A SEARCH FOR THE TYROSINASE-POSITIVE OCULOCUTANEOUS ALBINISM GENE USING LINKAGE ANALYSIS

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ABSTRACT

Tyrosinase-positive oculocutaneous albinism (ty-pos OCA) is an autosomal recessive disorder of the melanin pigmentary system, characterised by generalised hypopigmentation, with the accumulation of phaeomelanin pigments with increasing age. Southern African ty-pos OCA individuals occur with two distinct phenotypes, with or without ephelides. These phenotypes are apparently concordant within families, suggesting that there is more than one mutation at the ty-pos OCA locus. Elucidation of the basic defect(s) in ty-pos OCA would be aided by a definitive localisation of the ty-pos OCA gene. Linkage studies have been carried out in 41 families, using 52 markers, including 15 random polymorphic serogenetic markers and 16 random polymorphic DNA markers, as well as markers from two candidate genes, TYR and CAS2, and 19 polymorphic markers from 2 candidate chromosomal regions, on 11p and 15q. Pairwise lod scores were calculated using the MLINK program of the LINKAGE package. A significant lod score was initially obtained in linkage analysis between ty-pos OCA and two chromosome 15q11-q13 markers, in the Prader-Willi/Angelman Syndrome (PWS/AS) region. Linkage analysis with 13 other markers on chromosome 15, confirmed this localisation. A genetic linkage map of the proximal region of the long arm of chromosome 15 was constructed and shows that the most closely linked, flanking loci are the GABRB3 and D15S24. Linkage analysis has also shown no cross-overs between the D15S12 locus and ty-pos OCA in southern African Negroids, suggesting that this locus is very close to, or

part of, the disease locus. Caucasoid "ty-pos" OCA individuals are characterised by locus heterogeneity. Negroid ty-pos OCA families with and without ephelides were analysed separately at the tyrosinase and D15S12 (pIR10-1) loci. The summated lod scores at $\Theta=0.01$ were negative for the tyrosinase locus and positive for the D15S12 locus, suggesting no evidence for locus heterogeneity in this population. Allelic association was found between the polymorphic alleles detected at the D15S12 (pIR10-1) locus and ephelus status, which suggests that there were multiple origins of the mutations at the ty-pos OCA locus. The results of this thesis show that the ty-pos OCA gene is located on chromosome 15q11-q12. The gene is postulated to be the human homologue, *P*, of the mouse *pink-eyed dilution* gene, *p*, although definitive proof awaits the detection of mutations in this gene in individuals with ty-pos OCA.

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. The analyses in this thesis have formed part of a collaborative study within the Department of Human Genetics and include some laboratory practical results generated by other postgraduate students, but analysed by myself. It has not previously been submitted for any degree or examination at any other university and the Committee for Research on Human Subjects has cleared the protocol for this project (Clearance certificate reference number R14/49, for protocol number 12/4/89, in the author's maiden name, Colman).

MKedda

Mary - Anne Kedda

25th day of August, 1993

To my husband, Sean, and my family.

For all their patience

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Clearance certificate : Committee for Research on Human Subjects

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ABBREVIATIONS

A	Adenine
acd	Acid citrate dextrose
ACTC	Cardiac muscle actin (Gene)
AD	*Anno domini*
ALB	Albumin (Gene)
ANCR	Angelman chromosomal region
AS	Angelman Syndrome
ATCC	American *Type Culture* Collection
bp	Base pair(s)
C	Cytosine
°C	Degrees Celcius
cen	Centromere
cDNA	Complimentary DNA
Ci	Curie
cm	Centimetre(s)
cpm	Counts per minute
CsCl	Caesium chloride
CT	Calcitonin (Gene)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dup	Duplication
EBV	Epstein Barr Virus
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid
etc	*et cetera*
FISH	Fluorescence *in situ* hybridisation
g	Gram(s)
G	Guanine
G-banding	Giemsa banding
inv	Inverted
kb	Kilobases
kg	Kilogram(s)
LA	Luria-Bertani agar

LB	Luria-Bertani broth
lod	Logarithm of the odds
mA	Milliamps
ml	Millilitre(s)
mM	Millimolar
M	Molar
MPO	Myeloperoxidase glycoprotein (Gene)
mwt	Molecular weight
ng	Nanogram(s)
nm	Nanometer(s)
OD	Optical density
p	Short arm of chromosome
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFGE	Pulsed-field gel electrophoresis
pg	Picogram(s)
PIC	Polymorphism information content
PWCR	Prader-Willi chromosomal region
PWS	Prader-Willi Syndrome
q	Long arm of chromosome
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rpm	Revolutions per minute
SAA	Serum amyloid A (Gene)
SDS	Sodium dodecyl sulphate
SCH	Somatic cell hybrid(s)
SSC	Sodium citrate solution
SS DNA	Salmon sperm DNA
STS	Sequence tagged site
T	Thymidine
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl) aminomethane
TSB	Transformation and storage buffer

TYR	Tyrosinase (Locus)
Ty-pos OCA	Tyrosinase-positive oculocutaneous albinism
U	Units
UV	Ultraviolet
V	Volts
VEP	Visual evoked potential
WT	Wilms tumour (Locus)
YAC	Yeast artificial chromosome
μCi	MicroCurie
μg	Microgram(s)
μl	Microlitre(s)
%	Percentage

PUBLICATIONS

Publications arising as a result of this study:
(Most publications are in the author's maiden name, Colman)

JENKINS T, HEIM RA, DUNN DS, ZWANE E, COLMAN M-A, RAMSAY M, KROMBERG JGR (1990) In quest of the tyrosinase-positive oculocutaneous albinism gene. Ophthal Paediatr Genet 11 (4): 251 - 254

COLMAN M-A, SEGALO P, RAMSAY M, JENKINS T (1991) A third TaqI allele is detected by the probe pTD3-21 (D15S10) in southern African chromosomes. Nucleic Acids Res **19**: 5097

COLMAN M-A, SHIBAHARA S, KWON B, JENKINS T (1991) Two allele XbaI RFLP at the catalase 2 locus. Nucleic Acids Res **19**: 960

CHINTAMANENI CD, RAMSAY M, COLMAN M-A, FOX MF, PICKARD RT, KWON BS (1991) Mapping the human CAS2 gene, the homologue of the mouse brown (*b*) locus to human chromosome 9p22-pter. Biochem Biophys Res Commun **178**: 227-235

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COLMAN M-A, STEVENS G, RAMSAY M, KWON B, JENKINS T. Exclusion of two candidate pigment loci, *c* and *b*, a chromosomal region, part of 11p, and 33 random polymorphic markers as the locus for tyrosinase-positive oculocutaneous albinism. Hum Genet (in press)

MARQUES I, COLMAN M-A, STEVENS G, JENKINS T, RAMSAY M. Genetic variation at seven polymorphic loci in southern African populations. (In preparation)

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CHAPTER ONE

1.0 INTRODUCTION

Albinism is a heterogeneous group of conditions, characterised by a distinctive phenotype, usually involving generalised hypopigmentation and ocular abnormalities. Of the ten autosomal recessive types of albinism, tyrosinase-negative (ty-neg) and tyrosinase-positive (ty-pos) oculocutaneous albinism (OCA) are the most commonly described types occurring in Caucasoids and Negroids. The gene for tyrosinase-negative albinism has been mapped to the tyrosinase locus on 11q14-q21, but the gene for tyrosinase-positive oculocutaneous albinism, the commonest type of albinism, was not known until recently. Since tyrosinase-positive oculocutaneous albinism occurs with a high frequency in southern African Negroids, a search for the disease gene was greatly facilitated by the availability of affected individuals and their families. The first chapter of this dissertation includes a literature review of albinism and the rationale behind the investigation of linkage to tyrosinase-positive oculocutaneous albinism.

1.1 An historical overview

The observable racial differences in pigmentation which have been studied by anthropologists depend upon skin, hair and eye colour. Theories to explain the causes of distinctively different patterns of geographic variation in skin colour are numerous (Riley 1972). Although the evidence is indirect, it is likely that natural selection is important (Livingstone 1969) and it has been proposed that increased pigmentation in areas of high solar radiation protects the skin from burning and from the carcinogenic effects of this radiation (Ainsworth-Harrison 1973).

The classical work of Edwards and Duntley (1939), showed that melanin distribution affects skin colour. Since melanin is synthesised by melanocytes in the basal layer of the epidermis, the hereditary factors regulating melanin

synthesis and melanocyte distribution must determine racial colour variation, although external environmental factors such as ultraviolet light must also have an effect on skin colour (Ainsworth-Harrison 1973; Ortonne 1990). It has been postulated that 3 to 4 genes are responsible for racial differences between black and white skin (Stern 1970), although there may be more (Ainsworth-Harrison 1973), and it is likely that the genetics of normal skin colour may be best related to a polygenic model with several major genes.

The human pigmentary system is a complex set of dynamic cellular interactions and within this system there are multiple sites for dysfunction, each potentially resulting in a disorder of pigmentation. Albinism comprises a heterogeneous group of heritable disorders of the melanin pigmentary system. All forms of albinism are characterised by hypoplasia of the fovea, translucent irides, photophobia, nystagmus, decreased visual acuity, absence of binocular vision due to abnormal decussation of optic neurons at the chiasm, and decreased melanotic pigment in the skin, hair and eyes (oculocutaneous albinism) or eye (ocular albinism) (Witkop *et al.* 1989).

Albinism has been observed and described in Africa for nearly 2000 years. In the first century AD, Pliny (1942), referred to the *Leucoaethiopes* living in north Africa. The term "albino" is derived from the Latin adjective "albus", meaning "white", and was first applied to white Negroes in Africa by Belthazar Tellez (cited in Witkop *et al.* 1989). The intense visual sensitivity of albinos to sunlight led the eighteenth century Swedish naturalist, Linnaeus, to describe Negroid albinos as "*Homo nocturnus*", confined to their huts until twilight (Robins 1991). There are references to albinos in the Bible and in 1908, Sir Archibald Garrod speculated that albinism was an inborn error of metabolism. By 1913, albinism was a well characterised condition (Pearson *et al.* 1913).

Albinos are often referred to as *nkau* ("monkey") in southern Africa and by other derogatory terms elsewhere in Africa (Okoro 1975), although the origins of many

of these terms are unclear. Myths surround albinos, suggesting that they never die, have special divine protection and have particular medicinal skills. In the past, infants considered to be handicapped were often killed and infanticide of albino infants has been widely practised in Africa (Kromberg *et al.* 1987). More recently, however, attitudes are changing and superstitions have decreased as albinos become more widely accepted.

Ten different disorders of albinism can be distinguished on the basis of clinical, ultrastructural and biochemical criteria, by their tyrosinase activity, by their genetic characteristics as determined by matings of albinos of different genotypes, and by distribution in various populations (Witkop *et al.* 1989).

1.2 Pigment cell biology

Figure 1.1 illustrates the epidermal cells in human skin, including the melanocytes, keratinocytes and various epidermal cell layers, which are associated with melanin distribution.

1.2.1 The melanocyte

The melanocyte, derived from melanocyte precursor cells (melanoblasts), is a dendritic cell in the skin in which melanin biosynthesis occurs. The pigmented cells of the retina originate from the optic cup of the forebrain and all other pigment cells are derived from precursor cells in the embryonal neural crest. These cells migrate during melanogenesis, prior to birth, into hair follicles in the skin and into the uveal layer of the eye. Melanocytes are found predominantly within the basal layer of the epidermis and the matrix of the hair follicle, but are also found in the dermis, mucous membranes, the leptomeninges, the retina and uveal tract of the eye, and the cochlear and vestibular labyrinth of the ear (Witkop *et al.* 1989).

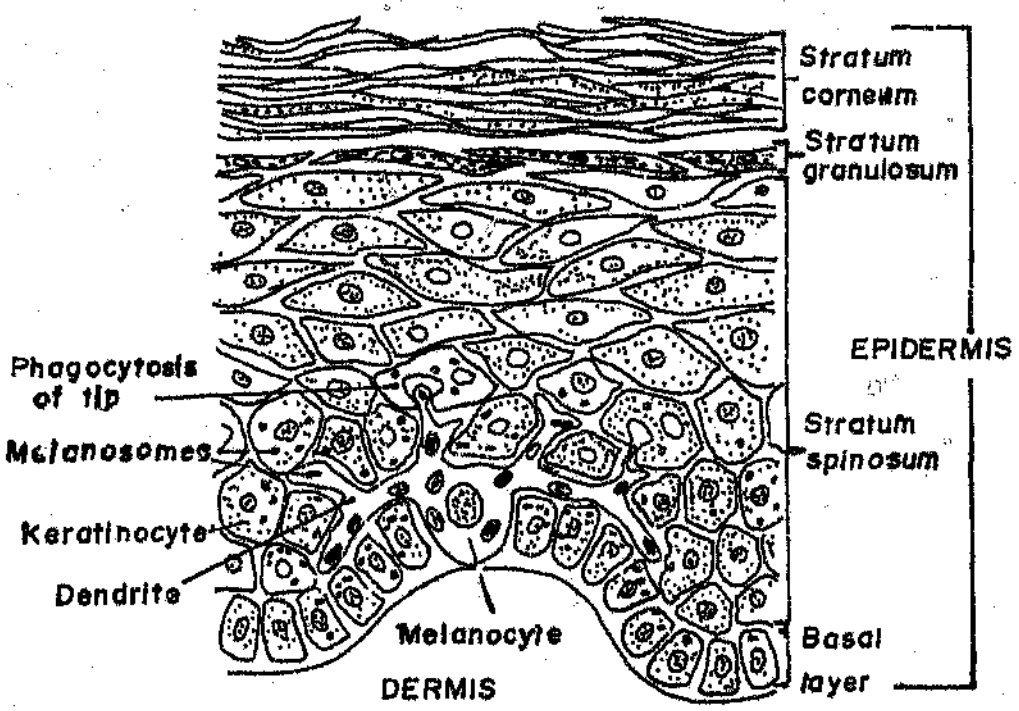

Figure 1.1 The epidermal melanin unit (Bolognia and Pawelek 1988).

It has been established that the same genes are responsible for pigment formation in the skin and eyes (Silvers 1979). Melanin pigments are formed within melanosomes, in the cytoplasm of the melanocyte. Deposition of melanin in the retina occurs primarily during embryogenesis, but in the skin it is a continuous process (Bolognia and Pawelek 1988). In the human epidermis, each melanocyte supplies melanin to an average of 36 keratinocytes in an "epidermal melanin unit" (Figure 1.1). Melanosomes are transferred to the cytoplasm of the keratinocyte when it phagocytoses the dendritic tip of the melanocyte and skin pigmentation is primarily determined by the amount of melanin transferred to the keratinocyte (Bolognia and Pawelek 1988).

Structural studies have shown little difference in the distribution of melanocytes in black and white skin. The intensity of pigment produced by the melanocytes, rather than the number of melanocytes, is thought to determine racial pigmentation differences (Riley 1972; Witkop *et al*. 1989).

1.2.2 The melanosome

The melanosome is a cytoplasmic organelle, in the melanocytes and keratinocytes of the epidermis, in which melanin pigment biosynthesis occurs.

Melanosomes contain tyrosinase and several structural matrix proteins. They are formed by the incorporation of tyrosinase, synthesised on the ribosomes, and structural proteins, synthesised in the rough endoplasmic reticulum, into small, membrane-bound vesicles, budding from the endoplasmic reticulum. The structural proteins form a lamellar matrix within the melanosome upon which melanin is deposited (Bolognia and Pawelek 1988). The mature melanosomes migrate into the dendritic processes of the melanocyte.

There are four stages in melanosomal development as the melanosome progresses from the perinuclear area of the melanocyte to the dendrites (Witkop *et al.* 1989). Stage I melanosomes are spherical and contain filaments and show no evidence of melanin deposition. Stage II melanosomes are oval and have numerous parallel longitudinal filaments, with little or no melanin deposition. Stage III melanosomes show tyrosinase activity and the internal structure is partially obscured by melanin deposition. Stage IV melanosomes show dense melanin pigmentation. Darkly pigmented skin has been shown to have more numerous, larger, singly dispersed, darker melanosomes than lighter skins (Bolognia and Pawelek 1988; Robins 1991). Tanning in normally pigmented skin results from an increase in the size of the melanosomal complexes and an increase in the amount of melanin in the melanosomes.

In normally pigmented skin there are two types of melanosome associated with different melanin pigments. Spherical phaeomelanosomes contain red/yellow phaeomelanin and ellipsoid eumelanosomes contain brown/black eumelanin (Bolognia and Pawelek 1988)

1.2.3 Melanin biosynthesis

Melanin is present in all animal species, fungi, bacteria and higher plants and appears to have many functions. A primary function is photoprotection, protecting against UV radiation by dissipating light energy as heat. Other functions may include thermoregulation and protection of other cell metabolites from photodestruction.

Melanin, and the metabolic roles of its precursors, are known to affect a number of biochemical, developmental, physiological and sensory-neural systems (Creel 1980). The melanin biosynthetic pathway is shown in Figure 1.2. Tyrosinase catalyses the hydroxylation of tyrosine to 3,4 dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone.

Dopaquinone is then further metabolised to either yellow/red phaeomelanin or to black/brown eumelanin (Hearing and Jimenez 1989). In addition to tyrosinase, a dopachrome conversion factor, converting dopachrome to 5,6 dihydroxyindole-2-carboxylic acid (DHICA), has been recognised (Barber *et al.* 1984; Jackson *et al.* 1992; Tsukamoto *et al.* 1992).

1.2.4 Tyrosinase

Tyrosinase (monophenol monooxygenase, EC 1.14.18.1)is a multifunctional, copper-containing monophenol dihydroxyphenyalanine: oxygenoxidoreductase enzyme, with a dual catalytic activity. It catalyses two major reactions within the phaeomelanin and eumelanin biosynthetic pathways (Halaban *et al.* 1988; Takeda *et al.* 1989), as illustrated in Figure 1.2.

Anagen hairbulbs contain a local concentration of melanocytes and can be incubated in L-tyrosine or L-3,4-dihydroxyphenylalanine (L-dopa) to stimulate *in vitro* tyrosinase activity. The tyrosinase activity in incubated hairbulbs of differently pigmented individuals can be measured quantitatively using an assay measuring the tyrosine hydroxylase activity of tyrosinase, by the rate of formation of ^3HOH with the oxidation of L-tyrosine to L-dopa (King and Witkop 1976). Tyrosinase activity can also be measured qualitatively by measuring the degree of darkening of the incubated hairbulb (Van Dorp *et al.* 1982). Tyrosinase activity has been found to vary considerably among normal individuals, both with the same and with different hair colour.

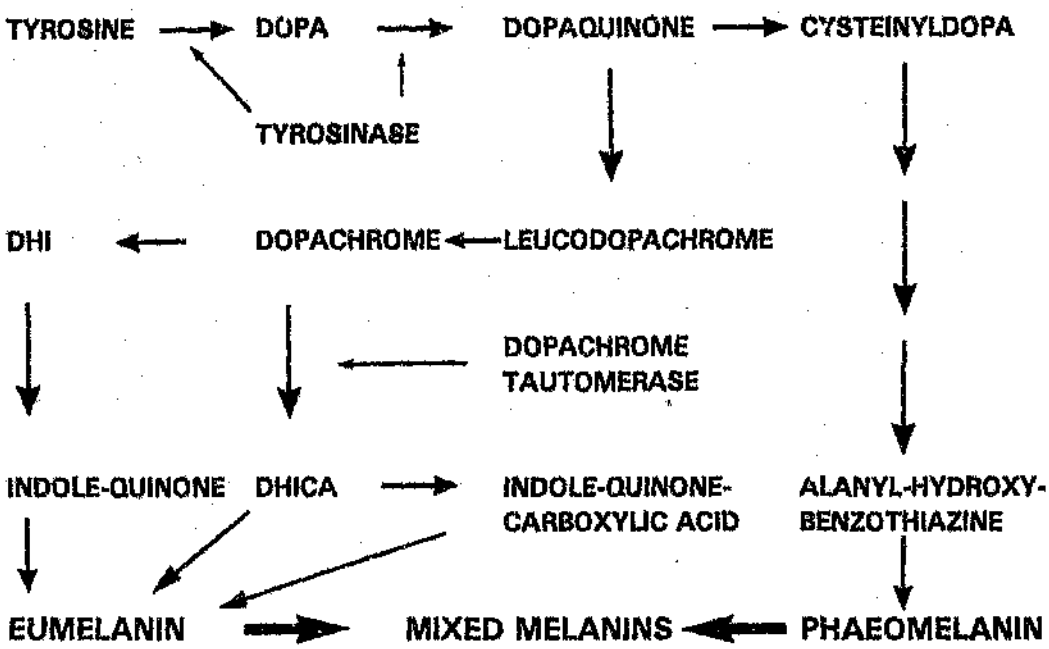

Figure 1.2 The melanin biosynthetic pathway (Tsukamoto *et al.* 1992).

Tyrosinase assays are often used to distinguish different types of OCA. Ty-neg OCA hairbulbs show no tyrosinase activity while ty-pos OCA hairbulbs contain moderate to large amounts of tyrosinase. Quantitative tyrosinase assays show broadly overlapping values between variously pigmented individuals and are thus not always a reliable indicator of tyrosinase or pigmentation status. Similarly, the qualitative test is not a particularly accurate diagnostic tool since there is variation in the degree of darkening among anagen hairbulbs of the same patient (Kugelman and Van Scott 1968; Van Dorp *et al.* 1982). Therefore, the hairbulb test alone is insufficient for classifying albinos (Van Dorp *et al.* 1982).

1.3 Ocular Albinism (OA)

Ocular albinism usually only involves the eyes, but may be associated with generalised decreased pigmentation.

X-linked ocular albinism (XOA) is a relatively rare heriditary eye disorder, characterised by hypopigmentation of the retina, decreased visual acuity, iris translucency and foveal hypoplasia in affected males (Witkop *et al.* 1989). Heterozygous females frequently show a mottled pigmentation in the periphery of the fundus as a result of Lyonisation (Bergen *et al.* 1991; Schnur *et al.* 1991) and occasionally a female will be as severely affected as a male (Witkop *et al.* 1989). This condition is due to a hereditary inborn error of pigment cell melanogenesis and linkage analysis places the XOA locus in the Xp22.2-p22.3 region (Schnur *et al.* 1991)

Features of the autosomal recessive ocular albinism (AROA) syndrome occur in both males and females and include decreased visual acuity, hypoplasia of the fovea, photophobia, nystagmus, strabismus and asymmetric monocular visual evoked potentials. Skin pigmentation is usually light but within normal limits (Witkop *et al.* 1989). XOA and AROA are not allelic since a mating between a

male with XOA and a female with AROA produced an X-linked heterozygote with normal pigmentation. An interstitial deletion of 6q13-q15 has been associated with AROA and may thus be a candidate region for this condition (Rose *et al.* 1992). It has been postulated that AROA in Caucasoids represents the same genetic defect as the brown OCA seen in Negroids (Witkop *et al.* 1989).

1.4 Oculocutaneous Albinism (OCA)

Ten different types of OCA have been described (Witkop *et al.* 1989), of which 9 are autosomal recessive conditions, including 4 syndromes associated with metabolic defects other than a biochemical defect in the melanin biosynthetic pathway. The Hermansky-Pudlak Syndrome (HPS) and Chediak-Higashi Syndrome (CHS) both present with lysosomal defects and OCA. The black locks, albinism and deafness syndrome (BADS) and Cross Syndrome are characterised by an absence of melanocytes. Autosomal dominant OCA may represent a melanosomal structural protein defect (Witkop *et al.* 1989). The most common autosomal recessive types of OCA are types I and II. Type II, or tyrosinase-positive, OCA occurs more frequently than type I (tyrosinase-negative OCA) in both Caucasoids and Negroids.

1.4.1 Type I OCA

1.4.1.1 Type IA/Tyrosinase-negative oculocutaneous albinism (ty-neg OCA)

This type of albinism represents the typical Garrodian phenotype, with no clinically detectable pigment, no tyrosinase activity and only stages I and II melanocytes. Most type IA OCA individuals are phenotypically similar and although this type of albinism is the second most common in type in Caucasoids, it is rare in southern African Negroids. Ty-neg OCA in

humans is allelic to c-locus albinism in mice (Section 1.6.2.1.5).

Type IA OCA results from deficient tyrosinase activity. Several human tyrosinase cDNAs have been cloned (Kwon *et al.* 1987; Shibahara *et al.* 1988; Bouchard *et al.* 1989; Giebel *et al.* 1991a) and the 529 amino acid sequence of the protein is known. The tyrosinase gene is located on human chromosome 11q14-q21 (Barton *et al.* 1988) and consists of 5 exons and 4 introns, spanning more than 50kb of DNA (Tomita *et al.* 1989; Giebel *et al.* 1991a). Genetic linkage has been demonstrated between the tyrosinase gene and ty-neg OCA in families with the condition (Giebel *et al.* 1990a).

A tyrosinase-related sequence on chromosome 11p contains the sequences corresponding to tyrosinase exons 4 and 5 and the surrounding noncoding regions, and shows 2.6% homology with the tyrosinase gene (Giebel *et al.* 1991a). This segment may be part of a gene expressed in cells other than pigmented cells, or it may be a truncated and translocated tyrosinase pseudogene (Giebel *et al.* 1991a).

Many mutations have been identified in the tyrosinase gene of patients with typical ty-neg OCA (Tomita *et al.* 1989; Giebel *et al.* 1990a; Kikuchi *et al.* 1990; Spritz *et al.* 1990a; Chintamaneni *et al.* 1991a; Giebel *et al.* 1991b; Oetting *et al.* 1991; Spritz *et al.* 1991; Oetting *et al.* 1992; Tripathi *et al.* 1992). Giebel *et al.* (1991c) and King *et al.* (1991), have described a novel type I OCA in which the tyrosinase is thermosensitive, losing activity at 35-37°C, causing melanin distribution to be correlated with local body temperature. This phenotype appears to be the human homologue of the Siamese and Himalayan phenotypes.

Most mutations associated with type I OCA cluster in 2 relatively small segments of the tyrosinase gene, homologous to the positions of clusters of murine mutations (Muller *et al.* 1988; Oetting *et al.* 1992), suggesting that these 2 sites code for the catalytic sites of the polypeptide (Tripathi *et al.* 1992a). It has been shown that all mutations resulting in type IA OCA occur within the evolutionarily conserved region of the tyrosinase gene and mutations resulting in decreased pigment production occur within less well conserved regions of the tyrosinase gene.

1.4.1.2 Type IB oculocutaneous albinism/Yellow mutant

Type IB OCA was first descibed in an inbred Amish kindred (Nance *et al.* 1970). Affected individuals have yellow/yellow-red hair and mildly tanning, cream coloured skin, with decreased tyrosinase activity and melanin production. The hairbulb test is usually minimal and stage III melanocytes show little, uneven pigmentation (Witkop *et al.* 1989). The existence of type IA/IB compound heterozygotes and the occurrence of ty-neg OCA and yellow mutants within a single pedigree, suggested that these types are allelic (Hu *et al.* 1980; Giebel *et al.* 1991d) and tyrosinase gene RFLP analyses confirmed allelism of these types of albinism. Sequence analysis of unrelated type IB albinos showed different tyrosinase alleles associated with this condition, producing "leaky" *in vivo* phenotypes, with greatly decreased tyrosinase activity (Giebel *et al.* 1991d).

1.4.1.3 Type IC oculocutaneous albinism/Minimal pigment/Platinum mutant

The minimal pigment (King *et al.* 1986) and platinum (Witkop *et al.* 1989) conditions are probably identical. Affected individuals are characterised primarily by a type IA phenotype, with the development of minimal amounts of iris pigment and phaeomelanin pigments in the hair with

increased age. Electron microscope studies show stage II premelanosomes with normal morphology. Tyrosinase activity is absent, so it has been postulated that a further allele at the tyrosinase locus is responsible for this phenotype, resulting in altered tyrosinase function and melanin distribution in the iris and hair (King *et al.* 1986).

1.4.2 Brown oculocutaneous albinism

Brown OCA is an autosomal recessive condition occurring in Negroids, and is characterised by a distinctive brown coloured skin which tans, brown hair and blue or brown eyes (Kromberg *et al.* 1990), and the ocular features of albinism. Little pigment accumulates with age in these individuals and hairbulb tyrosinase is normal, indicating that this condition is distinctly different from type I OCA. The melanocytes are structurally normal, although stage IV melanosomes contain reduced amounts of melanin. The phenotype and melanosome morphology suggest a block in the distal eumelanin pathway. It has been suggested that brown OCA in Negroids may be allelic to AROA in Caucasoids (Witkop *et al.* 1989).

1.4.3 Rufous oculocutaneous albinism

Red or rufous albinism is a recessively inherited condition first described by Pearson (1913). This condition has been observed in New Guinea (Walsh 1971), Africa and in AfroAmericans (Witkop *et al.* 1989). It is characterised by distinctive brick-red to mahogany skin, hazel to brown eyes and fair, ginger to red hair. Nystagmus and photophobia are present in about 10% of red albinos and visual evoked potentials (VEPs) are generally normal. The prevalence of rufous albinism is reported to be 1 in 8580, but may be higher in southern African Negroids. These individuals have normal melanosomes and melanocytes on electron micrograph studies (S. Kidson personal communication). Basal cell carcinoma has not been described in these

individuals (Kromberg *et al.* 1990). The rufous hairbulbs show tyrosinase activity and thus the condition is not expected to be allelic to ty-neg OCA.

1.4.4 Type II/Tyrosinase-positive oculocutaneous albinism (ty-pos OCA)

1.4.4.1 Clinical features of ty-pos OCA

Ty-pos OCA is the commonest type of OCA in Africa, characterised by fair skin and an accumulation of phaeomelanin pigments with age, to give yellow/tan hair and light hazel/brown eyes as pigment accumulates in the irides. These individuals often have esotropic strabismus, and always present with nystagmus and photophobia (Figure 1.3). Visual acuity is decreased but is usually better than in ty-neg OCA. Matings between ty-pos albinos and ty-neg albinos are complementary, illustrating that ty-pos OCA and ty-neg OCA are not allelic (Witkop *et al.* 1970).

Ty-pos OCA individuals in southern Africa present with two distinctly different phenotypes. Affected individuals in a family will be characterised by the presence or absence of clearly demarcated, pigmented patches or ephelides, particularly on sun-exposed areas (Kromberg *et al.* 1989). The presence of ephelides has been correlated with a lower risk of developing skin cancer, presumably as a result of the presence of melanin pigments offering photoprotection (Kromberg *et al.* 1989). These phenotypes are illustrated in Figures 1.3 and 1.4.

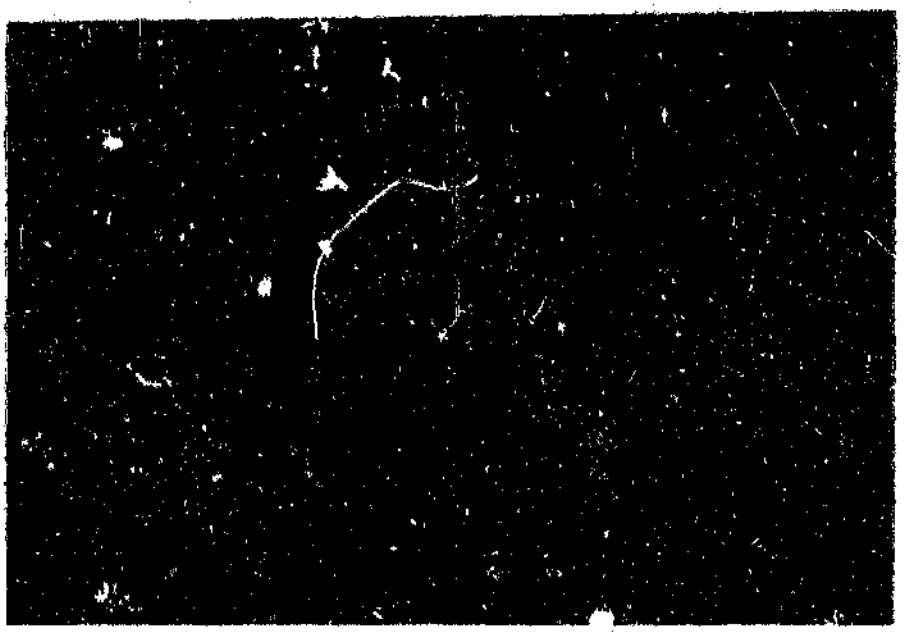

Figure 1.3 A family with three affected ty-pos OCA individuals, illustrating the fair skin and cream coloured hair, strabismus and light coloured eyes, as compared to the normal Negroid phenotype.

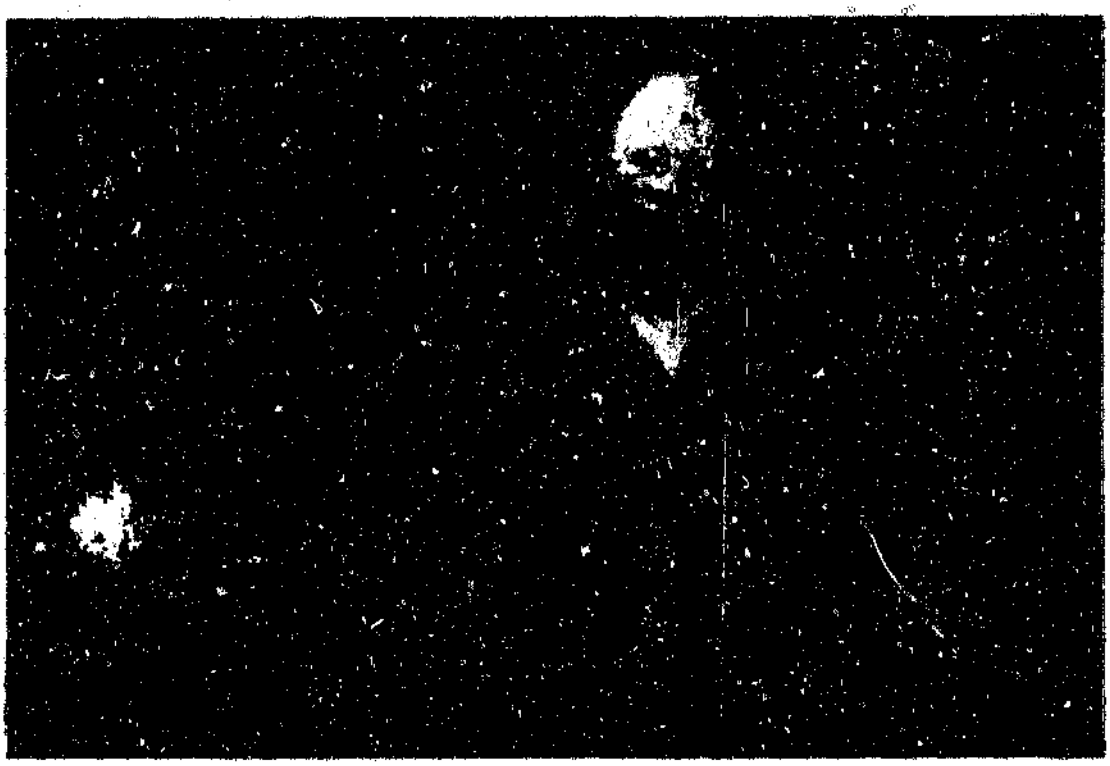

Figure 1.4 Two phenotypically different ty-pos OCA individuals. The individual on the right has ephelides on the sun-exposed areas of skin, while the individual on the left has no eumelanin pigmentation, despite obvious sun exposure.

Quantitative and qualitative hairbulb incubation tests show evidence of tyrosinase activity and frequently show a few discernible pigment grains in stage III melanosomes. Ty-pos OCA heterozygotes are not clinically or biochemically detectable, although skin reflectance studies suggest that ty-pos OCA heterozygotes have lighter skins than normal controls. However, this test is also not sensitive enough to be applied in the diagnosis of the non-carrier state (Roberts *et al.* 1986). Tyrosinase assays show that normally pigmented ty-pos OCA heterozygotes may have markedly reduced or absent tyrosinase activity in hairbulb melanocytes. It is postulated that these individuals do not have soluble tyrosinase but that all the tyrosinase is membrane-bound and thus the heterozygous individual is normally pigmented (King and Witkop 1977). Ty-pos OCA heterozygotes cannot be identified reliably using the hairbulb tyrosinase assay, due to the overlap between normally pigmented controls and heterozygotes (Van Dorp *et al.* 1982; King and Olds 1985).

Until recently, the basic defect in ty-pos OCA was unknown. Serum copper levels and serum β-MSH levels are normal, as are the serum levels of tyrosine and phenylalanine. Tyrosinase activity in the hairbulb demonstrates normal uptake of the substrate by melanocytes and tyrosinase activities of hairbulbs range from normal to a 4-fold increase from hairbulbs of normally pigmented individuals (Witkop *et al.* 1989). If the defect is biochemical, it would appear to exist in the distal eumelanin pathway (King and Olds 1985).

1.4.4.2 Prevalence of ty-pos OCA

The overall prevalence of ty-pos OCA is approximately 1 in 3900 in southern African Negroids. The prevalence varies amongst populations, rising to as high as 1 in 2000 in the Tswana and the south Sotho, and to 1 in 4500 in the Zulu (Kromberg and Jenkins 1982). The high rates of

albinism in the Swazi and Sotho-Tswana people can be correlated with the high rate of consanguinity practised by these groups. In the Zulu people, consanguineous marriages are discouraged. Obligatory heterozygotes for ty-pos OCA are often lighter skinned than the general population and, since light skin colour is a desirable characteristic in some ethnic groups, carriers may be at a selective advantage. Grandparents of albinos have been shown to have slightly increased fertility, while albinos generally have lower fertility, which may be the result of physiological problems or due to difficulties with social acceptance (Kromberg 1987).

1.4.4.3 Visual anomalies

Individuals with ty-pos OCA have characteristic visual disturbances (Creel 1980), including congenital nystagmus, strabismus, photophobia and monocular vision (Apkarian *et al.* 1983). The monocular vision is secondary to abnormalities in the optic pathways connecting the retina and the geniculate nuclei. In vertebrates with binocular vision, there is an increase in the number of ipsilateral fibres from the temporal retina, allowing impulses from corresponding points on the two retinas to project to the same area of the brain (Drager 1986). In vertebrates with all types of albinism, there is a decrease or absence of the homolateral fibres (Figure 1.5). Electrophysiological VEP tests (Creel *et al.* 1974; Apkarian *et al.* 1983; Kriss *et al.* 1990) reveal nondecussated optic tracts in albinos and may be used to confirm a diagnosis of albinism.

It has been postulated that embryonic optic routing in albinos may be affected by: an absence of the active role of melanin at the embryonic optic chiasm in optic development; an error in ontogenic timing when retinal ganglion fibres initially course through the chiasma; a lack of genetic control which is correlated with melanin pigment; chemical influences on the neurons in the optic stalk (Creel *et al.* 1990).

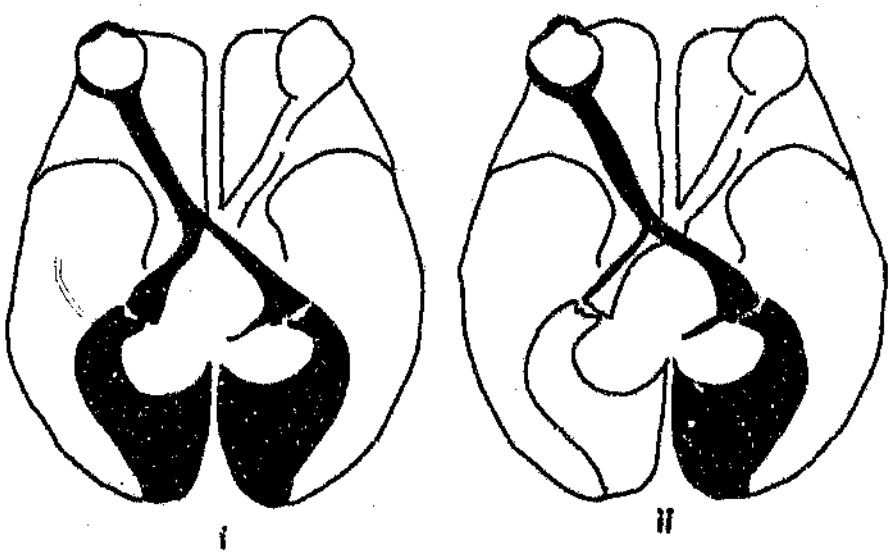

Figure 1.5 Schematic representation of i) normal routing of optic nerve fibres and ii) albino routing of optic nerve fibres (Creel *et al.* 1990).

Cats, heterozygous for the *c* (*albino*) gene, have similar decussation abnormalities as cats homozygous for this gene, thus it has been suggested that visual pathway anomalies in the presence of an albino gene may not be the result of hypopigmentation, but a pleiotropic effect of the gene. However, individuals with X-linked and autosomal recessive ocular albinism have identical visual pathway abnormalities to those in OCA, as do some Prader-Willi/Angelman Syndrome patients, suggesting that lack of pigment is the cause of fibre misrouting (Creel *et al.* 1990). The decrease in homolateral fibres in albinos is thought to occur as a result of lack of pigment in the optic stalk during embryonal development (Lund 1986; Creel *et al.* 1990).

Excess light scatter results in photophobia and the presence of hypoplastic fovea decreases visual acuity and causes nystagmus (Bolognia and Pawelek 1988). Retinal image quality is adversely affected by the presence of nystagmus, high refractive errors and intra-ocular light scatter, as well as neuroanatomical abnormalities, including increased spacing of the central cones, foveal hypoplasia and the misrouting of ganglion cell axons at the chiasma (Abadi *et al.* 1990; Creel *et al.* 1990; Guillery 1990). Stabismus is likely to occur as a result of misdirected ocular reflexes (Witkop *et al.* 1982).

Obligatory ty-pos OCA heterozygotes seldom, if ever, have detectable visual anomalies, despite the finding that these carriers have significantly lighter skins than normal controls (Castle *et al.* 1988). Visual acuity is reduced in ty-pos OCA and although visual function within a family is usually consistent, it may have variable expression, and thus homozygous affected relatives may not always be identified by reduced visual acuity and nystagmus (Summers *et al.* 1991).

1.4.4.4 Auditory anomalies

There are many cases of an association between congenital abnormalities of the inner ear and abnormal body pigmentation (Creel 1980), including a report of a mild case of Angelman syndrome, light pigmentation and sensorineural hearing loss (Williams *et al.* 1989a).

Human albinos have abnormal auditory evoked potentials, localised to the brain stem relays of the auditory pathway (Creel 1980) and the nerve cells in the albino brain stem relays are abnormally small (Guillery 1990). It is not known whether the absence of melanin affects auditory pathway development (Witkop *et al.* 1982) or whether the auditory abnormalities are a pleiotropic effect of the ty-pos OCA gene.

1.4.4.5 Skin cancer

Apart from optical and auditory disorders, the major medical problems for the African albino are associated with hypopigmented skin and the consequent suseptibility to basal and squamous cell carcinomas (Okoro 1975; Kromberg *et al.* 1989). Susceptibility to skin cancer in southern Africa increases with age, with over 31% of albinos exhibiting lesions by the end of the second decade and 42% by the end of the third decade (Kromberg *et al.* 1989). The risk in sub-Saharan Africa appears to be less than at the equator. In Nigeria, it was found that every albino over 20 years of age had premalignant or malignant lesions (Okoro 1975).

1.4.4.6 Counselling issues

Psychosocial attitudes of southern African albinos appear to be reasonably positive and they seem to be well accepted in the present day communities. However, few albinos ever marry (Kromberg and Jenkins

1984) and some have psychosomatic symptoms, possibly related to their appearance differing from their normally pigmented contemporaries. The birth of an albino infant is often emotionally upsetting for the mother and it may take up to 2-3 months for bonding to occur between the mother and the albino child (Kromberg *et al.* 1987). It is widely believed among Negroid peoples that albinos do not die, but simply vanish. This myth may be related to the special signifiance some local Negro groups attach to the colour white (Kromberg and Jenkins 1984). Despite poor vision, the intelligence level of the ty-pos albino is normal (Kromberg 1987).

Counselling should include descriptions of the basic genetic defect, details of the phenotype including poor vision and possible skin lesions, and inheritance. Myths and superstitions should be discussed, along with community and social attitudes (Kromberg and Jenkins 1984). Clinics should encourage the use of anti-actinic cream, particularly to sun-exposed areas, the use of hats and long-sleeved clothes and trousers and avoidance of direct sunlight, in order to reduce the risks of skin cancer (Kromberg *et al.* 1989).

1.4.4.7 Prenatal diagnosis

Prenatal diagnosis of albinism may be based upon hairbulb fetoscopy between the 16th and 20th weeks of pregnancy, and detection of melanosome development. In the case of ty-neg OCA, it could be done by direct tyrosinase gene analysis and for ty-pos OCA, by linkage analysis to markers on chromosome 15. It is important to establish which type of OCA is present since there is locus heterogeneity of ty-pos OCA in Caucasoids (Tripathi *et al* 1992). In Australia, prenatal diagnosis has been requested and would presumably lead to termination of pregnancy in some cases, should an affected foetus be found (Haynes and Robertson 1981). In southern Africa, the cultural views on prenatal diagnosis and abortion

would have to be taken into account and investigated in some detail should the diagnostic test become available. However, with appropriate counselling and care, albinos should be able to live relatively normal lives (Taylor 1987) and prenatal diagnosis may not become a regular requirement.

1.5 Chromosomal localisation of a gene locus

The human haploid genome ($3x10^9$ nucleotides) is thought to contain 50 000 to 100 000 functional genes (Bodmer 1981). In an attempt to identify these genes, the human gene mapping project (the Human Genome Project) was initiated. The final goal of this project is to determine the sequence of the genome (Watson 1990), beginning with genetic maps and physical mapping and eventually generating the entire DNA sequence (Dawson and Singer 1990).

To date, the chromosomal positions of more than 4000 genes are known (Watson 1990) and recombinant DNA technology has enabled the cloning and analysis of a few hundred disease genes within the last 15 years. Most of the genes were isolated either by "functional cloning" or "positional cloning" (previously known as the "reverse genetics" approach). "Functional cloning" depends upon the availability of information about the protein product and/or function(s) of the responsible gene and mapping of this gene follows cloning. "Positional cloning" and determination of gene function occur after the gene has been mapped (Collins 1992).

Gene identification by positional cloning begins with the collection of pedigrees in which the gene responsible for a distinct phenotype segregates with a known pattern of inheritance. These families are studied with multiple polymorphic markers until linkage is found with one or more of these markers. Additional fine mapping with other markers in the region further defines the position of the locus. Then, using closely linked markers, a physical map may be generated using

pulsed field electrophoresis (Wicking and Williamson 1991), and eventually the gene of interest will be identified.

Positional cloning is often a time-consuming process (Friedmann 1991). The development of new techniques, such as cosmid and YAC cloning, have greatly increased the size of cloned sequences for analysis and thus decreased the time needed to find a gene. Coding sequences may be detected by cross-species homology, northern blotting and cDNA library screening. Once a coding sequence is identified, the DNA sequence can be determined and it will be possible to search for mutations or functionality, based upon previously sequenced genes.

1.5.1 Polymorphism in the human genome

Genetic polymorphism has been defined as the occurrence together in the same locality of two or more discontinuous forms of a species in such proportions that the rarest of these cannot be maintained merely by recurrent mutation (Ford 1940, cited in Harris 1980). More recently, the term has been defined as the occurrence of multiple alleles at a locus, where at least two alleles appear with frequencies greater than 1% (Bodmer and Cavalli-Sforza 1976). Alleles occurring with a frequency of less than 1% are called rare variants (Meisler 1983). Locus variation may have selectional significance, or be selectively neutral and over 2300 polymorphisms have been described in the human genome (Williamson *et al.* 1991).

The study of human variation provides information on our evolutionary history and is essential for genetic analysis (Bowcock and Cavalli-Sforza 1990). To date, most work on human evolution has been carried out on "classical" or non-DNA polymorphisms, including blood groups, serum proteins and red cell enzyme polymorphisms.

Variability in human DNA can be in the form of single nucleotide substitutions or involve more nucleotides in insertion/deletion rearrangements. It is often assumed that, since coding regions of the genes are conserved, most variation detectable at the DNA level occurs in the non-coding regions and is genetically neutral. Advantageous mutations are more likely to be fixed by natural selection. Therefore, the more variability found in a sequence, the less likely it is to be of functional importance (Bowcock and Cavalli-Sforza 1990).

Botstein *et al.* (1980), proposed that differences in the DNA sequence behave like allelic variants of a gene and may therefore be used as genetic markers. Using the technique described by Southern (1975), these differences can be detected by the use of restriction enzymes, cutting the DNA at specific sites and resulting in DNA fragments of different lengths which will migrate differentially on electrophoresis. These fragments are seen as bands in different positions, representing different alleles. Polymorphisms obtained in this manner are called restriction fragment length polymorphisms (RFLPs). Most RFLPs presumably have no physiological functions and are thus well-suited for use as genetic markers (Ott 1991). RFLPs are generally caused by single nucleotide changes, resulting in the loss or gain of a restriction endonuclease recognition site, but may result from a variable number of tandem repeats (VNTRs) of a relatively short oligonucleotide sequence (Nakamura *et al.* 1987a).

The human genome contains many repetitive DNA sequences which are polymorphic in length because of variability in the number of repeat units in a tandemly repeated sequence. Hypervariable regions with tandem repeats of more than two nucleotides are known as variable number tandem repeats (VNTRs) (Nakamura *et al.* 1987a) or isatellites (Jeffreys *et al.* 1985), by analogy to the larger, similarly structured segments of satellite DNA (Jarman and Higgs 1988). Hypervariable regions with tandem repeats of two

nucleotides are known as microsatellites or dinucleotide repeats (Litt and Luty 1989) or simple sequence length polymorphisms (Smeets *et al.* 1989).

Hypervariable regions have been shown to have a common core sequence with strong homology to a recombinational hotspot in *E. coli* (Jeffreys *et al.* 1985) and in mouse (Steinmetz *et al.* 1986). It is postulated that variability at minisatellite loci is generated by a recombination signal based on the minisatellite core sequence, promoting unequal crossing-over in mitosis and meiosis (Wolff *et al.* 1988). It is not clear to what extent interallelic unequal exchange is involved in generating variability. Unequal sister chromatid exchange and replication slippage may also result in minisatellite mutation (Jeffreys *et al.* 1985; Jeffreys *et al.* 1990).

Tandem repetitive minisatellite regions in human DNA frequently show substantial allelic variability in the number of repeat units and thus provide highly informative genetic markers. Allele patterns at several different minisatellite loci can be used to generate a DNA "profile" of an individual.

The usefulness of VNTRs (or short tandem repeats with a repeat unit of more than two nucleotides) is limited by the fact that they are not randomly distributed in the human genome, but are preferentially located in the telomeric regions of chromosomes, where there are high rates of recombination (Nakamura *et al.* 1988).

Microsatellites (or dinucleotide repeats) are evenly distributed throughout the human genome and each locus may show up to 99% informativity (Wong *et al.* 1987). Each microsatellite consists of a variable number of the dinucleotide repeat units $(dC\text{-}dA)_n$, or $(dG\text{-}dT)_n$, where n ranges from 10 to 60. Approximately 50 000-100 000 copies of these repeats are proposed to exist in the genome and, on average, are distributed every 30-60kb. Polymorphisms due to variable numbers of the CA dinucleotide, were first

described by Weber and May (1989) and Litt and Luty (1989). Alleles differ by 2bp and can be s n polyacrylamide gels after PCR amplification. Generally, the longer the CA repeat, the greater the probability that it is polymorphic and the higher the heterozygosity at that locus (Wolff *et al.* 1988; Weber 1990).

The role(s) of CA repeats are unknown, but they may be involved in gene conversion and chromosomal rearrangements and they are postulated to determine the formation of Z DNA (Hamada *et al.* 1984). Since tracts of $d(GA)_n.d(TC)_n$ are evenly distributed in most eukaryotic genomes, they may play a role in various biological processes, including replication or recombination (Wahis *et al.* 1990), transcription or chromatin organisation (Yee *et al.* 1991). These sequences may mediate their biological function *in vivo* by the formation of triple-stranded DNA structures or through direct DNA-protein interactions (Yee *et al.* 1991).

Hypervariable loci have been found to have high mutation rates, and minisatellite loci with heterozygosities of 99% have been reported to have mutation rates of up to 5% between 2 generations (Jeffreys *et al.* 1988b). CA repeat sequences may change due to the loss or gain of a dinucleotide or the insertion/deletion of a longer segment. Actual evolutionary mutation rates in CA repeat sequences may be deceptively low (Bowcock and Cavalli-Sforza 1990), depending if recurrent mutations give rise to identical alleles. However, some groups postulate that CA repeats may in fact be stable through evolution and are thus useful for population studies and in forensic applications (Sherrington *et al.* 1991).

Polymorphisms have been traditionally detected using serological and Southern blotting techniques. Recently, RFLPs and VNTRs have been shown to be more rapidly and accurately detected by the polymerase chain reaction (PCR), where high fidelity amplification of a single locus is possible (Jeffreys

et al. 1988a, 1990).

The PCR is an *in vitro* method of selectively amplifying many copies of a specific nucleic acid sequence in a reaction mixture, first described by Saiki *et al.* (1986). The amplification reaction involves denaturation of double-stranded DNA to separate the strands, annealing of the primers and extension of these primers by the DNA polymerase. These steps are repeated for many cycles, resulting in a linear accumulation of intermediately lengthed template and an exponential accumulation of the target sequence. such that only the latter is detected once the reaction is completed. It has been shown that fragments up to 3000 nucleotides are easily amplified under standard conditions, however, with longer extension steps, fragments as large as 10 200 nucleotides have been produced (Jeffreys *et al.* 1988a). PCR is designed to show great specificity to its intended target sequence and simultaneous use of multiple primer pairs allows for coamplification of multiple products (Bloch 1991).

Since PCR theoretically only requires a single molecule of DNA for successful amplification, it has been shown to have many varied uses, often with low quality, low concentration DNA samples, and the products can be visualised directly on both agarose and polyacrylamide gels after amplification. Disadvantages of PCR include the necessity of having known target flanking sequences, easy amplification of contaminants and infidelity of the *Taq* polymerase enzyme, which results in 0.3-0.8% accumulated mutations after 20-30 cycles (Keohavong and Thilly 1989). The latter is only of particular importance if the PCR product is to be used for cloning, although it may also be significant in the detection of CA repeat polymorphisms.

1.5.2 Genetic linkage analysis

The use of genetic data to determine linkage is a scientific inference procedure which draws information about the relative distance between two loci on a chromosome from the segregation of alleles in nuclear families or pedigrees (Tai and Song 1991).

If two alleles at two loci on a single chromosome are inherited independently of one another, then offspring receiving these two alleles in the same order in which they occurred in a single grandparent, are referred to as *nonrecombinants*. If two different grandparents contribute one allele at each of the two loci, then the offspring is called a *recombinant*. If these two loci appear to be genetically coupled, in other words, they are passed from grandparents to following generations with the same combination of alleles more frequently than expected, these loci are said to be *genetically linked*, or in *linkage disequilibrium* (not segregating at random within the population).

The extent of genetic linkage is measured by the *recombination fraction*, which is the probability that a gamete produced by a parent is a recombinant. The recombination fraction is traditionally referred to as Θ (*theta*). Loci segregating independently are *unlinked* and are characterised by a recombination fraction of $\Theta=0.5$, whereas *linked* genes are characterised by $\Theta<0.5$. Theta approaches zero for closely linked genes, when recombination occurs rarely between them.

Genetic linkage analysis investigates the genetic distance between disease genes and polymorphic marker loci (genetic entities following a Mendelian mode of inheritance) and requires i) linkage analysis in related individuals over several generations ii) informativity at a locus, where parents are heterozygous at that locus iii) distinctions between male and female recombination fractions, due to different recombination rates between the two

sexes, where crossing-over is usually more frequent in the homogametic (female, XX) sex (Haldane 1922, reviewed in Ott 1991).

A *linkage group* is defined as a set of loci in which each locus is linked with at least one other locus in the same set. Loci located on the same chromosome are said to be *syntenic*. In meiosis, homologous chromosomes pair and exchange genetic material in the process of *crossing-over*, to result in recombinant chromosomes which will be passed to the offspring. The occurrence of crossing-over is semi-random and can be used to construct genetic maps. The further apart two loci occur, the more likely they are to recombine. The *genetic map distance* (in units of Morgans) between two genes is defined as the expected number of cross-overs occurring between the two genes. Recombinations are the observable effects of crossing-over and may therefore be counted as cross-overs. Thus the recombination fraction between two loci is approximately equal to the distance (in Morgans) between them, for example, if the recombination fraction is 6%, Θ=0.06, then the loci are approximately 0.06 Morgans apart, or 6cM apart. Theta cannot be additive as a distance measure between multiple loci, as multiple cross-overs are likely to have occurred between these loci. Since map distance is a genetic distance, it is a measure of the observed number of cross-overs between two loci and does not necessarily correlate directly with physical distance.

A marker's usefulness for linkage analysis depends upon the number of its alleles and their frequencies (its degree of polymorphism). The degree of polymorphism may be measured in terms of *heterozygosity* (H), when

$$H = 1 - \Sigma p_i^2$$

and where p is the population frequency of the ith allele and H gives the probability that a random individual is heterozygous for any two alleles at a locus with allele frequencies p_i.

Another measure of the degree of polymorphism is the *polymorphism information content* (PIC) value (Botstein *et al.* 1980), calculated as

$$PIC = 2 \Sigma_i \Sigma_j p_i p_j (i - p_i p_j)$$

where Σ_i is the sum of i from 1 to n-1 and Σ_j is the sum of i+1 to n, p is the population frequency of the ith and jth allele and the PIC is the probability that the marker genotype of a given offspring will allow deduction of which of the two marker alleles of the parents it has received.

The number of families required to find linkage to a disease marker can be calculated using the various characteristics of the disease genotype, including mode of inheritance and penetrance. The number of meioses for which the phase is known and which will provide evidence for linkage, can be calculated knowing the mode of inheritance of the trait (Ott 1991). Unknown phase adds one offspring to each sibship and marker heterozygosity determines the frequency with which a parent is potentially informative for linkage (Ott 1991).

1.5.2.1 Two-point linkage analysis

The statistical techniques used in current linkage analysis are mostly based on maximum likelihood (Z) estimation and likelihood ratio testing (Ott 1991). The phenotypes in a pedigree imply the underlying genotypes in such a way that the recombination events can be scored unambiguously. If the parental phases are unknown, then the recombination fraction can be estimated using the maximum likelihood method (Ott 1991). This method calculates the probability of occurrence of the data for a variety of assumed values of Θ and selects that Θ value associated with the highest likelihood as the best estimate or odds for linkage. It is usually convenient to work with the logarithm of the likelihood or odds (*lod*) rather than the likelihood ratio.

Linkage programs permit relatively easy calculations of lod scores and take into account all variables, such as multiple alleles at a single locus, multigeneration pedigrees, varying penetrances and missing information. The first generally available program to compute likelihoods numerically in large pedigrees was LIPED (for LIkelihood in PEDigrees) (Ott 1974), which is based on the Elston-Stewart algorithm (cited in Ott 1991) and allows for the calculation of two point lod scores.

The LINKAGE programs (Lathrop *et al.* 1984) which were originally developed for gene mapping use the Elston-Stewart algorithm and are presently available for general pedigree analysis (Ott 1991). The MLINK program calculates likelihoods by stepwise variation of the recombination fraction in one of the interlocus intervals and it computes genetic risks. The number of loci which can be jointly analysed is limited by the available memory of the computer and, under MS-DOS on microcomputers, only allows for analysis of a total of 12 alleles simultaneously (Ott 1991). A major difference between the LIPED and LINKAGE programs is in the definition of penetrance. LIPED uses penetrance in a general sense, while in LINKAGE penetrance is the probability of being affected given a genotype (Ott 1991). These programs differ also in the use of male and female recombination fractions, with LIPED using both parameters directly and LINKAGE outputting both male and female thetas required.

Positive lod scores indicate evidence for linkage and negative lod scores indicate absence of linkage. When linkage exists, the lod scores are cummulative with the addition of more families. The critical value said to convey significant evidence for linkage is denoted by the maximum likelihood, Z=3 (Morton 1955). Two loci for which the lod score is less than or equal to -2 are not considered to be linked, in other words, linkage between these loci is excluded.

Traditionally, linkage analyses have been carried out as a sequence of pairwise (two-point) comparisons between a trait locus and each of a number of marker loci. However, with the linkage map becoming denser, the simultaneous use of several loci (multipoint analysis) is gaining importance. With many loci, the order of the loci is important and the number of parameters considered increases. The order of the loci is defined by the map distances or recombination fractions among them and the most likely order of the loci within a linkage group can be calculated. This ordering may be done by two point linkage analysis for each locus pair and the addition of new loci in a stepwise fashion (Ott 1991). Multi-point linkage is often limited by micro-computer capacity.

1.5.3 Gene Mapping

1.5.3.1 Genetic linkage maps

Genetic linkage maps are based on the coinheritance of allele combinations across multiple loci. Using two-point linkage data generated by the LINKAGE program, a genetic linkage map can be constructed around closely linked loci. This map is based upon the number of recombinations between the loci, which can be correlated with the genetic map distance between them (Section 1.5.2). Since a genetic linkage map is based upon recombination fractions, it may not necessarily correlate directly with physical maps, since some regions of the genome may be associated with higher rates of recombination than others, generating so-called "recombinational hotspots" (Ott 1991). Since the number of cross-overs is roughly correlated with the distance between two loci, increased recombination between these loci will place them genetically further apart than will a physical map. Thus genetic and physical maps should be constructed simultaneously in order to define a region accurately.

Once the position of a gene is known, the frequency of the alleles of the closest markers may be calculated in order to determine whether there is *allelic association*. Edwards (1980), has proposed the term "allelic association" for a deviation from gametic phase equilibrium for alleles in linkage disequilibrium. Tight linkage between a marker and a disease locus will maintain linkage disequilibrium. Linked loci tend to show allelic association when the recombination fraction between them is less than 2% (Ott 1991).

Closely linked polymorphic markers are inherited together in a certain physical order. The pattern of inheritance of alleles at these markers is called a *haplotype*. Haplotypes generated using RFLP markers around the ty-pos OCA locus in affected individuals, assuming these markers are selectively neutral and situated close together, may be used to assess whether the mutation at the locus arose once in a relatively recent common ancestor (if a single or a few haplotypes occur with a significantly high frequency) or whether the mutation had multiple origins (many haplotypes associated with the mutant allele). The latter may also represent a single mutation which arose in the distant past and around which the surrounding markers have since undergone more mutations, generating many different haplotypes associated with the original mutation.

Haplotypes increase the information content of a chromosomal region, as in the study of the cluster of polymorphisms surrounding the cystic fibrosis mutation (Estivill *et al.* 1987) and the polymorphic restriction sites in the vicinity of the β-globin gene cluster (Antonarakis *et al.* 1985). The sickle cell mutation, associated mainly with three β-globin haplotypes in Africa, has been used to show the multicentric origin of the sickle cell haemoglobin gene (Pagnier *et al.* 1984) and association with a single major haplotype in the Bantu-speakers provided the first biological evidence for the common ancestry of these people (Ramsay and Jenkins 1987).

1.5.3.2 Physical Maps

1.5.3.2.1 Use of somatic cell hybrids

The construction of a physical map of the human genome has been dramatically aided by the isolation of moderately sized regions of DNA within interspecific somatic cell hybrids (SCH) containing a single human chromosome or part of a chromosome. These hybrids are useful for DNA library construction from chromosomal regions (Warburton *et al.* 1990). Monochromosomal rodent-human hybrids often use aneuploid human cell lines as donors, although the human chromosomal complement must be carefully characterised, especially if rearrangements are present. Lymphoblastoid cell lines are good donors of human chromosomes, and do not undergo chromosomal rearrangements (Warburton *et al.* 1990).

In order to map a marker to a chromosome, a panel of SCH with overlapping and unique chromosomal complements is required. Hybridisation of restriction enzyme-digested DNA from this panel of SCH with the marker of interest, will show rodent-specific hybridisation patterns as well as human-specific hybridisation patterns. Using well-characterised SCH lines, a process of deduction will enable the marker to be localised to a particular chromosome. Once the chromosome of interest has been identified, hybridisation of the marker to SCH containing different, known parts of this chromosome can be used to further define the subchromosomal region to which it maps. Similarly, if the marker is thought to map within a syntenic region on a particular chromosome, SCH containing the whole chromosome or known parts thereof can be used to confirm or negate this theory.

1.5.3.2.2 Other mapping techniques

Physical maps can be cytogenetically or molecularly based. Cytogenetically based maps order loci with respect to visible banding patterns or relative position along the chromosome by SCH data and use of *in situ* hybridisation. Molecularly based maps directly characterise large regions of DNA by establishing molecular landmarks such as restriction endonuclease recognition sites or sequence tagged sites (STSs). These maps are usually constructed from data generated by pulsed field gel electrophoresis or other techniques which enable sizing and ordering of large restriction fragments of DNA. Characterisation of cloned DNA (in YACs or cosmids) results in the establishment of overlapping assemblages of large clones, called contigs (Stephens *et al.* 1990), which enable physical analysis of larger pieces of DNA, thus generating more information with less preparative work.

1.6 Approaches to the chromosomal localisation of the ty-pos OCA locus

When this study was initiated, the ty-pos OCA locus had not yet been localised and since the biochemical defect was unknown, a linkage study was undertaken. Three different linkage approaches were used: searching for linkage between ty-pos OCA and random serogenetic and DNA markers; determining linkage between candidate pigment genes and the ty-pos OCA locus; establishing linkage between candidate chromosomal regions and ty-pos OCA. Although the number of potential candidate cDNAs may be relatively small, the candidate gene approach often reveals the gene of interest without necessitating physical and genetic mapping (Collins 1992). Once linkage had been found between ty-pos OCA and any marker or chromosomal region, genetic and fine physical mapping would follow and, ultimately, the gene would be cloned.

The presence of visible cytogenetic rearrangements which interrupt or delete a disease locus greatly facilitate gene mapping of that locus (Wicking and Williamson 1991). Ty-pos OCA has been reported in an individual with a balanced translocation, 46, XY, t(2;4)(q31.2;q31.22), and it was postulated that there may be a locus for ty-pos OCA in the q31 region of either chromosome 2 or chromosome 4 (Walpole and Mulcahy 1991).

Gene mapping is often facilitated by the concurrence of two conditions within a single individual or within a family, which may indicate that the two conditions are genetically linked. It has been postulated that linkage exists between albinism and schizophreniform psychosis, on the basis of the concurrence of the conditions in a single family (Baron 1976). The occurrence of cerebellar ataxia and albinism in a single family may suggest that the genes responsible for these conditions are in synteny with one another (Bamezai *et al.* 1987). The oculocerebral syndrome (Cross Syndrome) and generalised hypopigmentation have been found to occur together in single individuals, suggesting that these conditions may be genetically linked (Castle *et al.* 1989; Pessagno *et al.* 1992). However, these conditions have not yet been mapped and it is not always clear which type of OCA was associated with each condition. Thus the association between the conditions and ty-pos OCA is still vague.

1.6.1 Random markers

Often, a linkage study will be initiated using random polymorphic markers scattered throughout the genome, in an attempt to establish linkage to a chromosome containing any of these markers. If linkage is found between a random marker and a disease, other markers in the region will be used, in an attempt to fine map the region surrounding the gene. With the introduction of the ty-pos OCA linkage study, several random serogenetic and DNA markers from different chromosomes were studied in the ty-pos OCA families.

1.6.2 Comparative mapping

Mutations in mice have been instrumental in the identification of a number of structural and regulatory genes involved in various aspects of human development, including pigmentation (Searle *et al.* 1989). Genes affecting coat colour and pigmentation in mice were among the first to be studied for Mendelian inheritance. More than 150 mutations at more than 50 pigment loci have been described in the mouse (Silvers 1979) and human pigmentation may be equally complex.

Those loci associated with decreased pigmentation in mice, and for which the phenotype could be correlated with the ty-pos OCA phenotype, include the *pink-eyed dilute* (*p*), *agouti* (*A*), *extension* (*E*), *dilute* (*d*), *albino* (*c*), *brown* (*b*) and *slaty* (*Slt*) loci (Silvers 1979). The proteins encoded by the last three genes (*c*, *b*, *Slt*) form a tyrosinase protein family (Jackson *et al.* 1992). The pigment genes follow a Mendelian pattern of inheritance and produce a wide range of specific effects on features including the origin and development of melanoblasts, the shape and size of melanosomes, the biosynthesis of tyrosinase and melanosome transfer (Robins 1991). The human homologues of all these genes may thus be investigated for their role, if any, in the ty-pos OCA phenotype.

1.6.2.1 Candidate loci

1.6.2.1.1 *Pink-eyed dilution* (*p*)

The *pink-eyed dilution* locus (*p*) is defined by one of the earliest known coat-colour mutations in the mouse. The wild-type allele (+) generates intense pigmentation in both the coat and eyes. At least 13 recessive alleles at this locus reduce eumelanin pigmentation in the coat and eyes, with little effect on phaeomelanin pigmentation (Silvers 1979). In

addition to pigmentation, some *p* allele effect reproduction, development and behaviour. It has been suggested that these pleiotropic effects may occur as a result of multilocus deletions altering both the *p* gene and other genes adjacent to it (Lyon *et al.* 1992). A nested set of deletions around the *p* locus has been characterised and used in complementation studies to construct a functional map of the region (Lyon *et al.* 1992).

A syntenic relationship has been demonstrated between gene markers closely linked to the *p* locus on mouse chromosome 7 and human chromosomes 11 and 15 (Figure 1.6, Section 1.6.2.2.1)(Searle *et al.* 1989; Saunders and Weldin 1990; Chaillet *et al.* 1991; Nadeau *et al.* 1991). The *p* region is of particular interest as a result of its homology with the Prader-Willi/Angelman Syndrome (PWS/AS) region on human chromosome 15q11-q13 (Nakatsu *et al.* 1992), since these syndromes are often associated with hypopigmentation (Section 1.6.2.2.2).The region of homology includes the GABRB3 gene mapped close to the *p* locus in mouse and in the PWS/AS region in man (Wagstaff *et al.* 1991). If the genes mediating the pleitropic effects of certain *p* mutations in mouse have homologues in a conserved linkage group on human chromosome 15q11-q13, they may also be involved in some of the pleiotropic effects of the PWS/AS region, including the common neurological features (Lyon *et al.* 1992). Mutations at the human homologue of the mouse *p* locus have been proposed to result in a ty-pos OCA phenotype (Brumbaugh *et al.* 1979; Witkop 1985; Brilliant *et al.* 1991; Nakatsu *et al.* 1992; Ramsay *et al.* 1992; Rinchik *et al.* 1993).

The *p-unstable* (p^{un}) allele

The *p-unstable* (p^{un}) allele apparently only affects pigmentation and produces a phenotype consisting of areas of dilute and intense pigmentation, which seem to occur as a result of a spontaneous reversion to wild-type at a relatively high frequency in all stages of development (Silvers 1979; Brilliant *et al.* 1991).

Brilliant *et al.* (1991) found the p^{un} mutation to be associated with a DNA duplication. Loss of this duplication resulted in a reversion of the p^{un} phenotype to the wild-type phenotype, suggesting that the p^{un} duplication disrupts the coding sequence of the *p* locus. Removal of the duplication restores the linear array of genetic information at this locus and leads to a reversion of phenotype (Brilliant *et al.* 1991). This phenotype may be correlated with that of ty-pos OCA with pigmented patches or ephelides (Ramsay *et al.* 1992).

1.6.2.1.2 *Agouti* (*A*)

The *agouti* locus is localised to mouse chromosome 2, in a region with synteny to human chromosome 15 (Searle *et al.* 1989). Seventeen alleles at this locus control the banding pattern of individual coat hairs by regulating a switch between eumelanin and phaeomelanin production (Silvers 1979). Some mutations at the *A* locus are associated with viral insertions (Hearing and Jimenez 1989). Allelomorphic *agouti* systems are present in most mammalian orders and the *A* gene may therefore have a fundamental role in mammalian development (Barsh and Epstein 1989).

Heterozygotes for the lethal yellow mutation (A^y) produce only phaeomelanin in hair follicle melanocytes, giving uniformly yellow hair

(Hearing and Jimenez 1989), a phenotype which may be correlated to that of ty-pos OCA. The human homologue of the *A* locus has not yet been mapped, but may occur on human chromosome 15.

1.6.2.1.3 *Extension (E)*

The *E* locus on mouse chromosome 8 determines the phaeomelanic nature of melanin products, resulting in uniform yellow hairs, a phenotype which may again be correlated with that of ty-pos OCA. Studies on these yellow mice have shown that the activity of the dopachrome converion factor (DCF) is significantly reduced (Lamoreux *et al.* 1986). It is known that the locus for the DCF is on mouse chromosome 14, however the gene product of the *E* locus and the DCF may be functionally and physically related, resulting in the phaeomelanic phenotype of *E/E* mice.

1.6.2.1.4 *Dilute (d)*

The *d* locus on mouse chromosome 9 dilutes intense pigmentation and affects melanosome production and distribution (Silvers 1979). It has been associated with viral insertions (Hearing and Jimenez 1989). A recombinant cDNA from the *d* locus has been cloned and encodes a novel myosin heavy chain protein, affecting melanosome shape and pigment distribution (Mercer *et al.* 1991). This locus occurs in a region with synteny to human chromosome 15 (Searle *et al.* 1989), but as the melanosomes in ty-pos OCA skin and hair appear to be normal (Sue Kidson, personal communication) it is unlikely that the *d* locus is the mouse homologue of the human condition.

1.6.2.1.5 *Albino (c)*

The *albino* or *c*-series of alleles is characterised by a deficiency or alteration in the structure of tyrosinase and phenotypically characterised by a severe decrease in pigmentation or complete absence of pigment (Section 1.4.1). The *c* locus has been localised to mouse chromosome 7 and the wild-type allele (*C*) results in full tyrosinase activity, dominant over all other alleles. The *c* locus on mouse chromosome 7 is homologous to the tyrosinase locus on human chromosome 11q. The *p* and *c* loci on mouse chromosome 7 constituted the first linkage group identified in mammals (Haldane *et al.* 1915, reviewed in Muller *et al.* 1988). Radiation-induced deletions surrounding the c locus have been useful in constructing detailed functional maps of a 6 to 11cM region on mouse chromosome 7 (Schmid *et al.* 1985; Tonjes *et al.* 199?).

Albino (*c/c*) mice have no pigment in their skin or eyes, since the genes at all other pigment loci (for example *agouti* and *brown*) are masked. These mice (*c/c*) produce amelanotic melanocytes, while other *c* alleles (for example, c^{ch} *chinchilla*), have reduced pigmentation due to altered tyrosinase activity (Silvers 1979). Alleles of the black wild-type *C* include *c*, *albino*; c^{ch}, *chinchilla*; c^{h}, *himalayan*; and c^{e}, *extreme dilution*. Mice heterozygous for these alleles have tyrosinase levels intermediate between those those of *C/C* and *c/c* mice.

Several independent mouse cDNA clones encoding tyrosinase and generated by alternative splicing of a single transcript have been isolated (Yamamoto *et al.* 1987; Muller *et al.* 1988; Shibahara *et al.* 1988; Kwon *et al.* 1988,1989; Terao *et al.* 1989). It was shown by deletion mapping that the full-length isolated gene was localised to the *c* locus (Kwon *et al.* 1987). Muller *et al.* (1988), used a human tyrosinase cDNA (Section 2.2.1.1) to isolate a mouse cDNA which,

when transfected into albino cells, coded for functional tyrosinase. The mouse and human cDNAs show 78% DNA sequence homology and the proteins encoded by these show 80% homology (Muller *et al.* 1988). Beerman *et al* (1990) and Mintz and Bradl (1991), constructed a human tyrosinase minigene (Beerman *et al.* 1991; Mintz and Bradl 1991) which was injected into fertilised eggs of an albino mouse, several of which developed into transgenic mice with pigmentation in both skin and eyes. The hair pigmentation often occurred in patterns of transverse stripes, demonstrating dorsoventral migration of melanoblasts. Homozygous descendants of these mice were darker than the hemizygotes and transmitted the basic pattern through many generations (Mintz and Bradl 1991). This rescue of the albino phenotype proved the hypothesis that the *c* locus encodes the structural gene for tyrosinase.

Other workers have rescued the albino phenotype *in vitro* in albino melanocyte cell lines, transfecting the cells with different full-length cDNAs and producing pigmented cells (Yamamoto et al. 1989; Larue and Mintz 1990). Differences in the expression and characteristics of tyrosinase from different *C* allele homozygous murine melanocytes strains grown in culture, also prove that the albino locus in mice encodes the structural gene for tyrosinase. Since most cell lines from mice with *c* locus alleles have similar amounts of tyrosinase mRNA, it has been postulated that all these mutants have decreased pigmentation due to abnormal post-transcriptional or post-translational modification of the enzyme (Halaban *et al.* 1988).

1.6.2.1.6 *Brown (b)*

Four mutations have been assigned to the *b* locus on mouse chromosome 4, where the wild-type B produces black pigment and *b* results in pale eumelanin deposition in small, round melanosomes (Silvers 1979). The *b* locus alleles are unlikely to have a demonstrable influence on phaeomelanin synthesis (Silvers 1979) and since tyrosinase activity is usually above normal in *b/b* mice, this condition cannot be allelic to the *c* locus.

Shibahara *et al.* (1986), isolated a pigment cell-specific mouse cDNA, pMT4, and provided evidence suggesting that it encoded tyrosinase. It had nearly 40% homology with a tyrosinase cDNA, but Muller *et al.* (1988), showed that pMT4 cDNA-transfected cells did not show tyrosinase activity. Jackson (1988), demonstrated that pMT4 maps to the *brown* (*b*) locus and termed the protein which it encodes, tyrosinase-related protein 1 (TRP1) (Zdarsky *et al.* 1990; Chintamaneni *et al.* 1991b). The *b* locus gene is approximately 15-18kb long and is organised into 8 exons and 7 introns (Jackson *et al.* 1991; Shibahara *et al.* 1991). The protein encoded by this gene is a glycoprotein (gp75) with catalase activity (Halaban and Moellmann 1990), homologous to the human melanoma antigen, GP75 (Vijayasaradhi *et al.* 1990). The *b* protein has also been shown to have tyrosine hydroxylase and dopaoxidase activities by immunorecognition of specific peptides (Hearing and Jimenez 1989). The human homologue of the mouse *brown* locus has been mapped to human chromosome 9 (Abbott *et al.* 1991; Chintamaneni *et al.* 1991b; Murty *et al.* 1992) and has been termed CAS2, CATB and TYRP (McKusick 1992).

The light allele at the *brown* locus (B^b) is dominant over the wild type *B*. This mutation results in the hair being pigmented at the tip and very

little at the base. The light base gets more extensive as the mouse ages. It is postulated that this phenotype occurs as a result of premature melanocyte death following pigment synthesis. Melanin production from tyrosinase involves potentially cytotoxic intermediate compounds, such as 5,6 dihydroxyindole. If the mutant *light* TRP1 protein is bound to the lumenal membrane of the melanosome, it may cause loss of membrane integrity, allowing spillage of toxic metabolites and resulting in melanocyte death (Johnson and Jackson 1992).

1.6.2.1.7 *Slaty (Slt)*

Jackson *et al.* (1992) and Tsukamoto *et al.* (1992), reported a cDNA clone with homology to pMT4 (*brown* cDNA) and tyrosinase, but encoding a different protein, termed TRP2. This protein has dopachrome tautomerase (DT) activity and has also been called a dopachrome conversion factor, dopachrome oxidoreductase (Barber *et al.* 1984) and dopachrome isomerase (Jackson *et al.* 1992). The gene is located on mouse chromosome 14 at the *slaty* locus. A single base pair change within one of the copper-binding sites of the protein results in a 3- to 4-fold increase in DT activity and a change in coat colour to dark grey/brown with an excess of eumelanin.

1.6.2.1.8 The tyrosinase protein family

Two immunologically cross-reactive molecules are expressed specifically in the melanocyte and map to the *c* and *b* loci (Shibahara *et al.* 1991). The conservation of the primary sequences between the proteins encoded by the *c* and *b* loci is striking, showing 40-52% amino acid homology (Zdarsky *et al.* 1990; Jackson *et al.* 1991). Fifteen of the 16 cysteine residues and 7 of the 8 tryptophan residues are conserved. This homology has also been maintained in the human tyrosinase gene. The

2 potential copper-binding sites are highly homologous, as are the signal sequences, transmembrane spanning domains and the glycosylation sites (Hearing and Jimenez 1989). This strong sequence homology indicates a common evolutionary ancestor (Zdarsky *et al.* 1990; Jackson *et al.* 1991).

The tyrosinase protein family (tyrosinase, TRP1 and TRP2) appears to be an old one, since the genes are dispersed and located on different chromosomes in mouse and man. A comparison of the gene structures suggests that there was an ancient duplication and the only existing gene homology is that giving amino acid homology (Jackson *et al* 1992). Jackson *et al.* (1992), propose that all 3 proteins colocalise at the inner melanosomal membrane and form a multienzyme complex which is responsible for eumelanin synthesis. Thus if any one of these proteins was functionally altered, eumelanin synthesis would be affected.

1.6.2.2 Candidate chromosomal regions

Use of mouse genome maps and determination of which chromosomal segments are conserved in the human genome, may enable predictions about where mutations are localised in one species and will map in the other (Nadeau 1989). Comparative mapping of candidate chromosomal regions is thus useful for developing mouse model systems to study human genetic diseases.

A second set of candidate chromosomal regions include those regions known to contain genes for diseases sometimes found to be associated with another phenotype, in this case involving pigmentation. Pigmentary defects and, occasionally, albinism, have been found to be associated with several phenotypically distinct conditions, including Prader-Willi syndrome and Angelman syndrome and some psychiatric disorders (Section 1.6.2.2.2).

1.6.2.2.1 The Hbb, *c* and *p* linkage group

It has been postulated that the *c* and *p* loci in lower mammals are equivalent to the ty-neg and ty-pos OCA loci in humans (Witkop 1985). In the rat, cat, mouse, deermouse and rabbit, it has been shown that the *c* and *p* loci are linked to the haemoglobin B locus (Hbb) and occur within linkage group 1 (French *et al.* 1971; Stolc and Gill 1983; Roderick and Davisson 1984; O'Brien *et al.* 1986; Sandberg and Anderson 1987). There is strong evidence against linkage between ty-pos OCA and the β-globin gene cluster on human chromosome 11p (Heim *et al.* 1988), suggesting either that the *c*, *p* and Hbb syntenic group has not been conserved in humans, or that ty-pos OCA is not linked to any of these loci on chromosome 11 in man.

Mouse chromosome 7 has a number of genes with homologues found on both the short and long arms of human chromosome 11, and on the long arm of chromosome 15 (Figure 1.6). The human chromosome 11 homologues are mostly clustered towards the telomeres, interrupted by the chromosome 15 linkage group. A single human 11p homologue (Hras-1) occurs between loci occurring on human chromosome 15q11-q13, and the tyrosinase locus, for which the human homologue occurs on chromosome 11q, is situated between two chromosome 15q homologues (Saunders and Seldin 1990). Thus the linkage groups are not well conserved between mouse and man and the human homologue of the *p* locus may be in a region of synteny on either chromosome 11 or 15.

Figure 1.6: Linkage map of mouse chromosome 7 and homologous genes on human chromosomes 11 and 15. Mouse locus [human locus] locus name: Tph [TPH] tryptophan hydroxylase; Ldh-1 [LDHA] lactate dehydrogenase A; Myod-1 [MYOD1] myogenic differentiation 1; Saa-1 [SAA1] serum amyloid A; D15F32S1h [MN7]; D7Hms1 [D7PWS]; D15S12h/D7Nic1 [DN10]; Gabrb-3 [GABRB3] γ-aminobutyric acid β3 receptor; p [?] pink-eyed dilution; D15S9h-1 [pML34]; Hras-1 [HRAS] Harvey rat sarcoma oncogene; Igf1r [IGF1R] Insulin-like growth factor 1 receptor; Fes [FES] Feline sarcoma oncogene; Tyr [TYR] tyrosinase; Idh-2 [IDH2] isocitrate dehydrogenase 2, mitochondrial; Calc [CALCA] calcitonin; Pth [PTH] parathyroid hormone; Hbb [HBB] haemoglobin, β-chain; Th [TH] tyrosine hydroxylase (Searle *et al.* 1989; Saunders and Seldin 1990; Chaillet *et al.* 1991; Nadeau *et al.* 1991; Nicholls *et al.* 1992).

MOUSE CHROMOSOME 7	HUMAN LOCUS	POSITION ON HUMAN CHROMOSOME
Tph	TPH	11p14.3-p15.1
Ldh-1	LDHA	11p14-p15.1
Myod-1	MYOD1	11p15.4
Saa-1	SAA1	11p14-p15.1
D15F32S1h	MN7	15q11-q13
D7Hms1	D7PWS	15q11-q13
D15S12h (D7Nic1)	DN1S	15q11-q13
Gabrb3	GABRB3	15q11-q13
p	?	?
D15S9h-1	pML34	15q11-q13
Hras-1	HRAS1	11p15.5
Igf1r	IGF1R	15q25-qter
Fes	FES	15q25-qter
Tyr (c)	TYR	11q14-q21
Idh-2	IDH2	15q21-qter
Calc	CALC	11p15.4
Pth	PTH	11p15.5-pter
Hbb	HBB	11p15.5
Th	TH	11p15

1.6.2.2.2The Prader-Willi/Angelman Syndrome chromosomal region (PWCR/ANCR)

Prader-Willi Syndrome (PWS) is generally sporadic and is characterised by infantile hypotonia, early onset childhood obesity, mental deficiency, short stature, small hands and feet, hypogenitalism and a characteristic face. Fifty to 60% of patients have a cytogenetically visible deletion of 15q11-q13 (Butler 1990). Hypopigmentation is a relatively newly appreciated feature of the PWS (Hittner *et al.* 1982; Butler *et al.* 1986; Pettigrew *et al.* 1987; Wiesner *et al.* 1987; Pembrey *et al.* 1989; Butler 1989, 1990; Magenis *et al.* 1990; Trent *et al.* 1991) and it has been reported that about half PWS patients with a 15q deletion are hypopigmented (Wiesner *et al.* 1984; Butler *et al.* 1986; Wiesner *et al.* 1987; Butler 1989), often with low tyrosinase levels (Wiesner *et al.* 1984). Three cases of PWS and OCA have been reported: Wiesner *et al.* (1984), reported a single case with slightly decreased tyrosinase levels, another report (Phelan *et al.* 1988) described a Negroid female with OCA and PWS and an interstitial deletion of 15q11.2 and Wallis and Beighton (1989), reported concurrence of these conditions in a Chinese girl with a normal karyotype.

The Angelman Syndrome (AS) is characterised by mental retardation, microcephaly, paroxysms of laughter and seizure disorders, ophthalmic abnormalities, a prominent jaw, large mouth, absent speech and characteristic "puppet-like" gait and other movements (Angelman 1965). Forty to 60% of AS patients have a 15q11.2-q13 deletion (Pembrey *et al.* 1989; Hamabe *et al.* 1991). Recently, hypopigmentation has been recognised as a common feature of AS (Pembrey *et al.* 1989; Williams *et al.* 1989b) and a case of deletion 15q11.2-q13 AS with OCA has been described (Shapiro Fryburg *et al.* 1991). This patient had low hairbulb tyrosinase activity.

Although apparently similar deletions occur on chromosome 15 in both the PWS/AS, the syndromes are generally clinically distinct, suggesting that the deletions may have some region of overlap but are not identical. It has been found that paternally derived deletions give rise to a PWS phenotype, while maternally derived deletions cause AS (Zori *et al.* 1990; Smeets *et al.* 1992), suggesting that differential gene expression or imprinting occurs in this region (Knoll *et al.* 1990; Magenis *et al.* 1990; Williams *et al.* 1990; Gregory *et al.* 1991; Hulten *et al.* 1991; Izumikawa *et al.* 1991; Knoll *et al.* 1991; Malcolm *et al.* 1991; Naritomi 1991; Hall 1992; Wagstaff *et al.* 1992).

A causal link between PWS/AS and hypopigmentation is the association of optic misrouting and defective retinal and optic tract pigmentation in many PWS/AS patients (Creel *et al.* 1986). If a gene with a role in melanin biosynthesis does occur in this region and is deleted in about 50% of PWS patients, it would result in a hemizygous state of this gene and PWS/AS deletion patients may be hypopigmented (Wallis and Beighton 1989; Butler 1990). It is interesting to note that obligate heterozygotes for both ty-pos OCA and ty-neg OCA are, however, normally pigmented.

Several polymorphic loci have been localised in the 15q11-q13 region and DNA markers in the PWS/AS region have been available for several years, but detailed molecular characterisation of the region has been difficult due to the relatively low polymorphism content for most markers. The physical mapping and ordering of loci in this region has primarily based upon deletion analysis of PWS/AS patient DNA using RFLP and quantitative Southern blot analysis. However, often several of these markers are deleted together, making definitive ordering difficult by deletion analysis alone (Kuwano *et al.* 1992). Multicolour fluorescence *in situ* hybridisation (FISH) analysis of interphase loci has

been combined with YAC contig information to provide a physical order of the markers in this region: centromere-D15S9-D15S11-D15S13-D15S10-GABRB3-D15S12-D15S24-telomere (Kuwano *et al.* 1992).

1.7 Aims of This Study

The aims of this study were:

i) To search for linkage between ty-pos OCA and several random polymorphic markers, candidate pigment loci and candidate chromosomal regions, in affected individuals from Negroid families in southern Africa.

ii) Once linkage had been established between any marker(s) and ty-pos OCA, more polymorphic markers in the same chromosomal region would be examined for linkage in order to determine the closest flanking markers and to increase the genetic information about the region.

iii) To construct a genetic linkage map of the region around the ty-pos OCA gene, using as many linked markers as possible.

iv) To test for allelic association between the closest linked marker(s) and the mutation(s) determining ephelus status in individuals with ty-pos OCA and to construct haplotypes of normal and affected chromosomes in an attempt to determine whether the ty-pos OCA mutation had a single origin or multiple origins.

The high prevalence of ty-pos OCA in southern Africa has provided many families who could participate this linkage study, thus generating statistically acceptable linkage results. This project has involved the use of several polymorphic markers, including blood groups, serum proteins, RFLPs and the

recently characterised hypervariable VNTR and dinucleotide repeat markers. Many of these markers have previously only been characterised in Caucasoids and with their use in the Negroid families in this study, have been shown to detect new alleles and, in some cases, increased the polymorphism information content in this population group.

The linkage project was initiated in the Department of Human Genetics in 1985 and some of the data obtained in the search for linkage between ty-pos OCA and random markers were reported in the PhD thesis of Ruth Heim (1988) and have been published (Heim *et al.* 1989). The project has been an ongoing one in the Department and the data presented in this dissertation include practical results obtained by the author, Gwynneth Stevens (another PhD student) and Prashiela Manga (an MSc student). When information was produced by a person other than the author, this is clearly stated. All data were analysed by the author. Presently, Gwynneth Stevens and Prashiela Manga are continuing with their studies on characterisation of the ty-pos OCA gene.

CHAPTER TWO

2. SUBJECTS, MATERIALS AND METHODS

A list of the abbreviations used is given on page xix. Appendix A, on page 176, gives the sources of the reagents used. Appendix B, on page 178, gives the composition and preparation of media and other solutions used.

2.1 Subjects

The subjects of this study were southern African Negroids from 41 nuclear families, ranging in size from 3 to 16 individuals, with at least one affected ty-pos albino member each. Some of these families were from Boputhatswana, but the majority were from the large urban satellite town of Soweto and from the Johannesburg and Pretoria environs, as well as from elsewhere in the Transvaal. Some families were traced through affected individuals at the Blind School in Katlehong and from the Cancer Clinic at Hillbrow Hospital. Where possible, blood from both parents and unaffected sibs was also collected. With the current social climate, family members were often widely dispersed and difficult to locate, resulting in the study of smaller family units. Family pedigrees were obtained in as much detail as possible when families were interviewed.

2.1.1 Identification of affected individuals

Individuals with the ty-pos OCA phenotype were identified by Dr George Nurse, Professor Trefor Jenkins, Professor Jennifer Kromberg and Sister E Zwane, of our unit, on the basis of the clinical features described by Witkop *et al.* (1989).

The chiefdom affiliation of each family was recorded and the clinical features of each affected individual were noted in detail. These features included hair colour, visual acuity and the occurrence of nystagmus and photophobia, the

presence or absence of ephelides and the evidence of any premalignant or malignant lesions on sun exposed areas of skin. Where possible, the ages of all available members of each family were recorded at the time of clinical investigation. Anagen hairbulbs were collected from some families for hairbulb incubation tests and electron micrograph studies to be conducted in Cape Town, in order to confirm that the condition was ty-pos OCA.

2.2 DNA markers

In this linkage study, two candidate pigment loci, the tyrosinase and *brown* loci, four markers in a candidate region on chromosome 11p thought to be the position of a syntenic region containing the human equivalent of the mouse *p* locus, and 10 random DNA markers, were tested. A candidate region on chromosome 15q, the PWS/AS region, was also examined, using the probes pTD3-21 and pTD189-1. Once linkage between these markers and ty-pos OCA had been established, another 13 markers on the proximal long arm of chromosome 15 were tested.

The practical work on thirteen of the markers tested was done by Gwynneth Stevens and on three of the markers, by Prashiela Manga. The details of these markers and the results obtained with them were used in the linkage analysis and are included in this thesis to complete the linkage data. In a study previously completed in this department, linkage analysis was carried out with 15 serogenetic markers and 7 DNA markers (Heim 1988; Jenkins *et al.* 1990). Although the results obtained with these markers are included in this study, the details of the markers have not been included and may be found in Heim (1988).

2.2.1 Characterisation of candidate pigment loci

The DNA probes used in this linkage study, are outlined in Table 2.1.

2.2.1.1 Pmel 34 (TYR)

This probe was isolated from a cDNA derived from a λgt11 human melanocyte cDNA library screened with antibodies against hamster tyrosinase. The deduced protein was found to be consistent with the structure of tyrosinase, which is a glycoprotein containing two copper-binding domains. Southern blot analysis of DNA derived from newborn mice carrying lethal albino deletion mutations revealed that Pmel34 mapped near or at the *albino* (*c*) locus, the position of the structural gene for tyrosinase (Kwon *et al.* 1987). This tyrosinase cDNA was used to map the human tyrosinase locus (TYR) to chromosome 11q14-q21 by Southern blotting analysis of SCH DNA and by *in situ* hybridisation to metaphase chromosomes (Barton *et al.* 1988). It was also found to detect tyrosinase-related sequences on the short arm of chromosome 11 (p11.2-cen) (Barton *et al.* 1988; Takeda *et al.* 1989). Pmel34 has been found to detect a *Bgl*II polymorphism (Spritz and Strunk 1990b), which was used in this study. It also detects a 2 allele *Taq*I RFLP in Caucasoids (Spritz *et al.* 1988) which was not informative in the Negroid families studied.

This probe was kindly supplied by Professor Byoung Kwon.

Table 2.1 Non-chromosome 15 DNA probes

PROBE	LOCUS	POSITION	VECTOR	INSERTION SITE[1]	INSERT SIZE (kb)	ENZYME DETECTING RFLP	ALLELE SIZES[2] (kb)	REFERENCES
CANDIDATE LOCI								
Pmel34	TYR	11q11-q21	pGEM	*Eco*RI	1.85	*Bgl*II	5.8/5.6	Kwon *et al.* 1987 Spritz *et al.* 1990b
pMT4	*Brown*	MOUSE 4	O-B[3]	*Pvu*II	0.25	*Xba*I	10/8.6	Shibahara *et al.* 1986 Colman *et al.* 1991c
Human cDNA	CAS2	9p22-pter	pGEM	*Eco*RI	2.0	*Xba*I	10/8.6	Chintamaneni *et al.*1991b Cohen *et al.* 1990
CHROMOSOME 11 MARKERS								
pHC36	CALCA	11p14-qter	pBR322	*Pst*I	0.66	*Taq*I	8.0/6.5 3/2.3(c)	Hoppener *et al.* 1984
pSAA82	SAA	11p-pter	pBR322	?	0.58	*Hind*III	4.6/2.8 3.6/1.1(c)	Kluve-Beckerman *et al.* 1986
pDH3.2 ($p^{A}\gamma$)	HBB	11p15.5	pBR322	*Hind*III	3.2	*Hind*III	$^{G}\gamma$ 7.8/7.1 $^{A}\gamma$ 3.4/2.8	Tuan *et al.* 1979
RANDOM LOCI								
DQα	HLA	6p21	pDCH1	*Pst*I	0.80	*Taq*I	1a-2.6 1b-5.8 1c-6.2 2-4.3 3-5.3	Bidwell *et al.* 1988
DXα	HLA	6p21	pDCH1	*Pst*I	0.80	*Taq*I	Upper Low	Bidwell *et al.* 1988

1 - All probes were amp^{R} and tet^{S}
2 - c "constant"
3 - Okayama-Berg Vector (Okayama and Berg, 198_)

2.2.1.2 CAS2/*brown* locus

In humans, this locus is also known as CATB and TYRP (McCusick 1992), but will be referred to as CAS2 for convenience. Two different cDNA probes were received, detecting the mouse *brown* locus and the human homologue. Primarily, the mouse probe was used to detect polymorphisms in the human DNA. Once the human probe had been received, it was used in the linkage study.

The mouse probe, pMT4, was isolated from a cDNA clone derived from a B16 mouse melanoma cDNA library by differential hybridisation. This DNA was originally thought to encode mouse tyrosinase by virtue of the fact that the gene product cross-reacted with monoclonal antibodies, TMH-1 and TMH-2, produced against mouse tyrosinase (Shibahara *et al.* 1986). However, it was found that pMT4 had only 40% homology with a cDNA found to encode a functional tyrosinase protein and it was postulated that it was a tyrosinase isozyme, with dopa oxidase activity (Shibihara *et al.* 1988). The protein encoded by pMT4 has since been found to map at or close to the mouse *brown* (*b*) locus on mouse chromosome 4 and has been provisionally termed tyrosinase-related protein 1 (TRP1) (Jackson 1988). The gene product has been found to be homologous to the human melanoma antigen GP75 (Halaban and Moellmann 1990; Vijayasaradhi *et al.* 1990).

Cohen *et al.* (1990), presented the nucleotide and deduced amino acid sequence of the cDNA coding for the human homologue of the mouse *b* locus. The cDNA was also isolated by B. Kwon (Chintamaneni *et al.* 1991b). This gene had not yet been mapped in humans, but it was known to be on mouse chromosome 4, for which there is a syntenic group on human chromosome 9. A SCH panel and chromosome 9-specific hybrids, as well as *in situ* hybridisation, were used to localise the gene to 9p22-pter

(Abbot *et al.* 1991; Chintamaneni *et al.* 1991b; Murty *et al.* 1992).

Both the human and mouse *b*-protein cDNAs were kindly supplied by Professor Byoung Kwon.

DNA polymorphisms detected by these probes were unknown in both mouse and human. Human DNA samples were thus screened with several restriction enzymes in order to search for a polymorphism in the Negroid population. A 2 allele *Xba*I RFLP was found to be detected by these probes in Negroids (Colman *et al.* 1991a).

2.2.2 Characterisation of markers on human chromosome 11p

Conservation between markers on mouse chromosome 7 and human chromosome 11 (Figure 1.6,Section 1.6.2.2.1)suggests that the mouse *p* locus (mouse chromosome 7) may occur within a syntenic region on human chromosome 11p (Silvers 1979). Markers in this region were analysed for evidence of linkage to ty-pos OCA.

The practical work on pHC36 and pSAA82 was completed by Gwynneth Stevens.

2.2.2.1 pHC36 (CALCA)

Calcitonin (CT) is a polypeptide hormone secreted by the C-cells of the thyroid. A small molecular probe from part of the human CT cDNA was used to assign the CT gene to 11p14-qter by SCH analysis. A *Sau*961 fragment of the gene was shown to detect a 2 allele *Taq*I polymorphism (Höppener *et al.* 1984).

This probe was provided by Dr Sue Chamberlain, from St Mary's

Hospital Medical School, UK.

2.2.2.2 pSAA82 (SAA)

A human serum amyloid A (SAA) cDNA was used as a probe in chromosomal SCH mapping studies to position this locus in the 11p-pter region. Screening of the human DNA from unrelated individuals by Southern analysis, using this probe, revealed *Hind*III and *Pst*I RFLPs. A 2 allele *Hind*III polymorphism occurring within intron 2 of the SAA2 locus was detected in Caucasoids, Negroids and San (Kluve-Beckerman *et al.* 1986) and another, Negroid-specific, 2 allele *Hind*III polymorphism, associated with the SAA1 locus, has been described (Stevens *et al.* Hum Genet in press). Haplotypes generated from the closely linked *Hind*III polymorphisms were used to determine linkage between this locus and typos OCA.

This probe was provided by Dr Sue Chamberlain, from St Mary's Hospital Medical School, UK.

2.2.2.3 pDH3.2 ($\psi^{A}\gamma$) (HBB)

In a previous study in this department (Heim 1988), the γ-globin/*Hind*III RFLP (Tuan *et al.* 1979) was analysed in 20 families. The remainder of the families have since been typed with this probe in an attempt to increase the linkage information on 11p.

This probe is a genomic probe isolated by J. Wainscoat (personal communication) and includes the $^{A}\gamma$-globin gene and flanking sequences. The $^{A}\gamma$-globin gene has extensive sequence homology with the $^{G}\gamma$-globin gene and the pHD3.2 probe detects 2 *Hind*III polymorphisms, one in each gene (Tuan *et al.* 1979).

2.2.3 Characterisation of random DNA probe DQα/DXα

A previous study (Jenkins *et al.* 1990) showed a positive lod score between ty-pos OCA and Bf at 6p21.3 (1.58 at Θ=0.1). Thus the polymorphic DQα probe, also localised to 6p21, was used in an attempt to confirm or refute linkage to a marker in this region of 6p.

The molecular organisation of the human class II major histocompatibility complex genes on human chromosome 6p has been studied extensively and Southern blot analysis has been used to interpret the allele-specific hybridisation patterns (allogenotypes) detected by the DQα probe (Auffray *et al.* 1984). This probe has homology with and cross-hybridises to the DXα gene and thus detects two separate allele systems with a single hybridisation (Bidwell 1988). DQα detects five DQα-specific alleles (DQα 1a, 1b, 1c, 2 and 3) and two alleles of the cross-hybridising DXα gene, DXα Upper (U) and DXα Lower (L).

This probe was purchased from the American *Type Culture* Collection (ATCC).

2.2.4 Characterisation of chromosome 15-specific DNA probes

The chromosome 15-specific DNA probes used are outlined in Table 2.2.

Probes pTD3-21, pTD189-1, pML34, pIR4-3R and pIR10-1 are cDNA segments from the proximal long arm of chromosome 15, isolated from a λ phage Charon 21A library, constructed from *Hind*III-digested DNA obtained from flow-sorted inv dup (15) chromosomes (Donlon *et al.* 1986) and subcloned into plasmids (Nicholls *et al.* 1989). The single copy inserts were initially mapped to chromosome 15 by hybridisation to DNA from Chinese hamster-SCH lines containing chromosome 15 as their only common human

chromosome. They were then mapped more precisely to 15q11-q13 by *in situ* hybridisation (Donlon *et al.* 1986).

All these probes were purchased from the ATCC.

The practical work on probes pIR10-1 and CMW-1 was completed by Gwynneth Stevens and on probes pJU2°1 and pDP151, by Prashiela Manga.

2.2.4.1 pTD3-21 (D15S10)

pTD3-21 was found to hybridise with reduced intensity in a PWS patient with a cytogenetically visible deletion of chromosome 15q11.2, indicating that this probe was deleted from one homologue of the patient and thus mapped to this region (Donlon *et al.* 1986; Tantravahi *et al.* 1989). pTD3-21 detects a two allele *Taq*I polymorphism in Caucasoids (Nicholls *et al.* 1989) and a third, Negroid-specific, *Taq*I allele (Colman *et al.* 1991b).

2.2.4.2 pTD189-1 (D15S13)

Probe pTD189-1 is polymorphic for many enzymes, however most enzymes (for example *Sac*I and *Taq*I) show an identical restriction fragment pattern and size difference between alleles, suggesting that a 2 allele insertion (+) or deletion (-) polymorphism is detected by this probe. *Bgl*II and some other enzymes, display a reversed pattern of alleles compared to most enzymes, as well as heterogeneous size differences. Genomic mapping was used to show that these results can be explained by the presence of recognition sites for these enzymes within a 1.8kb insertion in the (+) allele detected by pTD189-1 (Nicholls *et al.* 1989). The 2 allele *Taq*I RFLP detected by this probe was used in this study.

Table 2.2 Chromosome 15-specific DNA probes

PROBE	LOCUS	POSITION	VECTOR	INSERTION SITE[1]	INSERT SIZE (kb)	ENZYME DETECTING RFLP	ALLELE SIZES (kb)	REFERENCES
pTD3-21	D15S10	15q11-q12	pBR322	*Hin*dIII	2.2	*Taq*I	9/8.2/3.0	Nicholls *et al.* 1989 Colman *et al.* 1991b
pTD189-1	D15S13	15q11-q12	pUC18	*Eco*RI/ *Hin*dIII	0.9	*Taq*I	3.5/2.1	Nicholls *et al.* 1989
pML34	D15S9	15q11-q12	pBR322	*Hin*dIII	6.2	*Sca*I	6.5/6.3/ 6.1	Nicholls *et al.* 1989
pIR4-3R	D15S11	15q11-q12	pUC18	*Bam*HI/ *Hin*dIII	0.4	*Rsa*I	1.2/1.0	Nicholls *et al.* 1989
pIR10-1	D15S12	15q11-q12	pUC18	*Bam*HI/ *Hin*dIII	0.8	*Sca*I	17.5/16/ 12.5	Nicholls *et al.* 1989
CMW-1	D15S24	15pter-q13	pUC18	*Eco*RI	3.8	*Eco*RI	4.2-3.0 >6 alleles	Rich *et al.* 1988
pJU201	D15S3	15	pBR328	*Eco*RI/ *Hin*dIII	?	*Eco*RI	1.9/1.8/ 2.1/1.7	Cooper *et al.* 1985
pDP151	D15S2	15q15-q22	pBR322	*Eco*RI/ *Hin*dIII	2.6	*Eco*RI	11/9	Brissenden *et al.* 1986

1 - All probes amp^R and tet^S (except pJU201 - amp^R tet^R)

2.2.4.3 pML34 (D15S9)

pML34 was shown to detect a 2 allele *Sca*I RFLP in the Caucasoid population (Nicholls *et al.* 1989) and was used to screen the ty-pos OCA families.

2.2.4.4 pIR4-3R (D15S11)

pIR4-3R was found to detect a two allele *Rsa*I RFLP in Caucasoids (Nicholls *et al.* 1989) which was used in the linkage study.

2.2.4.5 pIR10-1 (D15S12)

Nicholls *et al.* (1989), reported a three allele RFLP detected by pIR10-1 with *Sca*I digests of Caucasoid DNA, which was also tested in this study. This RFLP is likely to result from two closely linked, independent *Sca*I site RFLPs (Nicholls *et al.* 1989). Heteroduplex analysis of the original λIR-10 insert, showed that it contained small inverted repeated segments, consistent with its belonging to the *Alu* family. These inverted repeat elements may explain many of the deletions and rearrangements involving band 15q11.2 in PWS, resulting from unequal sister-chromatid exchange or looping out of DNA around these repeats (Donlon *et al.* 1986).

2.2.4.6 CMW-1 (D15S24)

CMW-1 was isolated from a flow-sorted library cloned in Charon 21A, in the Los Alamos National Laboratory. It was mapped to 15q13 using a SCH regional mapping panel. Coordinate variation using multiple enzymes suggested that it detects a variable number of tandem repeats (Rich *et al.* 1988). The *Eco*RI RFLP detected by CMW-1 was used in the linkage study.

This probe was purchased from the ATCC.

2.2.4.7 pJU201 (D15S2)

pJU201 was isolated from flow-sorted chromosomes which had been digested to completion with *Bam*HI, cloned into phage and subcloned into the plasmid pBR328. The clone was shown to map to chromosome 15 using a panel of human-rodent SCH. pJU201 was originally reported to detect a 2 allele *Eco*RI polymorphism in Caucasoids (Cooper *et al.* 1985). It has also been shown to detect a third allele in Negroids and San, and a fourth allele in Negroids (data submitted to the Genome Database, Baltimore).

This probe was made available by Dr Sue Chamberlain, from St Mary's Hospital Medical School, UK.

2.2.4.8 pDP151 (D15S2)

pDP151 is a random DNA fragment isolated from a human genomic library (Lawn *et al.* 1978) and subcloned into the plasmid pBR322. Hybridisations of pDP151 to DNA from SCH showed it to map to chromosome 15. It was shown to detect a 2 allele *Eco*RI RFLP in Caucasoids (Brissenden *et al.* 1986).

This probe was made available by Dr Sue Chamberlain, from St Mary's Hospital Medical School,UK.

2.2.5 Characterisation of PCR markers detecting RFLPs

The random PCR markers used in this study detected both RFLPs and dinucleotide repeats and are detailed in Table 2.3.

The practical work on markers ALB, pMP6d and pJ3-11 was completed by Gwynneth Stevens.

2.2.5.1 ALB

The published sequence of the albumin (ALB) gene (4q11-q13) was used to select two 20mer oligonucleotide primers for PCR. These primers amplified a genomic fragment which contained a polymorphic *Hae*III site in intron VII, in Caucasoids, Negroids and San (Moolman *et al.* 1991).

2.2.5.2 pMP6d-9 (D7S399)

*Hind*III/*Msp*I subclones of pMP6d-9 (7q31) were sequenced to obtain oligonucleotides suitable for detection of an *Msp*I RFLP by PCR (Auth *et al.* 1989).

2.2.5.3 pJ3.11 (D7S8)

A subclone of a cosmid cloned from a human genomic library, using the pJ3.11 probe, was sequenced and oligonucleotides were designed to enable detection of *Pst*I and *Msp*I RFLPs by PCR. These RFLPs were originally described in unrelated Caucasoids (Northrup *et al.* 1989) and the *Pst*I RFLP was used in this study.

Table 2.3 PCR markers

MARKER	LOCUS	LOCATION	ENZYME DETECTING RFLP	PRODUCT SIZE[1] (bp)	DETECTION TECHNIQUE[2]	REFERENCES
RFLPs						
ALB	ALB	4q11-q13	*Hae*III	G1 418 G2 347 + 71 256 (c)	PAGE	Moolman *et al.* 1991
pMP6d-9	D7S399	7q31	*Msp*I	377 204 + 173	COMPOSITE GEL	Huth *et al.* 1989
pJ3.11	D7S8	7q31	*Pst*I	380 230 + 150	COMPOSITE GEL	Northrup *et al.* 1989
TYR/*Mbo*I	TYR	11q11-q21	*Mbo*I	334 247 + 87 177 (c)	COMPOSITE GEL	Giebel and Spritz 1990b
CA REPEATS						
2D9-1	D1S116	1p31.2	-	±97	PAGE	Sharma *et al.* 1991
Mfd3	APOA2	1q21-q23	-	±137	PAGE	Weber and May 1989
Mfd61	D6S105	6p	-	±131	PAGE	Weber *et al.* 1991
716/718	WT1	11p13	-	±144	PAGE	Little (pers comm)
Mfd15	D17S250	17q11.2-q12	-	±162	PAGE	Weber *et al.* 1990a
MPO	MPO	17q21-q23	-	±110	PAGE	Polymeropoulos *et al.* 1991
335/334	D21S168	21q22.3	-	±110	PAGE	Guo *et al.* 1990

1 - c "constant" 2 - PAGE "polyacrylamide gel electrophoresis" (6% gels); COMPOSITE "4% Nusieve:HGT (3:1) gel"

2.2.5.4 TYR/*Mbo*I RFLP

Two 20 mer oligonucleotides derived from exon 1 of the human tyrosinase gene (11q14-q21) were used to amplify DNA from this gene. *Mbo*I was found to detect a nonpathological 2 allele polymorphism within codon 192 of the amplified portion of the gene in Caucasoids (Giebel and Spritz 1990) and in Asians and Afrocarribeans, but not in Orientals (Johnston *et al.* 1992). To determine whether southern African Negroids are polymorphic at this site, and thus whether this polymorphism was potentially informative for the linkage study, 32 unrelated Negroids were screened for the presence or absence of the *Mbo*I site polymorphism.

2.2.6 Characterisation of PCR markers detecting dinucleotide repeats

2D9.1, Mfd3, Mfd61, Mfd15, MPO AND 335/334 are randomly distributed PCR markers. WT-1 is another marker on chromosome 11p.

The practical work on markers Mfd15, MPO and WT-1 was completed by Gwynneth Stevens.

2.2.6.1 2D9-1 (D1S116)

DNA sequences flanking a GT repeat in an *Alu*I subclone (2D9-1) of a cosmid obtained from a flow-sorted chromosome 1 library, were used to design PCR primers. The cosmid was localised to chromosome 1p31.2 by *in situ* hybridisation and was shown to reveal 7 alleles in Caucasoids (Sharma *et al.* 1991).

2.2.6.2 Mfd3 (APO A2)

The sequences used to design the two 20mer Mfd3 primers, flanking a CA repeat on 1q21-q23, were taken from GenBank (Version 54). The primers amplified a locus which had 6 alleles in Caucasoids (Weber and May 1989).

2.2.6.3 Mfd61 (D6S105)

PCR primers were constructed from the sequence of clone Mfd61, a human genomic *Sau*3AI fragment cloned into mp19 and selected by hybridisation to poly(dC-dA).poly(dG-dT). Clone Mfd61 was assigned to 6p using DNA templates isolated from panels of SCH. A panel of 6p radiation hybrids gave the order of tested markers as pter-D6S88-D6S108-D6S105(Mfd61)-HLA-cen. Mfd61 was thus used as another marker on 6p near the HLA locus. When tested in Caucasoids, Mfd61 revealed 10 alleles (Weber *et al.* 1991).

2.2.6.4 716/718 (WT-1)

The locus for Wilm's Tumour maps to 11p13 and was thus used to increase the information on human chromosome 11.

The primer sequences flanking a CA repeat in the Wilms Tumour gene, were kindly supplied by Dr Melissa Little (personal communication). The primers are: 716 5' AAT GAG ACT TAC TGG GTG AGG 3'

718 5' TTA CAC AGT AAT TTC AAG CAA CGG 3'

2.2.6.5 Mfd15 (D17S250)

These primer sequences were obtained from human genomic DNA fragments cloned into m13 and selected by hybridisation to poly(dC-dA).poly(dG-dT). This clone was assigned to chromosome 17q11.2-q12 using DNA templates isolated from panels of chromosomal and subchromosomal SCH. The primers were found to amplify 10 alleles in unrelated Caucasoids (Weber *et al.* 1990).

2.2.6.6 MPO

A polymorphic CA repeat was found in the human gene of light and heavy chains of the myeloperoxidase glycoprotein (MPO) (17q21-q23). Flanking primer sequences were designed to amplify this polymorphism and detected 4 alleles in unrelated individuals (Polymeropoulos *et al.* 1991).

2.2.6.7 335/334 (D21S168)

These primers were designed from the sequences flanking a GT repeat within an *Alu*I subclone of a cosmid, derived from a flow-sorted human chromosome 21-specific library. PCR of genomic DNAs from a SCH panel indicated localisation of the marker to 21q22.3. In Caucasoid DNA samples, 8 polymorphic alleles were reported (Guo *et al.* 1990).

2.2.7 Characterisation of chromosome 15-specific CA repeat polymorphisms

Details of the chromosome 15-specific PCR markers are shown in Table 2.4.

The practical work on markers D15S11, 635/636 and THBS1 was completed by Gwynneth Stevens and on marker MS14, by Prashiela Manga.

Table 2.4 Chromosome 15-specific PCR markers

MARKER	LOCUS	LOCATION	PRODUCT SIZE (bp) (±2bp)[1]	REFERENCES[2]
D15S10	D15S10	15q11-q13	±179	Lindeman *et al.* 1991
GABRB3	GABRB3	15q11-q13	±189	Mutirangura *et al.* 1992a
D15S11	D15S11	15q11-q13	±255	Mutirangura *et al.* 1992b
MS14	D15S97	15q11-q13	±178	Bowcock (pers comm)
635/636	ACTC	15q11-qter	±88	Litt and Luty 1989
THBS1	THBS	15q15	±165	Polymeropoulos *et al.* 1990
Mfd49	D15S87	15	±87	Weber *et al.* 1990b

1 - All products were detected by polyacrylamide gel electrophoresis on 6% gels

2 - pers comm = personal communication

2.2.7.1 D15S10

The probe pTD3-21 (15q11-q13) (Section 2.2.4.1) was used to screen a *Bam*HI-digested genomic library in λEMBL3. A subclone of a positive phage clone, which hybridised to poly(dC-dA).poly(dG-dT), was identified, sequenced and used to design PCR primers flanking a CA repeat. In unrelated Caucasoid samples 2 alleles were found (Lindeman *et al.* 1991), thus the marker was used to screen the ty-pos OCA families in an attempt to increase the informativity of this locus and to construct haplotype information from the 2 markers at the locus for linkage studies.

2.2.7.2 $GABA_A$ receptor β3 (GABRB3)

These PCR primers were designed from sequences flanking a CA repeat element in a subclone of a YAC, containing a human genomic clone complimentary to the rat Gabrb3 cDNA. Localisation of the clone on chromosome 15 was confirmed by PCR and SCH analysis and finer localisation to the PWS/AS region at 15q11-q13 was confirmed by *in situ* hybridisation. The marker was originally found to detect 11 alleles (Mutirangura *et al.* 1992a).

2.2.7.3 D15S11

An STS (sequence tagged site) was generated from the probe pIR4-3R (Section 2.2.4.4) and used to screen a total human YAC library. A positive clone was screened for CA repeats and subcloned. Fragments containing CA repeats were sequenced and then used to design PCR primers flanking these sequences. The localisation of the YAC to chromosome 15 was confirmed by *in situ* hybridisation and the dinucleotide repeat was confirmed to be on chromosome 15 by PCR of SCH. PCR with these primers revealed 10 alleles in unrelated individuals

(Mutirangura *et al.* 1992b). This marker was used to increase the amount of information obtained from this locus and to construct haplotypes from the two markers at the locus.

2.2.7.4 MS14 (D15S97)

The MS14 primer sequences were developed by Nick Dracopolis and kindly supplied by Anne Bowcock. The primer sequences are:

MS14-R 5' TCT CCC TCC AAT AAT GTG AC 3'
MS14-L 5' TGA GTC AAT GAT TGA AAT TAC TG 3'

This marker maps close to GABRB3 on 15q. It was not known whether it mapped proximal or distal to GABRB3 at 15q11-q13, nor how many alleles it revealed. Thus the marker was screened in the ty-pos OCA families and the most likely position of MS14, relative to both the ty-pos OCA gene and to GABRB3 was determined.

2.2.7.5 635/636 (ACTC)

The human cardiac muscle actin gene locus (ACTC) maps to 15q11-qter (Gunning *et al.* 1984). The published sequence (Hamada *et al.* 1982) and the GenBank DNA sequence database were used to generate primers flanking a GT repeat in intron 4 of the gene (Litt and Luty 1989; Watkins *et al.* 1991). The primer sequences constructed by Litt and Luty (1989), revealed 12 alleles in unrelated individuals.

(Mutirangura *et al.* 1992b). This marker was used to increase the amount of information obtained from this locus and to construct haplotypes from the two markers at the locus.

2.2.7.4 MS14 (D15S97)

The MS14 primer sequences were developed by Nick Dracoplis and kindly supplied by Anne Bowcock. The primer sequences are:

MS14-R 5' TCT CCC TCC AAT AAT GTG AC 3'
MS14-L 5' TGA GTC AAT GAT TGA AAT TAC TG 3'

This marker maps close to GABRB3 on 15q. It was not known whether it mapped proximal or distal to GABRB3 at 15q11-q13, nor how many alleles it revealed. Thus the marker was screened in the ty-pos OCA families and the most likely position of MS14, relative to both the ty-pos OCA gene and to GABRB3 was determined.

2.2.7.5 635/636 (ACTC)

The human cardiac muscle actin gene locus (ACTC) maps to 15q11-qter (Gunning *et al.* 1984). The published sequence (Hamada *et al.* 1982) and the GenBank DNA sequence database were used to generate primers flanking a GT repeat in intron 4 of the gene (Litt and Luty 1989; Watkins *et al.* 1991). The primer sequences constructed by Litt and Luty (1989), revealed 12 alleles in unrelated individuals.

2.2.7.6 THBS1

PCR primers flanking a polymorphic CT repeat in intron A of the human thrombospondin gene (THBS1) revealed 9 alleles in unrelated individuals (Polymeropoulos *et al.* 1990).

2.2.7.7 Mfd49 (D15S87)

Mfd49 primer sequences were designed from a human genomic *Sau*3AI fragment (Mfd49) cloned into mp19 and selected by hybridisation to poly(dC-dA).poly(dG-dT). The fragment was assigned to chromosome 15 by using DNA templates isolated from panels of SCH. PCR analysis revealed 11 alleles in Caucasoid samples (Weber *et al.* 1990b).

2.3 Preparation of probe DNA

2.3.1 Transformation

Two different methods of transformation were used. Originally, the Mandel and Higa (1986) method of transformation was used and, more recently, the Chung and Miller (1988) method of transformation was used.

When recombinant plasmid DNA was received, it was transformed into *E. coli* HB101, using one of two methods. Both methods require *E. coli* HB101 cells in log phase. *E. coli* HB101 cells from -70°C stocks were streaked out on a Luria agar (LA) (Appendix B) plate, and incubated at 37°C overnight. A single colony was used to inoculate 2ml of Luria-Bertani broth (LB) (Appendix B), which was then incubated overnight at 37°C with vigorous orbital shaking. One ml of this overnight culture was used to inoculate 100ml of LB, which was then incubated at 37°C with vigorous shaking for 2-4 hours, until the optical density (OD) at a wavelength of 550nm was 0.5 (the culture

was in log phase).

For the Mandel and Higa (1986), or heat-shock method, the HB101 culture was chilled on ice for 15 minutes, prior to a 10 minute centrifugation at 3000rpm. The cells were resuspended in 10ml of ice-cold $CaCl_2$:TrisHCl (50mM;10mM) solution by gentle pipetting up and down. $CaCl_2$ is thought to be responsible for a change in the cell wall of the bacterial cell, which improves DNA binding efficiency (Brown 1986). This mixture was placed on ice for 5 minutes and then centrifuged for a further 10 minutes at 3000rpm. The pellet was again resuspended in 1ml of ice-cold $CaCl_2$:TrisHCl solution and then aliquoted into 100μl amounts in labelled 1.5ml plastic Eppendorf tubes for use in the transformation procedure. The competent cells were kept for use for up to 48 hours at 4°C.

Approximately 5ng of recombinant plasmid DNA was mixed with a 100μl aliquot of competent *E. coli* HB101 cells and placed on ice for 30 minutes. The cells were then heat-shocked at 42°C for two minutes. Heat-shocking stimulates the movement of DNA into the competent cell by an unknown mechanism (Brown 1986). The samples were returned to ice for at least 10 minutes before the contents of the transformation mix were added to 3ml of LB and incubated at 37°C with vigorous shaking for 3 hours. One hundred microlitres of this culture were then plated onto appropriate antibiotic-containing selection LA plates and incubated overnight at 37°C.

For the Chung and Miller (1988) method of transformation, the *E. coli* HB101 cells were pelleted by centrifugation at ±2750rpm for 10 minutes at 4°C. These cells were resuspended in one tenth volume of transformation and storage buffer (TSB) (10% PEG [mwt 3350]; 5% DMSO; 20mM Mg^{2+} [10mM $MgCl_2$ + 10mM $MgSO_4$]; dissolved in LB [pH 6.1]). Polyethylene glycol (PEG) is a long chain polymeric compound which, in the presence of salt, absorbs water and thus concentrates the cells (Brown 1986; Sambrook

et al. 1989). This solution was placed on ice for 10 minutes and then aliquoted into 100μl amounts in cold 1.5ml Eppendorfs. These competent cells could be used immediately for transformation or could be stored at -70°C for future use.

For transformation, approximately 100pg of plasmid DNA was added to 100μl of competent cells, which were then placed on ice for 5-30 minutes. These cells were added to 900μl TSB + glucose (20mM) and were grown at 37°C with vigorous shaking for 60 minutes. The transformed cells were plated and grown overnight at 37°C on LA plates containing the appropriate antibiotic for selection of transformants.

2.3.2 Isolation of plasmid DNA

In plasmid DNA preparation, it is essential to separate plasmid DNA from the bacterial chromosomal DNA which is also present in the cells. Several methods are available for removal of bacterial DNA during plasmid purification. These methods are based upon the physical differences between plasmid and bacterial DNA, including size and conformation (Brown 1986). The procedures used in this study utilise these differences and combine different methods, based on the protocols outlined in Sambrook *et al.* (1989).

2.3.2.1 Large-Scale plasmid DNA preparation

A single colony from the plated transformants was streaked out onto a second antibiotic-containing plate and incubated overnight at 37°C to be used to inoculate 100ml of L.. directly, or used to inoculate 10ml of LB containing the appropriate antibiotic, and incubated overnight at 37°C with vigorous shaking. This broth culture was then used to inoculate 100ml of LB selection media which was again incubated overnight at 37°C with vigorous shaking.

The culture was centrifuged at 3000rpm for 20 minutes and the pellet resuspended in 6ml of lysozyme solution (25mM TrisHCl pH 7.5; 10mM EDTA; 15% sucrose; 2mg/ml lysozyme (Boeringer Mannheim) by gentle pipetting up and down, and placed on ice for 10 minutes. Treatment with EDTA and lysozyme is carried out in the presence of sucrose, which prevents spontaneous cell lysis and results in the formation of spheroplasts, retaining an intact, cytoplasmic membrane. Addition of 12ml NaOH:SDS (0.2M:1%) and gentle mixing, induces cell lysis (Brown 1986). This mixture was placed on ice for 10 minutes, then 7.5ml of 3M sodium acetate (pH 4.6) (20mM) was added, mixed by inversion and the solution placed on ice for a further 20 minutes to bring down the cellular debris, protein and chromosomal DNA.

The mixture was then centrifuged at 15000rpm for 15 minutes at room temperature to precipitate the cellular debris. The supernatant, containing the plasmid DNA, was transferred to another tube and 5μl of 1mg/ml RNase A was added to remove the RNA. The solution was placed at 37°C for 20 minutes to maximise the RNase action.

The solution was deproteinised by extracting the supernatant twice with phenol: chloroform: isoamylalcohol [25:24:1] and once with chloroform: isoamylalcohol [24:1]. Phenol (BRL) was melted at 65°C and equilibrated at least three times with 0.1M TrisHCl (pH 8.0), until the pH of the aqueous phase was approximately 7.0. Hydroxyquinolinol (0.1%) was added and the phenol was stored at 4°C for up to two weeks. The DNA was precipitated by addition of 2 volumes of ice-cold absolute ethanol. The solution was placed at -70°C for 30 minutes (or at -20°C overnight) and centrifuged at 3000rpm for 20 minutes. The pellet was washed with 70% ethanol, dried briefly at room temperature and dissolved in 1ml of TE (pH 8.0).

Plasmid DNA was purified by centrifugation to equilibrium in a caesium chloride-ethidium bromide gradient. Density gradient centrifugation is an important method for separating supercoiled plasmid DNA from linear bacterial DNA. Exactly 4.4g of CsCl were weighed into a plastic tube and dissolved in 3ml of TE (pH 8.0). The plasmid DNA solution was added to the tube and mixed gently but thoroughly by inversion. To this mixture 320μl of ethidium bromide (Boerhinger Mannheim) (to a final concentration of 740μg/ml) was added and mixed by inversion. The final density of the solution was 1.55g/ml with a refractive index of 1.3860 (Sambrook *et al.* 1989). The solution was then transferred to a Kwik-Seal centrifuge tube (Beckman, USA) and was centrifuged to equilibrium at 45000rpm in a VTi65 rotor at 20°C for 16-18 hours.

The high centrifugal force pulls the caesium and chloride ions towards the outside of the tube, creating a density gradient in which macromolecules will band at their buoyant density. Ethidium bromide binds to DNA molecules by intercalating between adjacent base pairs, causing partial unwinding of the double helix and thus decreasing the buoyant density of linear chromosomal DNA, while little ethidium bromide intercalates supercoiled plasmid DNA (Brown 1986). After centrifugation, two bands were evident: the upper band of bacterial chromosomal DNA and the lower band of plasmid DNA. The lower band was removed with a syringe inserted into the side of the tube.

The ethidium bromide was removed from the plasmid solution by repeated extractions with equal volumes of isoamylalcohol. The aqueous phase was stored at 4°C in CsCl, since DNA is thought to be more stable in CsCl at lower temperatures. When required for radiolabelling, 100μl was dialysed against TE buffer (pH 8.0) on a type VS: ϕ 0.025μm, 2.5cm Millipore filter for 1 hour.

The amount of plasmid DNA present was then estimated on an agarose gel by running a small amount of DNA against a marker of known concentration.

2.3.2.2 Small-scale plasmid DNA preparation

If small amounts of concentrated plasmid DNA were required, a small-scale plasmid preparation (or mini-preparation) procedure was followed.

A single transformant colony was selected and used to inoculate a 10ml overnight culture in LB selection medium. One and a half ml of the culture was centrifuged in an Eppendorf tube in a microfuge (Hägar designs, RSA) at 4°C for 5 minutes and the supernatant removed. The pellet was resuspended in 200µl ice-cold solution I (50mM glucose: 25mM TrisHCl, pH 8.0: 10mM EDTA) by pipetting thoroughly and then allowed to stand at room temperature for 5 minutes. Then 400µl of solution II (0.2M NaOH: 1% SDS) was added, mixed by inversion and the Eppendorf placed on ice. After 5 minutes, 300µl of ice-cold solution III (3M KOAc: 30mM NaAc) was added, mixed by inversion and the mixture placed on ice for 5 minutes. The solution was centrifuged in a microfuge at 4°C for 5 minutes and the supernatant was extracted twice with phenol: chloroform: isoamylalcohol [25:24:1] and once with chloroform: isoamylalcohol [24:1]. The DNA was then precipitated with 1ml of ice-cold absolute alcohol and centrifuged at 4°C for 10 minutes. The pellet was resuspended in an appropriate volume of TE (pH 8.0).

The amount of plasmid DNA present was then estimated on an agarose gel by running a small amount of DNA against a marker of known concentration.

2.3.2.3 Commercial mini-preparation

The Magic Minipreps DNA Purification System (Promega, RSA) provides a simple and reliable method for rapidly isolating plasmid DNA, using the techniques of Sambrook *et al.* (1989).

Each miniprep started from a 3ml overnight culture of transformed cells. These cells were pelleted by centrifugation of 1.5ml of culture in an Eppendorf tube for 5 minutes, removal of the supernatant and addition of a further 1.5ml of culture which was again centrifuged for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 200μl of Cell Resuspension Solution (50 mM TrisHCl, pH 7.5: 10mM EDTA: 100μg/ml RNase A) by gentle pipetting up and down. To the resuspended cells 200μl of Cell Lysis Solution (0.2M NaOH: 1% SDS) was added and mixed by inverting the tube several times until the cell suspension became clear. Then 200μl of Neutralisation Solution (2.55M KOAc, pH 4.8) was added to the tube and mixed by inversion. The solution was then centrifuged at 12000g for 5 minutes and the supernatant transferred to a clean Eppendorf to which 1ml of Purification Resin was added and mixed by inversion. For each Miniprep, 1 Minicolumn was attached to a 3ml syringe. The Resin/DNA mix was pipetted into the syringe and pushed into the Minicolumn. The DNA in the Minicolumn was washed with 2ml of Column Wash Solution and the Minicolumn transferred to a 1.5ml Eppendorf which was centifuged at 12000g for 20 seconds to dry the Resin. The Minicolumn was transferred to a new Eppendorf and 50μl of TE was added to the Minicolumn for 1 minute. The DNA was eluted by centrifuging the Minicolumn at 12000g for 20 seconds.

The amount of plasmid DNA present was then estimated on an agarose gel by running a small amount of DNA against a marker of known

concentration.

2.3.3 Restriction analysis of plasmid DNA

2.3.3.1 Checking the integrity of the human DNA insert

The size of the DNA insert in the plasmid was checked by digestion of 1μg of the plasmid DNA with the restriction enzyme used for insertion of the human DNA into the plasmid. The restricted DNA was then electrophoresed over a short distance against uncut plasmid DNA and a visible molecular weight marker, to determine the size of the insert.

2.3.3.2 Isolating the human DNA insert

In some cases it was found necessary to utilise the human DNA insert as the DNA probe, if the entire plasmid gave poor hybridisation results. A small amount (5μg) of plasmid DNA was digested with the appropriate restriction enzyme in order to release the human DNA insert. The restricted DNA was then electrophoresed on a 1% low melting point agarose gel (Seaplaque, FMC, Benmore Hospital Suppliers, RSA) at 4°C and at 80V, against uncut plasmid and a visible molecular weight marker. The gel was visualised over a UV transilluminator (Spectroline UV Transilluminator, Model TC-312A) and the insert was cut out of the gel, with as little excess agarose as possible. The gel slice was transferred to a preweighed 1.5ml Eppendorf tube. Water was added to the tube at a ratio of 3ml of water per gram of gel. This mixture was boiled for 3-5 minutes and aliquots of approximately 10-60ng of DNA were stored at -20°C until required for radiolabelling.

If it was found necessary to purify the DNA insert further and to remove the agarose, a phenol-freeze method of purification was used.

Approximately 300μl of agarose containing DNA insert was placed in a 1.5ml Eppendorf to which 500μl of phenol was added. This mixture was vortexed until all the agarose was in solution and then frozen rapidly in either liquid nitrogen or in an ethanol-NaCl ice bath for 2 minutes, before spinning in a microfuge at 4°C for 5 minutes. The vortexing, freezing and spinning steps were repeated twice more and then the aqueous top phase, containing insert DNA, was removed and placed in an Eppendorf. To this solution an equal volume of chloroform: isoamylalcohol [24:1] was added and the mixture was vortexed and spun in a microfuge for 5 minutes at 4°C. To the aqueous phase, one tenth volume 3M sodium acetate and 2 volumes absolute ethanol were added. The DNA was precipitated overnight at -20°C or for 1 hour at -70°C and spun in a microfuge at 4°C for 15 minutes. The supernatant was removed and the DNA pellet washed and spun in 70% ethanol for 15 minutes at 4°C. The ethanol was then removed and the dried pellet was resuspended in an appropriate volume of TE. The amount of plasmid DNA present was then estimated on an agarose gel by running a small amount of DNA against a marker of known concentration.

2.4 Venous blood sample collection and extraction of high molecular weight human genomic DNA from white blood cells

DNA was extracted from peripheral blood leucocytes. Thirty to 60ml of blood was taken by venipuncture, after informed consent, into vacutainer tubes (Radem Laboratory Equipment, RSA) containing acid citrate dextrose (ACD), as an anticoagulant. Approximately 5ml was taken into a plain vacutainer tube resulting in a clotted sample. The blood was centrifuged at 3000rpm for 10-15 minutes at 4°C in order to separate the different blood phases. Serum was removed from the clotted sample and used to test polymorphic serum markers. Plasma was removed from the ACD tubes and approximately 5ml was stored at -70°C for plasma protein studies. One or 2 ACD tubes from each individual were

used for EBV transformation, in the Cytogenetics Unit, in order to produce permanent cell lines for use should the DNA extracted from the leucocytes be finished. The buffy coats, or leucocytes, were collected into two plastic tubes and stored at -20°C until required for DNA extraction. The remaining red blood cells were mixed with an equal volume of preserving fluid (Appendix B) and stored at -20°C. When required for DNA extraction, one buffy coat sample from each individual was thawed at room temperature.

The method of Sykes (1983), with some modifications, was used for human DNA extraction. This technique requires freezing of the buffy coat before the extraction procedure is performed. Thawed samples were poured into 50ml plastic centrifuge tubes (Nunc) and an equal volume of 0.2% Triton X-100: 0.9% NaCl (vol/vol) was added and mixed thoroughly. Triton X-100 is a non-ionic detergent and lyses the cells by removing lipids from the membrane and releasing the cell contents into solution. The mixture was centrifuged at 3000rpm at 4°C for 15 minutes. The pellet was then resuspended in 30ml of fresh saline solution and respun at 4°C at 3000rpm to produce a whiter pellet after removal of the supernatant. If the pellet was too red, the Triton X-100: NaCl wash was repeated.

The pellet was then dispersed with a large diameter, sterilised glass rod in a few drops of lysing buffer (7M urea: 0.3M NaCl: 10mM EDTA: 10mM TrisHCl, pH 7.5). The pellet was gradually worked into the lysing buffer using the glass rod. Further small amounts of lysing buffer were added and the solution made up to a final volume of 10ml. Two ml of 10% SDS (Merck) was then added to the solution and the mixture was placed at 37°C for 10 minutes to maximise the lysis.

The proteins and cellular debris were removed using two organic solvents. Phenol and chloroform denature and precipitate proteins efficiently, leaving the nucleic acids in aqueous solution. Some RNA, especially mRNA, will also be removed by phenol treatment, but most will be retained in the aqueous layer

(Brown 1986). This RNA did not appear to interfere with the DNA analysis.

Deproteinisation was carried out by adding 10ml saturated phenol and 5ml chloroform: isoamylalcohol [24:1], followed by vigorous shaking and centrifugation at 3000rpm for 15 minutes. The aqueous phase was retained and the extraction repeated until the aqueous phase was clear. The aqueous phase was mixed with 10ml chloroform: isoamylalcohol [24:1] and centrifuged at 3000rpm for 10 minutes in order to remove the remaining protein and phenol from the solution.

DNA was then precipitated by addition of two and a half volumes of ice-cold absolute ethanol, and was spooled onto a sterile glass rod or removed with a sterile pipette. The alcohol was allowed to evaporate and the DNA was dissolved in 1ml TE (pH 8.0) for at least 24 hours.

The approximate DNA concentration of the samples was determined on an agarose gel by running small volumes (1-5μl) against known concentrations of lambda DNA. Aliquots of the DNA samples were then stored at 4°C for immediate use and the remainder at -70°C for more permanent storage.

2.5 Analysis of human genomic DNA

Molecular hybridisation of nucleic acids to membrane-bound DNA is perhaps the most common means of qualitatively assessing the presence of a particular polynucleotide sequence in a mixture of nucleic acids (Cannon *et al.* 1985). This is commonly achieved by the restriction endonuclease digestion of total human genomic DNA, agarose gel electrophoresis of the fragments, denaturation of the DNA, transfer to nylon membranes by Southern blotting, hybridisation with a specific radioactively labelled probe, and autoradiography.

2.5.1 Restriction endonuclease digestion of genomic DNA

The restriction endonucleases used in this study fell into the category of type II restriction endonucleases (Fuchs and Blakesley 1983). They are listed in Table 2.5,along with their recognition sequences and the manufacturers from whom they were purchased. The restriction enzymes chosen in the search for restriction fragment length polymorphisms (RFLPs) include those previously shown to reveal a high frequency of RFLPs in human populations (Barker *et al* 1984; Wijsman 1984; Feder *et al*. 1985).

Restriction enzyme digestions of the human DNA were carried out in 1.5ml plastic Eppendorf tubes. Approximately 5-10μg of each DNA sample was digested in a final reaction volume of 50μl, according to the manufacturer's specifications, using 2-3 units of enzyme per 1μg of DNA, and the appropriate buffer. Spermidine trihydrochloride (Sigma) was added to the restriction mixture to a final concentration of 4mM if the buffer solution used had an NaCl concentration below 50mM.

Reactions were incubated between 4 and 24 hours, after which approximately one tenth of the total volume was electrophoresed for a short distance at 60-80V on an agarose gel to assess whether it had digested to completion. The gel was visualised under UV light and a completely digested sample was represented by an even smear of DNA.

Reactions were stopped by placing the samples at 4°C or by the addition of one tenth volume of bromophenol blue loading dye. The chelation of Mg^{2+} by EDTA in the loading dye is an effective means of terminating cleavage.

Table 2.5 Type II restriction endonucleases

ENZYME	RECOGNITION SEQUENCE [1]	SUPPLIER
*Bam*HI	G/GATCC	Boerhinger Mannheim
*Bgl*II	A/GATCT	Anglian
*Eco*RI	G/AATTC	Anglian
*Eco*RV	GAT/ATC	Boerhinger Mannheim
*Hae*III	GCGC/	Boerhinger Mannheim
*Hpa*I	GTT/AAC	Anglian
*Hin*dIII	A/AGCTT	Anglian
*Mbo*I	/GATC	Bethsheda Research Laboratories
*Msp*I	C/CGG	Promega
*Pst*I	CTGCA/G	Boerhinger Mannheim
*Rsa*I	GT/AC	Boerhinger Mannheim
*Sca*I	AGT/ACT	Boerhinger Mannheim
*Taq*I	T/CGA	Boerhinger Mannheim
*Xba*I	T/CTAGA	Boerhinger Mannheim

1. / indicates the position of enzyme cleavage.

2.5.2 Agarose gel electrophoresis of DNA restriction fragments

Following restriction endonuclease digestion, DNA fragments were separated according to molecular weight by electrophoresis through horizontal submarine agarose gels. Agarose creates a matrix which acts as a filter, allowing small fragments to travel further than larger fragments which are slowed down. The migration of fragments is inversely proportional to the logarithm of the molecular weight. Fragments ranging from one to approximately 30kb can be effectively separated in agarose gels, whereas fragments smaller than 1kb are more effectively separated by polyacrylamide gel electrophoresis (Southern 1975).

Seakem HGT (FMS Bioproducts) agarose was used, the concentration ranging from 0.7% to 1%, depending upon the sizes of the restriction fragments to be separated. The agarose was dissolved in 1xTBE (Appendix B) buffer while stirring on a magnetic hot plate. When sufficiently cooled, ethidium bromide was added to a final concentration of 0.3μg/ml to facilitate visualisation of the DNA fragments after electrophoresis, just prior to pouring the gel into a horizontal perspex plate mould, 20cm by 18cm, to a depth of 1cm. The wells were made to be approximately 0.5cm wide and 1mm thick. The gel was submerged in 1xTBE running buffer and samples were loaded sequentially into the wells. Marker lanes were loaded with approximately 20ng of marker DNA, to enable fragment size determination. The marker DNA included bacteriophage lambda C1857 digested with *Hind*III, or *Eco*RV and *Hind*III, and was mixed with salmon sperm DNA (SSDNA) (10μg), which increased the volume and density of the marker solution so that the fragments ran through the gel at the same rate as the human DNA fragments in the other lanes. The gels were electrophoresed at 30-50V for 12-18 hours, depending upon the sizes of the fragments to be visualised. The DNA was visualised over a UV transilluminator and photographed on Polaroid Type 667 (positive) film, using a yellow filter.

2.5.3 Southern transfer of DNA fragments to a nylon membrane

The most common method of transferring DNA fragments from an agarose gel to a membrane is by capillary action or "Southern blotting". Originally, nitrocellulose membranes were used, however, there are several disadvantages associated with nitrocellulose, including membrane fragility. Thus several manufacturers have developed alternate hybridisation support matrices, including the nylon or chemically modified membranes (Cannon *et al.* 1985).

In this study, the method of Southern (1975) was slightly modified and used for the transfer of DNA fragments to a Hybond-N (Amersham) nylon membrane. The instructions supplied by the manufacturers of Hybond-N were used to produce high resolution and low background in the hybridisation reactions undertaken.

After the gel was photographed over the UV transilluminator, following electrophoresis, the gel was rinsed with distilled water to remove any remaining running buffer. The DNA in the gel was then denatured by the addition of denaturing solution (1.5M NaCl: 0.5M NaOH) and gentle orbital shaking at room temperature for 30 minutes. The denaturing solution was discarded and the gel rinsed with distilled water. The gel was neutralised in neutralising solution (1.5M NaCl: 0.5M Tris base) (pH 7.0) with gentle agitation for a further 30 minutes. The neutralising solution was discarded and the gel rinsed once more with distilled water. The gel was placed in 20xSSC (Appendix B) prior to blotting.

Hybond-N was cut to the same measurements as the gel. Approximately 500ml of 20xSSC was poured into a plastic container, a glass plate was placed across this container and a single sheet of Whatmann 3M filter paper was spread over the glass , ..c, with both ends in the solution, to act as a wick

for the salt solution. Any air bubbles under the wick were pressed out with a plastic pipette. The gel was placed on top of this wick and any air bubbles trapped underneath were rolled out. The Hybond-N membrane was carefully smoothed onto the gel with a pipette, excluding any air bubbles. The filter paper surrounding the gel was covered with plastic wrap to ensure that the salt solution passed only through gel. Two pieces of filter paper, cut to the size of the gel and soaked in 20xSSC, were placed on top of the Hybond-N membrane and four dry pieces were layered on top, followed by approximately 8cm of folded paper towels. A glass plate was placed over the paper towels with a 0.5-1kg weight to distribute the mass evenly over the gel area.

The transfer proceeded for 6-24 hours. Once complete, the paper towelling, filter paper, plastic wrap and gel were discarded and the membrane was labelled for orientation. The membrane was rinsed in 2xSSC, placed between dry filter papers and baked at 80°C for 1-2 hours. The blot was then stored, in the filter paper, in an air-tight plastic bag until required for use.

2.5.4 Radiolabelling of DNA probes

The most efficient method used to label DNA probes was found to be oligolabelling, using hexanucleotide primers (Feinberg and Vogelstein 1983). This procedure used ^{32}P deoxycytidine triphosphate (10mCi/ml, 3000Ci/mmol, Amersham, RSA) as the labelled nucleotide to be incorporated. Oligolabelling was used to label both whole plasmid (Pmel34, pMT4, Human *b*-protein cDNA, DQα, CALC, SAA, β-globin, pTD3-21 and pTD189-1) and restriction fragments (pML34, pIR4-3R, pIR10-1, CMW-1, pJU201 and pDP151), as described in Section 2.5.2.Specific activities of 10^9 dpm/μg may be attained using oligolabelling (Feinberg and Vogelstein 1983) and it is possible to attain up to 90% incorporation of the label under optimal conditions. The DNA to be labelled is first denatured by boiling for

3 minutes. Fragments in agarose were placed in a heating block (Hägar Designs, RSA) at 37°C to prevent the agarose solidifying.

The labelling reaction was performed in a total volume of 50μl. Up to 60ng of plasmid DNA was added to 50μCi of ^{32}P dCTP with oligolabelling buffer (Amersham), 5μl of primer solution (Amersham), and 2μl of enzyme solution (provided by Amersham). The mixture was incubated at 37°C for 5 minutes, after which time 1μl of 0.1M spermidine was added. The solution was then incubated for a further 15 minutes before the percentage incorporation was determined.

Incorporation was checked by spotting 0.5μl of reaction mixture onto a 2.5cm Whatman GF/C filter and total counts were measured using a scintillation meter (Series 900 mini-monitor, Weil Organisation, RSA). The filter was washed over a Millipore manifold attached to a vacuum pump, once with 10% trichloroacetic acid (TCA) and 3 times with 5% TCA. The acid precipitable counts were measured and the percentage incorporation was calculated as follows:

$$\text{Percentage incorporation} = \frac{\text{TCA precipitable counts}}{\text{Total counts}} \times 100\%$$

Incorporation of greater than 50% was acceptable for labelling whole plasmid, while incorporation above 30% was acceptable for smaller fragments due to the greater specificity of hybridisation of the insert. The reaction was stopped by placing the mix on ice.

In order to separate labelled DNA from unincorporated nucleotides, spun column chromatography was used. This method has advantages over conventional drip column chromatography and several samples can be handled simultaneously.

The spin columns were set up as described by Sambrook *et al.* (1989). The bottom of a 1ml disposable syringe was plugged with a small amount of sterile nylon fibre, or glass wool, which was firmly pressed into place. The syringe was filled with Sephadex G-50 (Fine: 20-80μm, Sigma), inserted into a 15ml disposable plastic tube and centrifuged (IEC Clinical Centrifuge, International Equipment Company, USA) for 5 minutes at room temperature. More sephadex was then added and the column recentrifuged until the volume of the packed column was approximately 0.9ml. The column was equilibrated by adding 100μl of TE (pH 8.0) and recentrifuging for a further 5 minutes. The volume of the probe mix was made up to 100μl with TE (pH 8.0) and applied to the column. The column was then centrifuged for 5 minutes and the effluent was collected into a decapped 1.5ml Eppendorf tube. Another 100μl of TE (pH 8.0) was applied to the column and recentrifuged into the same Eppendorf, to remove any remaining labelled DNA. The eluted DNA was then transferred to a capped 1.5ml Eppendorf tube and the volume made up to 1ml with TE (pH 8.0).

Prior to hybridisation, the probe was denatured by boiling for 5 minutes and placed on ice for at least 10 minutes before being added to the hybridisation solution.

2.5.5 Hybridisation of Hybond-N membranes

Hybond-N requires only a single solution for both prehybridisation and hybridisation. Prehybridisation is necessary to prevent non-specific binding of nucleic acids to the membrane containing the digested DNA, thus decreasing background hybridisation. Prior to prehybridisation, the membranes were soaked in 2xSSC. They were then placed in plastic bags, with 1-3 blots per bag, to which 10-20ml of 1xprehybridisation solution (10xSSPE; 10xDenhardt's solution; 1% SDS; 400μg/ml SSDNA), containing 50% deionised formamide, was added. Formamide is a chaotropic agent and

lowers the temperature at which DNA-DNA duplex formation occurs (Cannon *et al.* 1985) from 65°C to 42°C. The hybridisation solution contains SSPE salt solution (Appendix B), which masks the natural repulsion between the two highly charged DNA strands and thus enhances hybridisation (Meinkoth and Wahl 1984), and Denhardt's solution (Appendix B), which blocks non-specific DNA binding sites and acts with SSDNA which is added for the same purpose.

Membranes were incubated for 1-24 hours at 42°C with gentle orbital shaking before the labelled probe and labelled λ marker was added to the bag. Incubation was again carried out at 42°C, with gentle shaking for 18-48 hours.

After hybridisation, the membranes were removed from the bags for post-hybridisation washes and the hybridisation solution was either discarded or retained for use as a rehybridisation solution. To reuse the hybridisation solution, it was heated to 70°C to denature the DNA and then cooled on ice just prior to use.

2.5.6 Post-hybridisation washes of Hybond-N membranes

After hybridising, the membranes were washed to remove non-specifically bound probe, leaving only stable DNA-DNA duplexes on the blot. For normal strigency washes, the blots were washed twice in 2xSSPE: 0.1% SDS at room temperature for 15 minutes each wash, then washed twice in 1xSSPE: 0.1% SDS at 65°C for 30 minutes each wash, followed by rinsing in 0.1xSSPE at room temperature to remove any SDS which may cause background. If the signal was too faint, less stringent washes were used (increased salt concentration, for shorter wash times) and if there was background signal, more stringent washes were used (lower salt concentration, for longer wash times). The membranes were then sealed into plastic bags and set up for autoradiography.

2.5.7 Autoradiography

The sealed bags containing the nylon membranes were placed in Okomoto X-ray cassettes with Trimax calcium tungsten intensifying screens. AGFA Curix X-ray film was placed over the filter. The cassettes were placed at -70°C for 2-14 days, depending upon the expected intensity of the signal. The film was then removed and processed in Ilford (Phenisol) developer and fixed in Ilford (Hypam) fixer in an RP X-OMAT processor (Kodak).

2.5.8 Reuse of Hybond-N membranes

Hybond-N membranes can be successfully reused up to 20 times. In order to remove previously bound probe from filters, the filters were washed in denaturing solution (Appendix B) for 10 minutes, followed by two 10 minute washes in neutralising solution (pH 7.0) (special neutralising solution, Appendix B). Between each wash, the membranes were rinsed twice with distilled water. After the final wash the membranes were placed in 2xSSC until required for rehybridisation. If not used immediately, the filters were sealed singly in plastic bags and stored on a flat surface at 4°C.

2.6 PCR analysis of human genomic DNA

Where possible, the polymerase chain reaction (PCR) was utilised to detect polymorphisms, particularly because this technique requires very small amounts of DNA and results can be obtained relatively quickly.

All PCR primers were synthesised at the Biochemistry Departments of the University of the Witwatersrand or the University of Cape Town.

The PCR was used to amplify genomic DNA in standard amplification reactions in a Perkin-Elmer Cetus or Hybaid thermocycler. All reactions were carried out

in Treff PCR tubes, under mineral oil (Sigma), to prevent evaporation of the reaction mix.

2.6.1 RFLP analysis using PCR

Reactions were carried out in a total volume of 25μl containing: 25-50ng DNA, 5-50pmol each primer, 1.25mM each dNTP, 2U *Taq* polymerase (Promega), 2.5μl Promega buffer (10x), 0.5-1μl spermidine trihydrochloride (1 in 20 dilution of a 0.1M stock) (optional). Amplification was optimised for each primer set, for 25-30 cycles with denaturation at 94°C for 30-120 seconds, annealing at 47-62°C for 30-150 seconds, extension at 72°C for 60-120 seconds and a final extension at 72°C for 10 minutes.

Amplification products were digested by adding 5-10U of the restriction enzyme and buffer (10x) to the PCR tubes and incubating for 1-2 hours at the appropriate temperature. The restriction products were visualised on 4% Nusieve/HGT (3:1) agarose gels, run for 1-2 hours at 80V, against a visible molecular weight marker.

2.6.2 CA repeat analysis using PCR

Genomic DNA was amplified in a total volume of 25μl containing: 50-500ng DNA, 50pmol each primer, 200μM each dATP, dGTP, dTTP, 2.5μM dCTP, 1-2μCi α^{32}P[dCTP], 2U *Taq* polymerase (Promega, RSA), 2.5μl Promega buffer (10x). Amplification was optimised for each set of primers for 25-30 cycles with denaturation at 94°C for 30-120 seconds, annealing at 53-62°C for 30-150 seconds, extension at 72°C for 60-120 seconds and a final extension at 72°C for 10 minutes.

A volume of 1.5-3μl of each of the products was added to 2μl of formamide dye (Appendix B), heated to 100°C for 2 minutes and loaded onto 6%

nondenaturing polyacrylamide (Appendix B) gels. End-labelled *Hpa*II-digested pBR322 was used as a size marker in a single lane. Thereafter, a pre-sized reference sample was loaded in each run. Each gel was sequentially loaded 2-5 times and, depending upon the size of the amplified fragments, each loading was run for 30 minutes to 3 hours at 10-30mA (± 1200V).

Gels were transferred directly onto Whatman 3M filter paper, covered with plastic wrap and dried (Biorad M583 Dryer) under a vacuum (Savant RT100 Vacuum pump) for 15-30 minutes. The gels were autoradiographed at room temperature for 30 minutes to 5 days, depending upon the intensity of the signal.

2.7 Linkage studies

DNA was extracted from 245 individuals, representing 41 families. Affected sibs from 13 families had ephelides, 23 families had no ephelides and the ephelus status for 5 families was unknown. These individuals were typed for all markers. Details of the markers can be found in Section 2.2.

All data were entered into a data-base for easy access using the computer program DBase III Plus.

2.7.1 Linkage analysis

Linkage analysis was originally performed using the program LIPED, version 3 for personal computers (Ott 1974), by the computation of lod scores. The analyses in this study included the results obtained in two other studies (Heim 1988; Jenkins *et al.* 1990), which were also analysed using the LIPED program. All the results have been reanalysed using the MLINK program of the LINKAGE package (Lathrop *et al.* 1984). All pedigree data and marker data were entered into two separate MLINK files in the required binary

format, using an IBM compatible word-processing program. For linkage analysis between albinism and other markers, 100% penetrance in both sexes and an allele frequency of 0.001 was assumed for the disease trait. Allele frequencies were calculated for each marker. Since linkage to ty-pos OCA is established within each family for any marker, each family was scored separately for the hypervariable markers, irrespective of the actual allele sizes, in order to reduce the total number of alleles necessary for analysis. In this way it was possible to ensure that the computational capacity of the computer was not exceeded. All analyses were limited to a maximum of 12 alleles, irrespective of the number of loci, on the IBM-compatible micro-computer (with numeric processor).

2.7.2 Analysis of allelic association

Once the position of the ty-pos OCA gene was established, the frequency of the polymorphic alleles of the closest markers was calculated using the chi-square test in order to determine whether there was allelic association between these alleles and the ephelus-determining mutation(s) resulting in the absence or presence of ephelides.

It is assumed that a single locus is responsible for the ty-pos OCA phenotype and that different mutations at this locus give rise to the differences seen in the phenotypic manifestation of ty-pos OCA. One aspect of the phenotype which can be scored is the presence or absence of ephelides. When attempting to calculate the frequency of each of these mutations, using the phenotype to predict the genotype, then it is important to acknowledge that one of these allelic mutations may be dominant over the other and thus the true frequency of each of the mutations will be masked. For example, if the mutation(s) giving rise to a phenotype with ephelides is dominant over the mutation(s) resulting in an absence of ephelides, then both the homozygous and heterozygous carriers of the "ephelus mutation" will have ephelides. Only

those individuals homozygous for the mutation(s) giving rise to an absence of ephelides will have this phenotype, so the real frequency of each mutation will not be known as the number of heterozygotes cannot be calculated.

2.7.3 Haplotype analysis

Haplotypes were constructed for all families, using allele segregation in nuclear family units. The markers used included those which had a known physical order and two or three alleles, as well as the closely linked, 12 allele GABRB3 CA repeat system, for which the alleles could be scored unambiguously. Haplotypes were generated for non-OCA chromosomes, using known heterozygous individuals, and for chromosomes associated with the ty-pos OCA mutation. The haplotype patterns for affected individuals with and without ephelides were analysed separately in order to test for conserved patterns and to distinguish any obvious differences between the two groups.

The haplotypes were used in an attempt to establish whether a single origin to the ty-pos OCA mutation in southern African Negroids could be postulated. This could be done if there was a common allele pattern, particularly at loci closest to the ty-pos OCA gene.

Most of the hypervariable VNTR and dinucleotide repeat loci were not included in the haplotype analysis because, primarily, they are difficult to size without ambiguity, especially by Southern blot analysis. Similarly, as a result of their hypervariability, they are poorly conserved evolutionarily and are likely to have changed since the original mutation(s) occurred at the ty-pos OCA locus. The large number of alleles at these loci and their higher mutation rate (more frequent than the loci with only 2 or 3 alleles), would thus generate a large number of haplotypes, which would be unlikely to show any single common pattern in affected individuals.

2.8 Chromosomal and regional assignment of human cDNA probes

A small amount of SCH DNA was received from Sue Chamberlain (St Mary's Hospital Medical School, UK) and Sue Povey (Galton Laboratory, UK). This DNA was used to assign the human CAS2 cDNA marker to a specific region on human chromosome 9. These lines, PK-87-9 and 640-63a12, were originally obtained from Cynthia Jackson (Rhode Island Hospital, USA) and Carole Jones (Eleanor Roosevelt Institute, USA), respectively, and both occurred on chinese hamster backgrounds. PK-87-9 had chromosome 9 as its only human chromosome (Warburton *et al.* 1990) and 640-63a12 contained 9cen-qter as its only human DNA (C. Jones, personal communication).

The parental cell line used to indicate rodent chromosomes, was propagated by colleagues in the Cytogenetics Unit of the Department of Human Genetics, University of the Witwatersrand (Dos Santos 1986). The rodent parental cells were from Chinese hamster permanent cell line wg3-h (HGPRT[-]). The chromosome composition of the hybrid cell lines were determined cytogenetically by sequential G-banding techniques.

2.8.1 Extraction of high molecular weight DNA from SCH lines

Rodent parent cell lines were cultured by colleagues in the Cytogenetics Unit and DNA was isolated from the cells 10-14 days after planting, once confluent growth was obtained.

The culture medium was poured off and each flask of monolayer cells was washed twice with 5ml of ice-cold PBS (0.14M NaCl: 5mM KCl: 20mM phosphate, pH 7.5). Proteinase K was added to the lysis buffer (10mM TrisHCl, pH 7.5: 5mM EDTA: 0.5% SDS) to a final concentration of 50μg/ml. The flasks were arranged in piles of 3 each and 5ml of lysis buffer/proteinase K was added to the top flask in each pile. Each flask was

incubated flat in a 45°C waterbath for 10 minutes. The cells were poured off from this flask into the next flask in the pile. The incubation was repeated until all three flasks were lysed. The lysate was collected into a plastic 50ml tube (Nunc) and incubated at 68°C for 30 minutes, followed by a 90 minute incubation at 37°C. The solution was deproteinised by one phenol extraction and one phenol: chloroform: isoamylalcohol [25:24:1] extraction and two chloroform: isoamylalcohol [24:1]extractions. DNA was precipitated from the aqueous phase by addition of 5M NaCl, to a final concentration of 0.3M, and two and a half volumes of ice-cold ethanol. The DNA was spooled onto a glass rod, dried briefly and dissolved in an appropriate volume of TE (pH 8.0). Approximately 500-1000μg of DNA was isolated from 3 large confluent flasks.

2.8.2 Preparation of Southern transfers and hybridisations to DNA from SCH lines

Ten to 20μg of hybrid DNA was digested with restriction enzymes *Taq*I and *Xba*I, along with 10μg of DNA from normal human male and female controls and DNA from the rodent parental cell line. Digested DNA was electrophoresed on a 0.8% agarose gel, with a molecular weight marker, and was denatured and transferred onto a nylon Hybond-N membrane using the method of Southern blotting, as described in Section 2.5.3.

The probes to be mapped, pMT4 and its human homologue, human CAS2 cDNA, were radiolabelled with ^{32}P by oligolabelling, and were hybridised sequentially to the SCH DNA on the Southern blots. After autoradiography, the specific lanes of hybrid DNA to which each probe hybridised were noted and compared with the parental cell lines. Details of the preparations of Southern blots, radiolabelling, hybridising and autoradiography are given in Section 2.5.

CHAPTER THREE

3. RESULTS

3.1 Mapping the human CAS2 gene

The mouse *b* locus has been mapped to mouse chromosome 4, in a region with homology to human chromosome 9, thus it was likely that the human homologue, CAS2, would map to this human chromosome. The human CAS2 cDNA was hybridised to *Taq*I digests of human DNA, chinese hamster DNA and DNA from two chromosome 9 SCH. The hybridisation pattern is shown in Figure 3.1. Hybridisation revealed a 2 fragment (12kb and 10kb) pattern in human DNA, which was absent from chinese hamster DNA, and only occurred in the digested DNA from SCH PK-87-9, which contains the whole of human chromosome 9. SCH 640-63a12, which only contains human chromosome 9q, did not have these two bands, suggesting that the CAS2 gene is on human chromosome 9p. This result was confirmed by *in situ* hybridisation, which further localised the gene to 9p22-pter (Chintamaneni *et al.* 1991b).

3.2 The linkage study

3.2.1 Negroid families with ty-pos OCA

Forty-one families participated in this linkage study, of which the affected individuals from 13 presented with ephelides and from 23 presented without ephelides. The ephelus status in 5 families was unknown. There was 100% concordance with respect to ephelus status within families where all affected children were over 10 years of age. The structure and size of each family involved in the study is shown in Table 3.1 and the family data are summarised in Table 3.2.

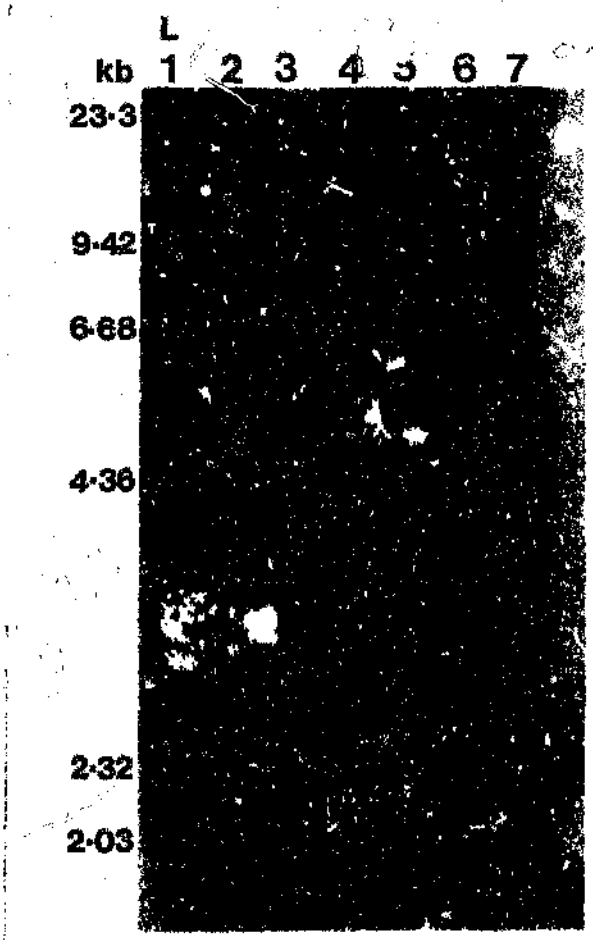

Figure 3.1 Autoradiograph showing hybridisation of human CAS2 cDNA to a Southern blot of *Taq*I digested DNA. Lane (L) 1 is a λ *Hin*dIII marker, for which the sizes are given to the left of the figure. Lane 7 is vastly overloaded, and contains a λ *Hin*dIII/*Eco*RI marker, for which accurate sizing was not possible. Lanes 2 and 3, respectively, contain human male and female genomic DNA. Lane 4 contains chinese hamster DNA. Lane 5 contains SCH PK-87-9 DNA and lane 6 contains SCH 640-63a12 DNA.

Table 3.1: Structure of the ty-pos OCA families used in the linkage study

FAMILY CODE	EPHELIS STATUS[1]	TOTAL NO INDIVIDUALS	NO OF GENERATIONS	NO AFFECTED INDIVIDUALS	NO UNAFFECTED SIBS
ZADA	-	10	3	3	4
ZADB	-	6	2	2	2
ZADC	-	6	2	3	1
ZADD	+	8	2	3	3
ZADE	+	7	3	3	1
ZADG	-	11	3	3	3
ZADH	-	13	3	4	6
ZADI	-	7	2	2	4
ZADJ	?	6	2	1	1
ZADK	-	5	2	3	1
ZADN	-	7	2	2	4
ZADP	-	4	2	1	1
ZADQ	+	13	3	4	3
ZADR	+	5	3	1	1
ZADT	+	5	3	1	1
ZADZ	+	4	3	4	0
ZAEB	+	5	3	2	1
ZAEE	+	6	2	2	2
ZAEF	?	4	2	2	0
ZAEG	-	4	2	1	1
ZAEJ	-	3	2	1	1
ZAEK	-	4	3	1	1
ZAEL	+	9	2	2	4
ZAEM	+	7	2	1	4
ZAEO	-	4	2	1	2
ZAEP	-	3	2	1	1
ZAES	+	3	2	1	1
ZAFA	-	3	2	1	1
ZAFC	+	4	2	1	1
ZAFF	-	5	2	1	2
ZAFI	-	16	3	4	1
ZAFH	?	4	2	3	1
ZAFI	?	3	2	1	0
ZAFJ	+	4	2	3	1
ZAFL	-	8	3	2	2
ZAFU	-	5	2	2	1
ZAFW	-	4	2	3	0
ZAFX	-	4	2	3	0
ZAFY	-	8	3	3	0
ZAFZ	-	6	2	2	2
ZAFA	?	5	3	2	0

Table 3.2: Summary of families with ty-pos OCA

	FAMILIES				INDIVIDUALS[2]				AFFECTED INDIVIDUALS			
EPHELUS STATUS[1]	+	-	?	TOTAL	+	-	?	TOTAL	+	-	?	TOTAL
NUMBER	13	23	5	41	80	147	22	249	21	48	7	76

1 : + With ephelides
- Without ephelides
? Ephelus status unknown

2 : Unaffected individuals from families where affected individuals present with (+) and without (-) ephelides

3.2.2 Two-point linkage analysis with random polymorphic markers

All two-point linkage analysis was done using the MLINK package of the LINKAGE program. For linkage analysis between ty-pos OCA and other markers, the following criteria were assumed for the disease trait: $\Theta_m = \Theta_f$, 100% penetrance and an allele frequency of 0.001.

3.2.2.1 Random serogenetic markers

Table 3.3 summarises the data obtained in two-point linkage analysis between ty-pos OCA and 19 random serogenetic markers, on 10 different chromosomes, as well as the polymorphism information content of each system in the Negroid population. These data were previously analysed using the LIPED program (Jenkins *et al.* 1990). From the data generated using the LINKAGE program, close linkage can be excluded between ty-pos OCA and PGM1, RH, PGD, FY, ACP1, GPX1, TF, GC, GYPA, GYPB, BF, GLO, GPT1, CA2, ABO and PEPA ($Z<-2$ at $\Theta=0.01$). Equivocal results were obtained between ty-pos OCA and AK, PI and HP, which did not show significantly negative lod scores at $\Theta=0.01$ ($-2<Z<3$). At $\Theta=0.2$, only RH could be conclusively excluded from linkage to ty-pos OCA ($Z=-2.67$).

Slightly positive lod scores were obtained between ty-pos OCA and BF ($Z=0.92$, $\Theta=0.2$), PI ($Z=0.72$, $\Theta=0.01$) and HP ($Z=0.42$, $\Theta=0.2$). However, these lod scores are not significantly positive ($Z<3$) and therefore do not suggest close linkage between the ty-pos OCA locus and any of these markers. The slightly positive lod score obtained for BF ($Z=0.92$, $\Theta=0.2$) was consistent with the positive lod score obtained previously (Jenkins *et al.* 1990) and thus other markers on chromosome 6p were tested for linkage.

Table 3.3: Two-point linkage analysis between 19 random serogenetic markers and ty-pos OCA

MARKER	CHROMOSOME POSITION	NO OF FAMILIES[1]	PIC[2]	LOD SCORES AT RECOMBINATION FRACTION θ					
				0.01	0.05	0.1	0.2	0.3	0.4
PGM1	1p22.1	18	0.53	-9.67	-4.38	-2.38	-0.83	-0.26	-0.05
AK	1p34	11	0.04	-0.003	-0.003	-0.003	-0.002	-0.001	0.00
RH	1p34.3-p36.1	24	0.47	-20.84	-10.57	-6.37	-2.67	-1.00	-0.23
PGD	1p36.13-p36.2	7	0.21	-3.09	-1.16	-0.47	-0.01	0.07	0.03
FY	1p21-q23	12	0.28	-5.85	-2.55	-1.31	-0.37	-0.07	-0.003
ACP1	2p23-p25	17	0.43	-4.20	-2.08	-1.20	-0.45	-0.15	-0.03
GPX1	3p13-p21.1	13	0.09	-2.23	-0.91	-0.43	-0.07	0.01	0.01
TF	3q21-q26.1	24	0.15	-2.21	-0.90	-0.41	-0.07	0.01	0.01
GC	4q12-q13	25	0.44	-13.12	-6.82	-4.18	-1.78	-0.66	-0.13
GYPA (MN)	4q28-q31	32	0.37	-12.84	-5.56	-2.83	-0.79	-0.14	0.01
GYPB (Ss)	4q28-q31	32	0.26	-7.52	-2.92	-1.24	-0.10	0.12	0.06
BF	6p21.3	24	0.47	-2.86	0.11	0.90	0.92	0.50	0.14
GLO	6p21.1-p21.3	16	0.32	-5.04	-1.60	-0.38	0.35	0.35	0.13
GPT1	8q13-qter	16	0.22	-4.58	-2.45	-1.53	-0.67	-0.26	-0.06
CA2	8q22	17	0.16	-4.39	-1.83	-0.92	-0.28	-0.08	-0.02
ABO	9q34	33	0.42	-20.65	-9.74	-5.38	-1.83	-0.54	-0.11
PI	14q32	32	0.17	0.72	0.68	0.61	0.45	0.26	0.08
HP	16q22.1	23	0.51	-1.54	-0.24	0.22	0.42	0.28	0.08
PEPA	18q23	14	0.20	-3.94	-1.95	-1.17	-0.50	-0.19	-0.04

1 : Number of informative families 2: Polymorphism information content

3.2.2.2 Random DNA markers

Two-point linkage analysis between ty-pos OCA and 15 random polymorphic DNA markers, on 8 different chromosomes, did not reveal any significantly positive lod scores (Table 3.4). At $\Theta=0.2$, none of the markers showed significantly negative lod scores (all $Z>-2$) and the only positive lod scores obtained were between ty-pos OCA and RH52 ($Z=0.08$, $\Theta=0.3$), DQα ($Z=0.44$, $\Theta=0.3$), Mfd61 ($Z=0.71$, $\Theta=0.3$) and MR3 ($Z=0.81$, $\Theta=0.1$). These results are not significantly positive ($Z<3$) and thus do not suggest evidence of linkage between ty-pos OCA and any of these random DNA markers. The slightly positive lod scores found between the serogenetic marker, BF, on 6p and ty-pos OCA, are also found with two of the other 6p DNA markers, DQα and Mfd61. However, since these lod scores are not significantly positive ($Z<3$) these markers are not likely to be linked to ty-pos OCA.

3.2.3 Two-point linkage analysis between ty-pos OCA and 2 candidate loci, and a candidate region on 11p

3.2.3.1 Tyrosinase locus

Table 3.5 summarises the results obtained with the human tyrosinase cDNA, Pmel34, showing a significantly negative lod score of -5.81 at $\Theta=0.01$. Thus tyrosinase can be excluded as a candidate locus for ty-pos OCA.

Table 3.4: Two-point linkage analysis between 15 random polymorphic DNA markers and ty-pos OCA

MARKER	LOCUS	CHROMOSOME POSITION	NO OF FAMILIES[1]	PIC[2]	LOD SCORES AT RECOMBINATION FRACTION θ					
					0.01	0.05	0.1	0.2	0.3	0.4
2D9-1	D1S116	1p31.2	24	0.38	-13.23	-5.71	-3.04	-1.13	-0.47	-0.17
Mfd3	APOA2	1q21-q23.2	34	0.79	-29.59	-12.39	-6.14	-1.69	-0.37	-0.04
RH52	RH52	1	32	0.37	-9.62	-3.79	-1.67	-0.23	0.08	0.05
ALB	ALB	4q11-q13	35	0.36	-13.63	-5.97	-3.01	-0.80	-0.15	-0.01
DXα	HLA	6p21	31	0.37	-13.56	-5.66	-2.75	-0.6.	-0.08	0.01
DQα	HLA	6p21	32	0.77	-19.77	-7.11	-2.74	0.02	0.44	0.19
Mfd61	D6S105	6p	38	0.83	-32.52	-11.92	-4.68	-0.03	0.71	0.31
pMP6d9	D7S399	7q31	33	0.37	-15.51	-7.30	-4.05	-1.42	-0.42	-0.07
pJ3-11	D7S8	7q31	31	0.35	-8.19	-3.46	-1.68	-0.42	-0.07	-0.01
pAW101	D14S1	14q32.1-32.2	6	0.69	-7.83	-3.29	-1.63	-0.42	-0.06	0.00
Mfd15	D17S250	17q11.2-q12	29	0.89	-21.83	-9.66	-5.01	-1.46	-0.29	-0.01
MPO	MPO	17q21.3-q23	38	0.72	-17.05	-6.42	-2.56	-0.03	0.36	0.15
MR3	MR3	17	14	0.37	-0.54	0.56	0.81	0.70	0.39	0.11
MR22	MR22	18	15	0.35	-5.48	-1.69	-0.41	0.31	0.31	0.11
335/334	D21S168	21q22.3	22	0.75	-22.83	-9.74	-4.84	-1.18	-0.11	0.05

1 : Number of informative families 2: Polymorphism information content

Table 3.5: Two-point linkage analysis between 2 candidate loci and ty-pos OCA

LOCUS	MARKER	CHROMOSOME POSITION	NO OF FAMILIES[1]	PIC[2]	LOD SCORES AT RECOMBINATION FRACTION θ					
					0.01	0.05	0.1	0.2	0.3	0.4
TYR	Pmel34	11q14-q21	14	0.20	-5.81	-2.83	-1.58	-0.53	-0.15	-0.02
CAS2	CAS2	9p22-pter	27	0.09	-6.51	-2.75	-1.25	-0.13	0.12	0.06

1 : Number of informative families 2: Polymorphism information content

In order to test for possible locus heterogeneity and to show exclusion of linkage to the tyrosinase locus in all families, the lod scores obtained at a recombination fraction Θ=0.01, were calculated for each family and are shown in Table 3.6. For each group, the summated lod score is negative, showing that there is no locus heterogeneity. The only positive scores obtained (families ZABK [Z=0.01], ZABN [Z=0.48], ZABR [Z=0.12] and ZACO [Z=0.12]), are not significantly positive and do not suggest locus heterogeneity. The relatively high lod score obtained for ZABN (Z=0.48), may be accounted for by a lack of information. No DNA was obtained from the father and the polymorphic alleles in the family are compatible with linkage, but also be explained by homozygosity in the absent father. Thus the tyrosinase locus is excluded from being the locus for ty-pos OCA in all groups.

The tyrosinase/*Mbo*I RFLP, present in Caucasoids, Asians and Afrocarribeans, but absent from Orientals, was not found in the 32 random Negroids screened for the polymorphism and is thus unlikely to be present in this population.

3.2.3.2 The CAS2 locus

Figure 3.2 shows the two allele *Xba*I RFLP detected by both the mouse *b* locus cDNA, pMT4, and the human CAS2 cDNA. In the Negroid population, the 10kb allele occurs with a frequency of 0.96 and the 8.6kb allele occurs with a frequency 0.04 (Colman *et al.* 1991a). Two-point linkage analysis between ty-pos OCA and the human CAS2 cDNA is summarised in Table 3.5. At Θ=0.01, Z=-6.51, thus excluding the CAS2 locus as a candidate locus for ty-pos OCA.

Table 3.6: Two-point linkage analysis between ty-pos OCA and the tyrosinase locus in each family at Θ=0.01

FAMILIES WITHOUT EPHELIDES	LOD SCORES AT Θ=0.01[1]	FAMILIES WITH EPHELIDES	LOD SCORES AT Θ=0.01[1]
ZABA	-	ZABD	-
ZABB	-	ZABE	-
ZABC	-	ZABQ	-
ZABG	-	ZABR	0.12
ZABH	-	ZABT	-
ZABI	-	ZABZ	-
ZABK	0.01	ZACB	-1.32
ZABN	0.48	ZACE	-
ZABP	-0.01	ZACL	-
ZACG	-1.28	ZACM	-
ZACJ	-	ZACS	-0.17
ZACK	-0.20	ZADC	-
ZACO	0.12	ZADJ	-
ZACP	-0.01	TOTAL	-1.37
ZADA	-		
ZADF	-	FAMILIES OF UNKNOWN STATUS	LOD SCORES AT Θ=0.01[1]
ZADG	-		
ZADL	-		
ZADU	-	ZABJ	-0.32
ZADW	-1.33	ZACF	-1.40
ZADX	-	ZADH	-
ZADY	-0.50	ZADI	-
ZADZ	-	ZAFA	-
TOTAL	-2.72	TOTAL	-1.72
TOTAL LOD SCORE FOR ALL FAMILIES = -5.81			

1 : "-"indicates family uninformative at this locus

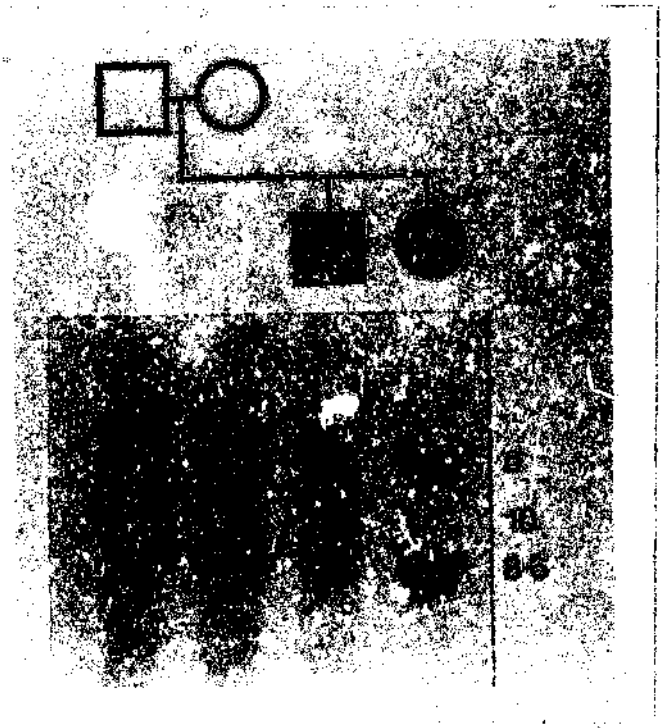

Figure 3.2 An autoradiograph showing the two allele *XbaI* RFLP detected by hybridisation to the CAS2 cDNA in family ZACF. The shaded symbols represent affected individuals with ty-pos OCA (c - constant).

3.2.3.3 Linkage analysis between ty-pos OCA and a candidate region on 11p

Two-point linkage analysis did not suggest evidence of linkage between ty-pos OCA and 4 markers on 11p, as shown in Table 3.7. At $\Theta=0.05$, all the lod scores were significantly negative ($\Sigma<-2$) and at $\Theta=0.2$, all the lod scores were slightly negative. These results exclude close linkage between four markers on 11p and ty-pos OCA.

3.2.4 Two-point linkage analysis between markers on 15q and ty-pos OCA

Positive lod scores were originally obtained when markers pTD189-1 (D15S13) and pTD3-21 (D15S10), on chromosome 15q11-q12, were tested for linkage to ty-pos OCA (Ramsay *et al.* 1992). Another 6 markers in the chromosome 15q11-q12 region and 2 markers in the chromosome 15q11-q13 region were also tested for linkage. Four markers in more distal 15q positions and a single marker, Mfd49, which had not been sublocalised on chromosome 15, were subsequently tested for linkage.

Figure 3.3 illustrates the inheritance of the 3 pTD3-21/*Taq*I alleles and Figure 3.4 shows the inheritance of the GABRB3 CA repeat, amplified by the PCR.

Table 3.7: Two-point linkage analysis between the human chromosome 11p candidate region and ty-pos OCA

MARKER	LOCUS	CHROMOSOME POSITION	NO OF FAMILIES[1]	PIC[2]	LOD SCORES AT RECOMBINATION FRACTION θ					
					0.01	0.05	0.1	0.2	0.3	0.4
pHC36	CALCA	11p14-p15.5	31	0.37	-9.08	-3.83	-1.87	-0.45	-0.06	0.01
pSAA82	SAA	11p11-pter	34	0.58	-16.45	-7.18	-3.72	-1.14	-0.30	-0.06
pDH3.2	HBB	11p15.5	32	0.60	-14.33	-6.29	-3.25	-0.99	-0.26	-0.04
716/718	WT1	11p13	17	0.76	-14.11	-5.53	-2.41	-0.29	0.18	0.12

1 : Number of informative families 2: Polymorphism information content

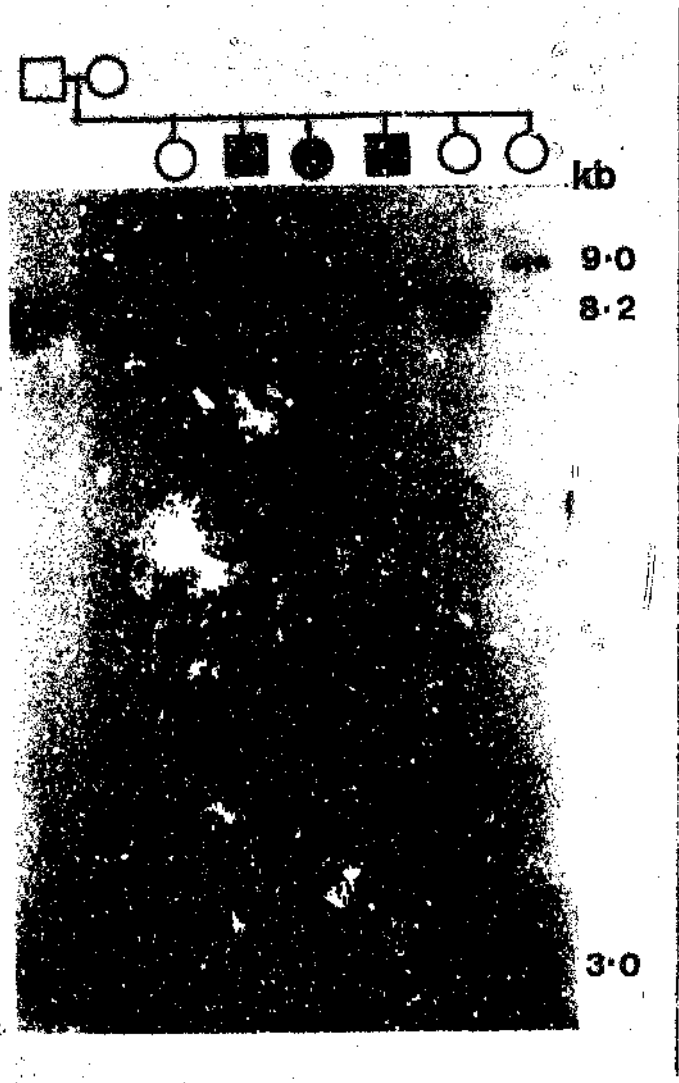

Figure 3.3 Inheritance of the pTD3-21/*Taq*I alleles in family ZABD. The 3.0kb allele is Negroid-specific (Colman *et al.* 1992). The shaded symbols represent affected individuals with ty-pos OCA. The alleles inherited by the second unaffected child illustrate a recombination between the disease locus and the marker locus since this individual has inherited the same pTD3-21/*Taq*I alleles as her affected sibs. The Θ_{max} between the pTD3-21 and ty-pos OCA loci is 0.1

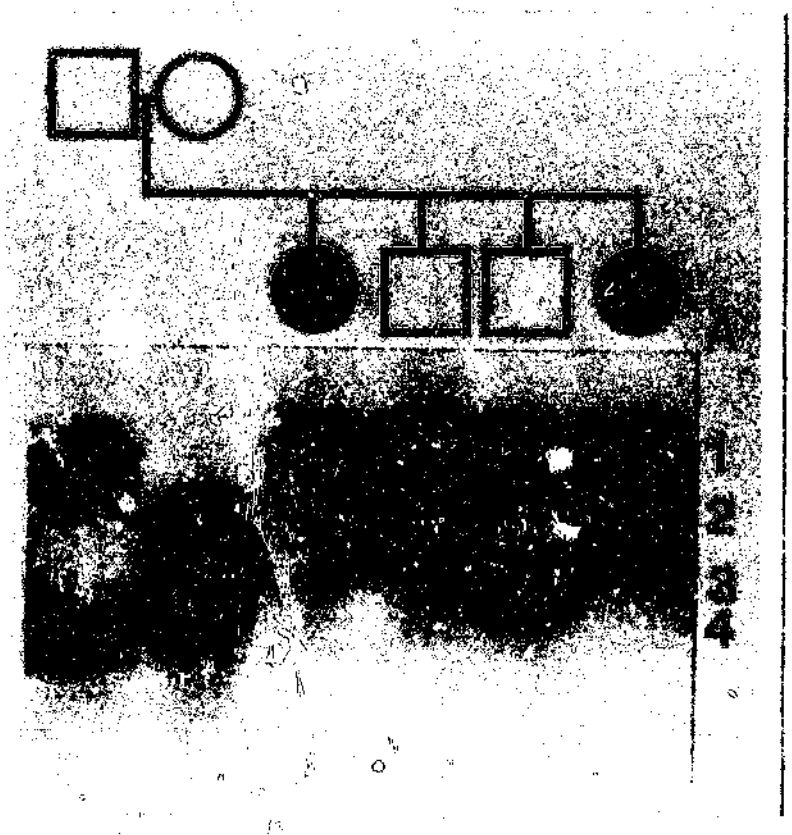

Figure 3.4 Inheritance of CA repeat alleles at the GABRB3 locus in family ZADZ detected by PCR, denaturing polyacrylamide gel electrophoresis and autoradiography. The darkest bands are the scored alleles (A) and the lighter bands on either side may represent products of slippage during replication or of *Taq* polymerase infidelity. The shaded symbols represent affected individuals. [GABRB3 is closely linked to the ty-pos OCA locus and thus the alleles inherited by the second child (first unaffected) illustrate an obligate cross-over in this individual. Both affected children have inherited bands 1 and 2, each of which must therefore segregate with the disease gene. Since the second child is unaffected, but also has alleles 1 and 2, there must be a cross-over between one of these alleles and the disease gene in this individual].

Table 3.8 summarises the results of two-point linkage analysis between ty-pos OCA and a total of 15 markers on the long arm of chromosome 15, confirming linkage to the 15q11-q13 chromosomal region. Significantly positive lod scores were obtained between ty-pos OCA and markers pTD3-21 ($Z=3.95$, $\Theta=0.1$), D15S10 ($Z=6.50$, $\Theta=0.05$), D15S11 ($Z=8.03$, $\Theta=0.05$), pIR10-1 ($Z=7.87$, $\Theta=0.01$), GABRB3 ($Z=12.17$, $\Theta=0.05$), MS14 ($Z=7.65$, $\Theta=0.05$) and CMW-1 ($Z=13.88$, $\Theta=0.01$). Positive lod scores were also obtained between ty-pos OCA and pIR4-3R ($Z=2.64$, $\Theta=0.1$) and ACTC ($Z=2.36$, $\Theta=0.1$).

Markers physically mapped to the 15q11-q13 region showed the most significantly positive lod scores, suggesting that the locus for ty-pos OCA is in or very near to this region. The THBS, D15S2, D15S3 and D15S87 loci did not show evidence of close linkage to ty-pos OCA ($Z<2$ at all Θ values). Table 3.9 shows the maximum lod scores (Z) and recombination values (Θ) between all closely linked chromosome 15 markers and ty-pos OCA, in the order of increasing recombination fractions (Θ).

The loci which did not show close linkage with ty-pos OCA - D15S2, D15S3, THBS and D15S87 - were not included in the maximum likelihood estimate table because of their insignificant lod scores ($Z<2$). In order to provide maximum linkage information, those markers which have been physically mapped to the same locus - pTD3-21 and D15S10, and pIR4-3R and D15S11 - have been analysed as haplotypes for the maximum likelihood analysis.

Table 3.8: Two-point linkage analysis between 15 markers on 15q and ty-pos OCA

MARKER	LOCUS	CHROMOSOME POSITION	NO OF FAMILIES[1]	PIC[2]	LOD SCORES AT RECOMBINATION FRACTION θ					
					0.01	0.05	0.1	0.2	0.3	0.4
pTD189-1	D15S13	15q11-q12	20	0.34	-0.79	1.46	1.96	1.70	0.98	0.29
pTD3-21	D15S10	15q11-q12	28	0.59	0.36	3.49	3.95	3.09	1.69	0.49
D15S10	D15S10	15q11-q12	38	0.80	3.82	6.50	6.34	4.34	2.15	0.58
pML54	D15S9	15q11-q12	24	0.58	-1.75	0.77	1.41	1.33	0.78	0.25
pIR4-3R	D15S11	15q11-q1[illegible]	32	0.37	1.11	2.53	2.64	1.99	1.08	0.31
D15S11	D15S11	15q11-q12	40	0.85	4.43	8.03	8.00	5.66	2.88	0.76
pIR10-1	D15S12	15q11-q12	23	0.55	7.87	6.98	5.86	3.72	1.85	0.50
GABRB3	GABRB3	15q11.2-q12	41	0.83	10.71	12.17	11.12	7.60	3.94	1.13
MS14	D15S97	15q11-q13	35	0.85	5.57	7.65	7.38	5.33	2.87	0.81
CMW-1	D15S24	15q13	38	0.86	13.88	12.45	10.58	6.85	3.54	0.95
635/636	ACTC	15q11-qter	40	0.80	-4.24	1.05	2.36	2.18	1.18	0.33
THBS1	THBS	15q15	40	0.75	-15.63	-3.92	-0.20	1.54	1.17	0.38
pDP151	D15S2	15q15-q22	5	0.37	0.61	0.58	0.52	0.38	0.22	0.07
pJU201	D15S3	15q26	12	0.75	-8.12	-2.99	-1.19	-0.03	0.16	0.07
Mfd49	D15S87	15	41	0.83	-25.44	-9.82	-4.24	-0.51	0.26	0.15

1 : Number of informative families 2: Polymorphism information content

Table 3.9: Maximum likelihood estimates (Z) and recombination values (θ) for linkage between chromosome 15 markers and ty-pos OCA

MARKER	LOCUS[1]	CHROMOSOME POSITION	NO FAMILIES[2]	Z MAX	θ
pIR10-1	D15S12	15q11-q12	23	8.09	0.00
CMW	D15S24	15q13	38	14.18	0.001
GABRB3	GABRB3	15q11.2-q12	41	12.20	0.04
MS14	D15S97	15q11-q13	35	7.70	0.06
D15S11[3]	D15S11	15q11-q12	32	5.90	0.08
D15S10[4]	D15S10	15q11-q12	32	8.97	0.07
pTD189-1	D15S13	15q11-q12	40	8.22	0.07
635/636	ACTC	15q11-qter	40	2.51	0.13
pML34	D15S9	15q11-q12	24	1.49	0.14
THBS1	THBS	15q15	40	1.55	0.22

1 : Loci given in the order of increasing recombination fractions (θ)
2 : Number of informative families
3 : D15S11 (CA repeat)/pIR4-3R RFLP haplotype
4 : D15S10 (CA repeat)/pTD3-21 RFLP haplotype

From genetic linkage analysis, the closest marker to the ty-pos OCA locus is D15S12 (pIR10-1) ($Z=8.09$, $\Theta=0.00$). Since there are no obligatory cross-overs between this marker and ty-pos OCA in southern African Negroids, the D15S12 locus is likely to be very close to, or form part of, the ty-pos OCA locus. Table 3.10 shows the lod scores obtained for each family with two-point linkage analysis between ty-pos OCA and pIR10-1 (D15S12), according to ephelus status. For each group, the summated lod score is positive, and the only negative lod scores obtained (ZABA [$Z=-0.002$], ZABI [$Z=-0.01$], ZABP [$Z=-0.04$] and ZACP [$Z=-0.13$]) are generated by a lack of data, rather than evidence for recombination, suggesting that there is no locus heterogeneity. Assuming D15S12 lies close to, or forms part of, the ty-pos OCA locus, then the closest flanking markers are GABRB3, in a proximal position, and D15S24 (CMW-1), positioned distal to the ty-pos OCA locus.

From physical mapping, D15S24 (CMW-1) is likely to lie distal to the ty-pos OCA locus. Similarly, physical mapping places the THBS and D15S3 (pJU201) loci distal to the ty-pos OCA locus, lying further along the chromosome than D15S24 (CMW-1). The pDP151 (D15S2) marker was only informative in 5 families and thus the lod score obtained ($Z=0.61$, $\Theta=0.01$) is insignificant. Therefore, this score cannot be used as an estimate of genetic distance, especially since the marker has been physically mapped to 15q15-q22. Markers pTD3-21 and D15S10 (CA repeat) physically map to the same locus, however, from the two-point linkage analysis with ty-pos OCA, they appear to map approximately 3cM apart. These results may have been generated because the CA repeat system, D15S10, is more informative than the pTD3-21/*TaqI* RFLP system, thus providing more genetic information in two-point linkage analysis with ty-pos OCA. Markers pIR4-3R and D15S11 (CA repeat) also physically map to the same locus, but appear to map approximately 1cM apart from two-point linkage analysis with ty-pos OCA. The CA repeat, D15S11, is more informative than the pIR4-3R/*RsaI* RFLP, which may affect the analysis of the genetic linkage data generated.

Table 3.10: Two-point linkage analysis between ty-pos OCA and pUK10-1 (D15S12) in each family at Θ=0.01

FAMILIES WITHOUT EPHELIDES	LOD SCORES AT Θ=0.01[1]	FAMILIES WITH EPHELIDES	LOD SCORES AT Θ=0.01[1]
ZABA	-0.032	ZABD	1.45
ZABB	0.55	ZABE	-
ZABC	0.09	ZABQ	0.33
ZABG	-	ZABR	0.125
ZABH	-	ZABT	0.125
ZABI	-0.01	ZABZ	-
ZABK	0.81	ZACB	0.73
ZABN	-	ZACE	0.25
ZABP	-0.04	ZACL	0.60
ZACG	0.13	ZACM	0.12
ZACJ	0.13	ZACS	-
ZACK	0.13	ZADC	0.125
ZACO	-	ZADJ	-
ZACP	-0.13	TOTAL	3.87
ZADA	0.13		
ZADF	-	FAMILIES OF UNKNOWN STATUS	LOD SCORES AT Θ=0.01[1]
ZADG	0.19		
ZADL	0.25		
ZADU	-	ZABJ	0.07
ZADW	0.15	ZACF	0.60
ZADX	0.73	ZADH	-
ZADY	0.14	ZADI	-
ZADZ	-	ZAFA	0.30
TOTAL	3.25	TOTAL	0.97
TOTAL LOD SCORE FOR ALL FAMILIES = 8.09			

1 : "-"indicates family uninformative at this locus

On the basis of the two-point linkage analysis and recombination values between ty-pos OCA and each of the markers on chromosome 15q, a genetic linkage map can be constructed and compared to the physical map, as shown in Figure 3.5.

3.2.5 Pairwise linkage analysis between all markers on chromosome 15q

Table 3.11 summarises the results of two-point linkage analysis between markers on chromosome 15q which show linkage to the ty-pos OCA locus [excluding D15S2 (pDP151), D15S3 (pJU201) and D15S87 (Mfd49)], showing the recombination fraction (Θ) at the maximum likelihood (Z) for each marker-marker combination. Linkage analysis could only be carried out between markers with fewer than 6 alleles each, due to the limitations imposed by the LINKAGE program for personal computers. For marker-marker analysis, the allele numbers were reduced for all systems with multiple alleles, while maintaining full linkage information in each family.

The informativity of one system relative to another is likely to affect the genetic distances between the markers generated by two-point linkage analysis. Thus two-point marker-marker linkage analysis may generate a different genetic map of the positions of the markers on chromosome 15q, relative to one another, than two-point marker-disease linkage analysis. For example, using the known physical map (Kuwano *et al.* 1992), and assuming pIR10-1 (D15S12) maps close to or at the ty-pos OCA locus, then the genetic map generated from two-point linkage analysis between ty-pos OCA and each marker (Table 3.9) should be: D15S9-D15S13-D15S10(CA)-D15S10(RFLP)-D15S11(CA)-D15S11(RFLP)-D15S97-GABRB3-**(D15S12/TY-POS OCA)**-D15S24-ACTC-THBS1.

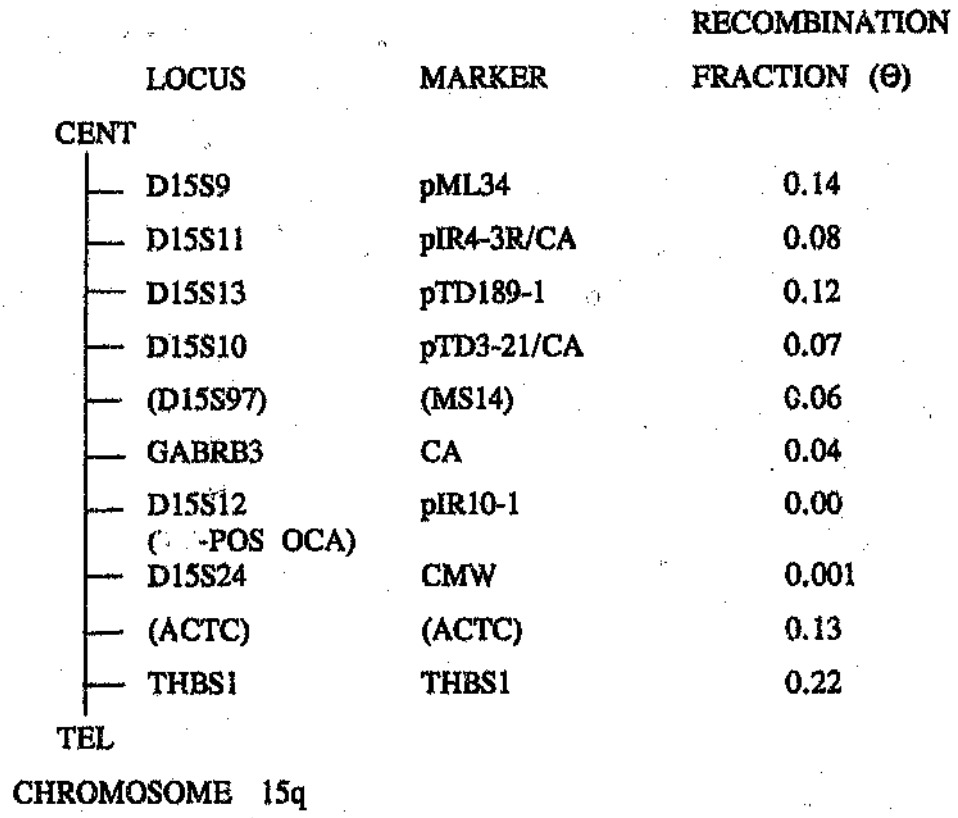

Figure 3.5 The physical map of chromosome 15q markers closely linked to ty-pos OCA (Kuwano *et al.* 1992) and the recombination fractions between these markers and ty-pos OCA. Loci ACTC and D15S97 have not yet been physically mapped and have been provisionally positioned using the genetic distances (Θ) between each locus and ty-pos OCA. The D15S10 and D15S11 loci have been analysed as haplotypes. (1: CA = CA repeat polymorphism)

Table 3.11: Pairwise recombination results (θ) and maximum likelihoods (Z) between all markers on chromosome 15q[1]

Z \ θ	D15S12 pIR10-1	D15S24 CMW	CA[2] GABRB3	D15S97 MS14	D15S11 pIR4-3R	CA[2] D15S11	CA[2] D15S10	D15S10 pTD3-21	D15S13 pTD189-1	CA[2] ACTC	D15S9 pML34	CA[2] THBS1
D15S12/pIR10-1		0.16	0.13	0.12	0.06	0.24	0.21	0.11	0.13	0.22	0.14	0.17
D15S24/CMW-1	1.19		ND	ND	0.06	ND	0.07	0.13	0.15	0.13	0.08	ND
GABRB3/CA[2]	3.43	ND		ND	0.05	ND	0.06	0.03	0.08	0.22	0.14	ND
D15S97/MS14	2.82	ND	ND		0.07	ND	0.08	0.05	0.05	0.21	0.19	ND
D15S11/pIR4-3R	2.22	3.99	9.23	2.99		0.04	0.09	0.05	0.04	0.26	0.09	0.24
D15S11/CA[2]	0.54	ND	ND	ND	7.67		0.09	0.05	0.03	0.19	0.09	ND
D15S10/CA[2]	0.91	8.90	16.58	9.76	3.08	9.36		0.01	0.05	0.17	0.07	0.25
D15S10/pTD3-21	1.58	3.45	17.93	9.37	5.78	12.74	13.45		0.02	0.17	0.12	0.22
D15S13/pTD189-1	1.31	1.16	7.10	5.47	3.64	7.04	5.26	7.53		0.30	0.04	-
ACTC/CA[2]	1.11	5.39	4.77	2.11	0.70	3.28	3.39	2.62	0.12		0.17	0.18
D15S9/pML34	0.62	3.74	3.14	0.85	2.75	4.58	3.01	2.09	2.27	1.23		0.22
THBS1/CA[2]	2.32	ND	ND	ND	0.96	ND	1.65	1.87	-	3.81	0.72	

1 : ND "Not done" (microcomputer analysis limited by number of alleles)
"-" No genetic linkage (Z negative for all values of θ)
2 : CA = CA repeat

However, pairwise linkage analysis between pIR10-1 (D15S12) and each marker (Table 3.11), generates another genetic map: D15S11(CA)-D15S10(CA)-D15S9-D15S13-GABRB3-D15S97-D15S10(RFLP)-D15S11(RFLP)-(D15S12/TY-POS OCA)-D15S24-ACTC-THBS1, presumably as a result of the varying informativity of each marker.

Marker-marker pairwise linkage analysis places D15S10 (CA repeat) and pTD3-21 only 1cM apart (Table 3.11), as compared to an apparent distance of 3cM genetic between them, generated by two-point linkage analysis between each marker and ty-pos OCA (Table 3.9). There is no detectable linkage between D15S87 (Mfd49) and D15S9 (pML34) (results not shown), and thus it is likely that D15S87 maps distal to the ty-pos OCA locus, closer to THBS1, although linkage analysis could not be carried out between D15S87 and THBS1 as they each detect 6 alleles. Markers D15S3 and D15S87 appear to be linked ($Z=2.30, \theta=0.16$) (results not shown) and thus it is unlikely that D15S87 would be linked to other markers in the 15q11-q12 region.

Two-point linkage analysis places markers D15S11 (CA repeat) and pIR4-3R approximately 4cM apart (an obligate cross-over was observed in ZADG). Marker D15S2 (pDP151) was only informative in 5 families and thus the pairwise linkage analysis between this marker and all the other chromosome 15q markers is unlikely to reach statistical significance, although it does show an equivocally positive lod score with GABRB3 ($Z=1.74, \theta=0.001$).

However, pairwise linkage analysis between pIR10-1 (D15S12) and each marker (Table 3.11), generates another genetic map: D15S11(CA)-D15S10(CA)-D15S9-D15S13-GABRB3-D15S97-D15S10(RFLP)-D15S11(RFLP)-(**D15S12/TY-POS OCA**)-D15S24-ACTC-THBS1, presumably as a result of the varying informativity of each marker.

Marker-marker pairwise linkage analysis places D15S10 (CA repeat) and pTD3-21 only 1cM apart (Table 3.11), as compared to an apparent distance of 3cM genetic between them, generated by two-point linkage analysis between each marker and ty-pos OCA (Table 3.9). There is no detectable linkage between D15S87 (Mfd49) and D15S9 (pML34) (results not shown), and thus it is likely that D15S87 maps distal to the ty-pos OCA locus, closer to THBS1, although linkage analysis could not be carried out between D15S87 and THBS1 as they each detect 6 alleles. Markers D15S3 and D15S87 appear to be linked ($Z=2.30, \Theta=0.16$) (results not shown) and thus it is unlikely that D15S87 would be linked to other markers in the 15q11-q12 region.

Two-point linkage analysis places markers D15S11 (CA repeat) and pIR4-3R approximately 4cM apart (an obligate cross-over was observed in ZADG). Marker D15S2 (pDP151) was only informative in 5 families and thus the pairwise linkage analysis between this marker and all the other chromosome 15q markers is unlikely to reach statistical significance, although it does show an equivocally positive lod score with GABRB3 ($Z=1.74, \Theta=0.001$).

3.3 Allelic association between pIR10-1 (D15S12) and the ty-pos OCA mutation(s)

Since there was no observed recombination between the alleles at the D15S12 (pIR10-1) locus and ty-pos OCA in southern African Negroids, it may be assumed that D15S12 maps close to, or at, the ty-pos OCA locus. Thus there may be an association between one or more of the three alleles detected by the pIR10-1 (D15S12)/*Sca*I RFLP and different alleles of, or mutations at, the ty-pos OCA locus. There are assumed to be at least 3 different types of alleles or mutations at the ty-pos OCA gene - one associated with the normal or non-ty-pos OCA allele (determined from known carriers), one associated with ty-pos OCA presenting with ephelides and a third associated with ty-pos OCA without ephelides. Thus each of these groups of chromosomes is thought to be associated with a different ty-pos OCA gene sequence.

Table 3.12 shows the numbers and frequencies of the alleles detected by the pIR10-1/*Sca*I RFLP in the three different groups. When using the chi-square test to compare the observed number of each pIR10-1 allele between these groups (Table 3.13), at the 1% significance level, only the numbers of the pIR10-1 alleles observed in the affected groups with and without ephelides differed significantly ($p<0.01$). At the 5% significance level, a comparison of the numbers of each of the pIR10-1 alleles between the normal ty-pos OCA gene and those associated with a lack of ephelides, was significant ($p<0.02$). The number of each of the pIR10-1 alleles observed between the normal and affected (with and without ephelides) were not significantly different.

Table 3.12: pIR10-1 (D15S12) allele frequencies

CHROMOSOME TYPE[1]	pIR10-1 ALLELES (FREQUENCY) 1	2	3	TOTAL
NON-OCA	31 (0.60)	13 (0.25)	8 (0.15)	52
OCA + EPHELIDES	14 (0.58)	3 (0.13)	7 (0.29)	24
OCA - EPHELIDES	33 (0.87)	4 (0.10)	1 (0.03)	38
TOTAL OCA (+/-)	56 (0.78)	7 (0.10)	9 (0.12)	72

Table 3.13: Comparison of numbers of pIR10-1 (D15S12) alleles[a]

pIR10-1 ALLELES[b]	χ^2	p	r[c]
Non-OCA vs OCA (+ and -)	4.237	0.120	0.136
Non-OCA vs OCA (+)	2.804	0.246	0.136
Non-OCA vs OCA (-)	8.295	0.016	0.215
OCA (+) vs OCA (-)	9.655	0.008	0.279

a : 2 degrees of freedom
b : OCA refers to ty-pes OCA
Ephelus status: + With ephelides
- Without ephelides
c: r = correlation coefficient

These results suggest that there is an association between the alleles at the D15S12 locus and the mutation(s) determining the presence or absence of ephelides with ty-pos OCA. The number of observed alleles show that allele 3 is more frequently associated with the presence of ephelides (0.29), than with an absence of ephelides (0.03). Allele 1 is the commonest in all groups of chromosomes.

3.4 Haplotype analysis

Since D15S12 (pIR10-1) is very close to, or part of, the ty-pos OCA locus, and the physical order of all the closely situated 2 and 3 allele markers, and the 12 allele GABRB3 locus, has been established, haplotypes can be constructed using these markers. Haplotypes were constructed using D15S12 and 4 other markers, creating more complex haplotypes by the consecutive addition of each marker lying further from the ty-pos OCA locus (and closer to the centromere). The 2 and 3 allele markers were used in haplotype construction because the allele sizes could be scored unambiguously and the haplotypes generated were easily analysed. Ideally, CMW (D15S24) should be used in the haplotype analysis as a closely linked flanking marker, however, since it is a VNTR analysed using Southern blotting, the alleles could not be clearly determined between gels. Haplotypes were also constructed using the D15S12 locus and the GABRB3 CA repeat system, which is physically and genetically close to the ty-pos OCA locus and for which the allele sizes could be clearly scored.

Tables 3.14-3.17 illustrate the haplotypes generated using the physically ordered markers D15S12-D15S10-D15S13-D15S11-D15S9. With the addition of each marker to a haplotype, the pattern becomes more complex and the number of informative chromosomes becomes less, because results are not available for every system on every chromosome. Table 3.18 shows the haplotypes generated using the D15S12 and GABRB3 loci. The number and frequency of each haplotype associated with the normal ty-pos OCA gene and with the disease

mutation(s) can be calculated. The numbers of each haplotype in these groups were compared using the chi-square test, with the Yates correction factor (for cells <5).

The number of 2 marker haplotypes, using D15S12 and D15S10 (Table 3.14) only differs significantly at the 1% significance level, between the groups with and without ephelides ($p=0.01$), suggesting that these groups are associated with different haplotypes. The group without ephelides shows a cluster of haplotypes associated with allele 1 of the D15S12 RFLP, while the normal chromosomes and the group with ephelides show a spread of haplotypes. Although the studies of allelic association show that all 3 pIR10-1 (D15S12) alleles are represented on both ty-pos OCA chromosomes and non-OCA chromosomes, with haplotype construction, some data are lost when there is information missing for any system on every chromosome.

The number of 3 marker haplotypes, D15S12, D15S10 and D15S13 (Table 3.15), only differ significantly between the groups with and without ephelides ($p<0.02$), with a clustering of haplotypes associated with allele 1 of the D15S12 RFLP in the group with ephelides, and a scatter of haplotypes associated with the normal chromosomes and the group with ephelides.

The number of 4 (D15S12-D15S10-D15S13-D15S11) (Table 3.16) and 5 (D15S12-D15S10-D15S13-D15S11-D15S9) (Table 3.17) marker haplotypes show no significant difference between any of the groups. There may be no significant difference because fewer informative chromosomes can be scored, reducing the number of haplotypes in each group to very small numbers.

The number of haplotypes using D15S12 and GABRB3 (Table 3.18), did not differ significantly between any of the groups, with a spread of haplotypes in each group.

Table 3.14: Haplotype analysis using loci D15S12 and D15S10

HAPLOTYPE		TY-POS OCA STATUS[1]		
D15S12	D15S10	+	-	N
1	1	8 (0.50)	16 (0.53)	13 (0.30)
1	2	2 (0.13)	11 (0.37)	10 (0.43)
1	3	-	3 (0.10)	2 (0.05)
2	1	-	-	2 (0.05)
2	2	-	-	3 (0.07)
2	3	1 (0.13)	-	1 (0.01)
3	1	2 (0.13)	-	5 (0.12)
3	2	3 (0.19)	-	6 (0.14)
3	3	-	-	1 (0.01)
TOTAL		16	30	43

Chi-square comparison

Ty-pos OCA Status[1]	χ^2	p (df)
+ vs - ephelides	15.03	0.010(5)
Normal vs + ephelides	1.586	0.991(8)
Normal vs - ephelides	8.356	0.400(8)
Normal vs affected	5.929	0.655(8)

1: + With ephelides, - Without ephelides, N Normal

Table 3.15: Haplotype analysis using loci D15S12, D15S10 and D15S13

HAPLOTYPE			TY-POS OCA STATUS[1]		
D15S12	D15S10	D15S13	+	-	N
1	1	1	6 (0.50)	6 (0.25)	7 (0.18)
1	1	2	-	5 (0.21)	4 (0.11)
1	2	1	-	5 (0.21)	4 (0.11)
1	2	2	1 (0.08)	5 (0.21)	4 (0.11)
1	3	1	-	1 (0.04)	-
1	3	2	-	2 (0.08)	2 (0.06)
2	1	1	-	-	2 (0.06)
2	2	1	-	-	2 (0.06)
2	2	2	-	-	1 (0.03)
2	3	2	1 (0.08)	-	1 (0.03)
3	1	1	2 (0.17)	-	2 (0.06)
3	1	2	-	-	1 (0.03)
3	2	1	2 (0.17)	-	3 (0.07)
3	2	2	-	-	2 (0.06)
3	3	2	-	-	1 (0.03)
TOTAL			12	24	36

Chi-square comparison

Ty-pos OCA status[1]	χ^2	p (df)
+ vs - ephelides	18.75	0.016 (8)
Normal vs + ephelides	4.21	0.989 (13)
Normal vs - ephelides	2.803	0.999 (14)
Normal vs affected	3.442	0.998 (14)

1 : + With ephelides, - Without ephelides, N Normal

Table 3.16: Haplotype analysis using loci D15S12, D15S10, D15S13 and D15S11

HAPLOTYPE				TY-POS OCA STATUS[1]		
D15S12	D15S10	D15S13	D15S11	+	-	N
1	1	1	1	2 (0.20)	4 (0.20)	2 (0.07)
1	1	1	2	3 (0.30)	2 (0.10)	3 (0.09)
1	1	2	1	-	1 (0.05)	-
1	1	2	2	-	1 (0.05)	4 (0.13)
1	2	1	1	-	3 (0.15)	2 (0.07)
1	2	1	2	-	1 (0.05)	2 (0.07)
1	2	2	1	1 (0.10)	1 (0.05)	1 (0.04)
1	2	2	2	-	4 (0.20)	-
1	3	1	2	-	1 (0.05)	-
1	3	2	2	-	2 (0.10)	2 (0.07)
2	1	1	1	-	-	1 (0.04)
2	1	1	2	-	-	1 (0.04)
2	2	1	1	-	-	1 (0.04)
2	2	1	2	-	-	1 (0.04)
2	2	2	1	-	-	1 (0.04)
2	3	2	2	-	-	1 (0.04)
3	1	1	1	1 (0.10)	-	2 (0.08)
3	1	1	2	1 (0.10)	-	-
3	1	2	1	-	-	1 (0.04)
3	2	1	1	2 (0.20)	-	1 (0.04)
3	2	2	2	-	-	1 (0.04)
3	3	2	1	-	-	1 (0.04)
TOTAL				10	20	28

Chi-square comparison

Ty-pos OCA status[1]	χ^2	p (df)
+ vs - ephelides	16.35	0.176 (12)
Normal vs + ephelides	5.293	0.998 (18)
Normal vs - ephelides	5.623	0.999 (20)
Normal vs affected	4.21	1 (21)

1 : + With ephelides, - Without ephelides, N Normal

Table 3.17: Haplotype analysis using loci D15S12, D15S10, D15S13, D15S11 and D15S9

HAPLOTYPE					TY-POS OCA STATUS[1]		
D15S12	D15S10	D15S13	D15S11	D15S9	+	-	N
1	1	1	1	1	-	1 (0.06)	-
1	1	1	1	2	2 (0.24)	1 (0.06)	-
1	1	1	1	3	-	-	1 (0.05)
1	1	1	2	1	1 (0.13)	1 (0.06)	1 (0.05)
1	1	1	2	2	1 (0.13)	1 (0.06)	-
1	1	2	1	3	-	-	1 (0.05)
1	1	2	2	1	-	-	2 (0.10)
1	1	2	2	2	-	-	1 (0.05)
1	2	1	1	2	-	3 (0.20)	2 (0.10)
1	2	1	2	1	-	-	1 (0.05)
1	2	1	2	2	-	1 (0.06)	1 (0.05)
1	2	2	1	2	1 (0.13)	1 (0.06)	-
1	2	2	2	1	-	2 (0.13)	1 (0.05)
1	2	2	2	2	-	2 (0.13)	-
1	3	1	2	2	-	1 (0.06)	-
1	3	2	2	1	-	1 (0.06)	2 (0.10)
1	3	2	2	2	-	1 (0.06)	-
2	1	1	2	2	-	-	1 (0.05)
2	2	1	1	2	-	-	1 (0.05)
3	1	1	1	2	-	-	1 (0.05)
3	1	1	1	2	1 (0.13)	-	-
3	1	2	1	2	-	-	1 (0.05)
3	2	1	1	2	-	-	1 (0.05)
3	2	1	*	3	2 (0.24)	-	-
3	2	2	3	1	-	-	1 (0.05)
3	3	2	1	2	-	-	1 (0.05)
TOTAL					8	16	20

Chi-square comparison

Ty-pos OCA status[1]	χ^2	p (df)
+ vs - ephelides	14.25	0.357 (13)
Normal vs + ephelides	7.875	0.996 (21)
Normal vs - ephelides	2.025	1 (23)
Normal vs affected	3.636	1 (25)

1 : + With ephelides, - Without ephelides, N Normal

Table 3.18: Haplotype analysis using loci D15S12 and GABRB3

HAPLOTYPE		TY-POS OCA STATUS[1]		
D15S12	GABRB3	+	-	N
1	1	-	3	1
1	2	-	3	1
1	3	5	3	4
1	4	1	3	8
1	5	3	3	3
1	6	1	4	1
1	7	-	3	4
1	8	4	7	6
1	9	-	2	2
1	10	-	2	1
1	11	-	2	3
1	12	-	1	-
2	2	-	2	2
2	3	-	2	1
2	4	-	-	1
2	5	-	-	3
2	7	-	-	1
2	8	3	-	2
2	9	-	-	1
3	1	-	-	2
3	2	-	-	1
3	3	-	-	3
3	4	1	-	1
3	5	3	1	3
3	6	1	-	2
3	7	-	-	2
3	8	2	-	1
3	9	-	-	1
3	11	-	-	1
TOTAL		24	42	62

Chi-square comparison

Ty-pos OCA status[1]	χ^2	p (df)
+ vs - ephelides	8.49	0.971 (18)
Normal vs + ephelides	9.77	0.999 (27)
Normal vs - ephelides	6.90	1 (28)
Normal vs affected	9.06	1 (28)

1 : + With ephelides; - Without ephelides; N Normal

Therefore, the haplotype analysis using these markers does not suggest a single origin for the ty-pos OCA mutation(s). However, the markers are physically and genetically positioned relatively far ($\Theta > 0.07$) from the ty-pos OCA locus. Thus they may not represent ancestral haplotypes, since many recombinations are likely to have occurred between the markers and the ty-pos OCA locus since the ty-pos OCA mutation(s) arose. The recombination fraction (Θ) gives a genetic distance between two loci. If Θ is greater than 0.07 (7% chance of cross-over between the loci), then it is unlikely that there will be an association between those loci. If Θ is less than 0.01 or 0.001, then there may be an association between two loci. Thus one may expect there to be an association between D15S12 and CMW and, perhaps, between D15S12 and GABRB3. However, a possible association between D15S12 and CMW cannot be tested with the existing data, and there is no significant association between D15S12 and GABRB3.

3.5 Summary of results

The human homologue of the mouse *brown* locus, CAS2, has been mapped to chromosome 9q22-qter by SCH mapping and *in situ* hybridisation.

Forty-one families were used in the linkage study. There were 13 families in which affected individuals had ephelides and 23 families in which they lacked ephelides. Ephelus status in 5 families was unknown. Two-point linkage analysis excluded linkage between ty-pos OCA and 41 polymorphic markers, including 19 random serogenetic markers and 15 random DNA markers, 2 candidate loci, TYR and CAS2, and a candidate region on 11p. Strong evidence for linkage was obtained between 7 markers on chromosome 15q11-q13 and ty-pos OCA (Table 3.9). A genetic linkage map of the 15q region was constructed using two-point linkage analysis and correlated to the physical map.

No recombination was found between pIR10-1 (D15S12) and ty-pos OCA, suggesting that the D15S12 locus maps very close to, or at, the ty-pos OCA locus. The frequency of each of the pIR10-1 alleles associated with chromosomes from individuals with ephelides differed significantly from the frequency of each of the alleles associated with chromosomes from individuals without ephelides, suggesting that the two phenotypes may be associated with different, but possibly overlapping, sets of mutations. Haplotype analysis showed diverse associations in each group, but did not often show statistical significance. This may be explained by the large genetic distance between the markers ($\Theta > 0.07$), which may also be physically too far away from the disease locus to be used in haplotype analysis for the purpose of establishing the origin(s) of the ty-pos OCA mutation(s).

CHAPTER FOUR

4. DISCUSSION

Tyrosinase-positive oculocutaneous albinism (ty-pos OCA) (type II OCA) is the most common type of albinism in Caucasoids and Negroids (Witkop *et al.* 1989). In southern Africa, the overall prevalence is about 1 in 3900, giving a carrier frequency of about 1 in 32, making this a common autosomal recessive disorder. Mapping the ty-pos OCA locus through linkage studies will contribute in a small way to the construction of the genetic map of the human genome. Characterisation of the locus responsible for the ty-pos OCA phenotype will help to identify the gene product and to determine its function(s). Since normal melanin production is disturbed in individuals with ty-pos OCA, more information may be gained about the melanin biosynthetic pathway and, possibly, the functioning of the cells involved in pigment synthesis.

In the absence of a clear understanding of the biochemical defect causing ty-pos OCA, or any protein defect in individuals with this condition, linkage studies were necessary to identify the chromosomal localisation of the gene locus. Once this has been accomplished, the gene may be cloned and characterised.

4.1 Ty-pos OCA in southern African Negroids

Since ty-pos OCA is the most common type of oculocutaneous albinism occurring in southern African Negroids, the search for the gene has been greatly facilitated by the large number of individuals and families who presented with the condition and who were willing to participate in the study.

The subjects involved in this study came from 41 families, the majority of whom are resident in the Transvaal region. Detailed clinical information included the ephelus status of the affected individuals, where ephelides were clearly demarcated pigmented patches, found particularly on the sun-exposed areas of the skin. It was found that there was 100% concordance within families with

respect to the presence or absence of ephelides. Most of the affected individuals in this study did not have ephelides (from 56% of the families), suggesting that the mutation(s) at the ty-pos OCA locus which gives rise to these pigmented patches may occur with a lower frequency than the mutation(s) associated with an absence of ephelides.

4.2 Novel information on polymorphic markers

The Negroid population often shows unusual polymorphic DNA fragments a d allele frequencies at loci which have previously only been characterised in Caucasoids (Nurse *et al.* 1985; Marques *et al.* in preparation). The PIC values have been calculated for each system from a random sample of unrelated Negroid individuals (for unlinked markers, both chromosomes from each parent in all the families were used to calculate the allele frequency for each system, and for all markers on chromosome 15q, the alleles occurring on the normal chromosome in each parent were used to calculate the allele frequencies for the polymorphic systems). These PIC values were often found to differ from those which have been published (the latter usually from the Caucasoid population), and have been included with the results obtained in this study. It has been found that polymorphic systems are often, but not always, more informative in Negroids, presumably because this ethnic group is evolutionarily older than the Caucasoid ethnic groups and has thus had more time in which to accumulate neutral (and more rarely advantageous) mutations at all loci.

In the Negroid population, the highly polymorphic dinucleotide repeat loci are often characterised by a higher number of alleles than the Caucasoid population, for example, at the D15S10 (CA) repeat locus, there are 9 polymorphic alleles in Negroids and only 4 in Caucasoids, and at the THBS1 locus, there are 14 polymorphic alleles in Negroids and 5 in Caucasoids (Marques *et al.* in preparation). This increased number of alleles usually increases the PIC value, making these loci particularly useful for population and forensic studies, as well

as for paternity testing in the Negroid population. In the search for polymorphism at different loci, new polymorphisms may be revealed, as with the 2 allele CAS2/*Xba*I RFLP (Colman *et al.* 1991a). Occasionally, polymorphic loci detected by RFLP/Southern blot analysis are associated with additional population-specific alleles, as in the case of the third Negroid-specific pTD3-21 (D15S10)/*Taq*I RFLP allele (Colman *et al.* 1991b). On the other hand, polymorphisms identified in Caucasoids may not be present in Negroids, for example, the TYR/*Mbo*I RFLP (Giebel and Spritz 1990b) was absent from the Negroid population in the present study.

4.3 Two-point linkage analysis

In this linkage study two-point linkage analysis was carried out between ty-pos OCA and 10 random markers, two candidate pigment loci (tyrosinase and CAS2) and four markers on chromosome 11p, thought to be the position of a syntenic group including the human homologue of the mouse *pink-eyed dilution* locus. The study also included reanalysis of linkage between ty-pos OCA and 15 serogenetic markers and 7 random DNA markers previously analysed by Heim (1988). A total of 15 markers in the Prader-Willi/Angelman syndrome chromosomal region on 15q11-q13, a candidate region often associated with hypopigmentation and more rarely with OCA, were also tested for linkage.

Close linkage was excluded between ty-pos OCA and 15 random serogenetic markers tested, and between ty-pos OCA and a total of 16 random polymorphic DNA markers. Slightly positive lod scores were obtained between ty-pos OCA and markers on chromosome 6p (BF [$Z=0.92$ at $\Theta=0.2$], DQα [$Z=0.44$ at $\Theta=0.3$]and Mfd61 [$Z=0.71$ at $\Theta=0.3$]),suggesting that this area may be of some interest. However, the lod scores were not signficantly positive ($Z<3$) and, since positive linkage results were then obtained between ty-pos OCA and chromosome 15q markers, it was considered unnecessary to continue with further studies on chromosome 6p.

Few candidate genes for pigment biosynthesis have been isolated and characterised, but some which have been identified and are polymorphic were tested for evidence that they may be the locus for ty-pos OCA. Two melanocyte-specific cDNA clones were isolated, and both were originally ascribed to tyrosinase (Kwon *et al.* 1987; Kwon *et al.* 1988; Kwon *et al.* 1989; Shibahara *et al.* 1986; Yamamoto *et al.* 1989). However, one of these clones has now been mapped to the *c* locus on mouse chromosome 7 (Kwon *et al.* 1987) and is the tyrosinase gene, whereas the other has been assigned to the *brown* (*b*) locus on mouse chromosome 4 (Jackson 1988). Thus, although there is extensive DNA sequence homology between the two genes, the localisation of the *c* gene to mouse chromosome 7 and the *b* gene to mouse chromosome 4, indicates that these two genes are different and involved in different functions (Chintamaneni *et al.* 1991), although they may be related (Jackson *et al.* 1992).

The mouse *b* locus maps to mouse chromosome 4 in a region of homology with human chromosome 9, thus it was likely that the human homologue would map to chromosome 9. However, the mouse *b* locus occurs in a region of synteny split into several segments by the insertion of human 9q loci into an area of 9p homology (Searle *et al.* 1989), making prediction of the exact position of the human homologue difficult. Using *in situ* hybridisation and human-rodent SCH, Abou *et al.* (1991), Chintamaneni *et al.* (1991) and Murty *et al.* (1992), have localised the human gene, CAS2, to chromosome 9p. It has been postulated that the *b* locus protein is a tyrosinase-related protein (Jackson 1988) or a catalase (Halaban and Moellmann 1990) and it is known to be homologous to the human melanosomal protein GP75 (Vijayasaradhi *et al.* 1990). A Negroid-specific *Xba*I RFLP was found with the CAS2 cDNA (Colman *et al.* 1991a) and two-point linkage analysis between ty-pos OCA and the CAS2 locus excluded the latter from being the locus for ty-pos OCA ($Z=-6.51$ at $\Theta=0.01$).

The human homologue of the mouse *c* gene is the tyrosinase gene (TYR) on human chromosome 11q14-q21. Mutations at the TYR locus give rise to a

tyrosinase-negative OCA phenotype, and the phenotypes associated with the yellow and temperature-sensitive mutants. Ty-pos OCA and ty-neg OCA are not allelic because matings between individuals with these conditions produce normally pigmented offspring (Witkop *et al.* 1970). However, in a group of clinically defined "ty-pos" OCA Caucasoids, IndoPakistanis, middle eastern Arabs and Jews, 6 out of 17 individuals had mutations in the tyrosinase gene, which were shown not to be simple polymorphisms. It is postulated that the "ty-pos" OCA phenotype in these individuals arose from homozygosity or compound heterozygosity of 2 relatively mild tyrosinase allele mutations (Tripathi *et al.* 1992b), rather than from a mutation in the ty-pos OCA gene. Thus it has been suggested that "ty-pos" OCA is a heterogeneous condition with a distinctive clinical phenotype, which may be associated with mutations in the tyrosinase gene, as well as mutations in other genes. In other words, "ty-pos" OCA in Caucasoids is characterised by locus heterogeneity. To test for locus heterogeneity in the Negroid ty-pos OCA families participating in this study, a polymorphism detected by a tyrosinase cDNA (Pmel34) was analysed in all ty-pos OCA families and in families with and without ephelides. The summated lod score for each group was negative (Table 3.6), effectively excluding the tyrosinase locus from being the locus for ty-pos OCA and also showing that there is unlikely to be locus heterogeneity in the Negroid ty-pos OCA families. The most positive lod score obtained for a single family at Θ=0.01 (ZABN, Z=0.48), can be explained by a lack of information, rather than by locus heterogeneity.

Mouse chromosome 7 has a number of homologuous genes found on both the long and short arms of human chromosome 11 (Figure 1.6), as well as on human chromosome 15. The *pink-eyed dilution* (*p*) and *albino* (*c*) loci on mouse chromosome 7 constituted the first linkage group identified in mammals (Haldane *et al* 1915, cited in Witkop *et al.* 1989) and thus it was considered possible that the human homologue of the mouse *p* gene may occur on chromosome 11p. If the linkage group was not conserved, the homologue of the *p* gene may be present on chromosome 15. No evidence of linkage was found

between 4 markers tested on human chromosome 11p and ty-pos OCA, showing that the linkage groups on mouse chromosome 7 had not been conserved on human chromosome 11p. The other region to search for the human homologue of the mouse *p* gene was on human chromosome 15, where several genes homologous to mouse genes, both distal and proximal to the *p* gene, are found (Figure 1.6).

4.4 Linkage of ty-pos OCA to markers on chromosome 15q11-13

Chromosome 15 is an acrocentric chromosome with a satellite-rich heterochromatic centromere and stalk regions. Satellite-rich heterochromatin interferes with meiotic crossing-over and preserves linkage groups in regions near the centromere. Cytogenetic and molecular evidence, however, suggest that the proximal long arm of chromosome 15 is rich in palindromic or inverted repeat sequences, which may lead to instability (Donlon 1988; Tasset *et al.* 1988). A combination of classical genetic data and PFGE techniques suggest the presence of recombinational "hotspots" in this region (Kirklionis *et al.* 1991). Buiting *et al.* (1990), predict that a high density of genes exists on the long arm of chromosome 15 and if the density of coding sequences in the genome is 1 per 30kb (100 genes in 3000kb), the current PWS/AS critical region could contain more than 20 genes.

The PWCR and ANCR have been localised to chromosome 15q11-q13 and both syndromes are often associated with hypopigmentation (Hittner *et al.* 1982; Butler *et al.* 1986; Pettigrew *et al.* 1987; Wiesner *et al.* 1987; Pembrey *et al.* 1989; Butler 1989, 1990; Williams *et al.* 1989b; Magenis *et al.* 1990; Trent *et al.* 1991). Two polymorphic RFLP markers in the 15q11-q13 region (pTD3-21 [D15S10] and pTD189-1 [D15S13]) were tested for linkage to ty-pos OCA. Once linkage had been established between these markers and the ty-pos OCA locus (Ramsay *et al.* 1992), another 13 polymorphic markers on chromosome 15q were tested for linkage. Two-point linkage analysis confirmed linkage between ty-pos OCA

and all markers tested in the PWS/AS region.

A genetic linkage map was created using two-point linkage analysis and was correlated with the known physical map (Kuwano *et al.* 1992) (Figure 3.5). The genetic map correlates fairly well with the physical map, although the physical order of the D15S11 and D15S13 loci is inverted relative to that suggested by the genetic map. The genetic distance of the D15S11 locus from the ty-pos OCA locus is based upon haplotype analysis at the D15S11 locus (D15S11 CA repeat and pIR4-3R/*Sca*I RFLP), while the genetic distance between ty-pos OCA and D15S13 is based upon analysis of a single RFLP (pTD189-1/*Taq*I). The latter is less informative than the haplotype and a paucity of information may influence the genetic distance obtained between ty-pos OCA and each of the systems. The genetic linkage map may also represent varying recombination rates between the marker loci of fixed physical distance, especially if this region has an increased frequency of cross-overs due to the existence of recombinational hotspots. The genetic map places D15S97 (MS14), which has not yet been physically mapped, distal to the GABRB3 locus. Bowcock (1992), has shown that there is a significant excess of recombination between the D15S97 and GABRB3 loci in females. This result could not be tested in the ty-pos OCA families, since the Θ_m was assumed to be equal to the Θ_f for all LINKAGE analyses. Both the D15S97 and GABRB3 loci were analysed using CA repeat markers, so two-point marker-marker analysis could not be carried out because there were too many alleles for microcomputer analysis. From the genetic map, the ACTC locus may map either distal or proximal to the ty-pos OCA locus. Rogan (1992), has shown that it is physically mapped distal to D15S24 (CMW-1), which does correlate with the results obtained in this study since the ACTC locus appears to be closer to D15S24 (CMW-1) ($Z=5.39$ at $\Theta=0.13$), than to the more proximal markers (all $\Theta>0.17$).

The linkage study has failed to reveal any obligatory cross-overs between ty-pos OCA and the marker pIR10-1 (the D15S12 locus). This suggests that the D15S12

locus is either very close to, or forms part of the ty-pos OCA locus. In order to test for locus heterogeneity, each group of ty-pos OCA families (with and without ephelides) was analysed. The summated lod score for each group was positive, with no cross-overs (Table 3.10), confirming that this locus may be the locus for ty-pos OCA, with no evidence of locus heterogeneity.

4.5 Allelic association between the D15S12 locus and ty-pos OCA mutations

Allelic association was found between the D15S12 (pIR10-1) locus and the mutation(s) determining the presence or absence of ephelides with ty-pos OCA. Allele 3 of the D15S12 locus was more commonly associated with the presence of ephelides than with an absence of ephelides. This suggests that the mutation(s) which causes ephelides, may have originally arisen in a chromosome which was characterised by allele 3 of the D15S12 locus. Alternatively, the somatic mutation(s) associated with the presence of ephelides may have arisen spontaneously and the predisposition to this spontaneous mutation may be related to allele 3 at the D15S12 locus. Since there is allelic association between the D15S12 locus and the presence or absence of ephelides, it seems that there was more than one origin of these mutations.

Allelic association was only assessed with the alleles at the D15S12 locus. Ideally, however, the alleles occurring at the D15S24 locus, a closely flanking marker ($Z=14.18$ at $\Theta=0.001$), should also be analysed for allelic association. The D15S24 RFLP is analysed using Southern blots, and the allele sizes are difficult to size accurately between gels, thus, with the current data, allelic association studies were not possible.

Haplotype analysis did not confirm or refute whether there was a single origin or multiple origins of the ty-pos OCA mutation(s). It is likely that the markers used in the haplotypes were genetically ($\Theta>0.07$) and physically too far away from the ty-pos OCA locus for accurate analysis since many cross-overs are likely

to have occurred between each marker and ty-pos OCA since the original mutation(s) occurred.

4.6 Mouse pigment genes

Genes affecting pigmentation in mice were among the first to be studied for Mendelian inheritance and some of the mutations associated with these pigment loci have been used to identify structural and regulatory genes involved in human development and pigmentation.

In an attempt to localise the ty-pos OCA locus, several mouse pigmentation loci, with phenotypic expression which could be correlated to that of ty-pos OCA in humans, were investigated as candidates for ty-pos OCA. Mouse chromosome 2 contains the *agouti* (*a*) locus, chromosome 7 contains the *pink-eyed dilution* (*p*) and *albino* (*c*) loci and chromosome 9 contains the *dilute* (*d*) locus. The human homologue of the *c* locus, tyrosinase, is known to map to human chromosome 11q14-q21, while the other three loci, with phenotypic effects which could be broadly correlated with those of ty-pos OCA, all occur in regions with homology to human chromosome 15 (Searle *et al.* 1989). Of the three loci, the phenotype produced by the *p* locus correlates best with that of ty-pos OCA.

The mouse *p* locus, on chromosome 7, has long been associated with defects of skin, hair and coat pigmentation. The wild-type allele generates intense pigmentation in both coat and eyes, while recessive mutations at *p* cause a reduction of the brown/black eumelanic pigments and have little effect on the red/yellow phaeomelanins (Silvers 1979). This locus maps close to the Gabrb3 gene in mouse, and since GABRB3 maps close to the PWS/AS region on human chromosome 15q, it has been suggested that the human homologue of the *p* locus may also map in this region (Nicholls *et al.* 1991; Gardner *et al.* 1992; Nakatsu *et al.* 1992; Ramsay *et al.* 1992), together with another three evolutionarily conserved loci (*D15S9h-1*, *D15S12h* and *D15SF32S1h*), comprising

a region of synteny between human chromosome 15q11-q13 and mouse chromosome 7 (Rinchik *et al.* 1993). The *p* region of mouse chromosome 7 is a complex, multilocus region (Nakatsu *et al.* 1992) and a radiation-induced complementation map of the *p* region predicts the location of at least 4 loci which affect development, reproduction, behaviour and pigmentation. These loci have homologues on human chromosome 15q11-q13 which may mediate the pleiotropic effects of the PWS/AS (Lyon *et al.* 1992).

A mutation at the *p* locus, the *pink-eyed unstable* mutation (p^{mu}), is associated with a duplication within the *p* gene. The p^{mu} phenotype is characterised by areas of light and dark pigmentation, due to spontaneous loss of the duplication at this locus, resulting in a reversion to wild-type (Brilliant *et al.* 1991; Nakatsu *et al.* 1992). This phenotype is similar to that of the ty-pos OCA individuals with ephelides. In p^{mu} mice the genetic reversion event results in the loss of duplicated sequences, which restores the normal linear array of coding information in the *p* gene (Gardner *et al.* 1992). The human cDNA, DN10 (which was used to identify the pIR10-1 [D15S12] cDNA), detects an abberant, large mRNA in skin from p^{mu}/p^{mu} mice and a correctly sized mRNA in partially pigmented skin from revertant mice. The spontaneous reversion to the wild-type phenotype in revertant mice occurs together with a loss of the aberrant transcript, as a result of a loss of the duplicated sequence, and appearance of a normal sized transcript. Since reversion to wild-type pigmentation is accompanied by a restoration of the normal DN10/*D15S12h* transcript, it has been suggested that the DN10 cDNA corresponds to the *p* gene itself (Gardner *et al.* 1992; Rinchik *et al.* 1993). Rinchik *et al.* (1993), have performed inter-specific mouse backcross analysis, to show that there is no recombination between *p* and *D15S12h*, implying a map distance of less than 1cM between these loci. This pigment gene is expressed in melanocytes and all tissues containing melanocytes (Gardner *et al.* 1992; Rinchik *et al.* 1993). No mRNA transcripts were detected by a DN10-derived probe in human pancreas, kidney, skeletal muscle, liver, lung, placenta, heart cells, or adult brain and it has been suggested that the DN10 cDNA is

derived from the human homologue of the mouse *p* locus, or *D15S12h* (Rinchik *et al.* 1993).

4.7 The Prader-Willi/Angelman chromosomal region

Many PWS/AS patients have hypopigmentation and optical misrouting, similar to those individuals with ty-pos OCA. Linkage between markers in the PWS/AS region and ty-pos OCA confirms that there is a pigment locus close to the critical region(s) of PWS and AS (Weisner *et al.* 1987). Since there is no recombination between ty-pos OCA and the D15S12 locus (the human homologue of the mouse *D15S12h* locus), it has been postulated that the D15S12 locus (close to the PWCR/ANCR on chromosome 15q11-q13) forms part of the gene for ty-pos OCA and is likely to be the human homologue, *P*, of the mouse *p* locus (Ramsay *et al.* 1992; Rinchik *et al.* 1993). Deletions of D15S12 have been correlated with hypopigmentation. It is possible that deletions of the *P* gene (D15S12) may be responsible for the hypopigmentation found in PWS/AS patients with a 15q11-q13 deletion (Gardner *et al.* 1992). Hamabe *et al.* (1991), showed that 77.4% of individuals with IR10 (D15S12 locus) deletions were hypopigmented, while only 10.5% of individuals with normal amounts of IR10 were hypopigmented. The latter may have genetic lesions in other parts of the D15S12 locus (Gardner *et al.* 1992), which were not tested. Pigmentation has been found to be normal in a single individual with PWS and a deletion of all loci tested on chromosome 15q11-q12 except the D15S12 locus (Rinchik *et al.* 1993). It is likely that hypopigmented PWS/AS patients are hemizygous or homozygous for mutant alleles of a gene responsible for ty-pos OCA and a patient with PWS and ty-pos OCA was found to have a partial deletion of the *P* locus, inherited from his mother, and an entire deletion of this locus from his father (Rinchik *et al.* 1993).

The PWS/AS region on human chromosome 15q11-q13 is subject to imprinting effects. The mouse *D15S9h-1* locus maps 9cM away from the *p* locus and is

imprinted, thus the human homologue of this locus, D15S9, may play a role in the phenotypes of PWS and AS. Gabrb3, the mouse homologue of the human GABRB3 locus, a closely flanking marker of the D15S12 locus, is equally well expressed from maternal and paternal chromosomes in brain tissue and is thus not imprinted in mice, raising doubts about a role for this gene in PWS and AS (Nicholls *et al.* 1992).

The *p* gene in mice is not likely to be imprinted (Gardner *et al.* 1992), but the *P* gene in humans occurs on chromosome 15q11-q13, a region which is known to be affected by imprinting. Deletions and/or mutations of the *P* gene have been implicated in both PWS/AS and ty-pos OCA. It may be postulated that in these conditions, apparent "heterozygotes" have different phenotypes while "homozygosity" results in OCA. Obligate carriers of ty-pos OCA are normally pigmented whereas some individuals with PWS/AS and apparent heterozygosity at the *P* locus are hypopigmented. This may be related to a "leaky" phenotype associated with imprinting effects in the 15q11-q13 region. OCA in PWS/AS may be associated with a deletion of the PWCR/ACR on the maternal or paternal chromosome and a mutation of the ty-pos OCA locus on the other chromosome, which results in effective "homozygosity" at this locus, similar to autosomal recessive ty-pos OCA.

4.8 The ty-pos OCA locus

Physical mapping studies, together with the linkage data presented in this study, suggest that the locus for ty-pos OCA is on human chromosome 15q11-q13 and it is postulated that the human homologue, *P*, of the mouse *pink-eyed dilution* gene, *p*, is the ty-pos OCA gene.

The DNA sequence of the *P* gene cDNA and the deduced amino acid sequence of the gene product have been determined (Rinchik *et al.* 1993). It has been proposed that the *p* locus encodes a structural protein present in the

melanosomal organelles of melanocytes, because mutant *p* melanosomes exhibit structural abnormalities and have a reduced capacity to bind or accumulate melanin, with abnormal melanogenesis (Gardner *et al.* 1992). The sequence of the putative human *P* polypeptide predicts an integral membrane protein, with at least 11 transmembrane domains, which may be a component of the melanosomal membrane involved in the transport of tyrosine (Rinchik *et al.* 1993) and may also affect the development of other organ systems (Gardner *et al.* 1992). More work is needed to define the precise biochemical functions of the *P* polypeptide.

The p^{m} mutation in mice is characterised by a phenotype with areas of light and dark pigmentation, as a result of spontaneous reversion to wild-type with the loss of a duplicated sequence from the *p* gene and restoration of normal gene function. The presence of ephelides in some individuals with ty-pos OCA may also occur as a result of a spontaneous reversion to wild-type, which may be related to sun-exposure, especially since most ephelides occur on sun-exposed skin. The possible role of gene duplication in ty-pos OCA must be considered.

Gene duplication has been recognised as a cause of several human diseases and has been suggested to play a role in organism evolution (Ohno 1970). Molecular characterisation of genes and their products has supported this view (reviewed in Hu and Worton 1992). It is generally assumed that gene duplication allows organisms to acquire novel biological functions as beneficial mutations may accumulate in one copy while another copy carries out the original function (Hu and Worton 1992), but gene duplication may be pathogenic, especially when part of an exon or a subset of exons are duplicated. The duplication may change the protein conformation, resulting in an unstable or dysfunctional protein, or it may result in a reading frame-shift in the mRNA, producing a truncated and usually dysfunctional protein (Hu and Worton 1992).

Partial gene duplication has been implicated in a number of diseases, including Duchenne muscular dystrophy and Becker muscular dystrophy (Den Dunnen *et al.* 19989; Hu *et al.* 1990) and Charcot-Marie-Tooth disease Type 1A (Lupski *et al.* 1991). Unequal crossing-over, either between homologous chromosomes or between sister chromatids of the same chromosome, has been postulated to be a mechanism by which a tandem gene duplication arises. This mechanism also predicts the formation of a deletion as a product of reciprocal exchange. Another mechanism which has been postulated to explain the tandem duplication of a limited number of nucleotides is the intra-strand slipped-mispairing model (Roth *et al.* 1985), where breakage of single-stranded DNA, mispairing between short homologous sequences and repair synthesis and replication will generate a tandem duplication. Both homologous and nonhomologous recombination have been proposed to lead to duplications and deletions. Molecular characterisation of genes and gene families indicate that the mechanisms leading to gene duplication are not uncommon in organism evolution, however, deletions appear to be more common than duplications (Hu and Worton 1992).

In summary, linkage analysis and comparative mouse-human mapping have localised the ty-pos OCA locus to human chromosome 15q11-q13. It has been postulated that the D15S12 locus forms part of the ty-pos OCA gene and it has been suggested that this gene is the human homologue, *P*, of the mouse *pink-eyed dilution* gene. The identification of mutations responsible for the ty-pos OCA phenotype and the presence or absence of ephelides will provide definitive proof that the *P* gene is in fact the ty-pos OCA gene.

CHAPTER FIVE

5.0 CONCLUSION

Oculocutaneous albinism (OCA) is a heterogeneous group of conditions, clinically defined by hypopigmentation of the skin, hair and eyes. Tyrosinase-positive (ty-pos) OCA is the most common type of OCA in Negroids, occurring with a average prevalence of 1 in 3900 in s[illegible] Africa. This condition is usually characterised by generalised hypopigmentation, with the accumulation of phaeomelanin pigments with increasing age, and, in some individuals, by the presence of pigmented patches or ephelides. The presence or absence of ephelides is apparently consistent within a family. Assuming that there is a single locus for ty-pos OCA in Negroids, as is suggested by the data presented here, then the occurrence of two distinctly different phenotypes suggests that there is more than one mutation at this locus.

Linkage studies in 41 southern African Negroid families, each with at least one member affected with ty-pos OCA, has shown that there is no linkage between ty-pos OCA and 15 random polymorphic serogenetic markers and 16 random polymorphic DNA markers, as well as markers from two candidate genes, the tyrosinase gene on chromosome 11q14-q21 and the CAS2 gene on chromosome 9p, and 4 markers from a potential candidate region on chromosome 11p. Two-point linkage analysis between ty-pos OCA and 16 markers on chromosome 15q, showed close linkage between the disease locus and the Prader-Willi/Angelman syndrome region on chromosome 15q11-q13. This analysis showed no cross-overs between ty-pos OCA and the D15S12 locus in 41 southern African Negroid families, suggesting that the D15S12 locus is very close to, or part of, the ty-pos OCA locus. A genetic linkage map of the region surrounding the ty-pos OCA locus showed the two most closely flanking markers to be the GABRB3 locus in a proximal position and the D15S24 (CMW-1) locus in a distal position.

Haplotype analysis did not reveal whether there was a single origin or multiple origins of the ty-pos OCA mutation(s). Allelic association was demonstrated

between the alleles at the D15S12 locus and the mutation(s) associated with the presence or absence of ephelides. The number of each of the D15S12 alleles associated with these phenotypes differed significantly, suggesting that the phenotypes may be associated with different mutations which may have had different origins.

At least three mouse pigment loci, *agouti*, *dilute* and the *pink-eyed dilution* locus, on mouse chromosomes 2, 9 and 7, respectively, have homologues on human chromosome 15. Mice homozygous for an unstable mutation, p^m, at the *pink-eyed dilution* locus have a phenotype characterised by patches of light and dark pigmentation, closely resembling that of ty-pos OCA individuals with ephelides. Thus it was suggested that the homologue of the *p* gene was the ty-pos OCA gene. The linkage results obtained in this study have confirmed the presence of a pigment locus on chromosome 15q11-q12 in the PWCR/ANCR and this locus has been postulated to be the human homologue, *P*, of the mouse *pink-eyed dilution* gene (*p*) (Gardner *et al.* 1992; Rinchik *et al.* 1993).

Future studies

The *P* gene has been postulated to be the locus reponsible for ty-pos OCA, however the gene must be characterised in southern African Negroids to provide definitive proof that mutations at this locus cause a ty-pos OCA phenotype. The *P* gene has been mapped and cloned, thus it will be possible to confirm whether or not this is the ty-pos OCA gene by functional complementation studies. Characterisation of the locus in individuals with ty-pos OCA will include a search for gross structural rearrangements using Southern blotting techniques, as well as an examination of the gene itself, using single-stranded conformational polymorphisms (SSCPs) and sequencing, in order to analyse point mutations possibly giving rise to the ty-pos OCA phenotype. It will be of great interest to examine the possibility that some of the mutation(s) associated with the presence of ephelides in humans are related to the partial gene duplication responsible

for the type of albinism with pigmented patches in mice which is due to homozygosity for the p^{sm} mutation. The evolutionary significance and heterozygote advantage of the ty-pos OCA mutation, if any, could be examined in southern Africa. It would also be interesting to study ty-pos OCA in the rest of Africa in order to determine the overall frequency of this condition on the continent and to study the mutation(s) associated with it.

Practical implications of finding the ty-pos OCA gene

Once the gene for ty-pos OCA has been found, or it has been confirmed that the *P* gene is the gene for ty-pos OCA, then it will possible to detect heterozygote carriers in ty-pos OCA families and in the general population and to offer prenatal testing to at risk couples, should it be requested. It will also be possible to characterise the gene product and its function(s) and to examine its role in pigment formation and optic and auditory tract development. This information may eventually lead to gene therapy for the pigment defect, although it would be more difficult to correct the neural tract abnormalities.

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APPENDICES

APPENDIX A

Sources of reagents

Acrylamide	Biorad Laboratories
Agar	DIFCO Laboratories
Amberlite	Sigma
Ammonium persulphate	Biorad Laboratories
Ampicillin	Sigma
Bacto-tryptone	DIFCO Laboratories
Bacto-yeast extract	OXOID Laboratories
Bis	Biorad Laboratories
Boric acid	Merck
Chloroform	BDH Chemicals
CsCl	Boerhinger Mannheim
Denhardt's	Sigma
EDTA	BDH Chemicals
Ethanol	BDH Chemicals
EtBr	Boerhinger Mannheim
Formamide	Fluka Chemicals
HGT Agarose	FMC
Hydroxyquinolinol	SAAR Chemicals
Isoamylalcohol	Merck
Lysozyme	Sigma
NaAc	BDH Chemicals
NaCl	Associated Chemical Enterprises
$Na^{++} PO_4 . H_2O$	BDH Chemicals
NaOH	Synthon
Nusieve Agarose	FMC
PEG	Sigma
Phenol	Merck
Proteinase K	Boerhinger Mannheim
RNase A	Boerhinger Mannheim
SDS	Merck
Sephadex G-50 (Fine)	Pharmacia
Spermidine trihydrochloride	Sigma
SS DNA	Sigma
TCA	SAAR Chemicals

Temed	Biorad Laboratories
Tris Base	Associated Chemical Enterprises
Tris HCl	Boerhinger Mannheim
Triton X-100	Sigma
Urea	Sigma

APPENDIX B

Composition and preparation of media

40% acrylamide stock

For 100ml mix 38g acrylamide
2g Bis
5g amberlite
50ml H_2O

Stir at room temperature for 30 minutes, filter through Whatman No 1 filter paper, make up to 100ml with H_2O, store at 4°C for up to 2 weeks.

6% nondenaturing polyacrylamide gel

For 50ml mix 21g urea
5ml TBE (10x)
7.5ml polyacrylamide (40%)
15ml H_2O

Add 45µl Temed and 160µl ammonium persulphate (10%), pour immediately.

Denhardt's solution

Dissolve 2g Ficoll
2g PVP
2g BSA

in 100ml H_2O, filter sterilise, store at -20°C.

Deionised formamide

For 1l m 5g amberlite
1l formamide

Stir for 30 minutes, filter, store at -20°C.

Formamide/EDTA/XC/BPB gel loading buffer

Mix 10ml formamide
10mg xylene cyanol
10mg bromophenol blue
200μl 0.5M EDTA (pH 8.0)

LA

Add 1.4g agar to 100ml LB, autoclave. When cool, add antibiotic (25μg/ml amp; 12.5μg/ml tet). Pour into plates.

LB broth

Dissolve: 10g bacto-tryptone
5g bacto-yeast extract
10g NaCl

in H_2O, pH to 7.7, make up to 1l. Aliquot into 100ml, autoclave. Add antibiotic (25μg/ml amp; 12.5μg/ml tet)

Special neutralising solution

Dissolve 60.6g Tris base
175.4g NaCl

in 1l H_2O, pH to 7.0, autoclave.

20xSSC

Dissolve 175.4g NaCl
88.3g Na citrate

in 1l H_2O, autoclave.

20xSSPE

Dissolve 210.4g NaCl
27.6g $NaH_2PO_4.H_2O$
7.4g EDTA

in H_2O, pH to 7.7, autoclave.

1xTBE

Dissolve 108g Tris base
55g boric acid
7.44g EDTA (disodium)

in 1l H_2O, autoclave.

1xTE

Dissolve 2ml EDTA (0.5M)
10ml Tris (1M)

in H_2O, pH to 8.0, make up to 1l, autoclave.