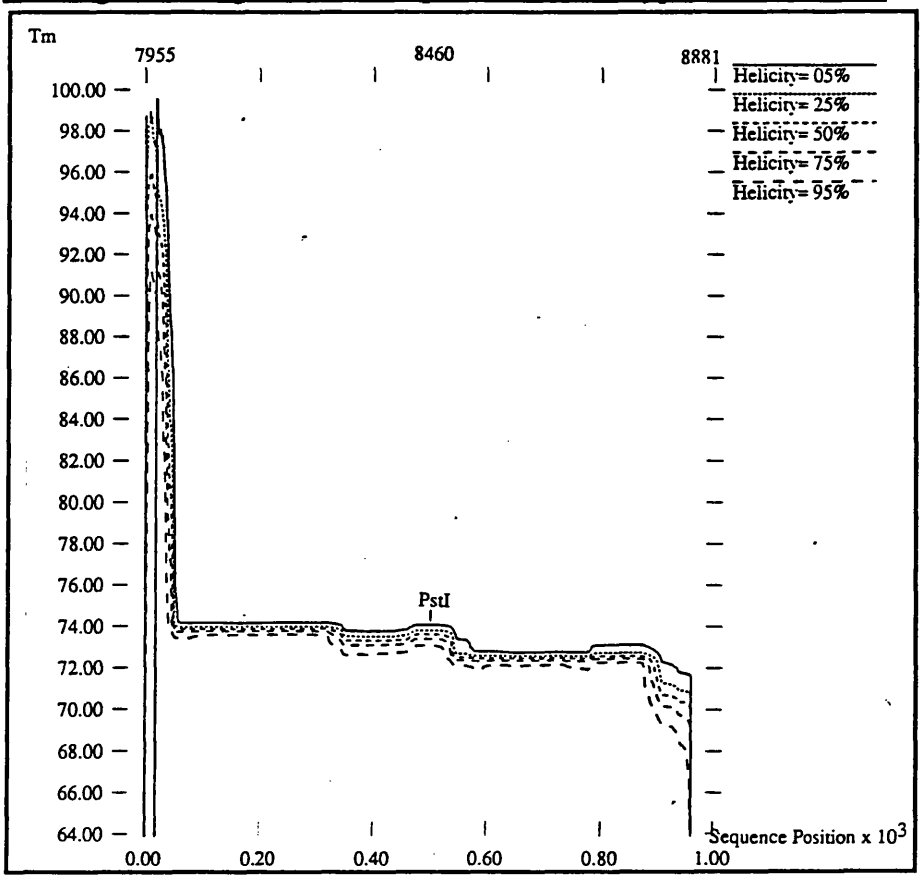

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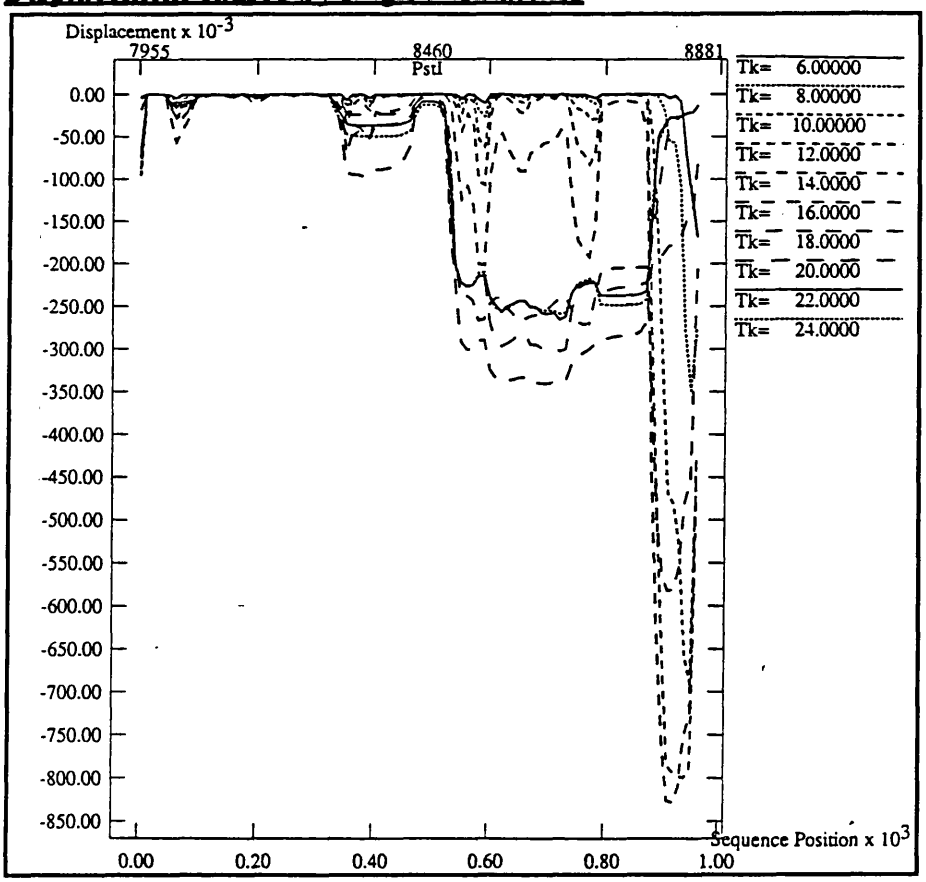

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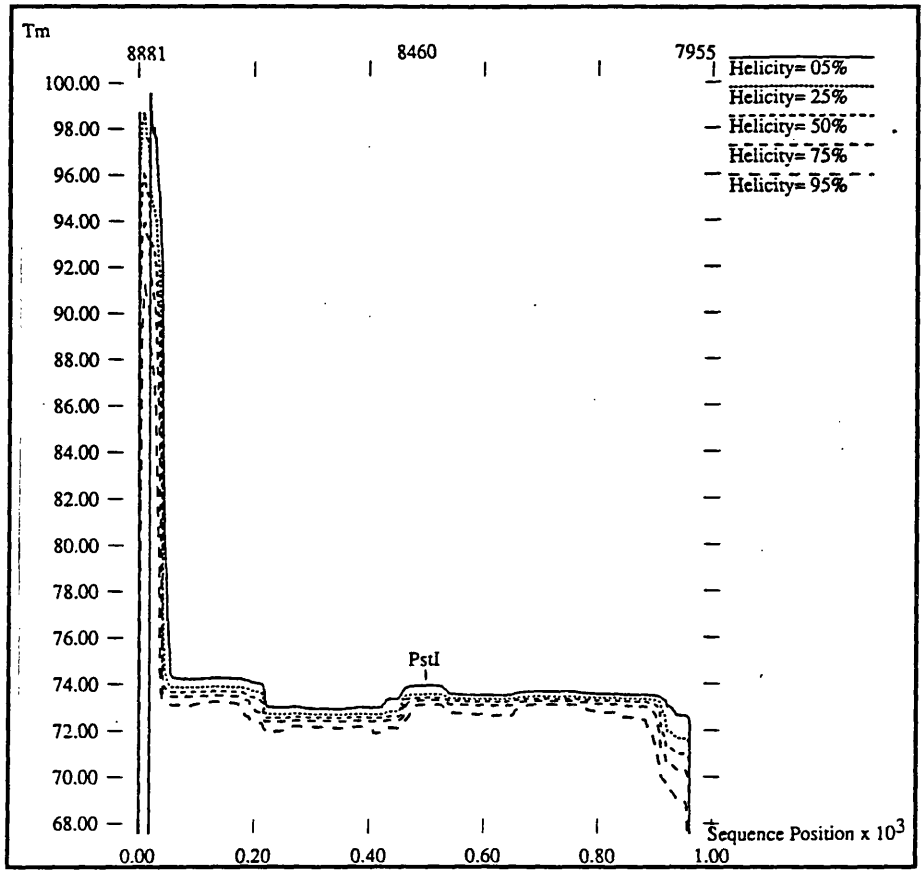

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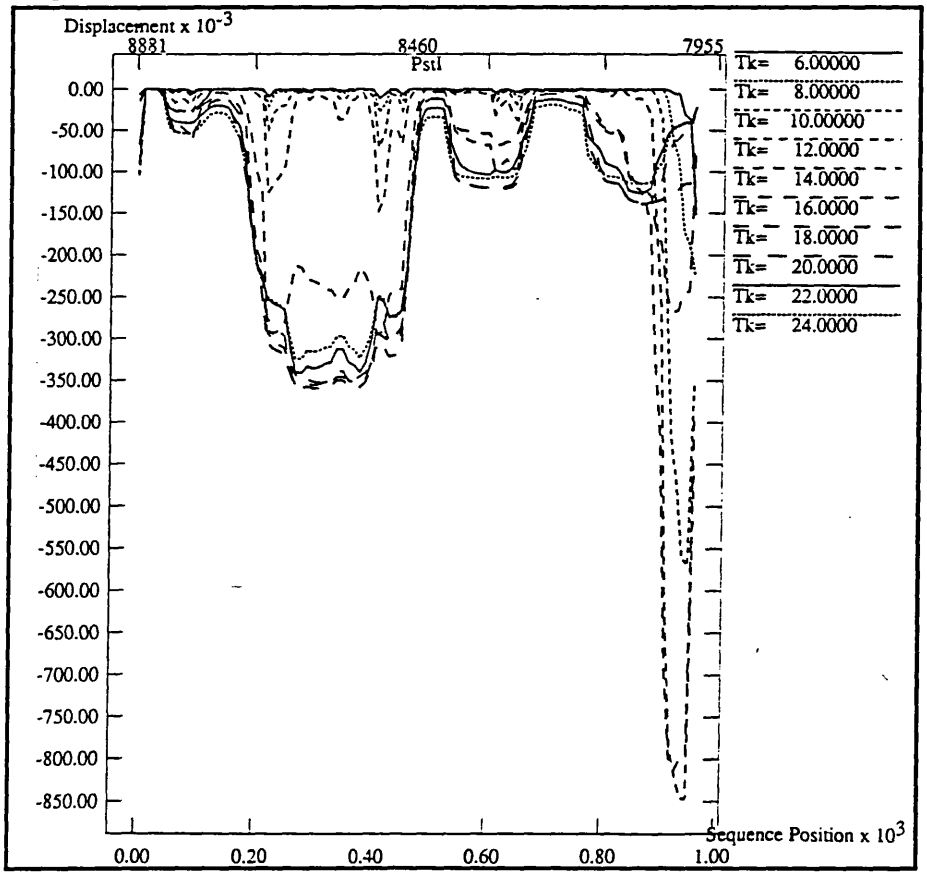

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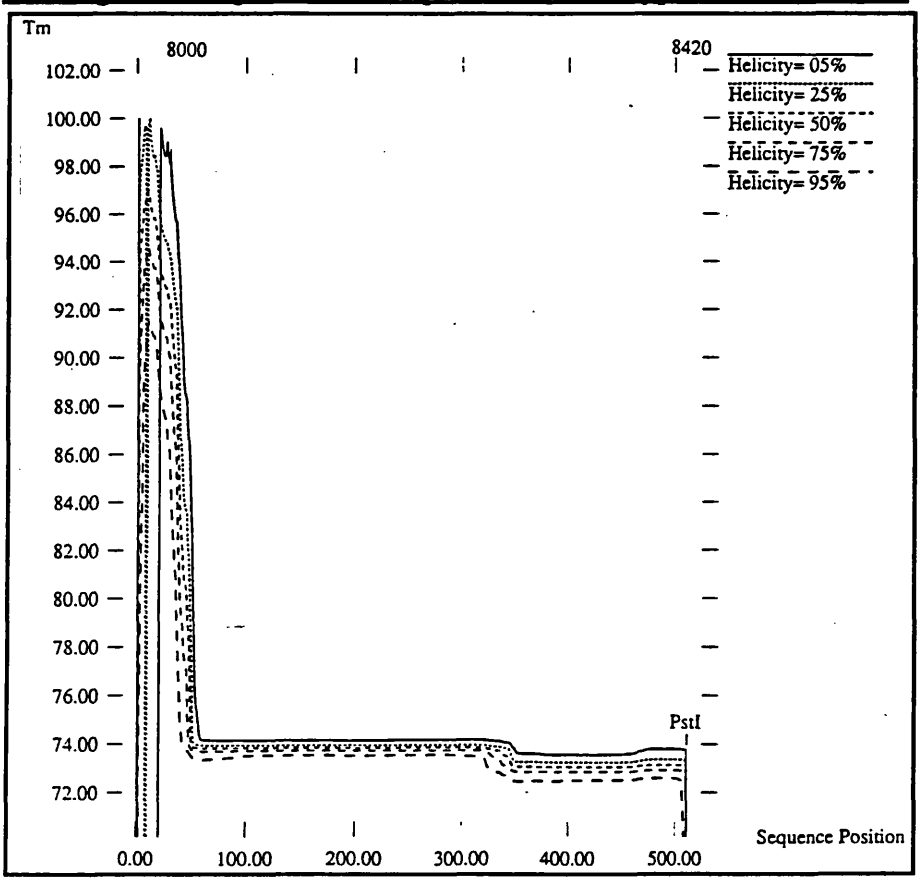

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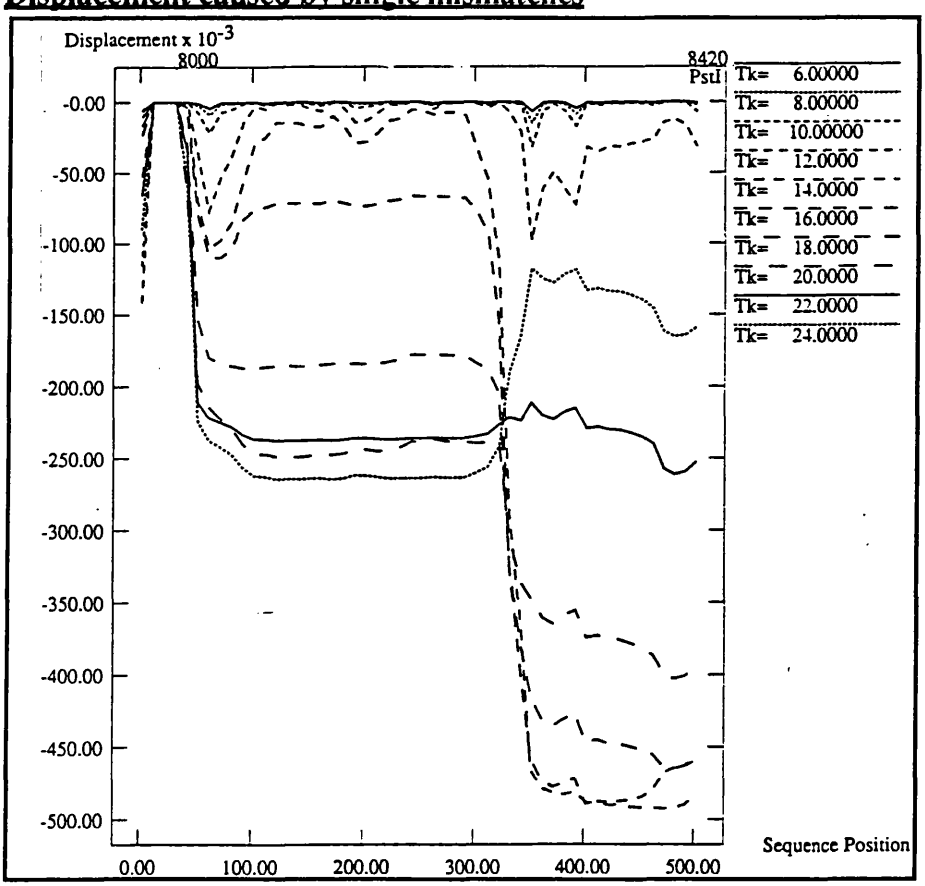

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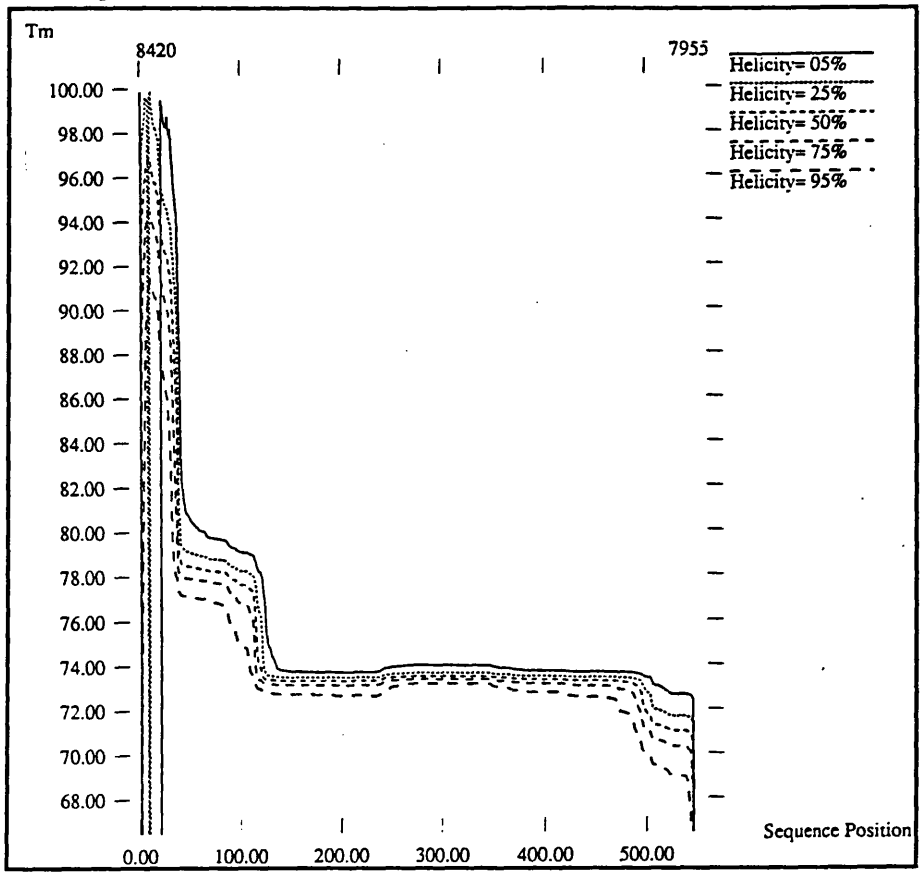

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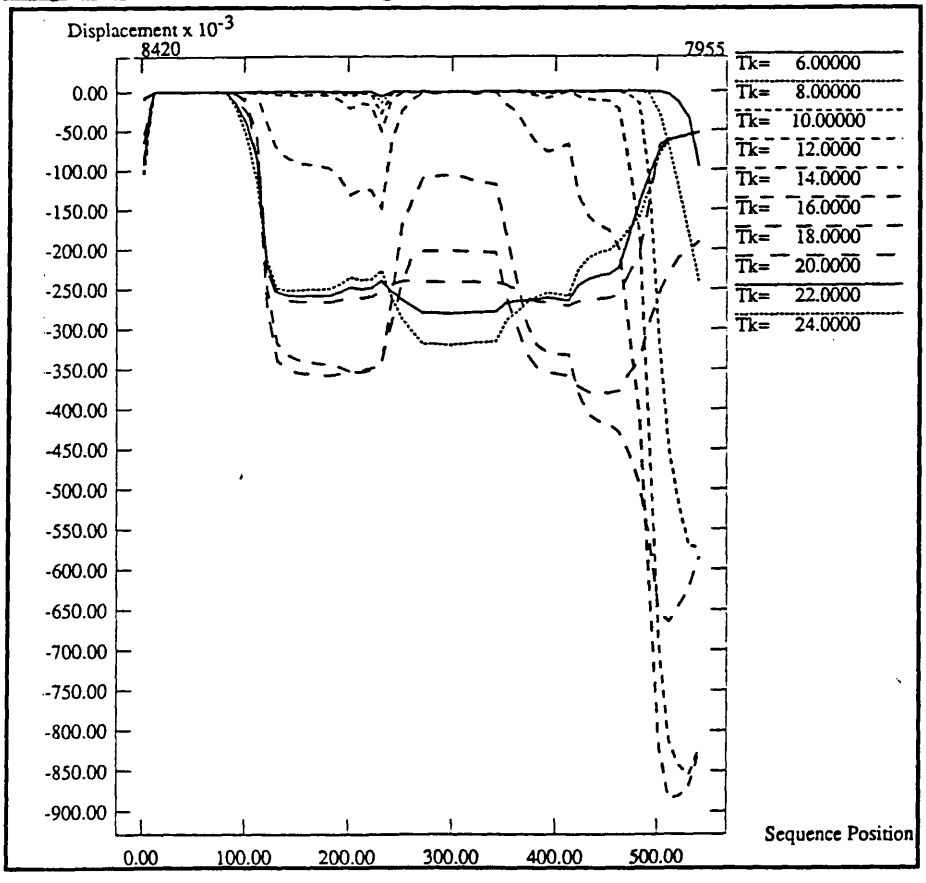

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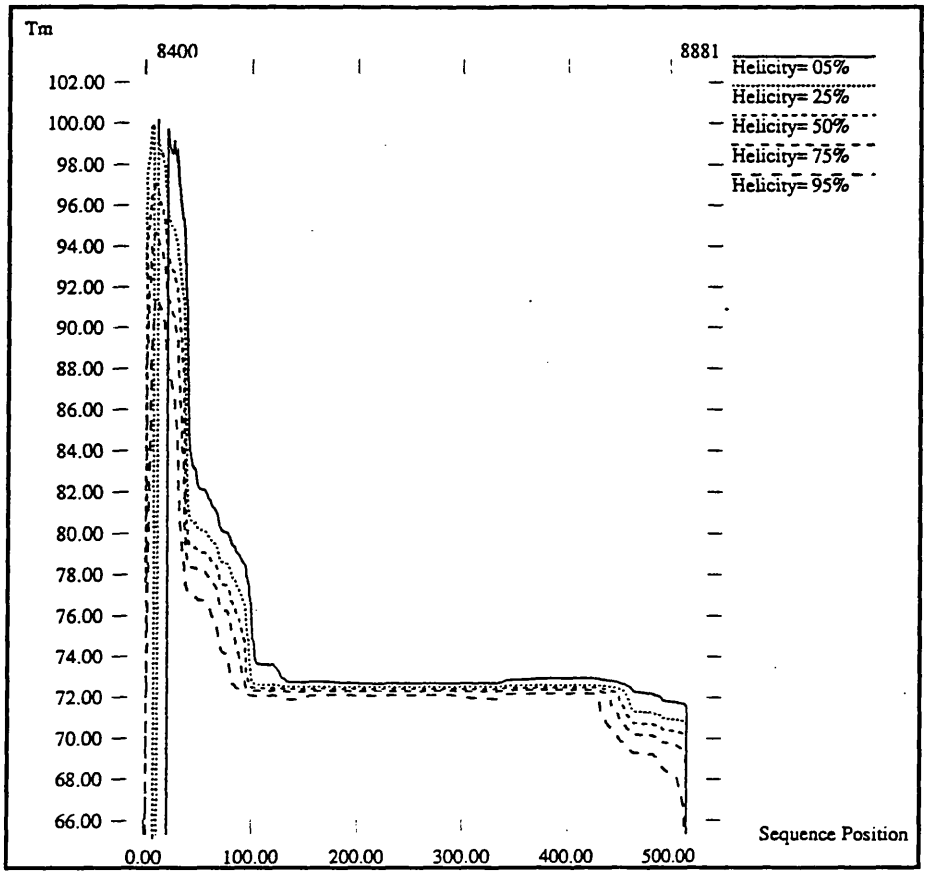

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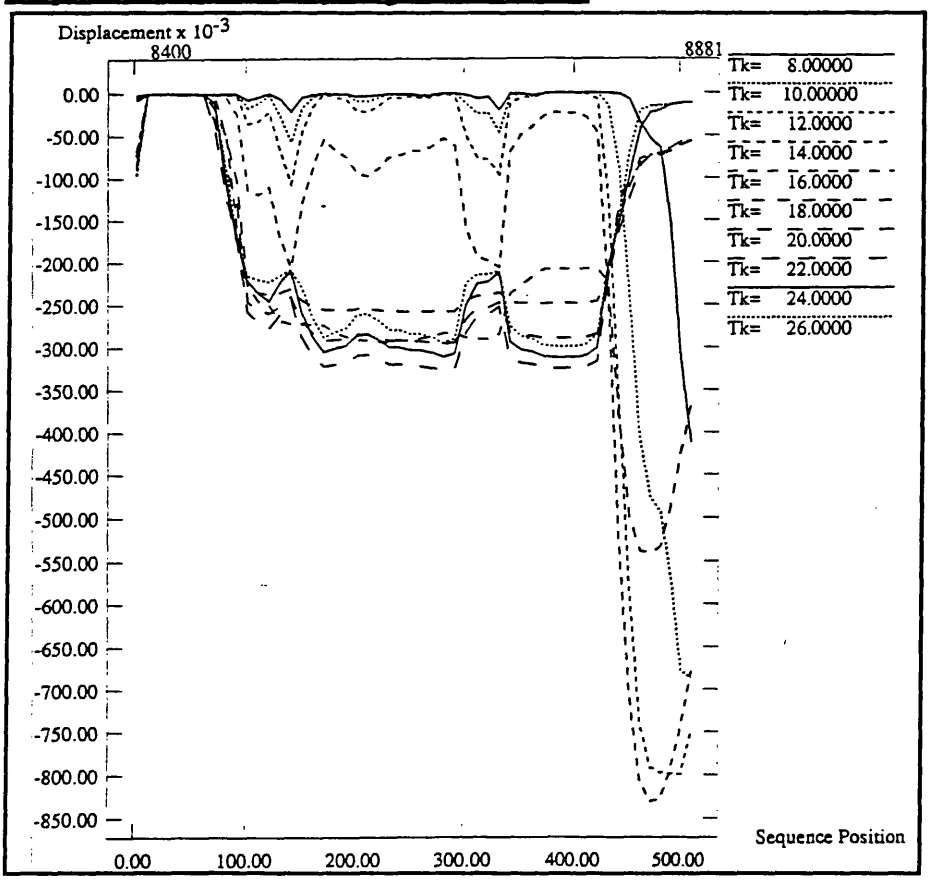

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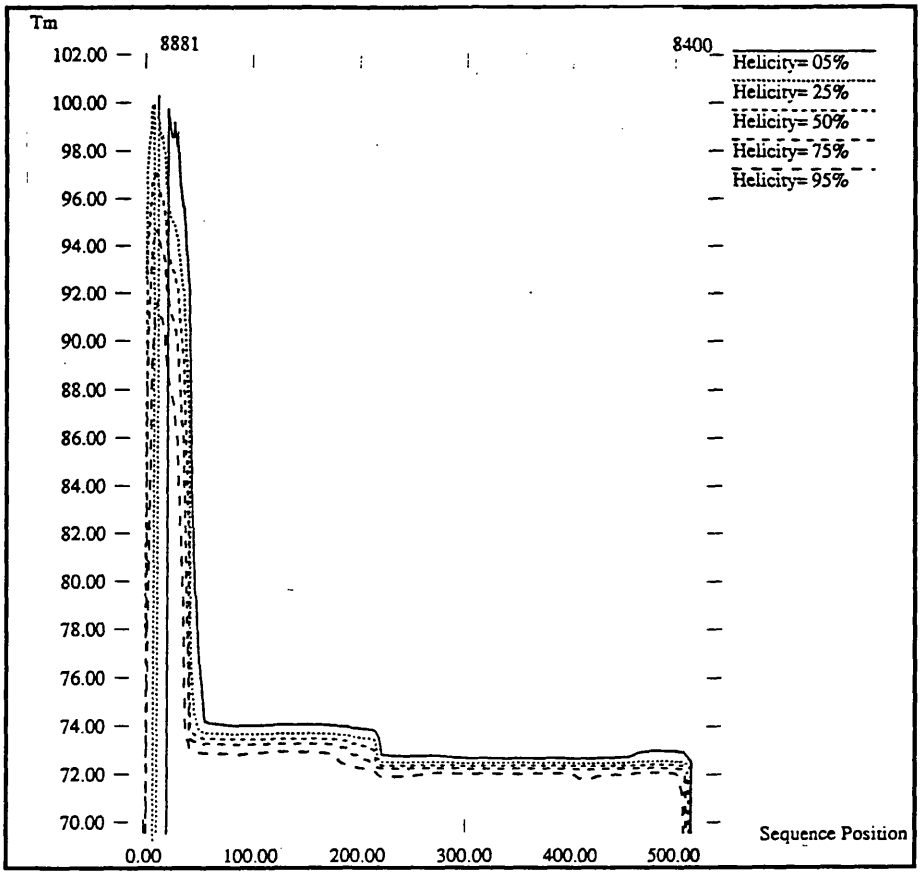

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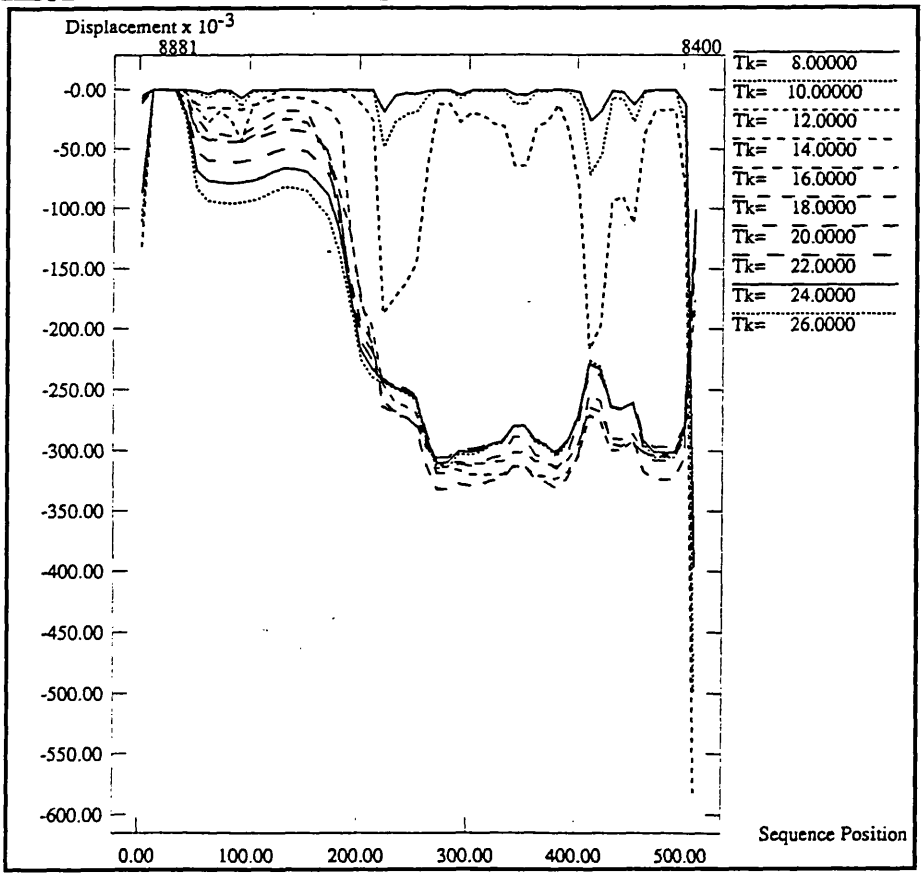
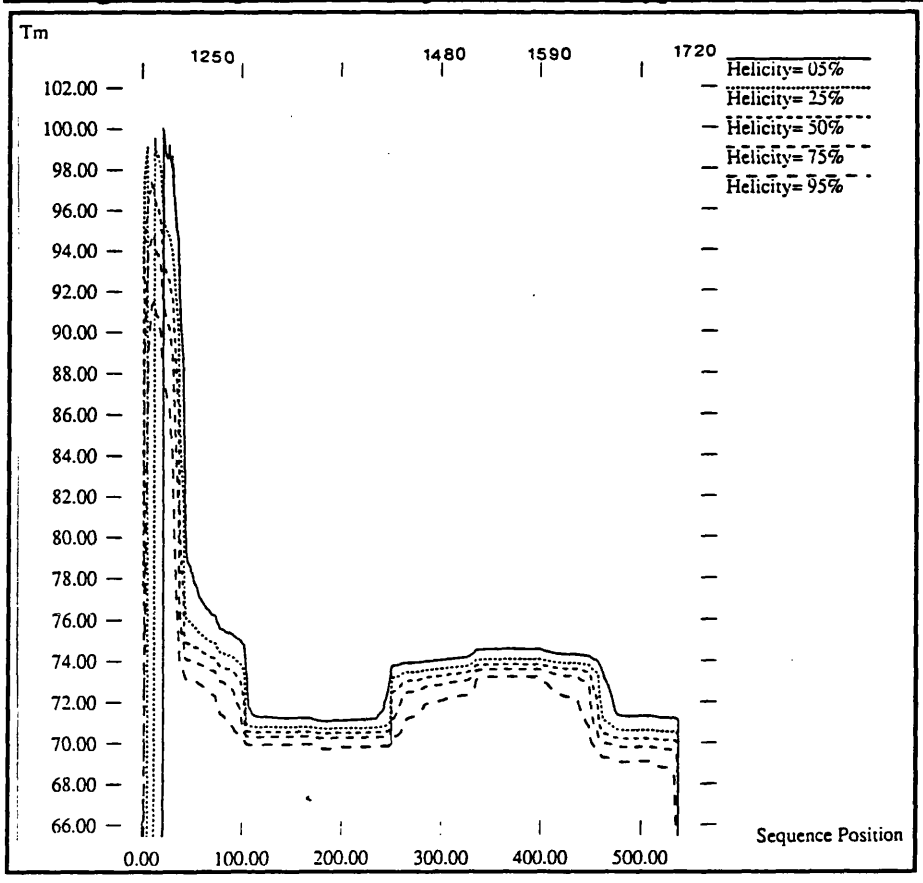
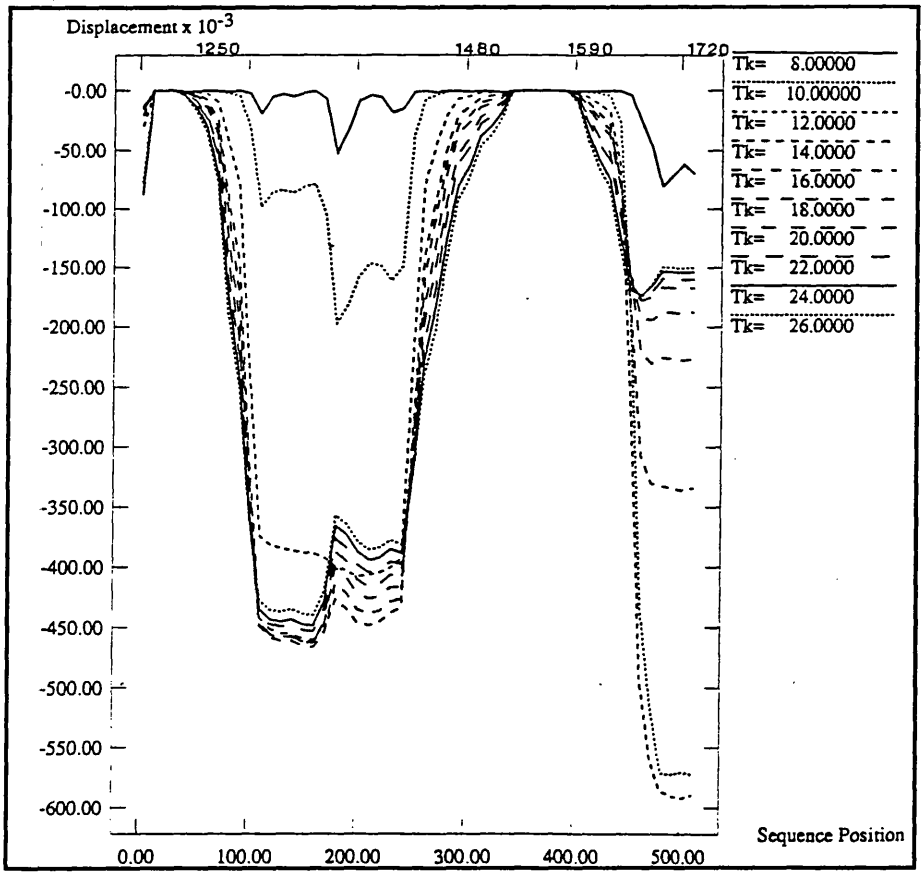


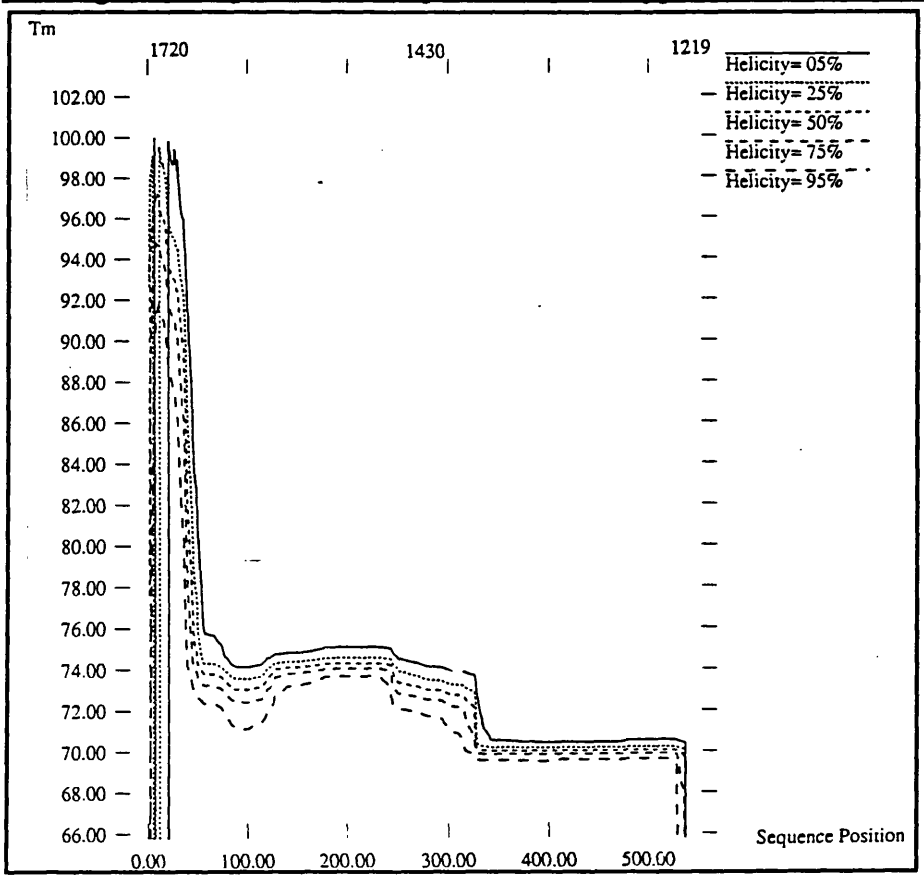
Melting contour plot across sequence 3ß-HSD type II exon 1/2 5'GC



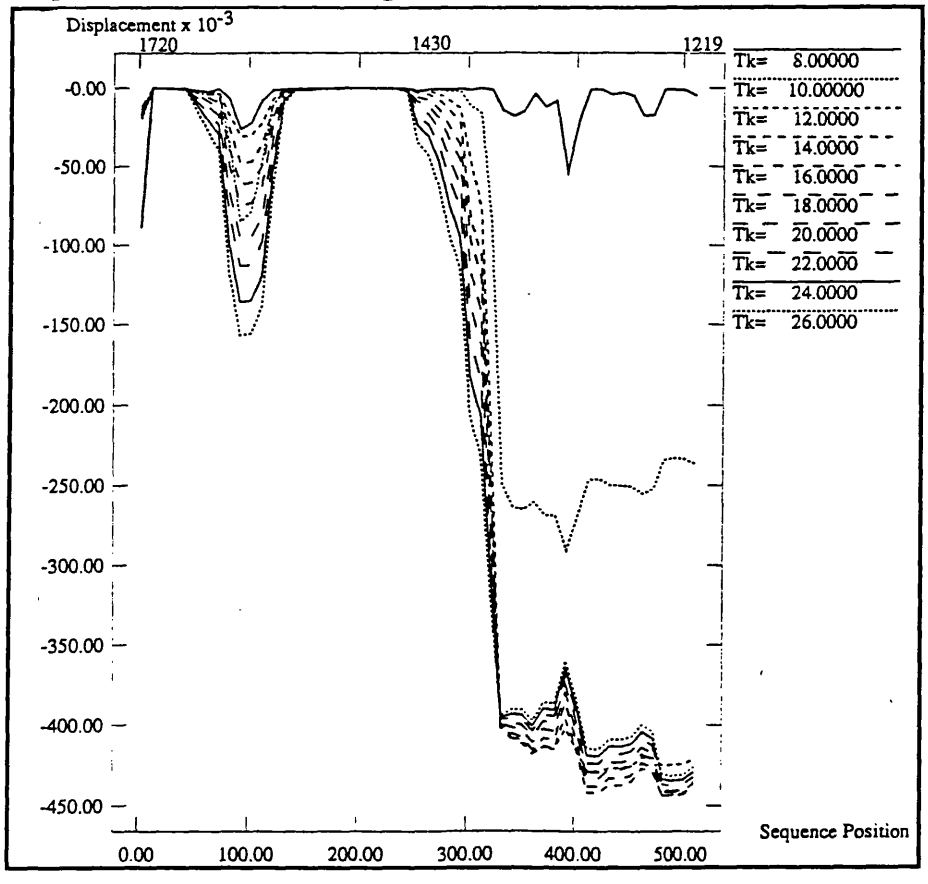
Displacement caused by single mismatches



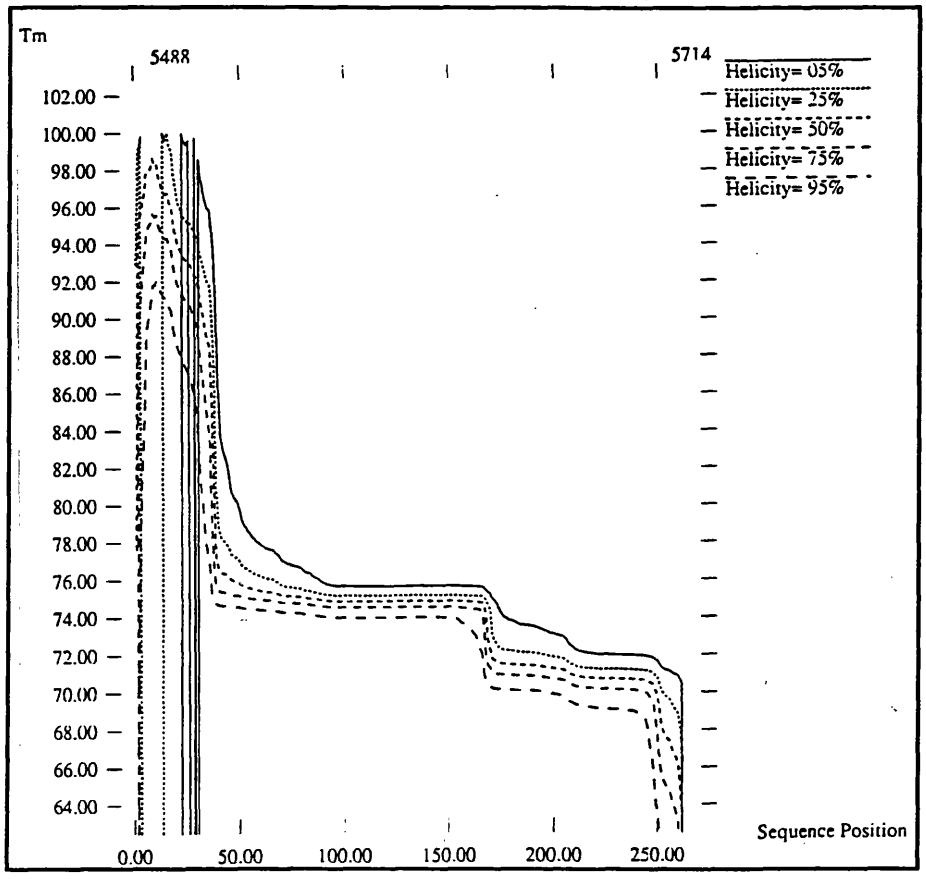
Melting contour plot across sequence 3ß-HSD type II exon 1/2 3'GC



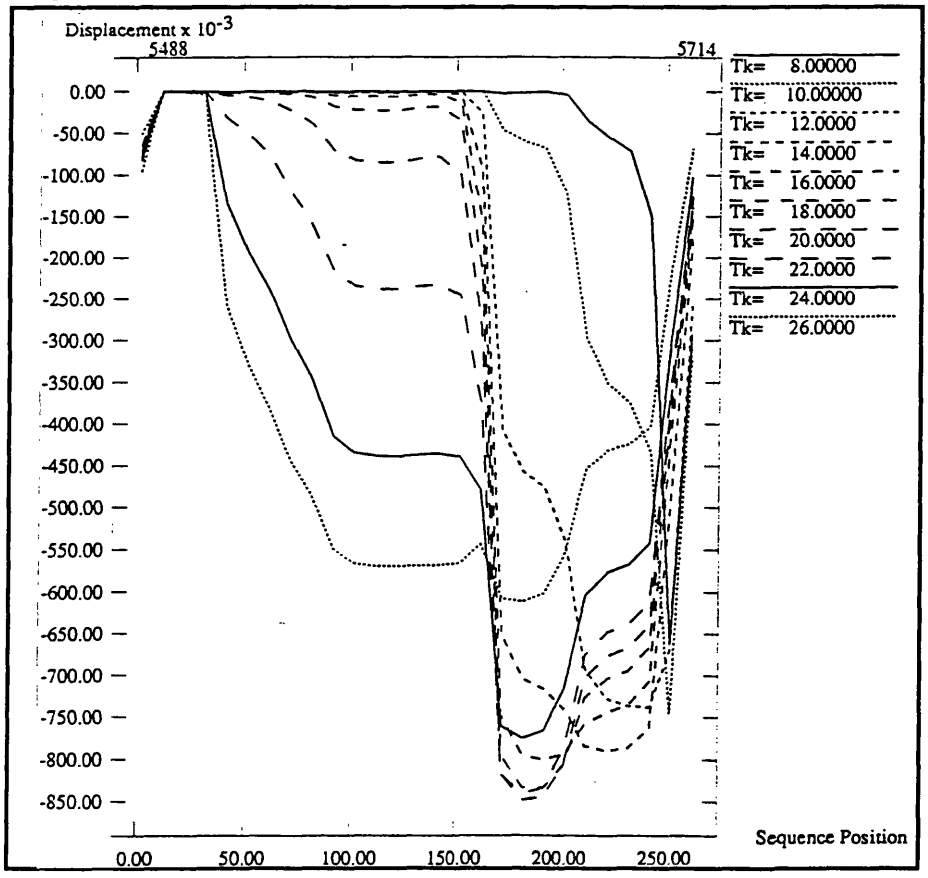
Displacement caused by single mismatches



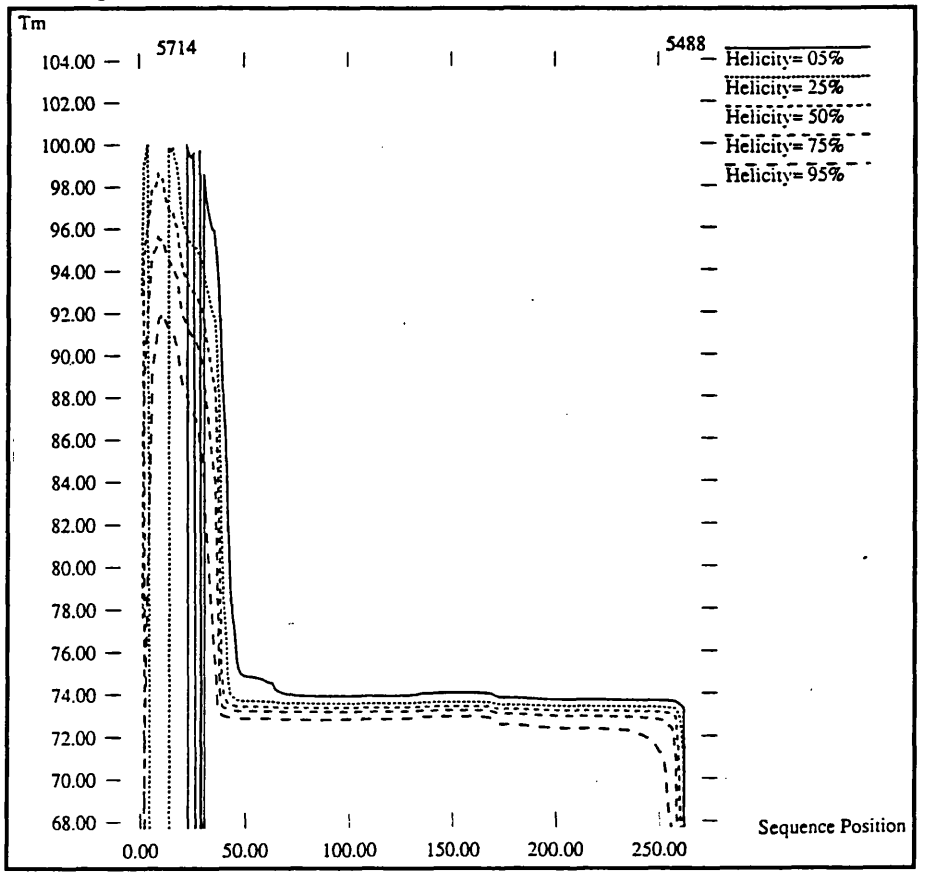
Melting contour plot across sequence 3ß-HSD type II exon 3 5'GC



Displacement caused by single mismatches



Melting contour plot across sequence 3ß-HSD type II exon 3 3'GC



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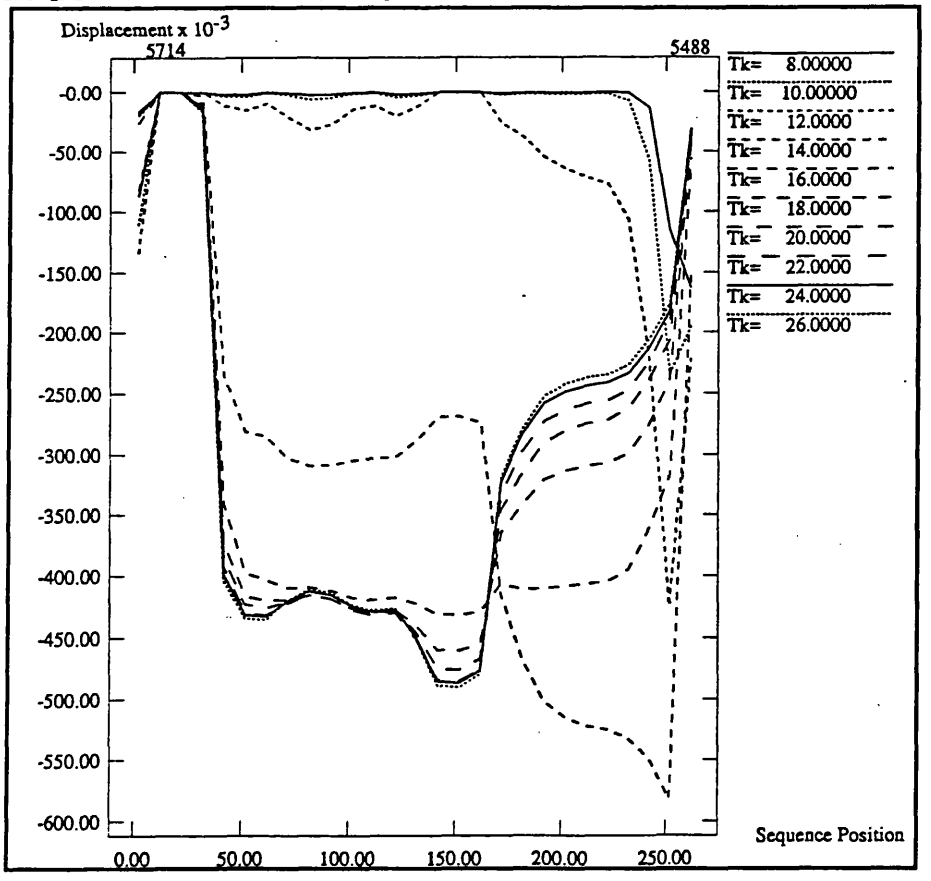
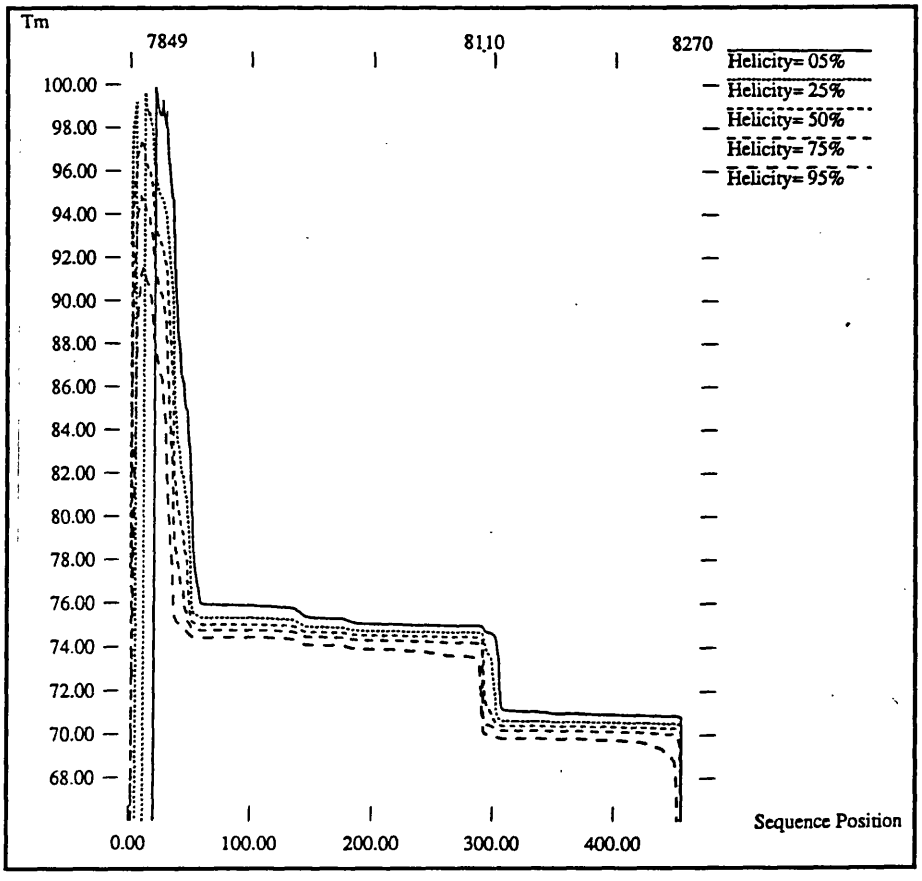
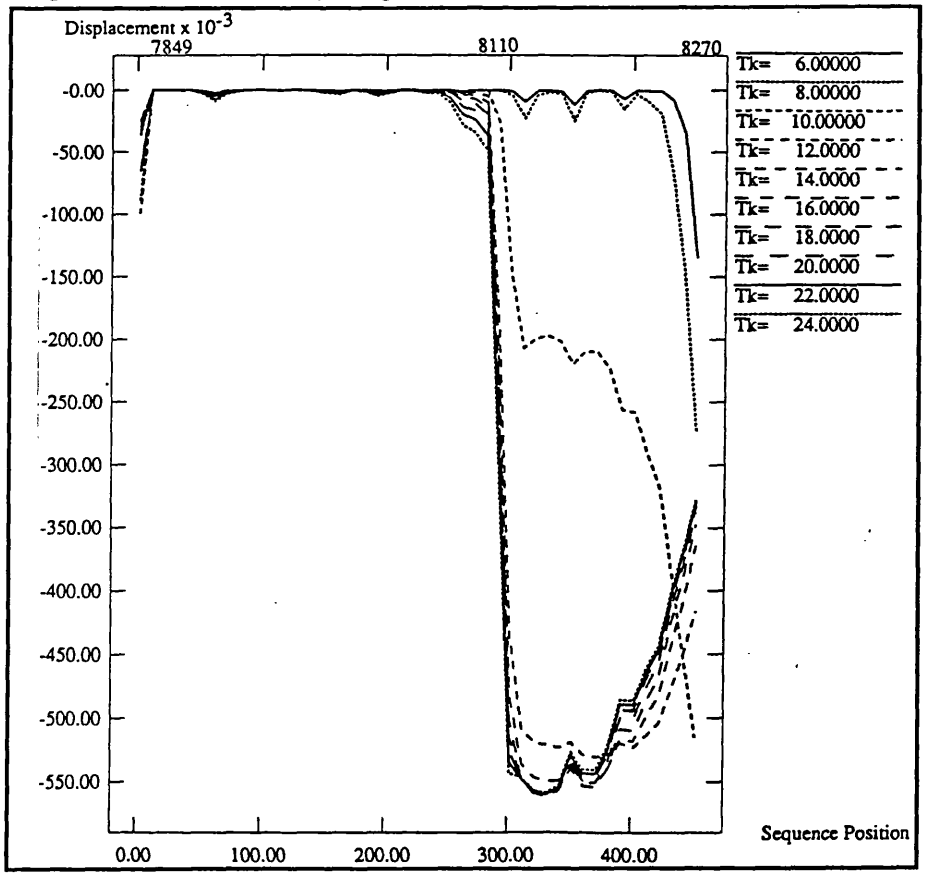


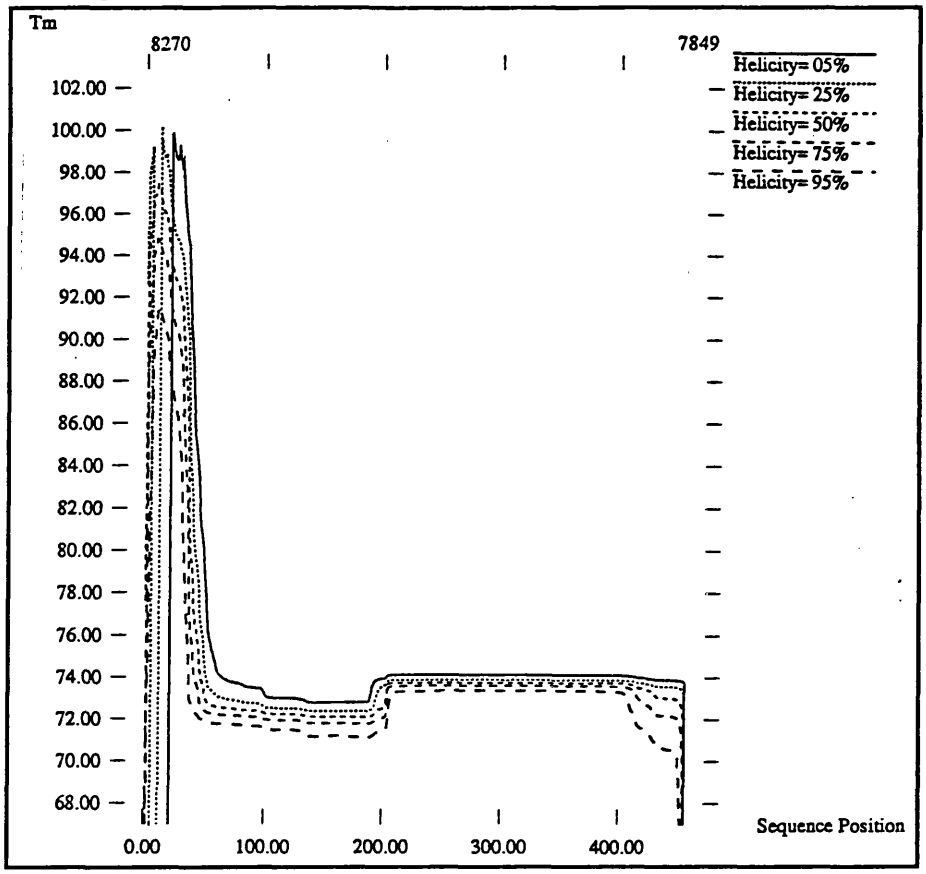
Melting contour plot across sequence 3ß-HSD type II exon 4A 5'GC



Displacement caused by single mismatches



Melting contour plot across sequence 3ß-HSD type II exon 4A 3'GC



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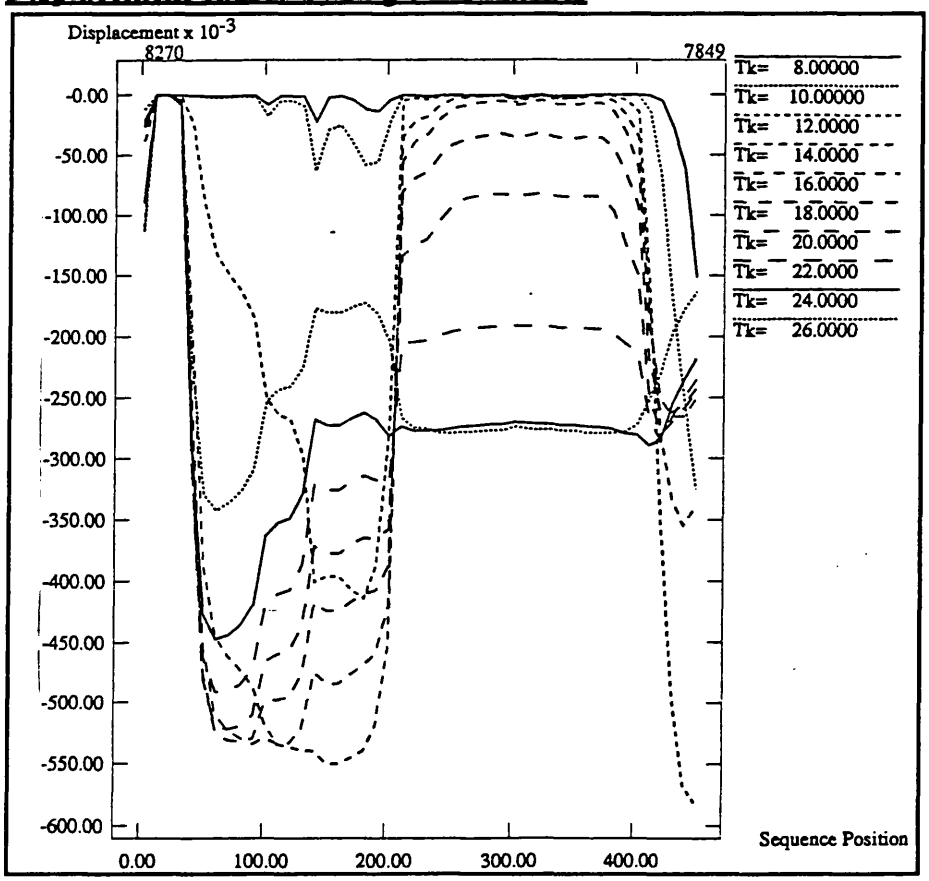
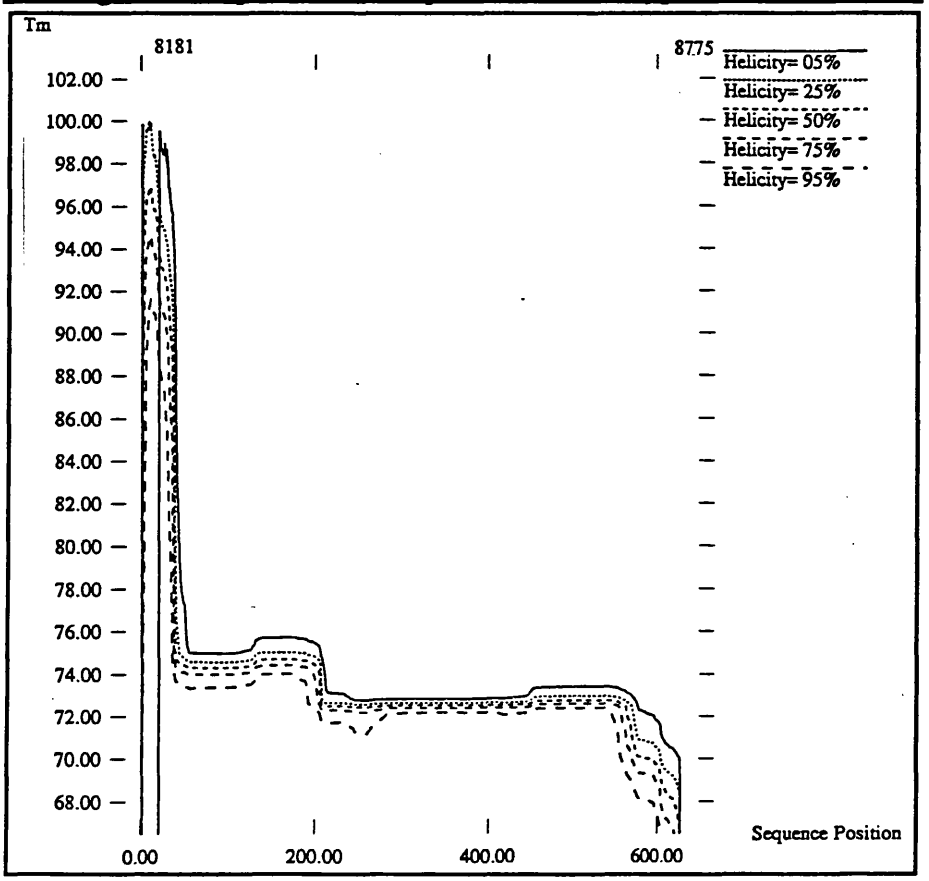
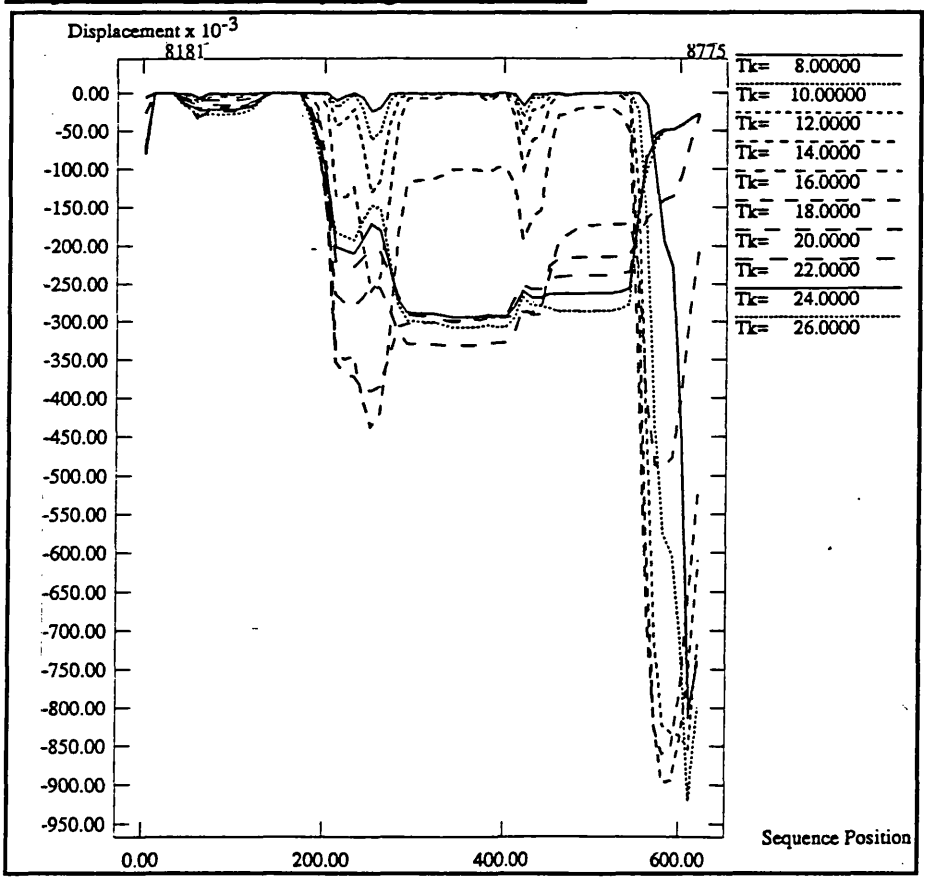


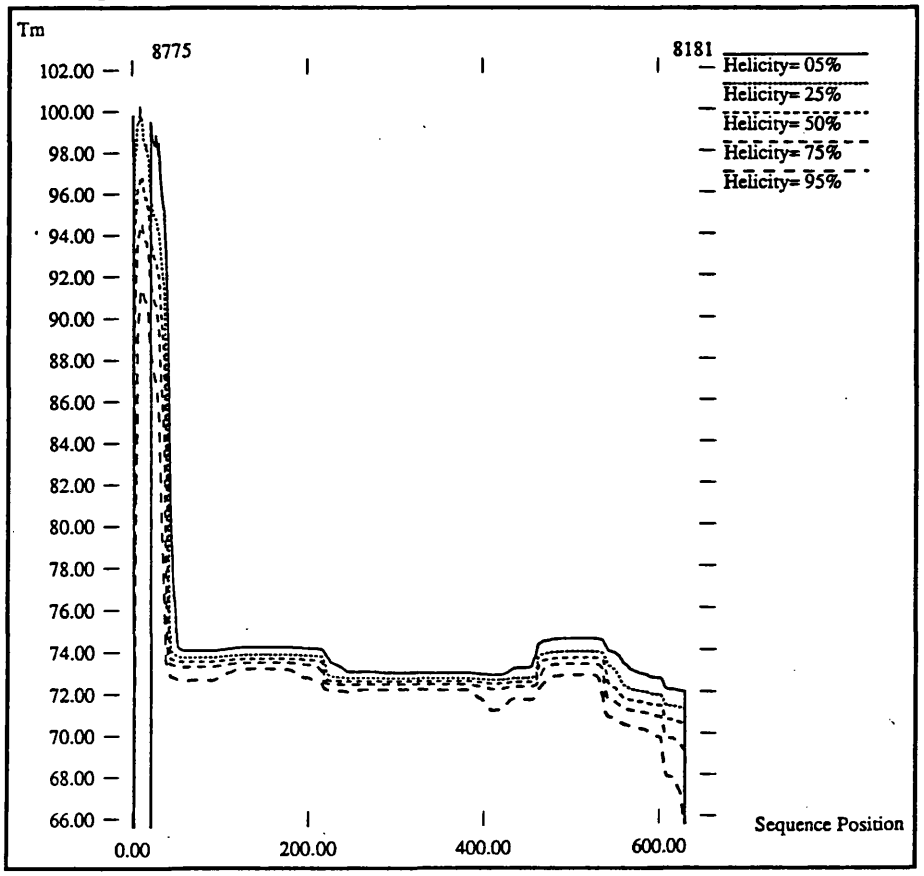
Melting contour plot across sequence 3ß-HSD type II exon 4B 5'GC



Displacement caused by single mismatches



Melting contour plot across sequence 3ß-HSD type II exon 4B 3'GC



Displacement caused by single mismatches