A MOLECULAR INVESTIGATION OF WAARDENBURG SYNDROME IN SOUTHERN AFRICA

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A thesis submitted to the University of Cape Town in partial fulfilment of the requirements for the Degree of Master of Science in Human Genetics

DECLARATION

I, Jennifer Leigh Butt, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work or any part of it, is being, or is to be submitted for another degree in this or any other University.

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Jennifer Leigh Butt
September 1994

The more you learn
the less you know

Petrus Waardenburg
1886-1979

Taken from *The Man Behind The Syndrome*,
(Beighton and Beighton, 1986.)

CONTENTS

ABSTRACT

Waardenburg syndrome (WS) is the most common form of syndromic deafness in schools for the hearing impaired in Southern Africa. It is an autosomal dominantly inherited disorder characterised by varying combinations of sensorineural deafness, pigmentary disturbances of the hair, skin and irides and dystopia canthorum. It has previously been demonstrated by linkage analysis that Waardenburg syndrome type I is linked to the *PAX3* gene at 2q35, while WS type II, where the phenotype does not include dystopia canthorum, is not linked to this locus. Families with WS type I have been shown to have different mutations in the *PAX3* gene, thereby confirming the linkage results and demonstrating allelic heterogeneity.

The aims of the study which forms the subject of this dissertation, were to determine the possibility of linkage of the disorder, as well as to elucidate the underlying molecular defect in the Southern African families with WS. Ten WS families were assessed clinically. 142 family members agreed to partake in the molecular study; 72 of these were affected. Of these families, 7 had WS I and 3 had WS II. A further 37 affected individuals with no family history were also incorporated into the molecular investigation.

The WS type I families were found to be potentially linked to *PAX3*, while the WS type II families were not linked. Mutation screening of the exons of *PAX3* has been performed in SA families in which the disorder was linked to this locus as well as the WS I sporadic individuals. A mutation in *PAX3* was confirmed in 3 families and is probable in another, although not confirmed. A silent polymorphism cosegregating with the disorder, was identified in another of the SA families. Molecular investigation proved that there was intragenic heterogeneity, with no correlation between the position of the mutation causing WS I and the resulting phenotype.

Linkage analysis was employed to locate gene loci which may be involved in the pathogenesis of WS type II. The genotypes of one of the type II families were indicative of linkage of the disorder in this family to a gene on chromosome 3, which had previously been located. Linkage analysis indicated a good probability of WS type II in another SA family being associated with markers on chromosome 20.

The elucidation of the pathogenesis of WS has introduced another level of complexity to the disorder. Three or more loci are responsible for the different types of WS and it is probable that these loci are all intragenically heterogeneous. This investigation provides the basis for further study of the genes which function during embryogenesis and the way in which the disruption of these genes causes intrafamilial variability of deafness and other phenotypic characteristics of WS.

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This research is a direct result of the surveys undertaken by the Departments of Human Genetics and Otolaryngology of the deaf schools in Southern Africa over the last 25 years. Without the continuing interest and thorough clinical patient examinations performed by Professor Sellars and Professor Beighton, this project could certainly not have been accomplished. This project was funded by the Leon Goldman Fund, administered by Professor Sellars to whom I am most grateful.

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FIGURES AND ILLUSTRATIONS

TABLES

ABBREVIATIONS

A	adenosine
aa	amino acid
$AgNO_3$	silver nitrate
Akp3	mouse alkaline phophatase gene
ALPP	human placental alkaline phosphatase gene
Ap	ampicillin
ASO	allele specific oligonucleotide
bp	base pair
C	cytosine
cDNA	complementary DNA
cM	centimorgan
dATP	deoxyadenosine triphosphate
dH_20	distilled water
DMSO	dimethylsulphoxide
EDTA	ethylenediaminetetra-acetic acid
EP	endocochlear potential
EtOH	ethanol
G	guanine
GCG	Genetics Computer Group
H_3PO_4	orthophosphoric acid
HuP2	Human paired box 2 gene
IPTG	isopropyl-ß-thiogalactopyranoside
KAc	potassium acetate
KCl	potassium chloride
LB	Luria broth
LiCl	lithium chloride
LOD	log of the odds of distance
Lp	Loop tail mouse mutant
$MgCl_2$	magnesium chloride
mi	Microphthalmia mouse mutant
min	minute
MMLV	Moloney murine leukaemia virus
$NaCO_3$	sodium carbonate
NAD	non-apparent dystopia
NaOH	sodium hydroxide

PAX3	Human paired box gene 3
PCR	polymerase chain reaction
PIC	polymorphism information content
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	room temperature
SA	Southern Africa
SDS	sodium dodecyl sulphate
Sp	Splotch mouse mutant
Sp^{2H}	radiation-induced Splotch allele
Sp^{d}	Splotch delayed allele
Sp^{r}	Splotch retarded allele
SSCP	single stranded conformational polymorphism
T	thymine
TBE	tris-borate-EDTA buffer
UV	ultraviolet
W	Ward index value
WS	Waardenburg syndrome
WS I	Waardenburg syndrome type I
WS II	Waardenburg syndrome type II
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
τMTMS	τ-methacryloxypropyltrimethoxysilane
Θ	recombination value

PLAN OF THE THESIS

Chapter 1 deals with the background to Waardenburg syndrome (WS) beginning with the perceptions of genetic deafness in the early 1800's and the original delineation of WS. The clinical characteristics are discussed with emphasis on the variability of the phenotype. The molecular background, which forms the basis for this dissertation, deals with the search for the genes involved in the pathogenesis of WS. As developmental abnormalities are fundamental to the manifestation of the phenotype, the embryonic environment is discussed to put into context the highly ordered events which are disrupted in WS.

The situation with regard to WS in Southern Africa (SA) is dealt with in chapter 2. The identification and clinical analysis of the families, which form part of the molecular investigation, are described.

Linkage analysis was necessary to distinguish which families were appropriate for mutation screening of the *PAX3* gene and to investigate the possibility of other gene loci. Chapter 3 describes the methodology of the linkage procedure and the results of this analysis.

The methods used for the analysis of the mutations underlying WS in the SA families are described in chapter 4. The elucidation of these mutations was the primary aim of this study. The results and discussion of the mutation analysis are presented in chapter 5.

The conclusions for each aspect of the study are discussed in detail at the end of each chapter. Chapter 6 provides a final comment on the study as a whole.

CHAPTER 1

INTRODUCTION

CHAPTER 1. INTRODUCTION

1.1. THE HISTORY OF INHERITED DEAFNESS

Until the middle of the 19th century heredity was not generally recognised as a cause of deafness. Information acquired from the Irish census of 1857 enabled William Wilde to observe that "the transmission of diseases by hereditary taint or family peculiarity...is very manifest among the deaf and dumb". In addition he concluded that the occurrence of deafness was comparatively higher when consanguineous marriage had taken place, and that there was an excess of males among congenitally deaf patients. He had thus unknowingly identified the three forms of inheritance of this disease - autosomal dominant, recessive and X-linked.

In 1882 Politzer wrote: "The most frequent causes of congenital deafness are: hereditary, including direct transmission from the parents as well as indirect transmission from forefathers, and marriage between blood relations" This statement, although not based on Wilde's work, confirmed the acceptance of the concept of hereditary deafness.

Some of the consequences of these findings were not favorable to the well being of those with genetic deafness. In a memoir, privately published and then presented to the National Academy of Sciences in 1883, Alexander Bell tried to discourage marriage between deaf individuals because he feared that "the intermarriage of such persons would be calamitous to their offspring" and would produce a "defective race of human beings". At the close of the 19th century, Love refuted a number of Bell's assumptions after much research into the deaf population. He concluded that there were many forms of deafness and that they were transmitted by various modes of inheritance.

It is now well recognised that there are numerous genetics disorders in which hearing loss is a major feature. In some, additional clinical manifestations facilitate precise diagnosis, as in the Waardenburg syndrome, which forms the subject of this dissertation. In others, which are catergorised as undifferentiated or non-syndromic deafness, the only common feature in the phenotype is hearing loss; these disorders

are usually classified by their mode of inheritance. There is little doubt that there is considerable heterogeneity in many genetic deafness syndromes and it can be foreseen that the application of molecular biology will elucidate this situation.

1.2. THE CLINICAL ASPECTS OF WAARDENBURG SYNDROME

1.2.1. Delineation of the syndrome

A deaf and mute child was presented by Klein at a meeting of the Swiss Society of Genetics in 1947. Her clinical phenotype involved depigmentation of the hair and skin, blue irides and malformations of the arms. In 1948 Waardenburg presented a patient with similar clinical manifestations to the Ophthalmology Society of Utrecht. This 72 year old man had abnormally spaced palpebral fissures and pale blue irides, as well as deafness. The similarity of this patient to the girl presented by Klein prompted Waardenburg to undertake a diagnostic survey in several institutions for deaf-mutes in the Netherlands. Of a total of 1050 patients examined, 13 were found to have the condition. Family studies were initiated through these individuals and in 1951 Waardenburg published his findings and delineated this genetic disorder. He described the syndrome as a combination of six characteristics:

1. Lateral displacement of the medial canthi, combined with dystopia of the lacrimal puncta and blerpharophimosis;
2. prominent broad root of nose;
3. growing together of the eyebrows, with hypertrichosis of their medial portions;
4. white forelock, as a form of partial albinism;
5. partial or total heterochromia of the irides;
6. deaf-mutism or some degree of congenital deafness.

Waardenburg concluded that the syndrome was inherited as an autosomal dominant trait, with variable expressivity and incomplete penetrance.

1.2.2. The variable phenotype

Waardenburg syndrome (WS) has been described in populations all over the world and occurs in about 2 - 3% of all children attending special schools for the deaf. The question of heterogeneity was addressed when Arias (1971) differentiated two separate forms of WS, distinguishable by the presence (type I) or absence (type II) of dystopia canthorum. To define dystopia quantitatively, the WARD index was formulated by Arias (1971) and Arias and Mota (1978). Using interocular measurements, as shown in Figure 1.1, this provided an objective biometric index.

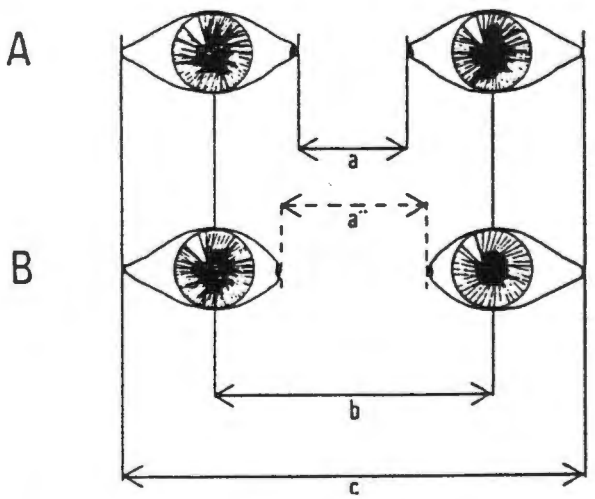


Figure 1.1. The anatomical basis for the interocular measurements used for the quantification of dystopia canthorum. A Normal situation; B dystopia canthorum (Taken from Hageman and Delleman, 1977).

The WARD index is calculated as follows:

$W = X + Y + a/b$

where $X = \frac{2a - (0.2199c + 3.909)}{c}$

and $Y = \frac{2a - (0.2497b + 3.909)}{b}$

a = inner canthal distance

b = interpupillary distance

c = outer canthal distance

Dystopia canthorum is said to be present when $W \geq 2.07$. Non-apparent dystopia (NAD) is diagnosed when the phenotype is clinically ambiguous, the patient is related to a typical WS I case, other characteristics of WS I are present and the W

index $\geq 1.87 < 2.07$. Non-penetrance occurs when the phenotype is as for NAD, but $W \leq 1.87$, ie. in the non-dystopic range.

By quantitating the dystopia canthorum with biometric parameters many "non penetrant" obligate carriers were shown to have non-apparent dystopia (Arias and Mota, 1978). Arias and Mota also demonstrated, using the W index, that the penetrance for dystopia canthorum is almost 100% for WS type I pedigrees.

The distinction between the two types of WS is important as the frequency of deafness for families with WS I is approximately 20%, whereas the frequency in WS II is 50%. This situation has considerable implications for genetic counselling.

Deafness in WS is not only variable in penetrance, but is also, as with the other characteristics, inconsistent in expression. Four types of hearing loss have been described from audiological studies of WS patients. Fisch (1959) reported a bilateral hearing loss with residual hearing in the low frequencies and a moderate unilateral hearing deficit with loss in the middle and lower frequencies. Bilateral severe hearing loss in all frequencies and unilateral profound deafness were described in WS patients by Hageman (1977). Intrafamilial variability in phenotypic expression further complicates genetic counselling.

The fifth characteristic described by Waardenburg, that of partial or total heterochromia irides, has been broadened to include hypoplastic, isochromic blue irides. Waardenburg omitted these individuals from his study, but his belief that these occular changes were part of the syndrome was substantiated when "Negroid" individuals with blue eyes and other signs of WS were reported (Di George et al., 1960). Since blue eyes are extremely rare in the black population, their frequent association with WS indicates that this feature is an expression of heterochromia. The unusually pale blue colour is due to hypoplasia and structural alterations of the iris stroma.

The white forelock can be represented by a blaze of white hair or by just a few strands. Greying of the eyebrows and premature greying of the hair may represent the equivalent of a white forelock in some individuals. Depigmentation of the skin, presenting as patchy leukoderma, has also been reported (Hageman and Delleman, 1977; Wang et al., 1981).

Skeletal dysplasia, with fused joints and underdeveloped muscles in the upper limbs, has been associated with the typical features of WS and is known as Klein-Waardenburg syndrome or WS III. Sheffer and Zlotogora (1992) confirmed that this very rare syndrome exists as a separate entity to WS and has an autosomal dominant pattern of inheritance. WS IV (Waardenburg-Shah syndrome) manifests as Hirschsprung disease with pigmentary anomalies. The white forelock and depigmentation of the skin are similar to WS I, but the patients lack dystopia canthorum, deafness and the irides have an unusually light brown colour.

Abnormalities which are occasionally associated with WS are cleft lip/palate, spina bifida (De Saxe et al., 1984), Hirschsprung disease (with the usual type I criteria) and myelomeningocele (Carezani-Gavin et al., 1992). Like the other WS characteristics, these anomalies are variable in expressivity and manifest in some affected individuals in a family and not in others. Omenn and McKusick (1979) reported 4 instances of Hirschsprung megacolon in patients with WS. The overall population frequency of Hirschsprung disease is 2 in 10 000, while that of WS is approximately 2 in 100 000 and it can therefore be infered that the risk of joint occurrence of these syndromes would be 4 in a billion people. The reports by Omenn and McKusick (1979) alone exceed this frequency and indicate that there is some significance in this association.

1.3. MOLECULAR BACKGROUND

1.3.1. The search for the WS locus

WS is a typical example of the application of reverse genetics, where the gene and its location have been identified without prior knowledge of the gene product. This strategy involves associating the mutant locus with a chromosome map position and localising this position by intensive genome analysis. The identification of chromosomal rearrangements expedites the mapping of the mutant locus, while homologies with the mouse genome facilitate in the fine mapping and cloning of the mutant gene.

Loose linkage was proposed by Simpson et al. (1974) between WS I and the ABO blood group loci at 9p34. Arias and Mota (1978) could not confirm this linkage and the locus was excluded by Foy et al. (1990). A Japanese child with a *de novo* inversion (2)(q35-q37.3) and WS I was reported by Ishikiryama et al. (1989). The

child presented with heterochromia irides, leukoderma, deafness and dystopia canthorum. As no other abnormalities were noted, it was assumed that the inversion was cytogenetically balanced. This report suggested that the inversion inactivated the gene for WS I and implied that the gene was situated at either of the breakpoints: 2q35 or 2q37.3. Ishkiriyama et al. (1993) later confirmed the location of the gene at 2q35.

After the report of the chromosome 2 inversion (Ishikiriyama et al., 1989), Foy et al. (1990) applied a marker, ALP-1 for alkaline phosphatase gene, at 2q37 for linkage analysis in families with WS I. Close linkage was demonstrated in 5 informative families, with the peak lod score being 4.76 at a recombination fraction of 0.023. The probability of this being a false positive lod score is lowered because the marker was not chosen randomly. The first reason for 2q being a candidate is that the breakpoints for the inversion described by Ishikiriyama are at this location. The second, is the similarity of the WS I phenotype to the Splotch mouse. The region containing the Splotch locus on mouse chromosome 1 is highly conserved with its human counterpart on 2q. The cloning of the gene, which is the eventual aim of a reverse genetic strategy, is made a great deal easier by the availability of a possible animal model. Linkage of WS I to 2q was confirmed by Asher and Friedman (1991) with multipoint linkage analysis of RFLP's; 5 markers producing a peak lod score of 6.67.

1.3.2. Identification of the gene using the Splotch mouse animal model.

The *Splotch* (*Sp*) mouse was proposed as a model for WS I by Asher and Friedman (1990). *Sp* mouse mutants exhibit abnormalities of the neural tube and neural crest during development. *Sp* alleles are autosomal dominant/semidominant mutants with varying degrees of severity. *Sp* and Sp^{d} arose spontaneously and have the mildest phenotypes; both give rise to incomplete closure of the neural tube in the homozygous form. Heterozygotes have characteristic white belly spots. Sp^{r}, from X-ray irradiation, is the most severe form of the mutation. Homozygotes die before implantation and heterozygotes have white spotting of the belly and growth retardation.

Sp^{r} has a cytogenetically detectable deletion on mouse chromosome 1. Molecular analysis of the region was carried out to determine the subchromosomal location of

the deletion. The proposed gene order on chromosome 1 is *Len2-Fn-Tp1-Vil-Des-Inha-Akp3-Acrg-Sag*. Epstein et al. (1991a) found that *Vil*, *Des*, *Inha* and *Akp3* were deleted in the Sp^r mutant. As Foy et al. (1990) had demonstrated linkage between WS I and the placental alkaline phosphatase locus on human chromosome 2q, the results of Epstein et al. (1991a), which included the alkaline phophatase *(Akp3)* gene in the Sp^r deletion, further implied that Splotch was the mouse model for WS I.

Goulding et al. (1991) mapped an embryonically expressed gene, *Pax 3*, to mouse chromosome 1 in the region of the Splotch locus. The structure of *Pax 3* identifies it as a member of a group of developmental control genes. These genes contain the conserved protein motifs: the paired domain, homeodomain and octapeptide, which indicate that they are involved in DNA binding. Comparison of the conserved genes of the paired box group shows that *Pax 3* is most homologous to the human gene, *HuP2*, with identical paired box and octapeptide domains. This observation indicates that *Pax 3* is the murine homologue of the *HuP2* gene.

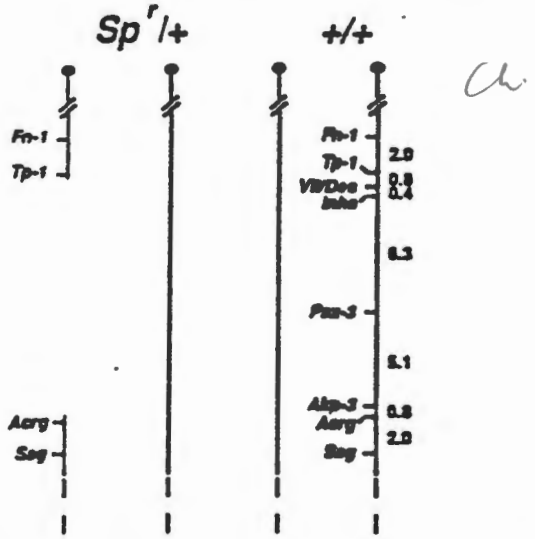


Figure 1.2. Chromosomal mapping of *Pax 3* on mouse chromosome 1. Intergene distances (cM) are marked indicated to the right of the wild type chromosome and the deleted segment in the Sp^r/+ heterozygote is shown to the left (Taken from Epstein et al., 1991b).

Using the Sp^r mutant as before, Epstein et al. (1991b) mapped the position of *Pax 3* with respect to the other loci assigned to this region of chromosome 1. The *Pax 3* gene was localised between *Inha* and *Akp3* as shown in Figure 1.2, confirming the tight linkage between Splotch and *Pax 3*.

Epstein et al. (1991b) examined the *Pax 3* gene in another Splotch mutant, Sp^{2H}, to investigate whether it was modified in any way. By Southern blot analysis it was determined that this mutant had an alteration in the *Pax 3* gene. Sequencing confirmed a 32 base pair deletion in the *Pax 3* homeodomain, interrupting the reading frame and creating a premature termination site. With the similarities in phenotype and the homologous map positions of Splotch and WS I, WS I was inferred to be the human homologue of the *Sp* mutant. Following this hypothesis, the human homologue of the *Pax 3* gene causing Splotch in mice, would be the gene causing WS I in humans, namely *HuP2*.

1.3.3. Mutations in the *HuP2* gene cause WS I

Mutations were identified in the *HuP2* gene by screening the coding exons (Tassabehji et al., 1992; Baldwin et al., 1992). Three exons had been identified and designated exons 2, 3 and 4, because of their homology to these exons in the mouse *Pax 3* gene. Primers were designed in the flanking introns for amplification of the exon sequences. Tassabehji et al. (1992) used heteroduplex analysis to test 17 unrelated WS individuals and found 6 to have alterations; 3 patients with variations in exon 2, 2 in exon 3 and 1 in exon 4. Three of these mutations were extended to include all family members. Mutant alleles from exon 2 of one of the families were cloned and sequenced and revealed an 18 bp deletion in the paired box region of *HuP2*. The translational reading frame remains unaltered, however, the loss of 6 amino acids in such a conserved region of the gene would probably cause a loss of protein function. Balwin et al. (1992) used the method of single stranded conformational polymorphism to identify the mutation in a large Brazilian kindred. A single base substitution, modifying the codon for proline to that for leucine, caused WS in this family. The mutation occurs in a hydrophobic region just upstream from the paired domain and is thought to influence the specificity of DNA binding in the protein. Other mutations have been described in WS type I families and, together with the methods for mutation analysis, are discussed in Chapter 3.

1.4. PAX 3 IN EMBYROGENESIS

Pax 3 is a member of a group of genes that regulate embryonic development. This family of homeobox containing genes is highly conserved from yeast to human, and control embryogenesis both temporally and spatially to ensure a highly ordered sequence of events imperative for normal development (Thorogood and Ferretti, 1992). Although Waardenburg and Splotch present a complex phenotype, this is to be expected since *Pax 3* is expressed early in embryogenesis and may regulate a number of diverse genes.

Sensorineural deafness is the most serious of the characteristics defining WS. It is necessary, therefore, to understand the development of the inner ear and the concurrent events during embryogenesis.

1.4.1. The development of the inner ear

The inner ear begins as the otic placode, a thickening of the ectoderm, which appears at approximately three weeks after conception. This tissue invaginates to form the otic pit which grows downwards into the mesoderm (Figure 1.3). The surface ectoderm then closes to form the otic vesicle (or otocyst).

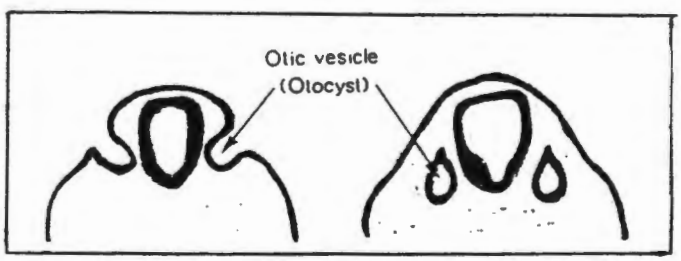


Figure 1.3. The development of the otic vesicle. (Taken from Beighton and Sellars, 1982)

The otic vesicle develops into two structures, the vestibular and cochlear parts. At six weeks the vestibular portion forms two pouches, which differentiate into the two vertical semicircular canals and the lateral canal. By nine weeks the vestibular component is well established, although the cochlear system is still undeveloped.

The cochlea grows from the sacculus as a spiral tube, completing two and a half turns by twelve weeks. A membranous labyrinth forms that separates the cochlea canal into three fluid-containing compartments: the scala vestibular, the scala media and the scala tympani. The epithelium of the cochlear duct differentiates into the tectoral membrane and the outer hair cells, while the organ of Corti begins to develop. The outer wall epithelium becomes glandular and underlying connective tissue undergoes vascularisation giving rise to the stria vascularis. The inner ear is fully formed twenty five weeks after conception.

As analysis of genes controlling development of the ear is not practical in humans and audiometric tests reflect only the type and severity of the hearing defect, it is a great advantage to acquire an animal model. This provides an ideal genetic background for studying normal development and the effects of individual gene mutations that may be involved in the pathogenesis of genetic deafness.

The mouse has provided a fitting model for analysing gene expression during development and many mouse mutants have been described with defects involving the ear. The inner ear of mice and humans is very similar, differing only in size, amount of bone surrounding the labyrinth and number of rows of hair cells (Steel, 1991).

Early investigation by Deol (1966) has shown that differentiation of the otic vesicle into a normal labyrinth depends on the influence of the neural tube. Two mouse mutants were studied, *Loop tail* (*Lp*) and *Splotch* (*Sp*), both having abnormalities of the neural tube. Homozygotes in these mutants die during middle of late gestation. Embryos of 10-13 days were examined and compared. In both mutants the neural tube is believed to be the site of gene action and the inner ear anomalies are secondary effects of the genes concerned.

1.4.2. *Pax 3* expression during embryogenesis

Goulding et al. (1991) studied the expression of *Pax 3* in the developing mouse embryo. By Northern blot analysis it was shown that *Pax 3* transcripts were only present from 9 -17 days *post coitum*. Detectable levels of *Pax 3* were not present in adult tissues, indicating a specific temporal expression.
The spatial distribution of expression in the developing mouse embryo was analysed by *in situ* hybridisation. The first transcripts were detected in the dorsal region of

the neural groove and in the neural tube. As the neural tube closes from the rostral to the caudal region, *Pax 3* expression moves from the neural groove to the dorsal neural tube and dorso-lateral somites, indicating a regulated temporal difference of expression in the posterior part of the embryo.

A multipotential population of cells, the neural crest, develops from the dorsal neural tube and migrates laterally to specific positions where the cells differentiate. (Figure 1.4.)

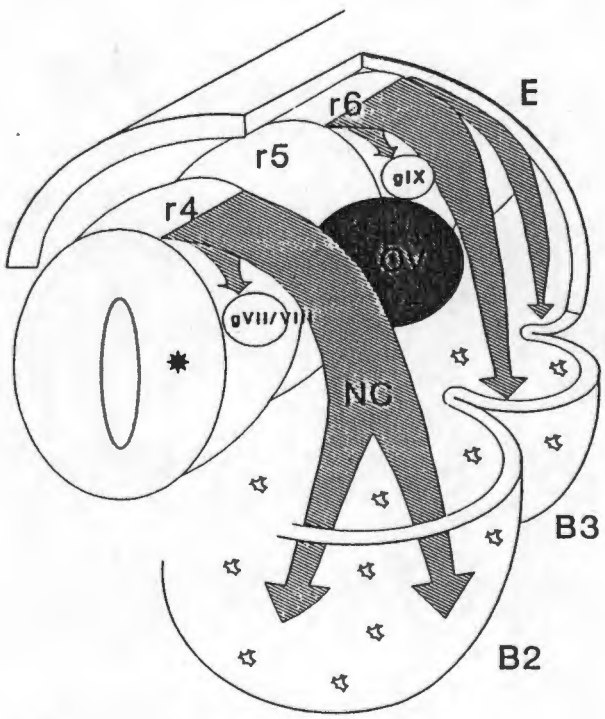


Figure 1.4. Migration of the neural crest cells (NC) from the neural tube of the segmented hindbrain into the forming branchial arches (B2, B3). The transected surface is marked with an asterisk. OV, otic vesicle; E, ectoderm covering dorsolateral surface of embryo; r4,r5,r6 rhombomere segmentations; gVII/VIII/IX are the cranial ganglia. (Taken from Thorogood and Ferretti, 1991)

Transplantation studies have shown that the invagination of the otic placode and the development of the otic vesicle are dependent on the close proximity of rhombomeres 5 and 6. The early morphogenesis of the vestibular and cochlea components is able to progress; spatial arrangement of these systems, however, requires the continued presence of the periotic mesychyme arising from the neural

crest (Noden and Van der Water, 1992). The neural crest is also fundamental in the formation of the middle ear ossicles and the outer ear as well as the positioning of the Eustachian tube and the tympanic membrane.

As well as being crucial to the formation of the ear, the neural crest contributes to most facial tissues, cranial ganglia, autonomic system ganglia, aortic arch arteries, pigment cells and other tissues. The phenotypic characteristics of WS can, therefore, be explained by a disruption in the migration of neural crest. The depigmentation in WS is due to a lack or absence of melanocytes in these areas (Ortonne, 1988), not the inability of the melanocytes to produce melanin. This indicates a deficiency in the ability of the melanoblasts to migrate to these regions. The occasional association of Hirschsprung disease and heart anomalies with WS may be explained by the failure of neural crest cell derivatives to uniformly populate the respective organs involved in these conditions. Kaplan and de Chaderevian (1988) reported a patient with deafness, patchy leukoderma, heterochromia irides, Hirschprung disease and pulmonary artery stenosis. Bowel biopsy analysis showed that ganglion cells were not absent from the bowel, but were abnormally distributed, as well as colonising regions usually devoid of ganglions. This is in contrast to the pathology of Hirschprung disease, where there is a completely aganglionic zone. Similarly tissue from the skin displayed abnormal distribution of melanocytes, which suggests disorderly migration of the neural crest.

Steel and Barkaway (1989) suggested another role for migratory melanocytes in the inner ear. They demonstrated that melanocyte-like cells in the stria vascularis were vital for the normal development of the stria and the production of an endocochlear potential (EP). In the *viable dominant spotting* mouse mutant, known to have a neural crest defect, there was an apparent absence of melanocytes in the stria as well as ultrastructural anomalies, which impaired the production of an EP by the stria. This observation could explain why mammals with spotting mutations often have associated deafness.

The expression of *Pax 3* is concurrent with the production and migration of the neural crest, initiating at the dorsal part of the neural tube and moving laterally. The homeodomain and the paired box sequence indicate that the *Pax 3* protein is involved in DNA binding, modulating transcription. The genes which it regulates are thus far unidentified.

CHAPTER 2

WAARDENBURG SYNDROME IN SOUTHERN AFRICA

CHAPTER 2. WAARDENBURG SYNDROME IN SOUTHERN AFRICA

2.1. INTRODUCTION

Since 1975 the departments of Human Genetics and Otolaryngology (University of Cape Town and Groote Schuur Hospital) have undertaken diagnostic surveys in an attempt to identify the cause of deafness in children attending special schools for hearing-impaired scholars in Southern Africa (Sellars et al., 1975; Sellars et al., 1976; Sellars et al., 1977; Sellars and Beighton, 1978; Sellars and Beighton, 1983a, 1983b; Beighton et al., 1987; Viljoen et al., 1988). Accurate diagnosis of the disorder underlying the deafness in these children is vitally important for prognosis and optimal management and, in the event of inherited deafness, for genetic counselling.

Of the 4452 scholars who were examined, a specific genetic syndrome could be diagnosed in 8% (Beighton et al., 1991). Waardenburg syndrome was found to be the most common form of congenital syndromic deafness manifesting in 2.7% of the total number of students. Although ethnic variation in prevalence was found, WS was present in children from all communities.

The families of several children attending special schools were investigated in order to provide the pedigree data and biological material which formed the subject of this study.

2.2. IDENTIFICATION OF SUBJECTS

The probands in this study were identified during aetiological surveys in special schools for the deaf and routine clinics by the departments of Human Genetics and Otorhinolaryngology. Each patient was examined clinically and assessed genealogically and, where appropriate, family members were visited and examined for WS. A positive diagnosis was made using the standard diagnostic criteria (Farrer et al., 1992; Winship and Beighton, 1992) as shown in Table 2.1. These criteria were identified and accepted by the Waardenburg Consortium (Farrer et al., 1992) for a collaborative study, in order to minimize misclassification of subjects. As part of the Consortium, the Southern African families were evaluated according to these guidelines.

For an unequivocal diagnosis an affected individual must have at least two major characteristics of WS or have one of the major criteria and two of the minor criteria. Rare associations were not used in establishing a diagnosis.

Many of the WS affected children attending special schools for the deaf and their families were re-examined by Winship and Beighton (1992), with strict attention paid to phenotypic characteristics. Measurements of periorbital structures were also taken at this time. The WARD index was calculated (Chapter 1, 1.2.2.) for the objective evaluation of dystopia canthorum in order to differentiate between WS type I and type II.

Blood samples were collected, with informed consent, in EDTA anticoagulant tubes. The blood was separated into 10 ml aliquots and DNA was extracted using the Biotest method (Biotest Diagnostics) or frozen at -70°C for later processing.

Table 2.1. Diagnostic criteria for WS

Major:

1. Sensorineural deafness.
2. Iris pigmentary abnormality:
    a. Two eyes of different colour
    b. Iris bicolour/segmental heterochromia
    c. Characteristic sapphire blue irides
3. Hair hypopigmentation
    a. White forelock
    b. White body hair
4. Dystopia canthorum*
5. First degree relative previously diagnosed with WS

Minor:

1. Congenital leukoderma
2. Synophrys
3. Broad nasal root
4. Hypoplasia of alae nasi
5. Premature greying of hair

Rare:

1. Hirschprung disease
2. Sprengel anomaly
3. Spina bifida
4. Cleft lip/palate
5. Limb defects#
6. Congenital heart anomalies
7. Abnormalities of vestibular function
8. Broad square jaw
9. Low anterior hair line.

* Indicates WS type I

Found in WS type III

2.3. RESULTS OF CLINICAL ANALYSIS

Ten families with WS were clinically assessed and consented to DNA studies. In total, 146 individuals agreed to provide blood specimens by venipuncture; of these persons, 72 were affected with WS. The pedigrees of the families are illustrated in Figure 2.1.

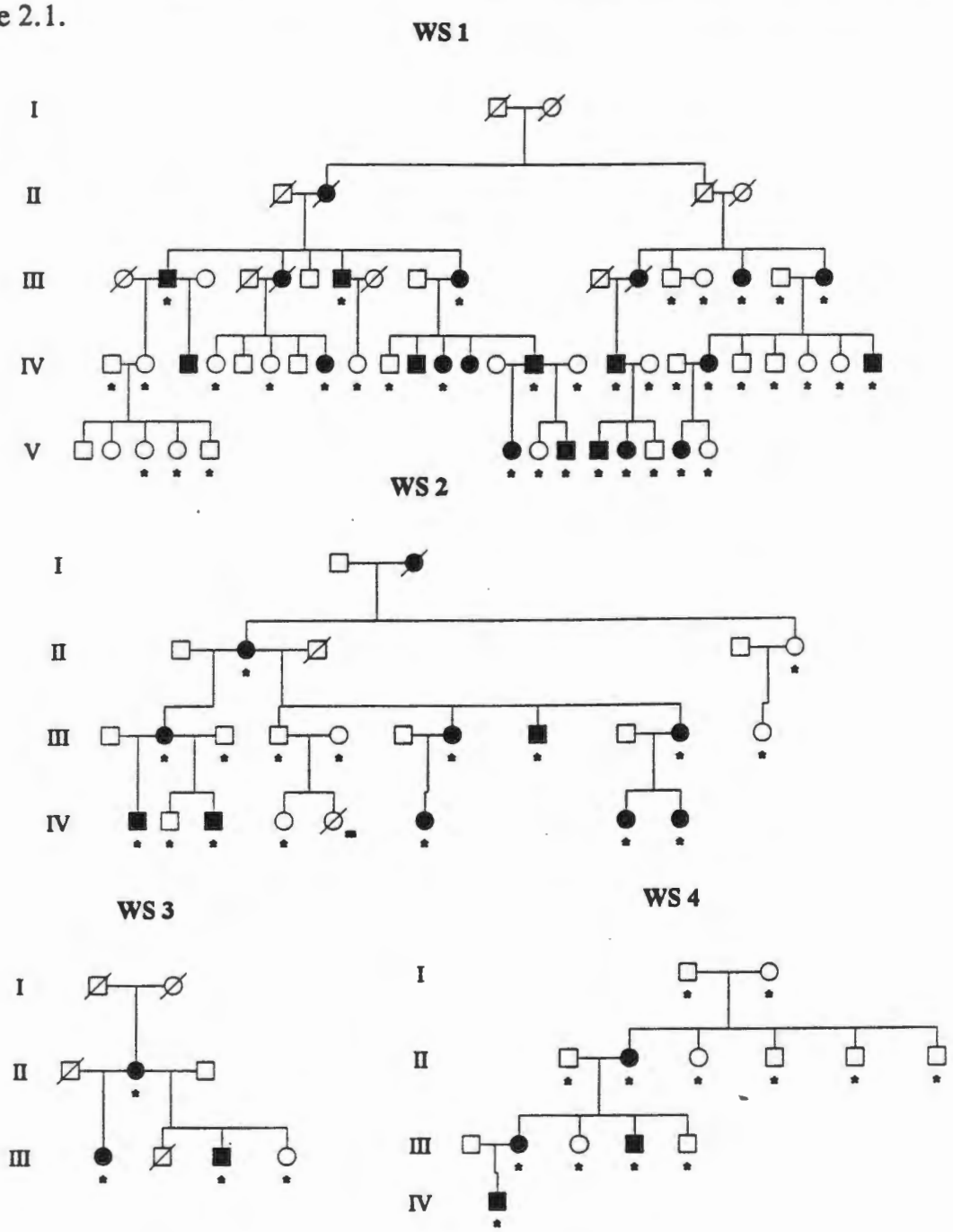


Figure 2.1. The pedigrees of the WS families in this study. □ = male, ○ = female, ■ affected male, ● affected female. An asterisk marks the individuals from whom blood was taken.

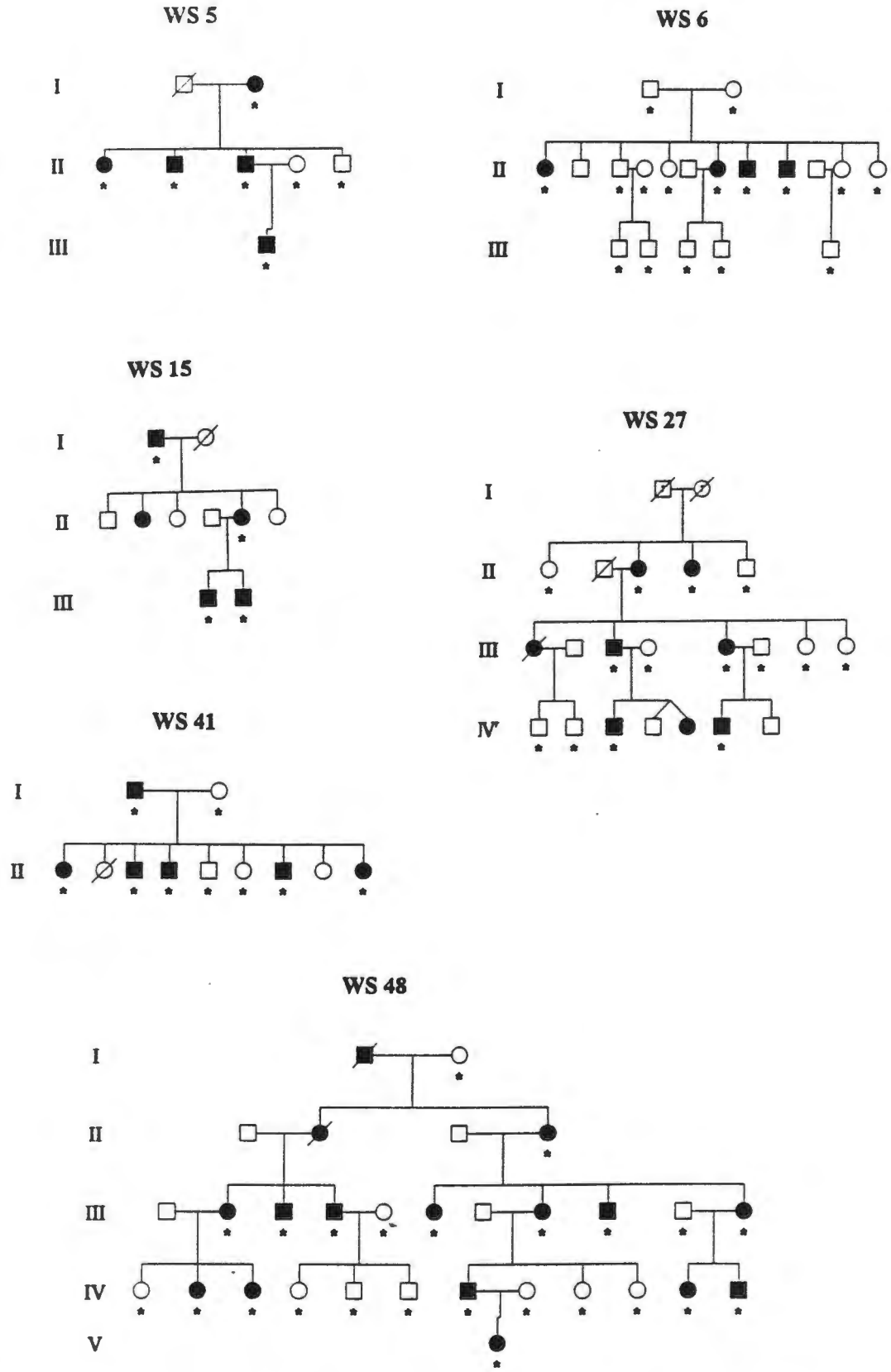


Figure 2.1. The pedigrees of the WS families in this study. □ = male, ○ = female, ■ affected male, ● affected female. An asterisk marks the individuals from whom blood was taken.

The characteristic stigmata of WS were present in these families with intrafamilial variability in their expression. The major features are depicted in Figures 2.2. - 2.5. A description of the phenotypic manifestations of the affected individuals in each family is presented in Table 2.2. A further 37 affected individuals, with no family history of WS or whose family history was unavailable, were also examined and agreed to take part in the molecular investigation.

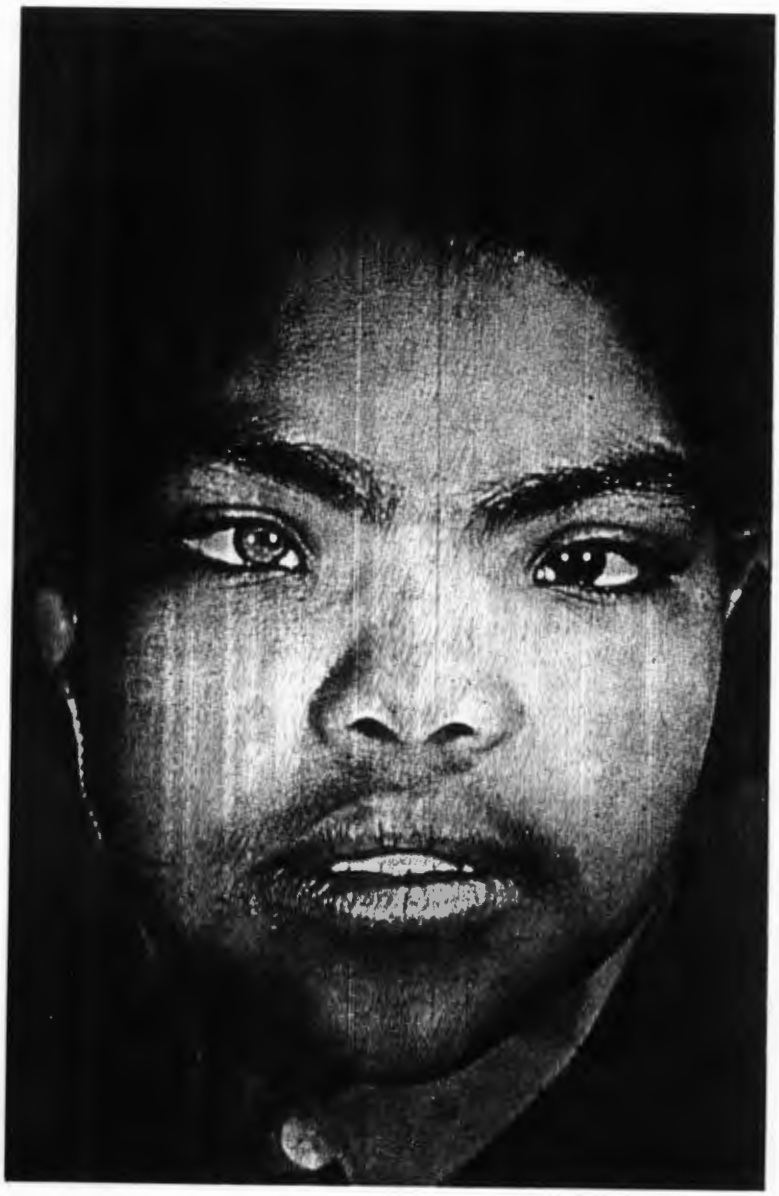


Figure 2.2. WS in a girl with profound perceptive deafness, heterochromia irides, synophrys and dystopia canthorum. (With permission, Beighton and Sellars, 1983).

Figure 2.3. Dystopia canthorum together with a white forelock, depigmentation of the medial portion of the eyebrows and patchy leukoderma. With permission, Beighton and Sellars, 1983).

Figure 2.4. Intrafamilial variability of the WS phenotype (pedigree WS 2). (With permission, Winship and Beighton, 1992)

Figure 2.5. Partial heterochromia and unusual skin depigmentation in a profoundly deaf boy. Dystopia canthorum is absent.

Table 2.2. Phenotypic characteristics of affected family members

Pedigee	Sensorineural deafness	Dystopia canthorum	Iris pigmentary anomalies	Hair hypo-pigmentation	Leukoderma	Synophrys	Early greying
Family 1							
III-2	-	+	+	+	+	+	+
III-7	-	+	-	+	-	+	-
III-10	-	+	-	+	-	-	+
III-17	-	+	-	+	-	-	+
IV-8	-	+	+	-	-	+	+
IV-17	-	+	-	-	-	+	+
IV-20	-	+	-	-	-	+	+
IV-25	-	+	-	-	-	+	+
V-6	-	+	-	-	-	+	-
V-9	-	+	+	+	+	+	-
V-10	-	+	+	-	-	+	-
V-12	-	+	+	+	-	+	-
Family 2							
II-2	-	+	+	+	-	+	+
III-2	+	+	+	-	-	+	+
III-7	+	+	+	-	+	+	-
III-8	+	+	+	+	-	+	-
III-10	-	+	+	+	-	+	-
IV-1	+	+	-	-	-	+	-
IV-3	+	+	+	-	+	+	-
IV-6	-	+	-	-	-	+	-
IV-7	-	+	-	-	-	+	-
IV-8	-	+	+	-	-	+	-

Pedigee	Sensorineural deafness	Dystopia canthorum	Iris pigmentary anomalies	Hair hypo-pigmentation	Leukoderma	Synophrys	Early greying
Family 3							
II-2	-	+	+	+	+	+	-
III-1	-	+	+	+	-	+	+
III-3	-	+	+	+	+	+	-
Family 4							
II-2	-	+	+	+	-	+	+
III-2	-	+	-	-	-	+	+
III-4	+	+	+	-	+	+	-
IV-1	-	+	-	-	-	+	-
Family 5							
I-2	-	+	-	-	+	+	+
II-1	-	+	+	+	-	+	+
II-2	-	+	-	-	-	+	+
II-3	-	+	-	-	-	+	+
III-1	-	+	+	+	+	+	-

Pedigee	Sensorineural deafness	Dystopia canthorum	Iris pigmentary anomalies	Hair hypo-pigmentation	Leukoderma	Synophrys	Early greying
Family 6							
II-1	+	-	+	+	+	+	-
II-7	+	-	+	+	+	+	-
II-8	+	-	+	+	+	+	-
II-9	+	-	+	+	+	+	-
Family 15							
I-1	-	+	+	-	-	-	-
II-5	-	-	-	-	-	-	-
III-1	+	+	+	-	-	+	-
III-2	+	+	+	-	+	+	-
Family 27							
II-3	-	-	-	-	-	-	+
II-4	+	-	-	+	-	-	+
III-3	+	-	+	+	+	-	-
III-5	+	-	+	+	+	-	-
IV-3	-	-	-	+	+	+	-
IV-5	+	-	+	+	-	+	-
IV-6	+	-	+	-	-	-	-

Pedigee	Sensorineural deafness	Dystopia canthorum	Iris pigmentary anomalies	Hair hypo-pigmentation	Leukoderma	Synophrys	Early greying
Family 41							
I-1		+	-	+	-	+	- -
II-1	+	-	+	-	+	-	-
II-3	+	-	+	-	+	-	-
II-4	+	-	-	-	+	-	-
II-7	+	-	+	-	+	-	-
II-9	+	-	-	-	+	-	-
Family 48							
II-4	-	+	-	+	-	-	-
III-3	-	+	+	+	-	+	+
III-4	-	+	+	-	+	+	+
III-6	-	+	-	+	-	-	+
III-9	+	+	+	+	-	-	-
III-11	-	+	-	-	-	+	+
IV-3	+	+	+				
IV-7	+	+	+	-	+	+	+
IV-12	+	+	+	-	+	-	+
V-1	-	+	+	-	-	-	-

From the table it is evident that 7 of the families can be designated as WS type I, while 3 kindreds were in the type II category (WS 6, 27 and 41). Where measurements were taken, the WARD index was calculated and it is noteworthy that subjective assessment was consistently in keeping with these indices. With the exception of one family member in WS family 15 (II-5), all affected individuals within the type I WS families had dystopia canthorum. The range of the WARD index in each family is shown in Figure 2.6.

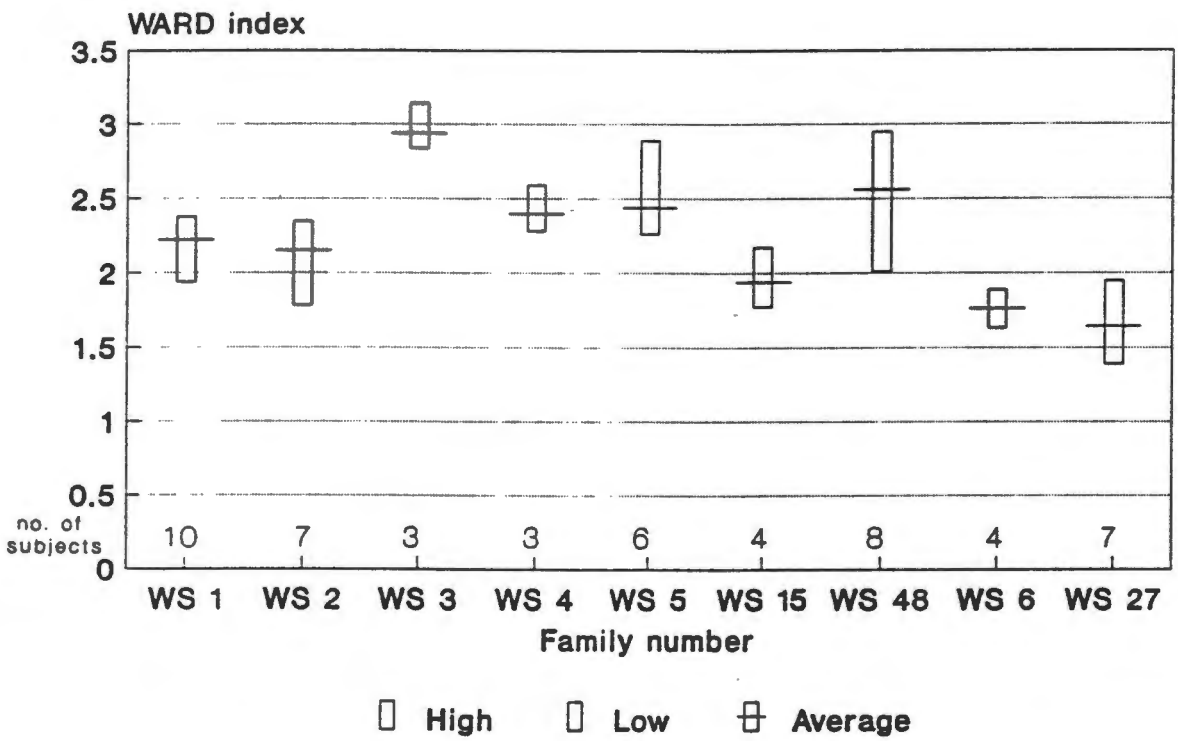


Figure 2.6. The range and mean values of WARD indices of affected individuals from each of the families.

Some persons with sporadic WS proved difficult to classify into type I and type II. In conjunction with the WARD index, 15 individuals could be distinguished as having WS type I and 13 as having WS type II. The categorisation of the remaining 9 persons was equivocal.

2.4. DISCUSSION OF CLINICAL ANALYSIS

With one exception, the pedigrees of the families with WS showed an autosomal dominant pattern of inheritance. In Family 6, however, the parents in generation I were clinically normal and none of the affected persons had produced offspring with the condition. This inheritance pattern could be explained by non-penetrance or autosomal recessive inheritance. Autosomal recessive inheritance is, however, not a recognised mode of transmission of WS and therefore it is unlikely. Another explanation could be that of germ-line mosaicism in one of the parents. This would account for the number of affected siblings in generation II. Germ-line mosaicism has previously been reported in WS by Kapur and Karam (1991).

The families with WS I conformed to the expected percentage of affected members with hearing impairment (10 out of 53) at 19%. Disparate figures were obtained for the type II families, with a high frequency of deafness at 88%; the accepted figure being only 50%.

Intrafamilial phenotypic variation was present as expected, with the exception of two of the type II families. The manifestations of WS in families 6 and 41 were strikingly uniform: all four affected siblings of WS family 6 had sapphire-blue eyes, white forelock, patchy depigmentation of the skin of the wrists and fore arms and profound deafness; all affected members of WS family 41 displayed unusual pigmentary changes in the skin, profound deafness and abnormalities of iris colour.

Rare associations with WS were encountered in only one family, WS 48. Hirschprung disease was present in two family members (III-4, IV-3) and a bilateral cleft palate in another. A small branch of this family had consistent heterochromia, with the right eye blue, and unilateral deafness on the right side. No correlation between heterochromia and deafness could be made in the rest of the family.

The WARD index was used to distinguish between families with WS type I and type II. Interocular distances, however, vary in affected and unaffected individuals and ethnic variation is also of importance. For this reason, it has been suggested that it is advisable to note the trend of the indices of affected relatives, rather than to assess only one individual (Farrer et al., In press). All type I families in the Southern African study had a mean WARD index ≥ 2.00, whereas the type II mean was ≤ 1.76. One member of WS family 15 seemed non-penetrant for all stigmata

of WS, as her father and two sons were clearly affected. When her index was excluded from the mean in this family, the average rose from 1.94 to 2.00. This change in the average is not significant, however it is prudent to exclude the W index of the non-penetrant individual from the calculation of the mean in this family.

Unequivocal diagnosis and the differentiation between WS type I and type II are not only important for genetic counselling, but also vital for the strategy appropriate for molecular testing and the analysis of the results.

CHAPTER 3

LINKAGE ANALYSIS

CHAPTER 3. LINKAGE ANALYSIS

3.1. INTRODUCTION

A gene for Waardenburg syndrome was localised to chromosome 2q37 by Foy et al. (1990), who obtained close linkage to the placental alkaline phosphatase (ALPP) locus at this position. The probe ALP-1 was used to detect an *Rsa I* polymorphism which, in five informative families, gave a combined lod score maximizing at 4.767 at a recombination fraction of 0.023. Four RFLP markers on 2q, including ALP-1, were used in a five-point linkage analysis by Asher et al. (1991). This analysis established significant linkage in a single WS I family, with a lod score of 6.667. By pooling the linkage results of 44 WS kindreds from different countries, the WS Consortium refined the location of the WS on 2q (Farrer et al., 1992). This collaborative study revealed that there was genetic heterogeneity in WS, with only 45% of all the families in the sample linked to the locus on 2q. The paired box gene, *HuP2* (now known as *PAX3*) at 2q35-37, was implicated in causing WS I when pathogenic mutations were identified in kindreds with this form of the disorder (Tassabehji et al., 1992; Baldwin et al., 1992).

To establish which of the South African families were linked to *PAX3*, linkage analysis was performed using microsatellite markers flanking the *PAX3* gene (Farrer et al., In press) The CA repeat markers that were selected have a higher polymorphism information content (PIC) than the RFLP-based systems used previously, as well as being in close proximity to the *PAX3* gene. Families that were unlinked to *PAX3* did not warrant mutation screening of this gene.

Those families that were not linked to *PAX3* were tested with other polymorphic markers surrounding three possible candidate loci that had not previously been reported. Markers on 10q were analysed because an interstitial deletion (10q11.2-q22.1) had been described in a boy with bright blue irides and other congenital abnormalities (Zenger-Hain et al., 1993). Furthermore, this region is close to the *RET* gene which, in the mouse, maps close to the microphthalmia (*mi*) locus. The *mi* mouse mutant was suggested to be a model for WS on the basis of phenotypic similarity. (Asher and Friedman, 1990).

The human homologue of the mouse *mi* gene, MITF, was mapped to 3p12.3-p14.1 (Tachibana et al., 1994). Hughes et al. (1994) established linkage of two WS type II families to markers on chromosome 3 in the region of the *MITF* gene. Markers at this locus, therefore, were also included in the linkage analysis of the WS type II families in this study.

A report of a chromosomal translocation t(7;20)(q22.1-q13.13) in a male infant with WS type II (Hood et al., 1989) led to the investigation of markers at the breakpoints of the translocation on chromosomes 7 and 20.

3.2. METHODS

Genotypes were interpreted from the allele amplification of highly polymorphic CA repeat polymorphisms in the region under investigation. PCR amplification of the CA repeat fragments was carried out in 25 μl volume reactions containing: 25 pmol of each primer, 200 ng DNA, 200 μM dNTP's (Boehringer Mannheim), 1 unit *Taq* polymerase (BRL) and 1 μCi α-^{32}P dCTP (Amersham) in a 1 x reaction buffer. Most of the reactions were optimized using the 10 x standard reaction buffer containing 500 mM KCl, 100 mM Tris (pH 8.4) and 15 mM $MgCl_2$. Some primers required the addition of 0.01 % gelatin, 0.1 % triton or 0.4 % ß-mercaptoethanol. An overlay of liquid paraffin was added to each sample to prevent evaporation during the reaction. Each PCR reaction was subjected to thirty cycles of amplification in a thermalcycler (Hybaid Omnigene).

Three microlitres of formamide loading dye (80% formamide, 10 mM EDTA, 0.02% bromophenol blue and 0.02 % xylene cyanol) was added to 2 μl of the amplified product and denatured on a heating block at 97°C for 3 min. The samples were placed on ice before loading on 6 % polyacrylamide gels, containing 5.7 % acrylamide, 0.3 % bisacrylamide, 7.65 M urea and 1 x TBE (0.089 M Tris, 0.089 M Boric acid, 0.002 M EDTA). The gels were electrophoresed at 60 Watts in a 1 x TBE running buffer, using the marker dyes in the loading dye to determine at which point the repeat fragments would be fully resolved. The gel was transferred onto Whatmann 3MM paper and dried on a Hoeffer Slab Gel Dryer at 80°C for 1 hour. Overnight exposure of the dried gel to an X-ray film (Agfa Curix) occured at -70°C. The X-ray film was developed in Cronex developer and fix solutions (Protea) and the genotypes ascertained.

The markers and PCR conditions used for linkage analysis in the region of the *PAX3* gene are set out in Table 3.1.

Table 3.1. Microsatellite markers used for linkage analysis on chromosome 2

Marker	Location	No. of alleles	PIC	PCR conditions	References
D2S102	2q	11	.87	94°C 30" 54°C 30" 72°C 30"	Genome Data base
D2S211	2q34-q37	8	.70	94°C 1' 50°C 1' 72°C 1'	Barber et al., 1993
PAX3	2q35-q37	9	.80	94°C 1' 55°C 1' 72°C 1'	Wilcox et al., 1992, Morrell personal communication

The markers that were used for linkage to loci other than *PAX3* are presented in Table 3.2.

Table 3.2. Markers used for linkage analysis in families that were not linked to *PAX3*

Marker	Location	PIC	Source
D10S199	10q11	0.84	Genethon
D10S220	10q11	0.83	Genethon
D7S479	7q21.3-q22	0.83	Research Genetics
D7S501	7q31	0.81	Research Genetics
PLC1	20q12-q13.1		Research Genetics
RPN2	20q12-q13.1		Research Genetics
D20S25	20q13		Research Genetics
D20S17	20q		Research Genetics
D20S120	20q	0.85	Research Genetics
D20S102	20q13-ter	0.64	Research Genetics
D20S52	20q		Research Genetics
D3S1217	3p14		Hughes et al., 1994
D3S1210	3p13		Hughes et al., 1994
D3S1284	3p13-p14	0.74	Hughes et al., 1994
D3S1285	3p14	0.72	Hughes et al., 1994

The PCR conditions were optimised for each marker individually and the reactions carried out as described for the chromosome 2 markers. The majority of markers functioned optimally at the cycling conditions of: 94°C for 1', 56°C for 1' and 72°C for 2'. D20S25, however, required an annealing temperature of 60°C.

Two point linkage analysis was performed using the MLINK Ver. 5.03 package (Lathrop and Lalouel, 1984). The lod scores between the markers and the WS loci were calculated using recombination fractions from 0.00 to 0.40. The penetrance was set at 93% with a gene frequency of 0.0001.

3.3. ANALYSIS OF LINKAGE

The results of the linkage analysis between the WS phenotype and markers on chromosome 2 are shown in Table 3.3. Two families (WS 1 and WS 48) were clearly linked to *PAX3*, with lod scores of 7.76 and 3.37, repectively, at $\Theta = 0.00$. A lod score of 1.0 or greater, maximising at $\Theta = 0.00$, was considered enough to indicate possible linkage in WS families 2, 4 and 5. In WS 4, PAX3 was uniformative due to the homozygosity of the alleles in an affected matriarch (II-2 on pedigree) and therefore D2S211 is a better indication of the probability of linkage. A low positive lod score was obtained at $\Theta = 0.00$ in 2 families (WS 3 and WS 15). These families could not be excluded from being linked to PAX3. On visual examination of the genotypes in WS 3 and WS 15 no obvious recombinants were observed between the WS phenotype and the marker PAX3. The lod scores obtained, therefore, were due to the small number of meioses in each of these families. In WS 6 one recombinant was observed; the lod score peaked at $\Theta = 0.10$ for the marker PAX3 and at $\Theta = 0.05$ for the other two markers. In the 2 remaining families, lod scores of less than -2.00, the accepted value for significant exclusion (Lester et al., 1990), at $\Theta = 0.00$, indicated that these families were not linked to PAX3.

Table 3.3. Pairwise lod scores between WS and markers in the region of *PAX3*.

Family	Locus	Recombination fraction (Θ) 0.00	0.05	0.1	0.2	0.3	0.4
WS 1	PAX3	7.76	7.10	6.41	4.93	3.30	1.54
WS 2	PAX3	1.15	1.05	0.94	0.72	0.48	0.24
	D2S102	1.20	1.24	1.20	0.99	0.67	0.29
	D2S211	-3.3	0.22	0.46	0.53	0.39	0.19
WS 3	PAX3	0.57	0.51	0.44	0.30	0.16	0.05
	D2S102	0.57	0.51	0.44	0.30	0.16	0.05
	D2S211	0.30	0.26	0.21	0.13	0.06	0.02
WS 4	PAX3	0.46	0.38	0.31	0.18	0.08	0.02
	D2S102	0.38	0.33	0.29	0.22	0.14	0.05
	D2S211	1.28	1.12	0.96	0.64	0.33	0.09
WS 5	PAX3	1.17	1.06	0.95	0.70	0.43	0.17
	D2S102	0.69	0.61	0.52	0.34	0.18	0.06
	D2S211	0.87	0.76	0.65	0.41	0.19	0.04
WS 6	PAX3	0.30	0.40	0.42	0.37	0.23	0.08
	D2S102	0.84	0.89	0.85	0.66	0.38	0.11
	D2S211	1.11	1.14	1.08	0.84	0.50	0.16
WS 15	PAX3	0.04	0.04	0.03	0.03	0.02	0.01
	D2S102	0.30	0.28	0.26	0.20	0.15	0.08
WS 27	PAX3	-6.04	-2.18	-1.57	-0.88	-0.47	-0.19
	D2S102	-5.95	-1.65	-0.99	-0.36	-0.08	0.02
	D2S211	-5.0	-1.18	-0.64	-0.17	-0.01	-0.01
WS 41	PAX3	-6.30	-1.24	-0.72	-0.28	-0.10	-0.02
WS 48	PAX3	3.97	3.62	3.26	2.52	1.74	0.90

The families that were unlinked at the *PAX3* locus were analysed for linkage using markers at other possible candidate loci. The resulting lod scores are presented in Table 3.4.

Table 3.4. Linkage analysis of families that were not linked to *PAX3*

Family	Marker	Recombination fraction (Θ) 0.00	0.05	0.10	0.20	0.30	0.40
WS 6	D7S479	-5.67	-1.10	-0.63	-0.23	-0.08	-0.02
	D7S501	-6.24	-1.39	-0.86	-0.35	-0.13	-0.03
	PLC1	-8.05	-1.39	-0.77	-0.26	-0.07	-0.01
	RPN2	-0.96	-0.67	-0.44	-0.17	-0.05	-0.01
	D20S17	-0.02	-0.04	-0.06	-0.09	-0.07	-0.03
	D3S1210	-4.48	-1.36	-0.74	-0.22	-0.05	-0.00
	D3S1217	-1.05	-0.73	-0.49	-0.20	-0.06	-0.01
WS 27	D7S479	-4.85	-0.29	-0.05	0.12	0.14	0.08
	D7S501	-4.63	-1.84	-1.21	-0.62	-0.31	-0.12
	D10S199	-8.66	-1.78	-1.02	-0.40	-0.15	-0.03
	D10S220	0.18	0.19	0.18	0.13	0.06	0.01
	PLC1	-6.76	-2.22	-1.38	-0.53	-0.14	0.02
	RPN2	-6.85	-1.50	-0.79	-0.17	0.04	0.07
	D20S17	1.56	1.41	1.25	0.93	0.58	0.25
	D20S52	1.56	1.56	1.47	1.17	0.76	0.34
	D20S102	0.72	0.60	0.50	0.32	0.14	0.02
	D20S25	-3.13	0.55	0.76	0.74	0.48	0.14
	D3S1210	-4.05	-2.22	-1.60	-0.83	-0.37	-0.11
	D3S1217	-0.51	-0.24	-0.10	0.02	0.04	0.02
WS 41	D3S1217	1.81	1.65	1.49	1.13	0.72	0.28
	D3S1284	1.81	1.65	1.49	1.13	0.72	0.28

As linkage had already been established to a locus on chromosome 3 in WS type II families (Hughes et al., 1994), a lod score of 1.81 is indicative of possible linkage in WS family 41. The markers are fully informative for linkage in this family as depicted by the genotypes shown on the pedigree illustrated in Figure 3.1.

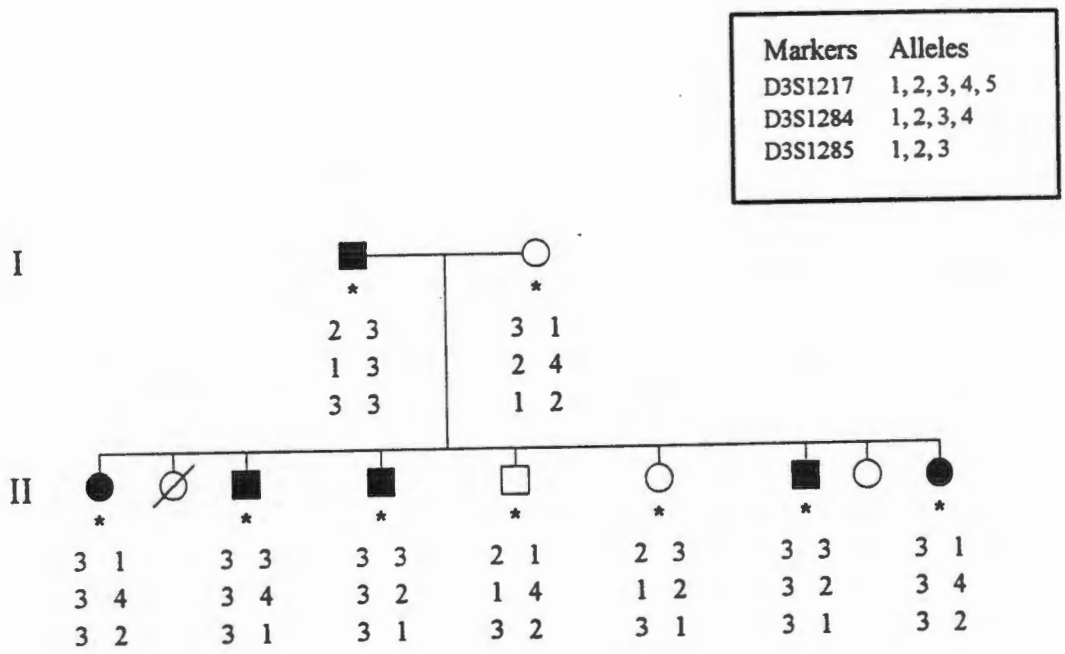


Figure 3.1. Pedigree of WS family 41 showing the genotypes of markers on chromosome 3. □ = male, ○ = female, ■ = affected male, ● = affected female.

The combination of genotypes of the markers analysed on chromosome 20 is illustrated on the pedigree shown in Figure 3.2. Haplotype analysis shows possible linkage of WS type II and this region on chromosome 20 in this family.

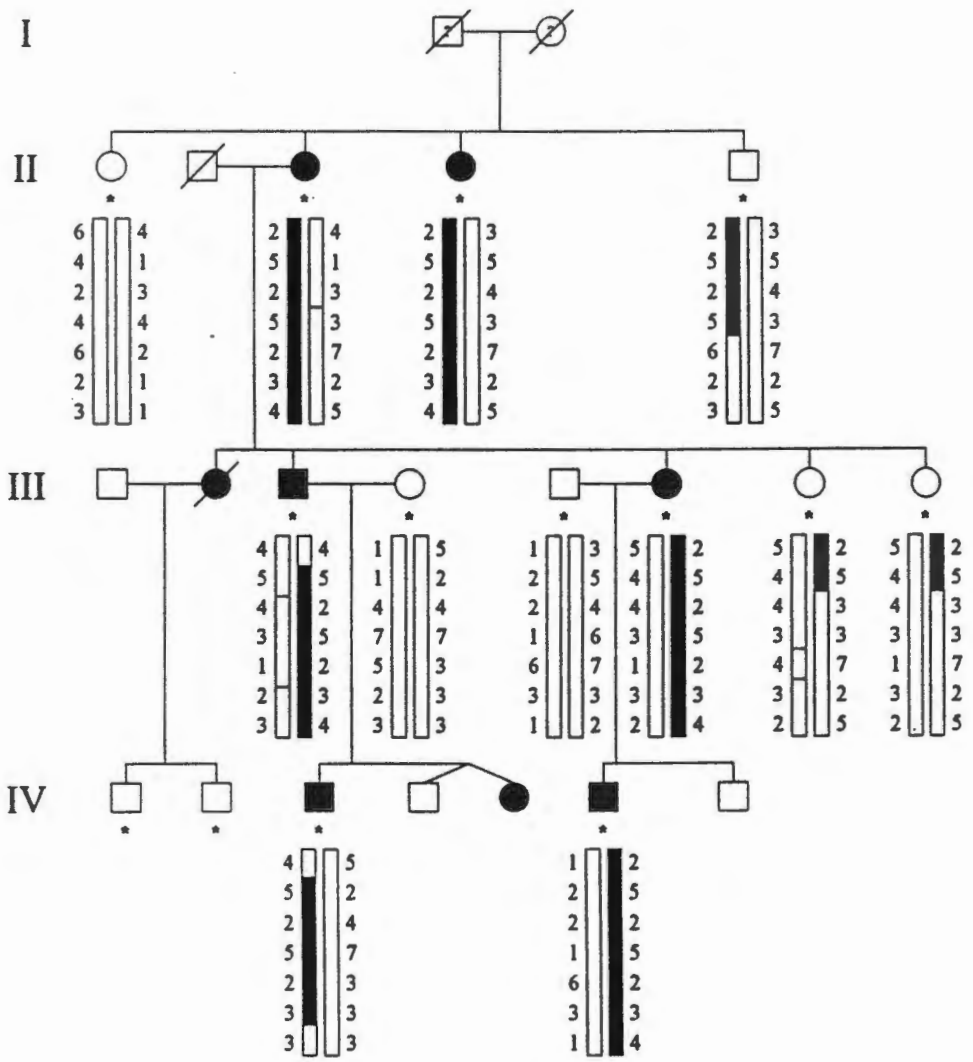


Figure 3.2. The pedigree of WS 27 with genotypes for each of the markers analysed on chromosome 20 Shaded bars represent haplotypes of the genotypes. The genotypes under each individual are in the following order: RPN2, PLC1, D20S17, D20S120, D20S52, D20S102, D20S25 (Personal communication, S. Marc, Genethon).

3.4. DISCUSSION OF LINKAGE ANALYSIS

Seven of the Southern African families, WS 1 - 6 and WS 27, formed part of a linkage study carried out by the Waardenburg Consortium to determine the precise proportion of families linked to PAX3. One of the conclusions of this investigation was that WS type I families were linked to PAX3, whereas type II families were unlinked (Farrer et al., 1994). Linkage results of the SA families in this study that were not included in the Consortium study are in accordance with their findings.

Of the seven WS type I families, five had adequate lod scores to be suggestive of linkage, while the remaining two were found to be equivocal. The SA WS type II families were found not to be linked to *PAX3* and therefore, only the WS type I families need be screened for mutations in *PAX3*. Sporadic individuals with dystopia canthorum could not be subjected to linkage analysis. However, the conclusive evidence of the disorder in WS type I families being due to alterations in the *PAX3* gene, warranted screening of sporadic WS I individuals for mutations within this gene.

A lod score of 1.81 at $\Theta = 0.00$ implied that WS family 41 may be linked to the type II locus on chromosome 3p. Because the family is small (9 members, 6 affected) with all meioses fully informative at the marker locus, a higher lod score was not attainable. The 2 other WS type II families in this study were unlinked at this position, confirming the suggestion by Hughes et al. (1994) that WS type II is genetically heterogeneous.

The disorder in family WS 27 may be linked to chromosome 20q13. This locus has been associated with WS II in the report of a chromosomal translocation involving this region (Hood et al., 1989). Pairwise lod scores are indicative of the probabilty of linkage and haplotype analysis strenghthens this proposal. Analysis of further markers in this region is required to confirm this hypothesis and to refine the position of the possible gene locus.

CHAPTER 4

MUTATION ANALYSIS

CHAPTER 4. MUTATION ANALYSIS

4.1. INTRODUCTION

4.1.1. The structure of the *PAX3* gene

The genes involved in the programming of early human development are thought to have evolved from primordial genes containing conserved protein domains. One of these genes, *PAX3* (or *HuP2*) was first described by Burri et al. (1989). The *PAX3* gene contains the paired domain, which is a highly conserved protein motif found in organisms from *Drosophila* to man. The coding sequence of the paired domain in *PAX3* was shown to be interrupted by two introns and contained an amphipathic α-helix in the first exon, a helix-turn-helix structure in the second and a conserved octapeptide in the third (Figure 4.1.). Goulding et al. (1991) described a homologous mouse gene, with identical exon-intron boundaries and protein domains. The sequence of this mouse gene (*Pax3*) was extended at the 3' end to include a paired type homeodomain and thus, based on the close similarity, the human counterpart was predicted to also contain this protein domain. The exons sequenced by Burri et al. (1989) were therefore numbered 2, 3 and 4 in keeping with the predicted sequence from the mouse homologue.

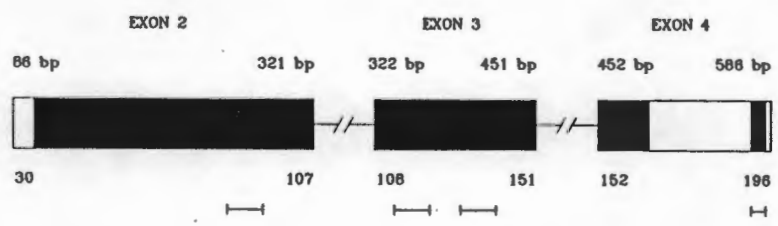


Figure 4.1. The structure of the paired domain of the *PAX3* gene The shaded region represents the paired domain and octapeptide, with the helices and octapeptide indicated below. The numbers shown above refer to the position of the exons with regard to the nucleic acid sequence, while the numbers below refer to the amino acid sequence.

Hoth et al. (1993) provided further sequence information and confirmed homology of the mouse and human gene structure at the 5' and 3' ends. Exon 1 proved to be small, while exon 5, contained the predicted homeodomain. In 1994 Tassabehji et al. determined, by systematic testing with multiple primers from the cDNA sequence, that three introns existed 3' of intron 4. The exon-intron boundaries were identified between exons 5, 6, 7 and 8. The homeodomain was split by intron 5 as illustrated in Figure 4.2.

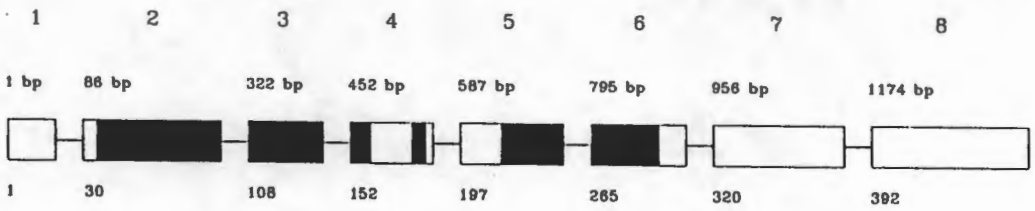


Figure 4.2. The structure of the *PAX3* gene with the 3' homeodomain. The shaded regions represent the paired domain, octapeptide and homeodomain. The numbers shown above refer to the position of the exons with regard to the nucleic acid sequence, while the numbers below refer to the amino acid sequence.

The determination of the exon-intron boundaries and the flanking intronic sequences was vitally important for mutation analysis of the *PAX3* gene in the pathogenesis of Waardenburg syndrome. Furthermore, the amino acid sequence predicted from the sequence of the DNA enabled the recognition of the functional domains of the proposed protein. Mutations, which do not lead to a truncated protein, may cause a loss of function as a result of their location in a functional domain.

4.1.2. Mutations in *PAX3*

PAX3 is expressed only during early human embryonic development making it impractical to analyse the protein or mRNA for alterations in size or structure in WS affected individuals. However, with the identification of the exon-intron

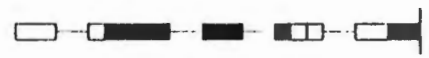
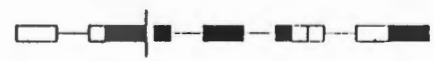
boundaries, the coding regions could be screened for mutations using genomic DNA. Amplified exons were screened by heteroduplex and single stranded conformational polymorphism (SSCP) analysis to identify the first mutations in *PAX3* causing WS (Tassabehji et al., 1992; Baldwin et al., 1992). SSCP analysis (Orita et al., 1989) is based on the differing mobility of single stranded DNA in a non-denaturing gel, as a result of its secondary structure or conformation. A mutation disrupting the secondary structure of a DNA fragment is clearly recognised by the difference in the banding pattern of the single strands. Heteroduplex analysis (White et al., 1992) involves the formation of heteroduplexes between normal and mutant alleles. The infidelity of binding retards the migration of the heteroduplex fragment on a non-denaturing gel.

Tassabehji et al. (1992) cloned and sequenced the mutant allele in one family showing an 18 bp deletion. Although this was an in-frame deletion, the 6 codons deleted include the last 3 amino acid codons for the amphipathic α-helix in a highly conserved region of exon 2.

Sequencing of the "SSCP" variant exon in a large affected Brazilian family showed a single-base substitution (Baldwin et al., 1992). The mutation occurred 17 amino acids (aa) into the paired domain, replacing a proline codon for a strongly hydrophobic leucine residue. This change may prevent DNA binding of the paired domain by altering the tertiary structure of the protein around the first α-helix. The phenotype in the family is variable and the frequency of deafness is unusually high (78 %).

The above in-frame mutations, although potentially pathogenic, leave most of the protein intact. A frameshift mutation, reported by Morrell et al. (1992), was caused by a deletion of 14 bp at the end of exon 2 in an Indonesian family with WS I. The shift in reading frame caused a premature termination codon 6 aa into exon 3. The truncated protein would not include the rest of the paired domain and lack the entire hŏmeodomain. The functional impairment of *PAX3*, as in the family reported by Baldwin et al. (1992), causes an unusually high penetrance (75 %) of sensorineural deafness in this type I family. WS mutations reported to date are listed in Table 4.1.

Table 4.1. Mutations in WS reported to date

Family	Exon	Amino acid change	Base change	Diagramatic representation	Reference
WS.055	2	Phe-Leu at 45	T-C		Tassabehji et al.,1994
BU 47	2	Asn-His at 47	A-C		Hoth et al.,1993
BU 26	2	Pro-Leu at 50	C-T		Baldwin et al.,1992
BU 35	2	Arg-Leu at 56	G-T		Hoth et al.,1993
BU 48	2	Val-Met at 60	G-A		Baldwin et al., GDB
WS.15	2	Gly-Ala at 81	G-C		Tassabehji et al.,1993
BU 5	2	Lys-Glu at 85	A-G		Baldwin et al., GDB
WS.009	2	Gly-Asp at 99	G-A		Tassabehji et al.,1994
BU 9	5	Arg-trm at 223	C-T		Baldwin et al., GDB
WS 4*	3/4	Splice at 151+1bp	G-A		Butt et al.,1994
WS.084	3/4	Splice at 151+1bp	G-T		Tassabehji et al.,1994
WS.003	3/4	Splice at 152-2bp	A-G		Tassabehji et al.,1994
WS.05	2	In frame	del 185-202		Tassabehji et al.,1992
WGM2	2	frameshift	del 266-279		Morrell et al.,1992
WS.06	2	frameshift	del 288		Tassabehji et al.,1993
WS.11	4	frameshift	del 556-557		Tassabehji et al.,1993
MSU 3	2	frameshift	ins 84+1		Morrell et al.,1993

A wide variety of mutations in the *PAX3* gene have been identified in WS I. A family with WS III was also found to have a single base substitution (See Table 4.1, family BU47) in the paired domain of *PAX3* (Hoth et al., 1993). WS I shows intragenic heterogeneity, with no correlation between the genotype at the mutation level and the resulting phenotype. The South African families with WS have been screened in order to characterise the mutations existing in our local population.

4.2. METHODS

The PAX3 gene was divided into smaller regions for PCR amplification using the exon-intron boundaries to mark the divisions. Each set of PCR primers was designed to include the whole of the coding exon and a small part of each of the flanking introns. In this way small fragments, which together covered the most of the coding region of PAX3, could be amplified for mutation screening.

Exons 2, 3, 4, 5, 6 and 7 of PAX3 were amplified using 100 - 200 ng genomic DNA in 25 μl reactions, containing 12.5 - 25 pmol of each primer, 200 uM dNTPs (Boerringer Manheim), 1 unit Taq polymerase (Gibco BRL) and a standard amplification buffer (See section 3.2) in a DNA thermalcycler (Hybaid). The reaction mix was overlaid with liquid parafin to prevent evaporation. The reactions were optimised for each primer set by adjusting the annealing temperature or addition of 0.01% gelatin and 0.1% triton to the standard reaction buffer. Details of the primers and the conditions for PCR are laid out in Table 4.2.

The size of exon 2 was not optimal for SSCP analysis and therefore internal primers (2-1b and 2-2b) were designed to amplify two overlapping fragments encompassing the whole of exon 2.

Table 4.2. Primers used for mutation analysis

Primer	Sequence	Size (bp)	PCR conditions	References
2F	5' tgtcgagcagtttcagcg	409	94 1'	Baldwin et al.
2R	5' cagtctgggagccaggag		63 1'	1992
			72 1'	
2F	as above	220	94 1'	
2-1b	5' tgtggcggatgtggttgg		56 1'	Own design
			72 2'	
2-2b	5' agggccgcgtcaaccagc	257	94 1'	Own design
2R	as above		56 1'	
			72 2'	
3F	5' cctgcccgcctgttctct	197	94 45"	Morell et al.
3R	5' cgactgactgtcgcgcct		63 45"	1992
			72 1'	
4F	5' agccctgcttgtctcaaccatgtg	241	94 45"	Tassabehji et
4R	5' tgccctccagtcacccagcaagt		61 45"	al.,1992
			72 1'	
5F	5' gtcggagagagaacttgg	325	94 30"	Own design
5R	5' tgtctggactgaagtagg		56 30"	
			72 1'	
6F	5' ttcatcagtgaaatc	264	94 30"	Own design
6R	5' tccaccagagaaatcgcc		46 30"	
			65 1'	
7F	5' catgatggttgacaatc	286	94 30"	Own design
7R	5' ctggtatacagcaaatcg		46 30"	
			65 1'	

4.2.1. Mutation screening

4.2.1a. Single stranded conformational polymorphism analysis

The method used for SSCP analysis was based on the technique developed by Orita et al. (1989) with modifications to allow for non-radioactive detection of the single-stranded nucleic acids by silver staining (Lohmann et al., 1992).

Preparation of electrophoresis plates

The glass plates were cleaned thoroughly with ethanol. The back plate was then wiped with acetone to prevent the gel sticking to this plate. The front plate was wiped with plate glue (500 μl τMTMS (τ-methacryloxypropyltrimethoxysilane), 50 ml absolute ethanol) and left to dry for 2 min before bathing in ethanol to remove excess plate glue. The bathing process was repeated twice and the plate wiped dry with paper towel. The glass plates were assembled with 0.35 mm spacers and a 0.35 mm well-forming comb.

Electrophoresis

SSCP analysis was performed by electrophoresis of the PCR product on a 0.5 x MDE gel (AT Biochem) in 0.5 x TBE buffer on a vertical gel apparatus. The samples were prepared by adding 5 μl of formamide loading dye (95 % formamide, 10 mM NaOH, 20 mM EDTA, 0.02 % Bromophenol Blue, 0.02 % Xylene Cyanol) to 5 μl of PCR product. Samples were denatured at 95°C for 5 min before loading. Gel electrophoresis took place overnight ($\pm$16 hours) at 4 - 6 W at room temperature (RT). Conditions were optimized for each PCR fragment.

Silver Staining

After termination of electrophoresis, the glass plates were prised apart, leaving the gel fixed to the glue treated plate. The gel was rinsed with distilled water and then soaked for 20 min in 0.1% $AgNO_3$ solution. The excess $AgNO_3$ was rinsed off and the gel placed in developing solution (1.5% NaOH, 0.01% Na borohydride, 0.15% fomaldehyde) for 10 min. To stop development, the gel was again rinsed with distilled water and fixed in a 0.75% $NaCO_3$ solution for 10 min. The banding pattern was noted and the gel transferred to Whatmann 3MM paper. A 20 min soak in 2% NaOH was occasionally required to remove the gel from the glass plate.

4.2.1b. Heteroduplex analysis

Preparation of glass plates

The glass plates were prepared as for SSCP analysis and assembled with 1 mm spacers and a 1 mm well-forming comb. A 1 x MDE gel (AT Biochem) was prepared with a 0.6 x TBE electrophoresis buffer. The heteroduplex technique was carried out according to the protocol provided with the Hydrolink MDE gel.

Sample preparation and electrophoresis

After completion of the PCR, EDTA was added to a final concentration of 5 mM. Samples were denatured at 95°C for 3 min and then allowed to cool to RT. MDE gel loading buffer (AT Biochem) was added to the sample before loading. The gel was electrophoresed overnight in a 1 x TBE running buffer, at a maximum voltage of 20 V per cm of gel, maintaining the gel temperature below 40°C. DNA bands were visualized by silver staining as described above.

4.2.2. Analysis of mutations

Once a polymorphism was detected, the relevant exon was investigated further to determine the nature of the mutation. The initial strategy was to clone the variant and the normal exons into a vector and then to distinguish between normal and mutant alleles before sequencing. This procedure proved time consuming and was superseded by a more direct method for sequencing PCR products.

4.2.2a. Sequencing of cloned PCR products

PCR products were cloned using the PCR-script cloning kit (Stratagene). The ligation reaction contained: 50 ng pCR-Script vector, 10 mM rATP, 10 units *Srfl* restriction enzyme, T4 ligase and a universal buffer, which were all provided in the kit. The PCR product (40 - 200 ng) was added to this reaction and incubated at room temperature for 1 - 2 hrs. The sample was then heated at 65°C for 10 min and stored on ice until the competent cells were ready.

The *Epicurian Coli* XL1-Blue MRF kan^r supercompetent cells, supplied with the cloning kit, were thawed on ice and aliquoted into prechilled Falcon 2059 polypropylene tubes. ß-mercaptoethanol was added to a final concentration of 25 mM to increase the transformation efficiency and the tubes placed on ice for 10 min, swirling every two min. The ligated vector-PCR was added and the mixture

incubated on ice for 30 min. The cells were heat shocked at 42°C for 45 sec followed by cooling by placing on ice for 2 min. SOC medium (Appendix A) was added for expression at 37°C with shaking for 1 hr. The expression mixture was plated on Luria agar (Appendix A) plates containing X-gal, IPTG and 50 μg/ml Ampicillin and incubated overnight at 37°C.

Plasmid extraction

White bacterial colonies (indicative of ß-galactosidase gene disruption) were picked and the plasmid extracted using the method of Ish-Horowicz and Burke (1981). Plasmids were isolated from a 5 ml overnight bacterial culture (LB + Ap, 50 μg/ml). The cells were harvested by centrifugation in an Eppendorf microfuge tube for 2 min. The pellet was resuspended in 0.2 ml Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0) and incubated at room temperature for 5 min. This was followed by the addition of 0.4 ml Solution 2 (0.2 M NaOH, 1% (w/v) SDS), the solution mixed well by vortexing briefly and placed on ice for 5 min. To precipitate the chromosomal DNA and cellular debris, 0.3 ml pre-cooled Solution 3 (5 M KOAc, pH 4.8) was added, mixed and left on ice for 5 min. This denatured DNA was removed by centrifugation for 10 min. The supernatant was transferred to a fresh tube, an equal volume of isopropanol was added and the plasmid DNA was precipitated by centrifugation for 10 min. The pellet was retained and washed with 70% ethanol, air dried and resuspended in 25 μl dH_20.

Plasmids underwent endonuclease digestion to verify the presence and size of the cloned insert in a reaction containing: 5 μl plasmid prep, 10 units *SacI*, 10 units *EcoRI*, 20 μg RNase A and a restriction buffer. The fragments were resolved by electrophoresis at 80 V in a 1.5% agarose gel with 1x TBE running buffer.

Pure plasmid preparations

Plasmids were prepared for sequencing using the Nucleobond AX PC-Kit 100. A 100 ml bacterial culture (LB, 50 μg/ml Ap) was grown overnight. The cells were pelleted by centrifugation and resuspended in 4 ml of buffer S1 (50 mM Tris/HCl, 10 mM EDTA, 400 μg RNase A/ml, pH 8.0). Four millilitres of buffer S2 (200 mM NaOH, 1% SDS) was added and the suspension mixed gently and incubated at room temperature for 5 min. Four millilitres of buffer S3 (2.80 M KAc, pH 5.2) was added and the solution mixed by shaking until homogeneous. The cell debris and genomic DNA was removed by centrifugation at high speed and 4°C for 38 min. The supernatant was removed carefully from the white precipitate and loaded

on a Nucleobond cartridge, equilibrated with buffer N2 (100 mM Tris/H_3PO_4, 15% ethanol, 900 mM KCl, pH 6.3). The cartridge was washed twice with 3ml buffer N4 (100 mM Tris/H_3PO_4, 15% ethanol, 1300 mM KCl, pH 6.3). The plasmid was eluted with 2 ml buffer N5 (100 mM Tris/H_3PO_4, 15% ethanol, 1000 mM KCl, pH 8.5). The purified plasmid was precipitated with 0.7 volumes isopropanol and pelleted by centrifugation, washed with 70% ethanol and resuspended in dH_20. All buffers were supplied with the kit.

Sequencing

The cloned insert was amplified, using the original primers and conditions for exon amplification. SSCP analysis was performed on the PCR products of these clones to distinguish between clones carrying inserts of normal alleles and those carrying the mutant alleles. Bacterial colonies of both the normal and mutant alleles clones were cultured to produce pure plasmids preparations for the purpose of sequencing.

Clones were sequenced using the Sequenase version 2.0 (USB) system. Three micrograms of purified plasmid DNA was denatured and added to an annealing mixture containing sequencing primer (one of the original set of primers used for the exon amplification) and reaction buffer. Annealing took place by heating the sample to 65°C for two minutes and then cooling slowly to 35°C. At this point the termination mixture for each nucleotide was aliquoted into 0.5 ml Eppendorf tubes and prewarmed to 37°C. The labeling mixture was prepared by adding labeling mix, [S^{35}] dATP and Sequenase polymerase to the annealing mixture. Radioactive labelling took place at 15°C for 5 min. Strand synthesis was terminated by adding labeling mixture to each of the four termination tubes and incubating at 37°C for 5 min. The reaction was stopped by the addition of Stop Solution. The samples were heated to 72°C for 2 min before loading onto a sequencing gel.

The sequencing gel apparatus was prepared by wiping both gel plates with ethanol and then with acetone to prevent the gel from sticking to the plates. The glass plates were assembled using 0.35 mm spacers and a 0.35 mm sharks tooth comb. A denaturing gel was made using Long ranger gel solution (AT Biochem) at 8% with 1.2 x TBE as the gel buffer and 0.6 x TBE as the running buffer to improve spatial resolution. Electrophoresis of the samples was carried out at 70 W constant power, which kept the gel temperature below 50°C. The gel was transferred to Whatmann 3 MM paper, dried and exposed to X-ray film (Agfa, Curix). Intensifying screens were removed from the X-ray cassette, which improved the sharpness of the bands,

but required longer exposure (2-3 days). The X-ray cassette was placed at - 70°C for the exposure time.

4.2.2b. Direct Sequencing of PCR products

Extraction of PCR product

Mutant and normal single-stranded alleles were excised from the silver-stained SSCP gel and eluted in 50 μl dH_20. Two microlitres of this eluent was used in a PCR reaction to amplify the allele. The PCR product was electrophoresed horizontally at 80 V on a 1.5% agarose gel in TBE buffer. Bands were cut out of the gel, macerated and placed in an Eppendorf tube. An equal volume of phenol (pH 8.0) was added and mixed with the agarose by shaking. The tube was placed on dry ice for 10 min and then centrifuged for 10 min to remove the agarose. The aqueous phase was transferred to a fresh Eppendorf tube and mixed with equal volumes of phenol and chloroform:isoamyl alcohol (24:1). The aqueous phase was removed after centrifugation and an equal volume of chloroform:isoamyl alcohol was added to eliminate any traces of phenol. After centrifugation, the DNA from the aqueous phase was ethanol precipitated with LiCl (1/10 volume 4M LiCl + 2.5 volumes of EtOH). The resulting DNA pellet was washed in 70% ethanol, dried and resuspended in 10μl dH_20.

Sequencing

Sequencing was accomplished using the direct incorporation protocol of the *fmol* DNA sequencing system (Promega). For each set of sequencing reactions, four tubes were designated for the appropriate nucleotide mix containing both the deoxy- and dideoxynucleotide triphosphate. Two microlitres of the purified PCR template was combined with *fmol* 5 x sequencing buffer (250 mM Tris-HCl, 10 mM $MgCl_2$), 10 pmol of one of the primers used for the exon being sequenced, α-^{32}P dCTP and 5 units of *Taq* polymerase (provided in the kit). The template mixture was dispensed into each of the four nucleotide tubes, liquid parafin layered on top and the tubes placed in a thermocycler. The primer length and GC-content determined the amplification conditions used for the PCR reaction. For a primer less than 24 bp or with a GC-content less than 50% the conditions were : 95°C for 2 min then, 95°C for 30 sec, 42°C for 30 sec, 70°C for 1 min for 30 cycles, then 4°C. For a primer greater than 24 bp or a GC-content greater than 50% amplification was a two step reaction: intial denaturation 95°C for 2 min then, 95°C for 30 sec, 70°C for 30 sec for 30 cycles, then 4°C. After the reaction was

completed *fmol* Stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) was added. The samples were heated to 70°C for 2 min before loading on a Long Ranger sequencing gel (preparation and electrophoresis as in 4.2.2a).

The difference in sequence between the normal and altered alleles was analysed to determine whether the amino acid sequence was disrupted at the site of the sequence change. With the aid of the map component of Genetics Computer Group (GCG) package, a mutation could be confirmed in a family by determining whether a restriction site was generated or obliterated by the change in sequence.

4.2.3. RNA Studies

4.2.3a. Transformations

Blood (10 ml) was collected in heparinised tubes. The blood was diluted 1:1 with growth media (Gibco Hams F10, 10% Gibco Fetal calf serum). Five millilitres ficoll (Histopaque 1077) was aliquoted into 15 ml tubes and 10 ml of the diluted blood layered on top. Separation of the blood phases was by low speed centrifugation for 20 min. The white blood cells were removed and transferred to a clean tube. Growth media was added to eliminate any remaining ficoll and the cells pelleted by low speed centrifugation. The supernatant was discarded and the washing repeated. The cells were resuspended in transformation media (2.5 ml growth media, 2.5 ml Epstein Barr virus, 4 μg/ml phytohaemogluttinin) and placed in a 50 ml tissue culture flask. Viral transformation occurred over three days in a 5% CO_2 incubator at 37°C. The transformation media was then removed and replaced with growth media. Incubation and feeding continued until a dense culture was obtained. The cells were then pelleted by centrifugation and washed once with growth media. The supernatant was discarded and the cells resupended in 6 ml freezing media (Gibco Hams F10, 20% Fetal Calf serum, 10% glycerol, 50 g/l penicillin, 30 g/l streptomycin, 25 g/l neomycin). Resupended cells were aliquoted in 1 ml amounts into 2 ml cryotubes. The cells were frozen and stored in liquid nitrogen for further use.

4.2.3b. RNA extraction from lymphoblasts

The extraction of RNA was by the method of Chomzynski and Sacchi (1987). Transformed lymphocyte cultures were centrifuged and the supernatant discarded.

The cells were washed in saline. 1 ml of solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol) was added with 0.1 ml 2 M sodium acetate, 1 ml water saturated phenol, 0.1 ml chloroform:isoamylalcohol 49:1). The tube was shaken vigorously and cooled on ice for 15 min. Centrifugation pelleted all the cell debris and left the RNA in the aqueous phase. The RNA from the aqueous phase was precipitated with ethanol on dry ice or stored in ethanol at -70°C until needed.

4.2.3c. cDNA synthesis and amplification

The reverse transcription reaction was carried out using 10 μg of total RNA. 1 μg oligo-dT primer (Genosys) and 1 μg random hexanucleotide primer (Genosys) were added to the RNA in a 10 μl volume with a final concentration of 5% DMSO and heated to 65°C for 3 min to denature the sample and then cooled on ice. A mixture was made containing 10 units RNasin, 200 units MMLV reverse transcriptase (Gibco BRL), dNTP stock to a final concentration of 200 μM and 5% DMSO in a 5 x dilution of the reaction buffer (0.25 M Tris-HCl, 0.375 M KCl, 15 mM $MgCl_2$ - Gibco BRL). This mixture was added to the RNA sample and incubated at 37°C for 1 hour. Following this, the sample was denatured at 95°C for 3 min and chilled on ice to prevent reannealing. A further 200 units of MMLV reverse transcriptase were added and the reaction incubated at 37°C for 1 hour. This step was repeated and reverse trancription was completed after the third hour of incubation. The first PCR amplification reaction, using the outside set of nested primers, contained 5-10 ul of reverse transcribed RNA, 10 pmol of each primer, 5% DMSO, 200 μM dNTP, 2 units *Taq* polymerase (Gibco BRL), 2 units

Taq extender (Stratagene) in a 1 x *Taq* extender reaction buffer (10 x: 200 mM Tris-HCl, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 1% Triton X-100, 1 mg/mlbovine serum albumin - Stratagene). The reaction underwent 30 cycles of amplification at 94°C for 30 sec, 56°C for 30 sec and 65°C for 1 min. Using 2 μl of this reaction, a further 30 cycles of amplification were carried out under the same reaction conditions, using the inside set of the nested primers. The primers used for *PAX3* amplification were: HUP2-4 5'-ggccagggccgcgtcaac and Pax3-1 5'-ctagaacgtccaaggctt (antisense) as the outside set (Hoth et al., 1993) and Ex2cF 5'-atacgtcctggtgccatcg and Ex6cR 5'-cttcctccatcttgcacgg (antisense) as the inside pair of nested primers (own design). The product was observed under UV light on a 2% agarose gel electrophoresed in TBE buffer.

CHAPTER 5

RESULTS AND DISCUSSION OF MUTATION ANALYSIS

CHAPTER 5. RESULTS AND DISCUSSION OF MUTATION ANALYSIS

5.1. MUTATION RESULTS

The results of the mutation analysis are described in the sequential order of the exons of *PAX3*.

A polymorphism in exon 2 was found to track with the disorder in WS family 2. Some of the affected individuals (IV-1 and III-8) seemed to be homozygous for this change as indicated on the SSCP gel in Figure 5.1.

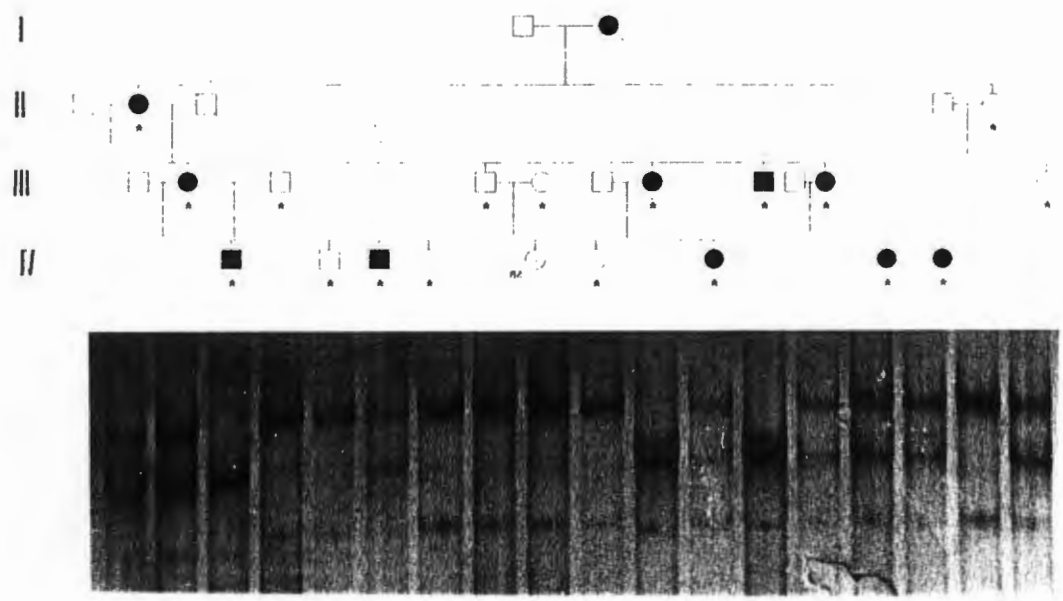


Figure 5.1. SSCP analysis of exon 2 in WS 2. All affected individuals inherit the polymorphic allele from an affected parent. An asterisk indicates the individuals from whom blood was taken. □ = male, ○ = female, ■ = affected male, ● = affected female.

Direct sequencing of the homozygous band as well as the heterozygous bands revealed a C to T change at codon 43 (Figure 5.2.). The change does not affect the amino acid sequence. This silent polymorphism has been previously reported by Tassabehji et al. (1994) and, although it is non-pathogenic, it confirms linkage of WS in family WS 2 to *PAX3* as the polymorphism tracks with WS I in this family.

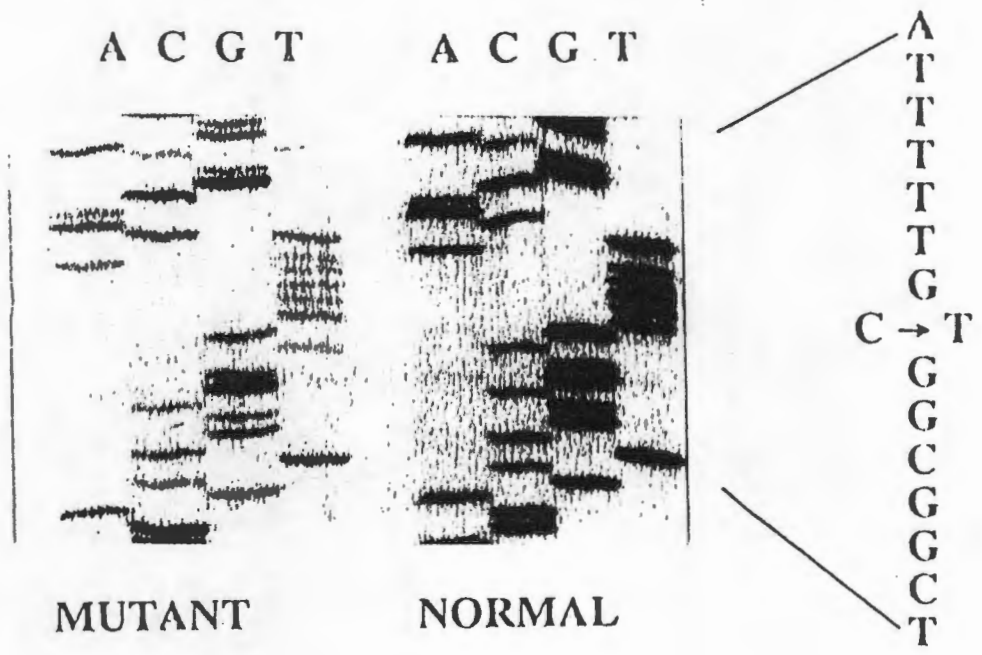


Figure 5.2. Direct sequencing of the normal and mutant exon 2 bands in WS 2.

The polymorphism can be used diagnostically in WS 2, provided the genotype of the affected parent and the spouse can be obtained. The SSCP shows that the mother of III-11, II-5, is negative for the polymorphism and is therefore unaffected. III-11, who is also unaffected, has the polymorphism which, therefore, must have been inherited from the father. Only a polymorphic allele inherited from an affected individual within the family is associated with an affected phenotype.

The primers designed to amplify two fragments encompassing exon 2, for optimal SSCP analysis of this exon, did not reveal any other changes, other than the

polymorphism in WS family 2, which was also detected using the orignal exon 2 primers.

SSCP analysis of exon 3 indicated changes in WS 4 and in a sporadic individual, WS 44.1. Expanding the analysis to include all the members of WS 4, revealed that the mutation arose in the second generation and was inherited with the disorder in the subsequent generations (Figure 5.3.).

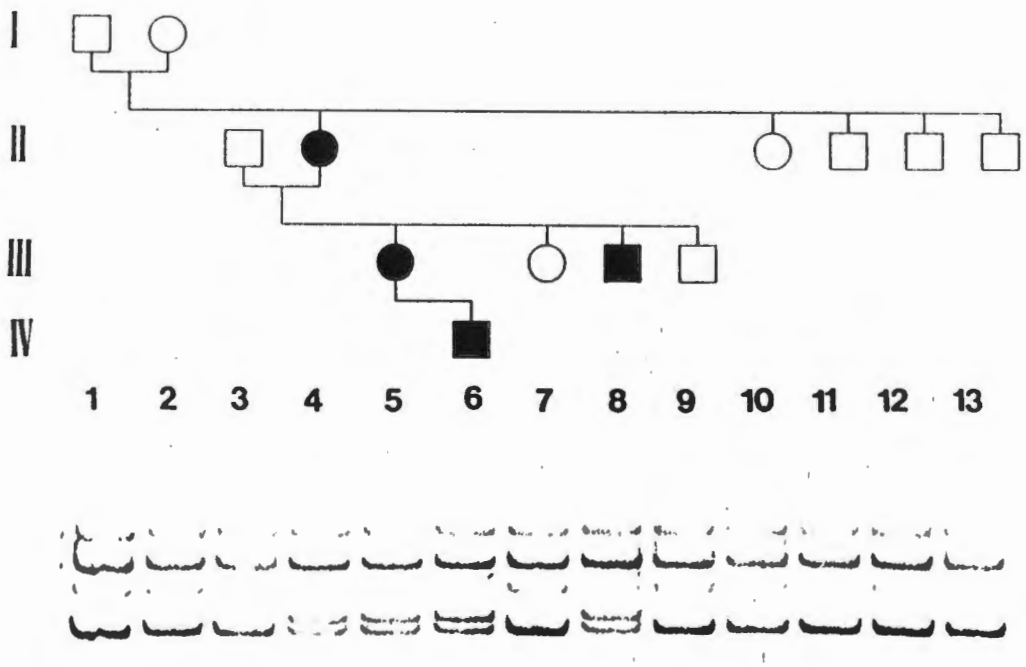


Figure 5.3. SSCP analysis of exon 3 in family WS 4. □ = male, ○ = female, ■ = affected male, ● = affected female.

For comparison, the results of heteroduplex analysis of WS 4 are shown in Figure 5.4. The difference in banding pattern between the normal and affected individuals in this family was more apparent when using SSCP analysis.

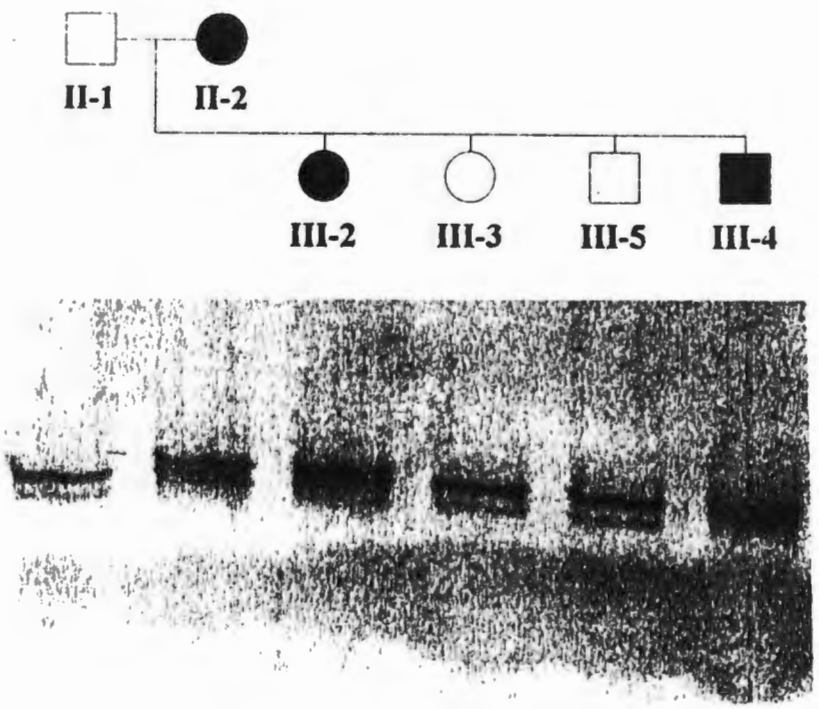


Figure 5.4. The results of heteroduplex analysis in WS 4. The generation numbers refer to the pedigree in figure 3.1. □ = male, ○ = female, ■ = affected male, ● = affected female.

The PCR fragment of exon 3, from unaffected and affected family members, was cloned. The clones were screened by SSCP analysis to determine which carried the variant allele and which the normal. Sequencing indicated a transition mutation, as shown in Figure 5.5, with guanine being replaced by cytosine.

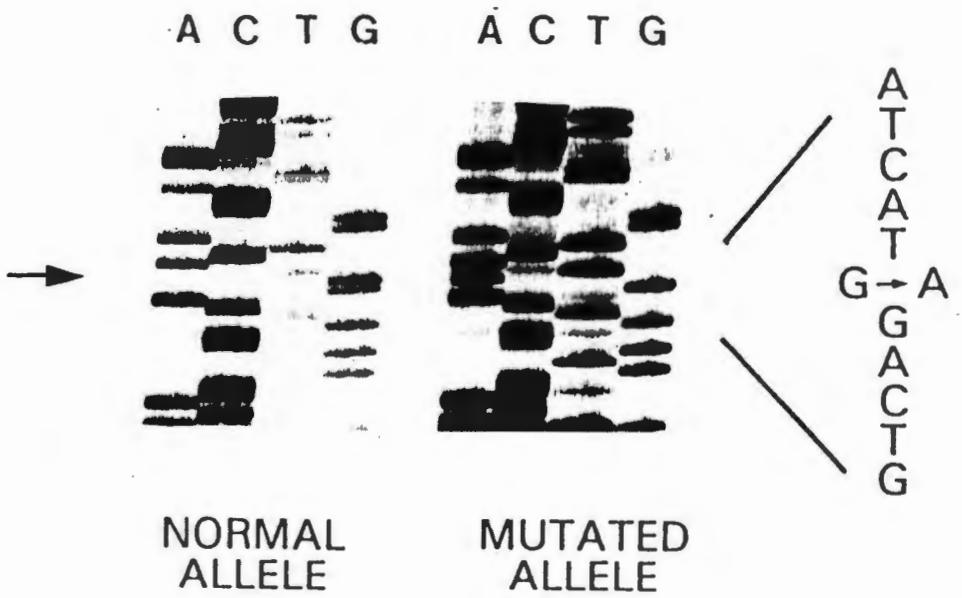


Figure 5.5. Sequencing of cloned alleles of exon 3 in WS 4.

The mutation could be confirmed in all affected individuals in this family as the base substitution interrupts an *RsaI* restriction site, inhibiting enzyme cutting of mutated alleles. All affected individuals were shown to carry a normal allele cut with *RsaI* and an uncut mutated allele as shown in Figure 5.6.

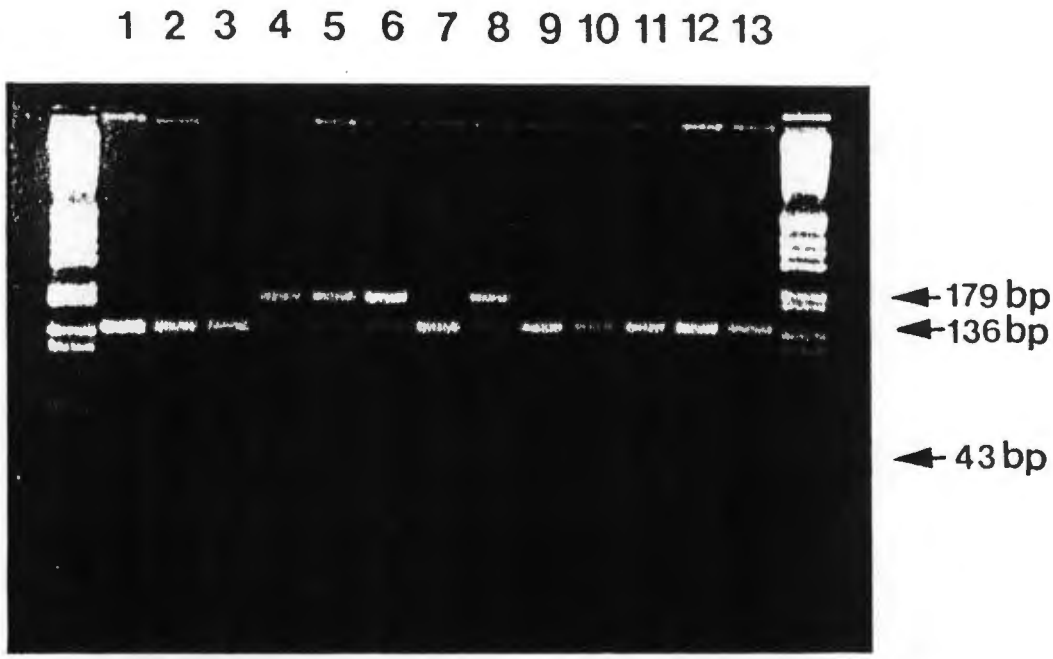


Figure 5.6. Endonuclease digestion of the exon 3 PCR fragment of WS 4 with *RsaI*. The numbers correspond to the pedigree in Figure 5.3.

The mutation was located at the exon 3-intron boundary, in the paired domain of PAX3 and has been described in Butt et al., 1994. The boundary between exons and introns contains splice site consensus sequences. Padgett et al. (1986) showed, in a survey of approximately 400 vertebrate genes, that the 5' consensus has a virtually invariant AG dinucleotide as the last 2 base pairs of the exon, and a highly conserved (100%) GU as the first dinucleotide of the intron. The mutated allele in affected WS 4 family members involves substitution of the first G of the intron dinucleotide for an A. This splice site consensus alteration has been reported to cause a number of other genetic diseases (Krawczak et al., 1992). The net effect of this intronic G to A transition is an aberration in mRNA processing.

The mutation in the sporadic individual (WS 44.1) in exon 3 was shown, by sequence analysis to occur at the same position as the mutation in WS 4. The splice site was disrupted by the insertion of 7 bp. The other members of this individual's family were all clinically normal and SSCP screening confirmed that they did not have the mutation. The banding pattern of the SSCP gel and the sequence analysis are shown in Figure 5.7.

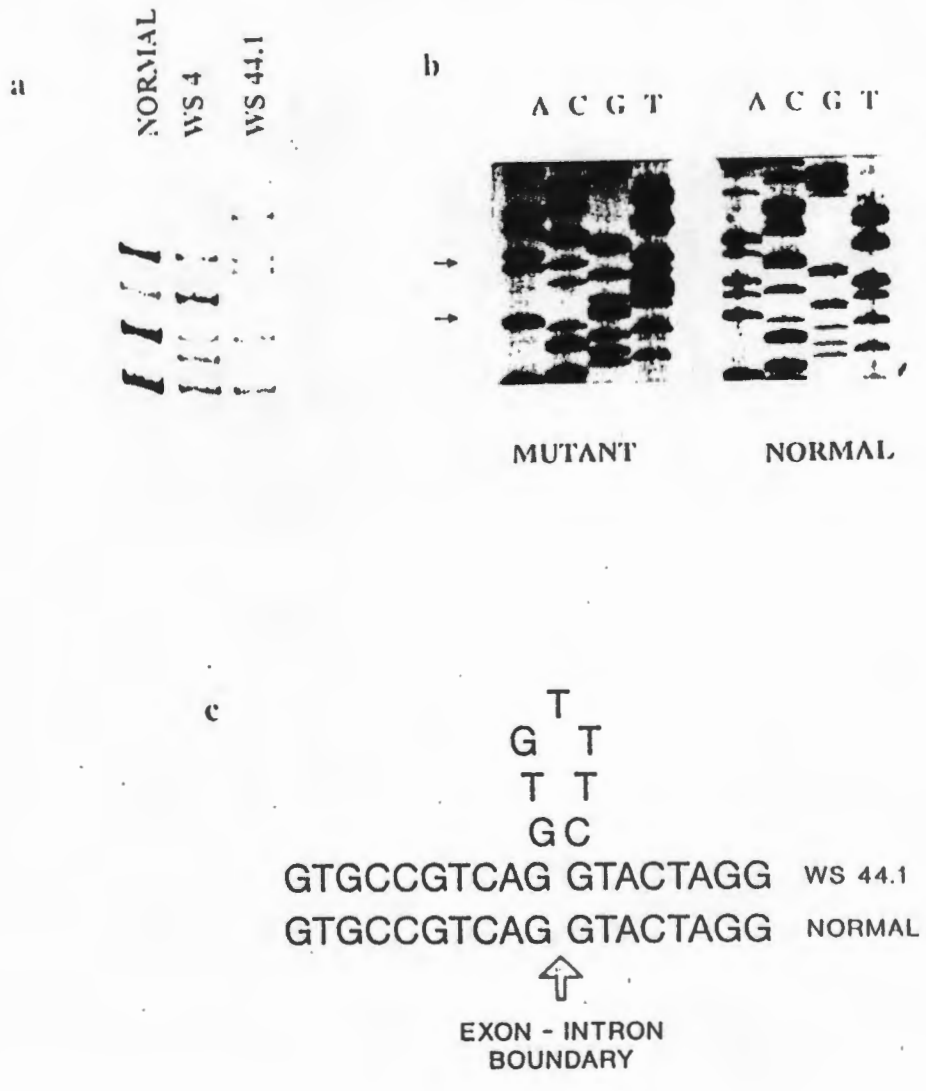


Figure 5.7. Analysis of exon 3 in WS 44.1. (a) A comparison of the SSCP banding patterns of exon 3 in a normal individual, WS 4 and the sporadic WS 44.1. (b) Sequencing autoradiograph showing the 7 bp insertion in the mutant allele of WS 44.1. (c) The site of the insertion disrupting the splice site at the exon 3-intron 3 boundary.

Another splice site mutation was located at the intron 3-exon 4 boundary in a sporadic individual. An A to G transition was identified in the 3' splice acceptor consensus sequence of intron 3, converting an invariant AG to GG as indicated in Figure 5.8. Only one affected member, WS 14.2, of this family was available for investigation.

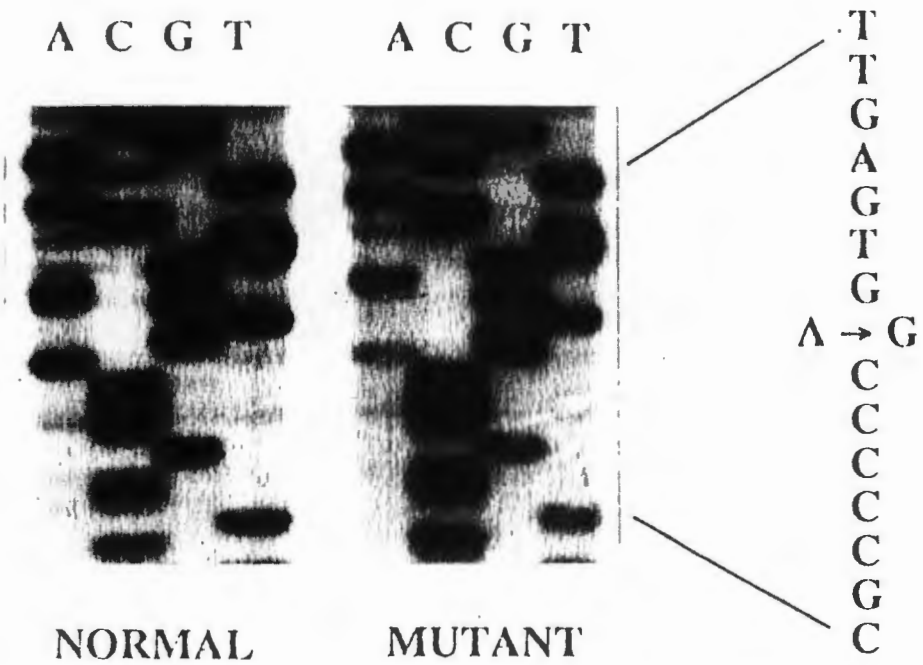


Figure 5.8. Sequence analysis of the intron 3-exon 4 boundary in a sporadic individual.

Mutation screening of the homeodomain was initiated after the exon-intron boundaries in this region had been identified (Tassabehji et al., 1994). SSCP screening localised the mutation in WS 1 to exon 5. The family pedigree with the mutation tracking with the affected members is illustrated in Figure 5.9.

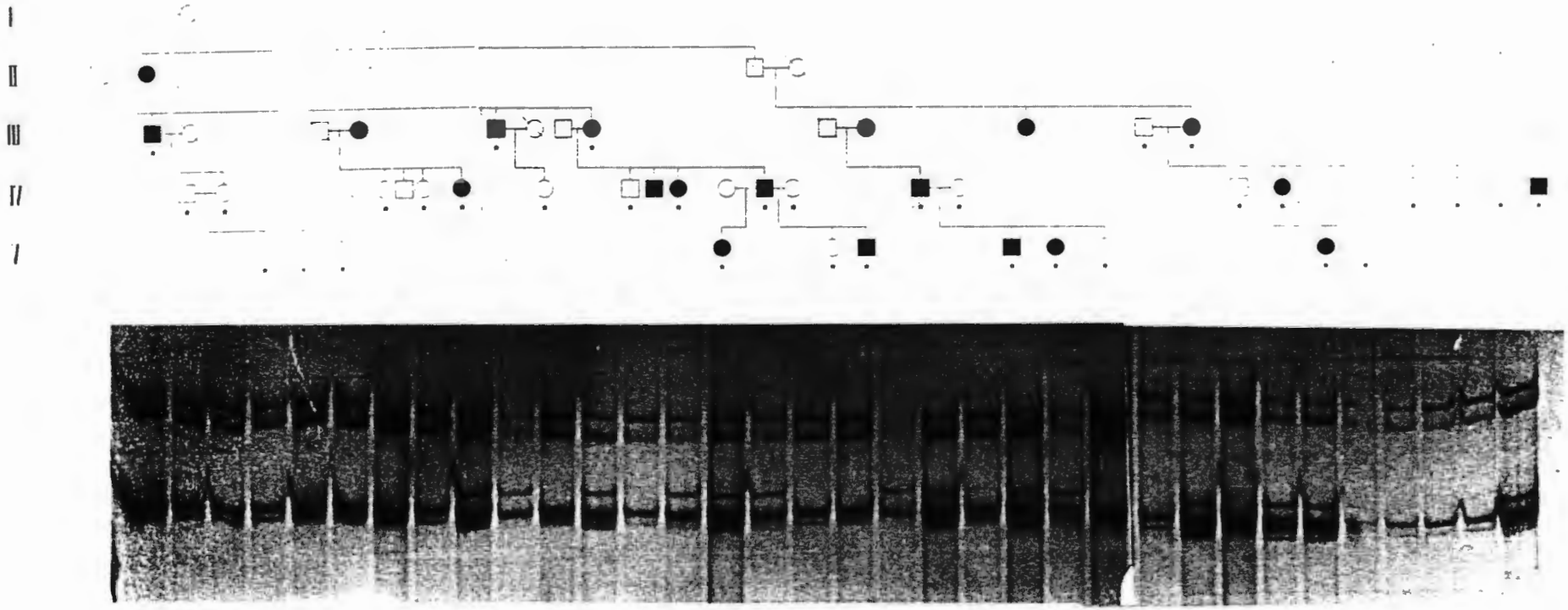


Figure 5.9. Pedigee of WS 1 indicating the SSCP change seen in exon 5 An asterisk indicates the individuals from whom blood was taken. □ = male, ○ = female, ■ = affected male, ● = affected female.

Direct sequencing showed a single base substitution (G to A) in the 5' region of the homeodomain, preceding the first helix (Figure 5.10.). The codon for an arginine residue at aa position 223 was converted to that for glutamine. This amino acid is 100% conserved in paired-type homeodomains genes, the murine Hox genes and in the *Drosophila Antennapedia*, *engrailed* and *bicoid* homeodomains.

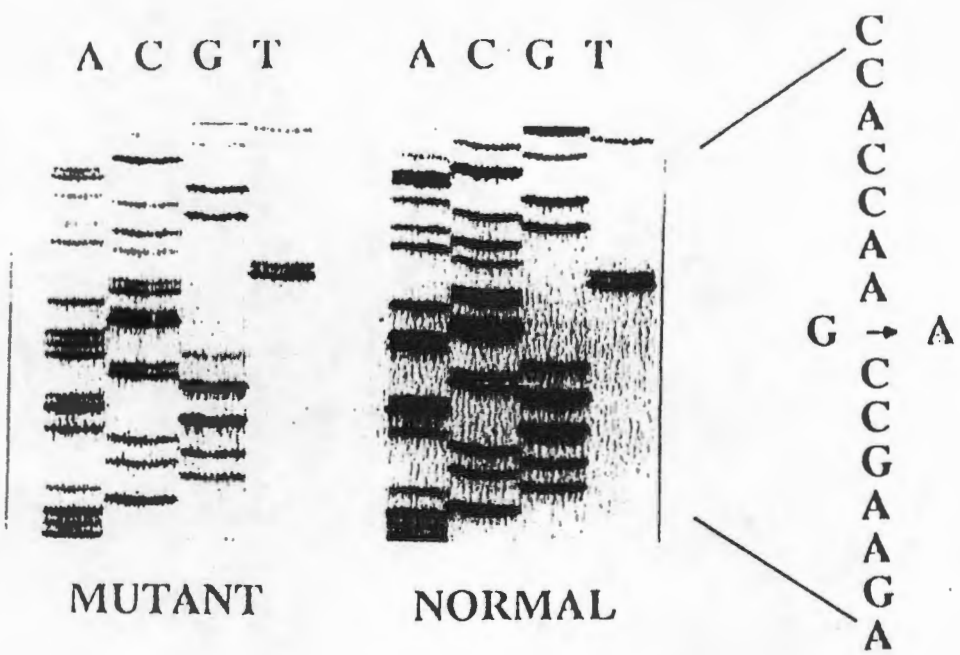


Figure 5.10. Sequence analysis of the mormal and affected alleles of exon 5 in WS 1

Restriction analysis using the GCG package indicated that no known restriction enzyme sites were abolished or created by the change. For confirmation of the mutation for diagnostic purposes, an allele specific oligonucleotide (ASO) could be designed to detect this mutation. The method has been described by Hoth et al. (1993).

Two families, WS 3 and WS 48, and a sporadic individual, were shown to have mutations in exon 6. The conformational change in WS 3 is shown in Figure 4.11.

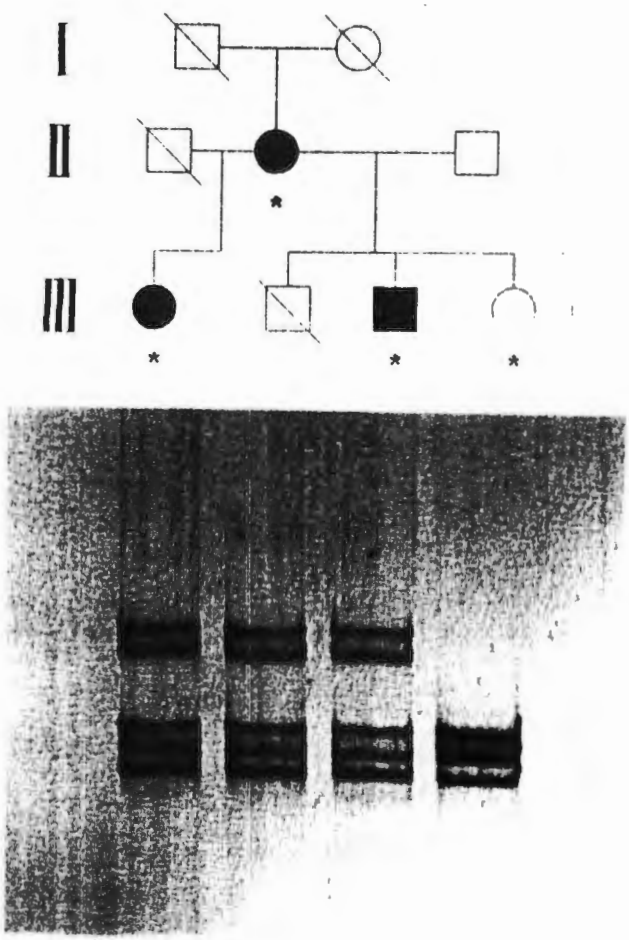


Figure 5.11. SSCP analysis of exon 6 in WS 3. An asterisk indicates the individuals from whom blood was taken. □ = male, ○ = female, ■ = affected male, ● = affected female.

Sequencing of the normal and mutant bands indicated the deletion of a single base pair (Figure 5.12.); the T at the first position of a phenylalanine codon (aa number 294). The codon reading frame is disrupted by the deletion, creating a nonsense message that is terminated prematurely by the creation of a stop codon in exon 7 at

aa 380. An *AvaII* site is created by the change and can be used as a confirmation of the sequencing results.

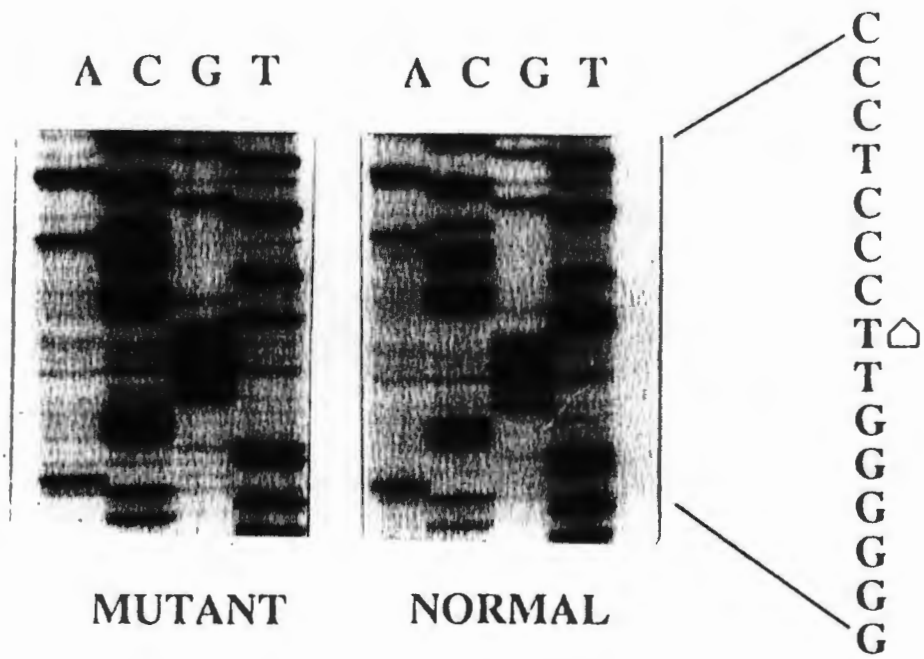


Figure 5.12. Sequence analysis of the mutant and mormal exon 6 alleles from members of WS 3.

The family, WS 48, in which rare phenotypic associations occured together with the usual WS characteristics, was found to have a conformational change in exon 6. The SSCP change co-segregates with the disorder in this family as shown by the results in Figure 5.13.

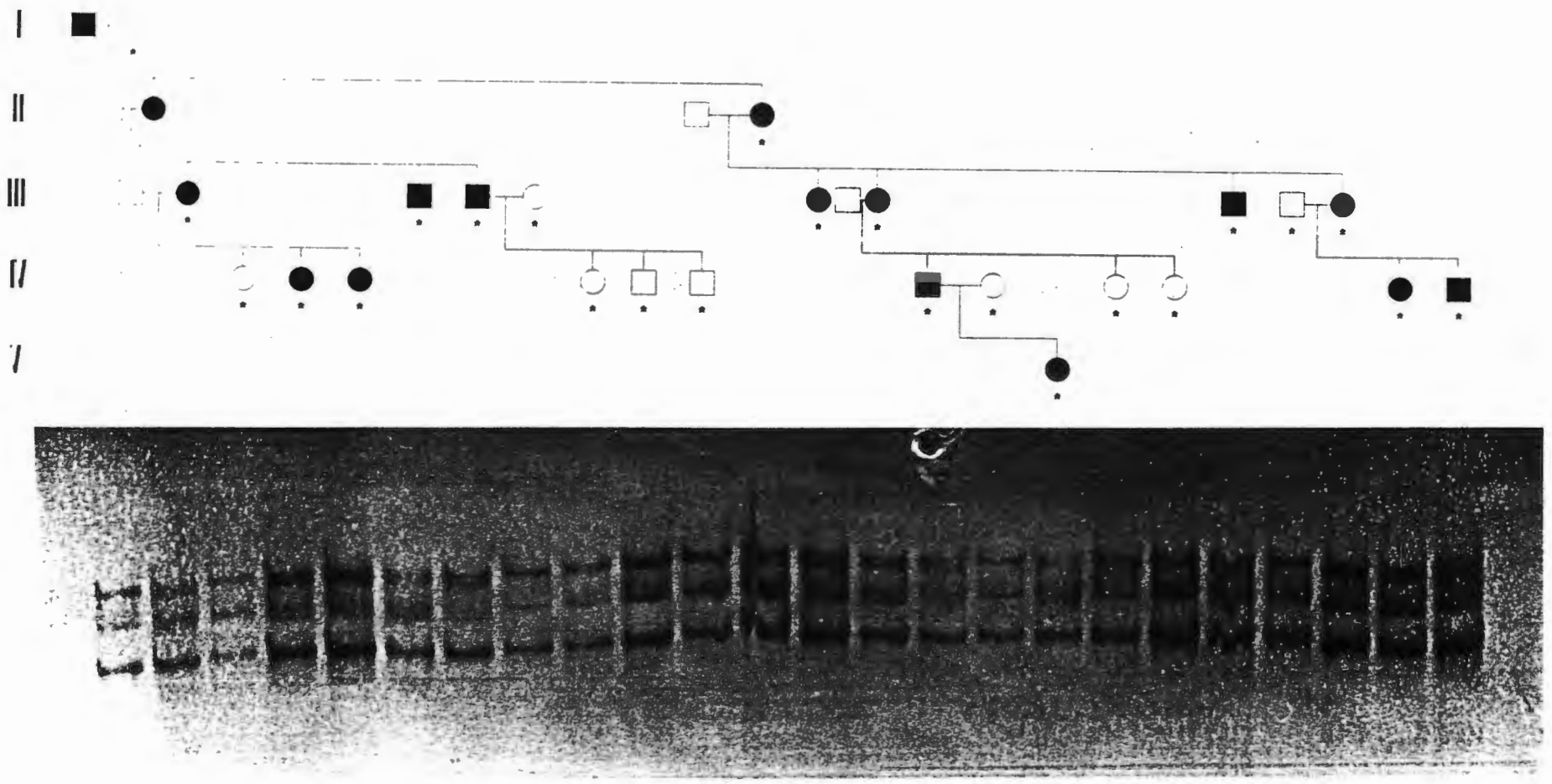


Figure 5.13. The conformational change in exon 6 co-segregating with the disorder in WS 48 as shown by SSCP analysis. The asterisk marks the individuals from whom blood was taken. □ = male, ○ = female, ■ = affected male, ● = affected female.

Initial sequencing of both the forward and the reverse strands of the normal and mutant alleles has failed to reveal a change in the DNA sequence.

A conformational change in exon 6 was also noted in a WS type I sporadic individual , WS 18.1. No other variant banding patterns were observed in exon 6 in 35 unrelated individuals with WS or in 10 normal, unrelated controls. SSCP analysis of exon 7 revealed only one individual with a variant banding pattern. This sporadic individual, WS 22.1, has a normal WS type I phenotype.

5.2. DISCUSSION OF MUTATION ANALYSIS

There is a high level of intragenic heterogeneity of mutations causing WS I. The exonic mutations in the Southern African families in this study are all unique when compared to those reported worldwide. The splice site mutations, however, show some analogies to mutations reported in both mouse and man.

A similar mutation to that found in the family WS 4 was reported by Tassabehji et al. (1994). The invariant GT donor splice site at the 5' end of intron 3 was abolished by the substitution of the G for a T, while in WS 4 reported here, the G is replaced by an A. In both cases, as shown in Table 4.1, section 4.1., abberant splicing is predicted to occur. The mRNA transcript would either exclude exon 3 or include intron 3 and utilize a cryptic splice site in the flanking DNA. Krawczak et al. (1992) noted that in 29 cases where the point mutation occurred at the 5' splice site, 16 showed exon skipping of the preceding exon, while in 7 cases a cryptic splice was used. The remaining 5 cases produced a mixture of exon-skipped and cryptically-spliced transcripts in differing proportions.

The original *Sp* mouse was shown to have a mutation at the 3' end of intron 3 (Epstein et al., 1993). This mutation abrogated the normal splicing of intron 3 by modifying the 3' acceptor consensus from the invariant AG to TG. The mutation identified in the sporadic individual, WS 14.2, and in a family reported by Tassabehji et al. (1994) modifies the 3' acceptor from AG to GG. The 3' splice site mutations analysed by Krawczak et al. (1992) indicate that this mutation would cause skipping of the downstream exon, exon 4, and/or utilisation of a cryptic splice site. The results of RNA studies on the *Sp/Sp* mouse by Epstein et al. (1993) showed that 4 transcripts were produced: 2 using cryptic splice sites in the

downstream exon and disrupting the reading frame, 1 retaining intron 3 and 1 transcript lacking exon 4. The only in-frame transcript produced was the exon 4 deletion. In this instance, the 3' end of the gene would still be functional; only exon 4 containing the octapeptide repeat would be missing. A similar consequence is expected with the human 3' splice site mutations.

An experiment to confirm this hypothesis was undertaken for WS 4 and WS 44.1. Detailed methods are given in section 4.2.3. Lymphocytes, isolated from veinous blood from affected individuals, were transformed with Epstein Barr virus in an endeavour to restore the production of *PAX 3* transcripts (Hoth et al., 1993). The RNA was extracted from the immortalised cells and was reverse transcribed. A nested PCR reaction was set up to amplify the *PAX 3* cDNA. Analysis of the size differences of the resultant PCR products would confirm the RNA processing dysfunction. Products of this PCR reaction were not of the expected sizes, though it has been observed that a number of transcripts are produced from cDNA from affected individuals in WS 4. These PCR products must be sequenced before any conclusions can be made.

Of the 7 Southern African WS type I families, 4 were characterised with regard to the pathogenic mutation in *PAX3* and one family showed a non-pathogenic polymorphism. This emphasises the fact that SSCP screening is not 100 % efficient in the detection of DNA alterations. Heteroduplex analysis was employed, but proved no more effective than SSCP analysis and an alteration in the banding pattern of SSCP's was more conspicuous than a heteroduplex banding pattern. SSCP was favoured as the screening method because of its simplicity and furthermore, the equipment available meant that far more samples could be screened by SSCP analysis than heteroduplex analysis.

CHAPTER 6

COMMENT AND FUTURE PROSPECTS

CHAPTER 6. COMMENT AND FUTURE PROSPECTS

The aim of this study was to elucidate the cause of WS in Southern African families. The findings are discussed in detail at the end of each chapter.

The WS type I families, differentiated from type II by the mean WARD index of the family, were shown to be linked to a developmental control gene, *PAX3*. Molecular screening of this gene elucidated the pathogenic mutations underlying the disorder in some of the type I families. Intragenic heterogeneity was demonstrated in this population, as it had been in others, by the fact that the mutations were widely spread over different exons of *PAX3*. Exon 2, which encodes part of the paired domain, contained most of the mutations reported by the International Waardenburg Consortium. In the SA sample of patients, there was no such bias; conversely the homeodomain was found to have more mutations than the 5' end of the gene. The sample size in this study is not large enough to warrant a general statement about the position of *PAX3* mutations causing WS, though it is worthy of comment. The position of the mutations, however, did not influence the phenotype and no correlations could be made as to the expressivity of one characteristic over another in respect to the location of the intragenic defect.

The SSCP and heteroduplex techniques have been employed for exonic screening in this study. Sequence heterogeneity was detected in 5 out of the 7 WS type I families. It has been shown that SSCP analysis detects 70 - 95% of mutations in PCR products of 200 bp or less (Michaud et al., 1992) and therefore it is not unexpected that mutations in *PAX3* have not been detected in some of the WS type I families. There is also the possibilty that the mutations in these families lie in exons 1 or 8, which have not been screened. A more efficient method for mutation detection, the Hydroxylamine Osmium Tetroxide chemical cleavage method (HOT), has been described by Cotton (1988). This method involves the use of highly toxic chemicals as well as τ-P^{32}. Saleeba et al. (1992) optimised the technique without using radioactivity, by visualising the DNA with silver stain. Although the HOT method is time consuming and involves a few different DNA manipulations, it is said to be 100 % efficient (Grompe, 1993). It is advisable to use SSCP analysis as an intial screen, but for those families where mutations have not been detected, it would be pertinent to employ the HOT method.

Families with WS type II were not linked to *PAX3*. The clinical distinction between WS type I and type II is of relative importance for genetic counselling, as type II individuals have a higher incidence of deafness. WS type II shows non-allelic heterogeneity, as reported by Hughes et al. (1994), when describing a gene (*MITF*) defective in some, but not all, WS type II families. The SA families were also found to be heterogeneous, with only one out of the three WS type II families having a strong probability of being linked to the *MITF* gene.

The likelihood of chromosome 20 containing a gene responsible for WS type II in a SA family was also investigated. Haplotype analysis of WS family 27 indicates a strong possibility of a gene being located at 20q13. Fine mapping of this region with further polymorphic markers is neccessary before this gene can be identified.

Intrafamilial variability of the phenotypic manifestations in both WS I and WS II is commonplace. This indicates that modifying genes, expressed at the same time as *PAX3* (or *MITF*) may be involved in determining the phenotype. Another possibility is that epigenetic factors may modify expression. The temporal and spatial expression of developmental genes, such as those involved in WS, is very precise. These genes form part of a cascade of regulated events, functioning in the control of expression of genes during development. The production of defective proteins early in the cascade would have a more widespread effect than proteins which may be produced late in development.

The conserved domains of *PAX3* indicate that it functions as a DNA binding protein. Prokaryotic proteins that control transcription, such as the *E.coli* cAMP receptor protein, have been structurally examined by X-ray crystallography to establish how specific DNA binding occurs (Schleif, 1988). It was determined that the helix-turn-helix motif angles the protein in such a way that contact with specific base pairs can be attained. The homeodomain, which consists of 3 helices separated by turns, is predicted to bind DNA in a similar fashion. A single amino acid in the third helix (residue 9) confers the DNA binding specificity of the homeodomain (Treisman et al., 1989). Proteins with the same residue 9 amino acid as *PAX3* have been shown to bind the *Drosophila* e5 recognition sequence. The e5 sequences have two recognition elements, one recognised by the paired domain and one by the homeodomain. High affinity binding is achieved when both protein domains are present; these domains, however, may also function individually and initiate transcription of other distinct genes (Goulding et al., 1991).

The *MITF* gene is also thought to encode a transcription factor (Hughes et al., 1993; Hodgkinson et al., 1993). The protein structure is that of a basic helix-loop-helix-zipper protein, which function as homodimers or heterodimers with related proteins to bind DNA. *MITF* is expressed in mouse embyros in the pigment layer of the retina, in cells surrounding the otic vesicle, in hair follicles and in the embryonic heart (Hodgkinson et al., 1993) and also in several adult tissues (Hughes et al., 1993). Like *PAX3*, the probable function of *MITF* during development is in transcriptional regulation of genes involved in controlling the migration, proliferation or diffentiation of melanoblasts.

Molecular genetics has played a vital role in locating genes involved in the pathogenesis of WS. Many of the mutations in these genes underlying the protein defect have now been characterised. Positive linkage and mutation detection make it possible to offer antenatal diagnosis, although, the severity of the phenotype can not be predicted.

Studies, such as this one, provide the basis for further investigation of the events that occur during embryogenesis and the genes that regulate these events during normal development. This appraoch, in turn, may provide clues as to the abnormalities during embryogenesis that give rise to neural tube defects and other medically important developmental anomalies.

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