A molecular investigation of the novel gene underlying autosomal dominant retinitis pigmentosa in a South African family

by

Soraya Bardien-Kruger

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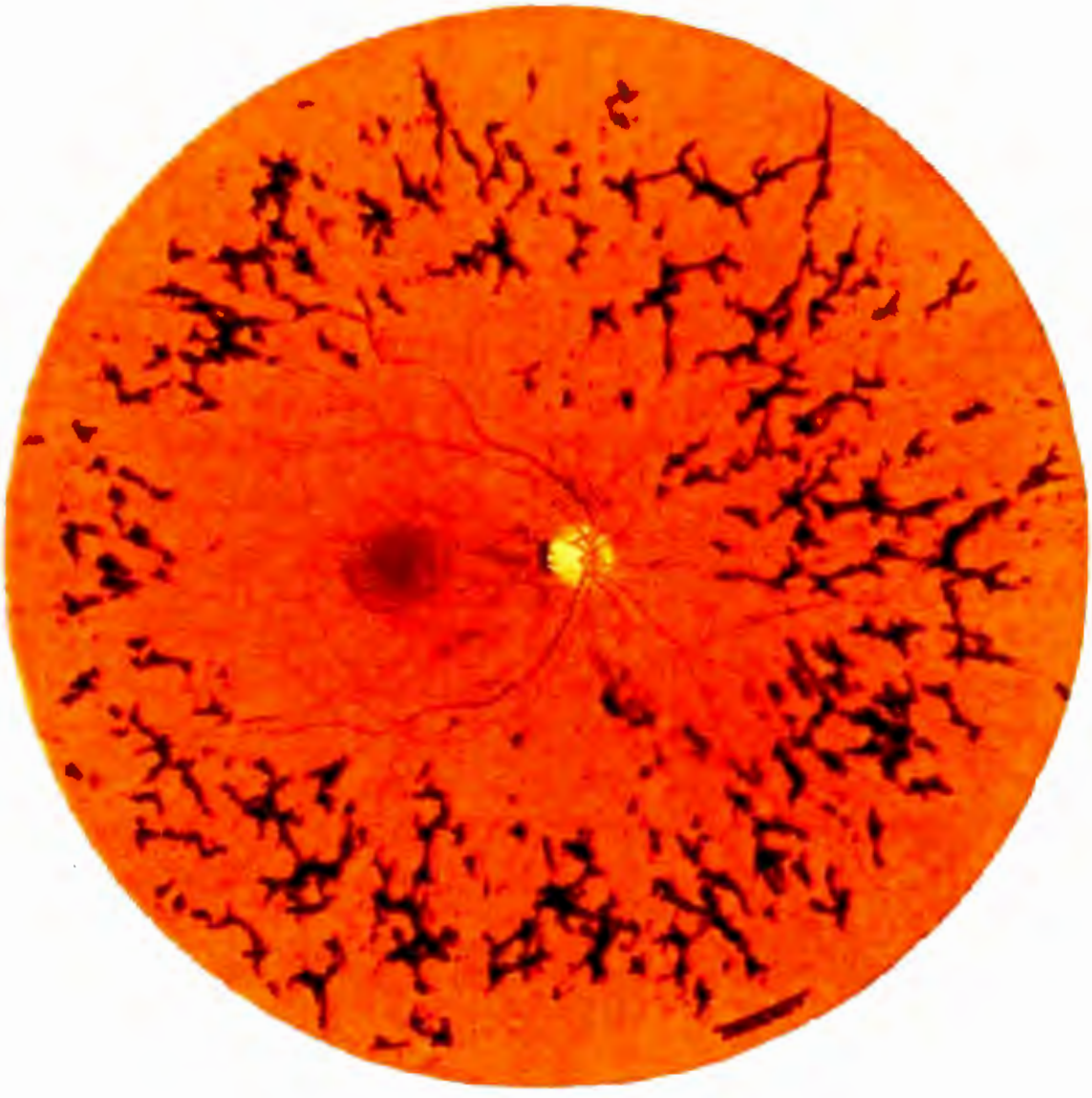
For my baby,
Justin



Fundus photograph of an individual affected with retinitis pigmentosa.

SEEING

They took away what should have been my eyes,
(But I remembered Milton's Paradise)
They took away what should have been my ears,
(Beethoven came and wiped away my tears)
They took away what should have been my tongue,
(But I had talked with God when I was young)
He would not let them take away my soul,
Possessing that, I still possess the whole.

Helen Keller

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ABSTRACT

The inherited retinal degenerative disorders are a common cause of severe visual handicap in the Western world. Retinitis pigmentosa (RP) is a group of retinopathies in which a primary feature is a progressive loss of photoreceptor and retinal pigment epithelium function. Over the last decade, investigations into the patho-physiology of RP have identified numerous disease-causing genes and loci (for a current listing refer to the web site http://www.sph.uth.tmc.edu/Retnet/).

A study of a South African family with an autosomal dominant form of RP (adRP) forms the basis of this dissertation. In this family, comprising 44 individuals, the first manifestation of visual disturbance is usually evident between 20 and 30 years of age. Subsequently, another South African adRP family, consisting of 25 members, was also incorporated into this investigation.

Genetic linkage analysis facilitated the mapping of the disease phenotype in the two South African adRP families to a 10 cM interval on chromosome 17q22. This novel locus, designated RP17, is the eighth identified for adRP. Haplotype construction in the two kindreds, in conjunction with multipoint analyses subsequently fine mapped RP17 to a 1 cM region between microsatellite markers D17S1604 and D17S948. Although the two families are from ethnically diverse population groups, they share the same disease-associated haplotype spanning 12 cM, which suggests that the disorder may be caused by the same pathogenic mutation in the same gene.

The positional cloning approach was utilised in an endeavour to identify the RP17 gene and an attempt was made to construct a physical map of the 1 cM critical region. A contig consisting of seven yeast artificial chromosome (YAC) clones was assembled using sequence-tagged-site (STS) content mapping. In order to close a gap in the YAC contig, a bacterial artificial chromosome (BAC) library was screened and the vectorette PCR technique was used to verify overlapping sequences.

This contig should provide a useful tool for the purpose of isolating genes or transcription units within the RP17 critical interval. In this regard, purified YAC DNA was isolated

using pulsed-field gel electrophoresis and the cDNA selection technique was employed to generate a transcription map. This approach was applied to YAC 751c12 using a foetal brain cDNA library, and two rounds of selection were performed to create a sub-library for enriched cDNAs derived from this clone. Screening for the presence of contaminating sequences in the 480 transformants revealed that (i) approximately 7% of the selected clones contain COT-1 DNA and (ii) none of the clones were contaminated with yeast AB1380 DNA. Ten randomly chosen clones were sequenced and subjected to BLASTN analysis, which revealed the presence of a 23 bp contaminant, known genes as well as novel transcripts.

In order to optimise efforts to isolate the adRP gene, four positional candidates residing on 17q were screened for evidence implicating them in the adRP phenotype in the two 17q22-linked families. The genes investigated were: *PDEG* (gamma subunit of rod phosphodiesterase), *TIMP2* (tissue inhibitor of metalloproteinases-2), *PKCA* (protein kinase C alpha) and *retinal fascin*. These candidates were chosen on the basis of (i) mapping to 17q, (ii) expression in the retina and/or (iii) potential involvement in the rod phototransduction pathway. Recombination events between the adRP locus and a single strand conformation polymorphism (SSCP) in *PDEG*, and a restriction fragment length polymorphism (RFLP) in *TIMP2* provided evidence for the exclusion of these candidate genes. A novel SSCP detected in the promoter region of *retinal fascin* was genotyped in the two adRP families and showed a lack of co-segregation with the disease locus. Furthermore, direct DNA sequencing of the coding regions as well as the promoter region of *retinal fascin* in RP affected family members did not reveal any pathogenic mutations. In addition, data is provided which suggests that *PKCA* does not reside on any of the YACs and BACs encompassing the RP17 critical interval. This gene is therefore unlikely to be responsible for the adRP phenotype in the two RP17-linked families.

Ultimately, the work reported in this thesis may contribute to the body of knowledge on inherited retinal degenerative disorders. Moreover, this investigation should provide the basis for further study of the aetiology of RP in all families linked to the RP17 locus on chromosome 17q22. The immediate application of these molecular findings is the potential for pre-symptomatic testing of at-risk members from the two adRP kindreds.

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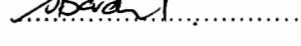

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SYMBOLS AND ABBREVIATIONS

°C	degrees Celsius
[χ^{32}P]ATP and [α^{32}P]dCTP	radio-isotope of phosphorus
μg	microgram
μM	micromolar
μl	microlitre
%	percentage
θ	theta
A	adenine
adRP	autosomal dominant retinitis pigmentosa
arRP	autosomal recessive retinitis pigmentosa
BAC	bacterial artificial chromosome
bp	base pair (s)
C	cytosine
Ca^{2+}	calcium ions
cDNA	complementary deoxyribonucleic acid
cen	centromere
CEPH	Centre d'Etudes du Polymorphisme Humain
cGMP	cyclic guanosine 3',5'-monophosphate
cM	centiMorgan
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
E-coli	Escherichia coli
ERG	electroretinography/ electroretinogram
EST	expressed sequence tag
FISH	fluorescent in-situ hybridisation
G or g	guanine
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HCl	hydrochloric acid
hr/ s	hour (s)
K^+	potassium ions

kb	**kilobase (s)**
KCl	**potassium chloride**
l	**litre (s)**
LA	**Luria-Bertani agar**
LB	**Luria-Bertani broth**
lod	**log of the odds**
M	**molar**
Mb	**megabase (s)**
$MgCl_2$	**magnesium chloride**
min/ m	**minute (s)**
ml	**millilitre**
mM	**millimolar**
mRNA	**messenger ribonucleic acid**
mV	**millivolts**
Na^+	**sodium ions**
NaCl	**sodium chloride**
NaOH	**sodium hydroxide**
ng	**nanogram**
NRL	**neural retina-specific leucine zipper gene**
O/N	**overnight**
ORF	**open reading frame**
PAC	**P1-derived artificial chromosome**
PCR	**polymerase chain reaction**
PDE	**cGMP-phosphodiesterase**
PDEA	**alpha subunit of PDE**
PDEB	**beta subunit of PDE**
PDEG	**gamma subunit of PDE**
pH	**log H^+ ions**
PKCA	**protein kinase C alpha gene**
PFGE	**pulsed-field gel electrophoresis**
pmol	**picomoles**
prcd	**progressive rod-cone degeneration**
RFLP	**restriction fragment length polymorphism**
RP	**retinitis pigmentosa**

RPE	**retinal pigment epithelium**
ROM1	**rod outer segment membrane protein 1 gene**
SA	**South Africa**
S. cerevisiae	**Sacchromyces cerevisiae**
SDS	**sodium dodecyl sulphate**
sec/ s	**second (s)**
SSCP	**single strand conformation polymorphism**
STS	**sequence-tagged-site**
T	**thymine**
TBE	**Tris-borate/EDTA**
tel	**telomere**
TIMP2	**tissue inhibitor of metalloproteinases-2 gene**
Trp	**tryptophan**
U	**units**
UK	**United Kingdom**
Ura	**uracil**
UCT	**University of Cape Town**
USA	**United States of America**
UTR	**untranslated region**
xlRP	**X-linked retinitis pigmentosa**
YAC	**yeast artificial chromosome**

■ □	**affected/unaffected male**
● ○	**affected/unaffected female**
⊠ ⊘	**deceased male/ female**

PLAN OF THESIS

This dissertation is divided into six chapters and the layout is as follows:

- **Chapter One** provides a background to hereditary retinal degenerative disorders, focussing on retinitis pigmentosa (RP). **Chapter Two** is a synopsis of the development of research on RP in South Africa, and includes an outline of the aim of the study and the experimental approach to the investigation.

- Chapters Three, Four and Five are the experimental chapters, each dealing with a different aspect of the molecular genetic investigation that forms the basis of this study. Each of these chapters is divided into four sections i.e. Introduction, Materials & Methods, Results and Discussion. **Chapter Three** describes the intensive genetic linkage study undertaken in a large South African family in order to map a gene underlying an autosomal dominant form of RP. **Chapter Four** deals with the construction of a physical map for the locus mapped in Chapter Three. In addition, this chapter describes the approach used towards generation of a transcription map for this region. The screening of positional candidate genes for RP17 is discussed in **Chapter Five**.

- The conclusions drawn for each aspect of this study are discussed in detail at the end of the relevant Chapter. **Chapter Six** provides a final comment on each of these facets and includes future research prospects, which are recommended based on the results obtained.

CHAPTER ONE

GENERAL INTRODUCTION

1

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GENERAL INTRODUCTION

1.1 THE BIOLOGY OF VISION

1.1.1 Structure of the eye

The human eye is a small yet complex organ, composed of three main layers (Fig. 1.1)[James et al. 1997]

(i) The tough outer **corneo-sclera** layer forms the transparent cornea anteriorly, and also provides sites for the attachment of ocular muscles and gives the eye its mechanical strength.

(ii) The **uvea**, the middle vascular layer, consists of the iris and ciliary body anteriorly which extend posteriorly into the choroid, a pigmented layer carrying a rich supply of blood vessels.

(iii) The **retina** is the innermost layer and it consists of two main components, the retinal pigment epithelium (RPE) and the multi-layered neural retina.

Bruch's membrane, an extracellular matrix composed of structural proteins, is situated between the RPE and the choroid.

1.1.2 Retina

The retina arises as an evaginated portion of the midbrain (the optic vesicle), which subsequently invaginates to form the two-layered optic cup (Freund et al. 1996). The outer layer of the optic cup evolves into the RPE and the inner layer forms the neural retinal tissue, which later differentiates into photoreceptor cells, intermediate neurons, ganglion cells and various supporting cells (Fig. 1.2). The cells in the mature retina do not divide. Photoreceptor cells are light sensitive and are responsible for the initiation of the visual response. These cells capture

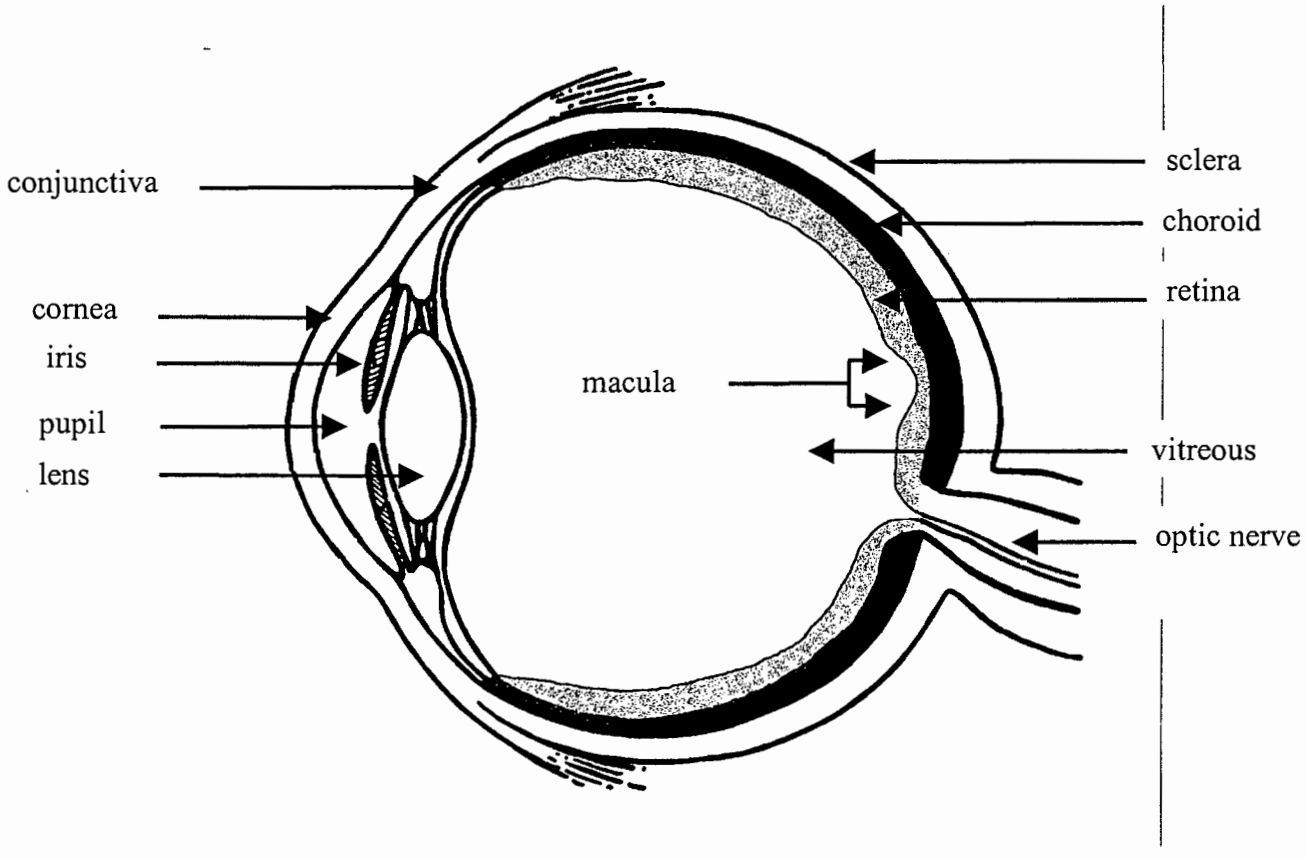


Figure 1.1 A schematic diagram depicting the structure of the human eye.

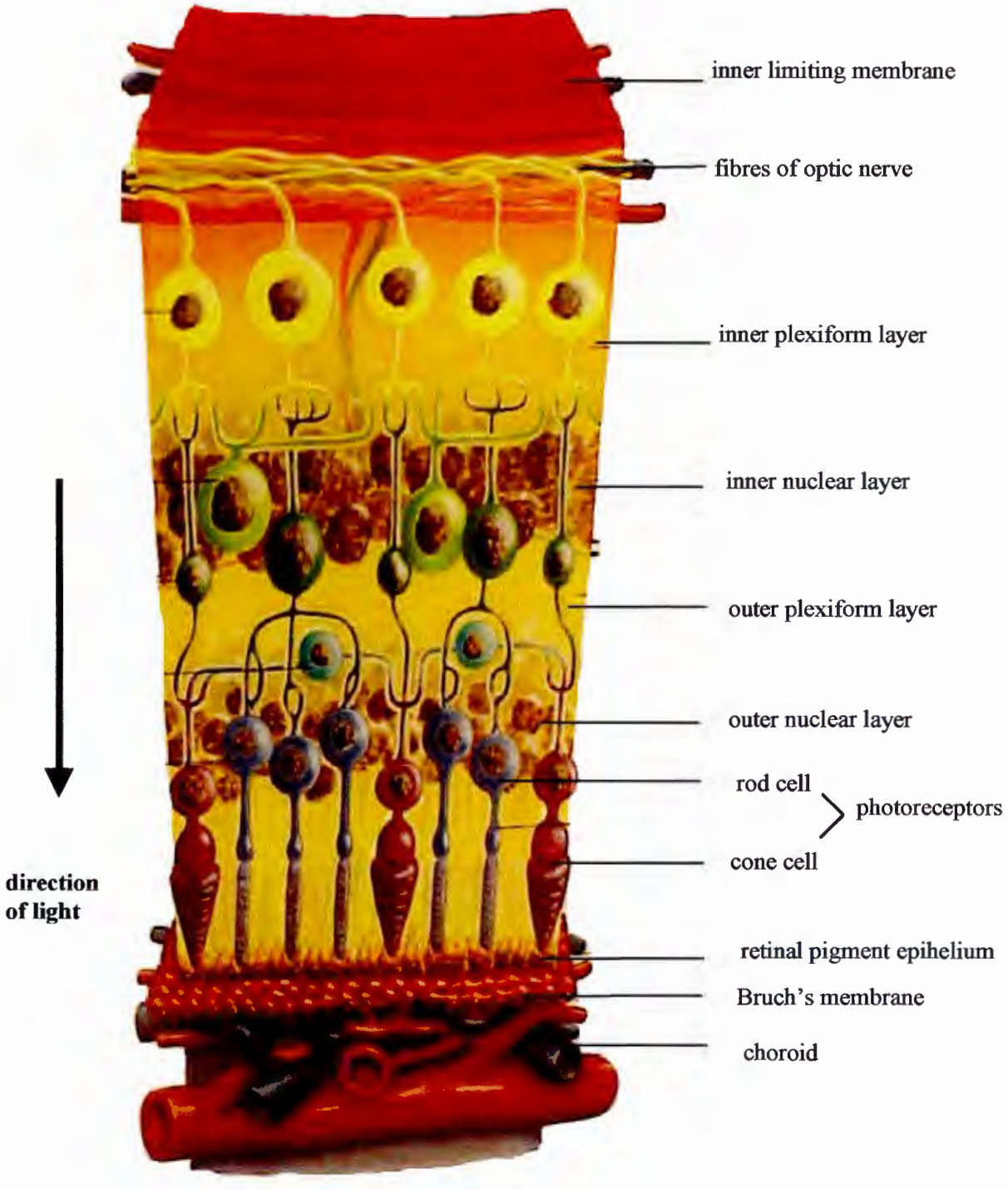


Figure 1.2 Schematic representation of the layers in the retina.
(Reproduced and modified from BioLone™, Germany)

photons of light and pass these in the form of electrical impulses to the intermediate neurons, where electrical processing takes place. The impulse is then transmitted to the ganglion cells, which have long fibres collectively forming the optic nerve. This nerve forms the pathway of visual information from the eye to the brain.

The two main morphological types of light-sensitive photoreceptor cells, the rods and the cones (Fig. 1.3) share many metabolites and cofactors but differ in their anatomical distribution within the retina. The biology of the photoreceptor cells is well-documented (Yau 1994) and an outline of their structure and function is given below:

There are approximately 6 million cone photoreceptor cells (Fig. 1.3a) in each eye and the macula lutea, the central region of the retina at which visual perception is most acute, is cone-rich. These photoreceptor cells are responsible for colour vision and are important for fine vision. The outer segments of cone cells consist of discs (flattened membrane sacs) that are continuous with the plasma membrane forming a highly convoluted surface membrane.

Rod cells (Fig. 1.3b), numbering about 120 million in each eye, are distributed throughout the retina, with the exception of the centre of the fovea. These cells are responsible for dim light vision, peripheral vision and contrast sensitivity. The outer segments of rod cells consist of a stack of discs separated by thin layers of cytoplasm and surrounded by the plasma membrane. Old discs are phagocytosed by the RPE and new discs are constantly resynthesised. The disc and plasma membranes form the site of the rod phototransduction pathway, which is discussed in the following section.

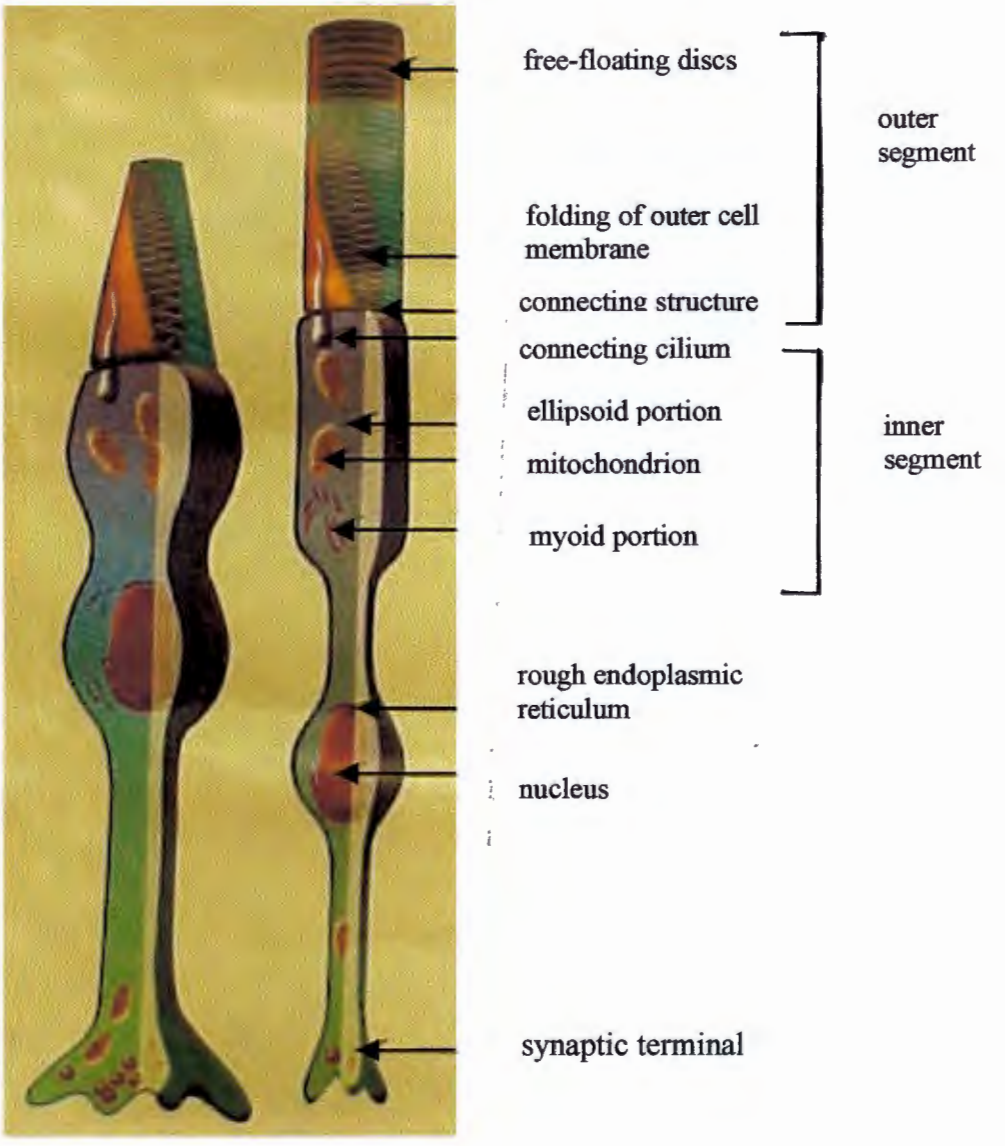


Figure 1.3 The two types of photoreceptor cells.

(a) cone cell (b) rod cell

[Reproduced and modified from VisiCare (Pty) Ltd., South Africa]

1.1.3 Rod phototransduction cascade

Sight depends fundamentally upon the detection of light and its conversion by synaptic responses into a form interpretable by the neurons. This biological process, termed the phototransduction cascade (Stryer 1991) takes place in the outer segments of the rod (Fig. 1.4) and cone cells. The pathway begins with a visual image entering the eye in the form of photons of different wavelengths of light, which strike the photoreceptor layer.

In dim light vision, rhodopsin, the visual pigment in rod cells, absorbs the photons, thereby inducing the isomerisation of the associated retinal chromophore from the 11-*cis* to the all-*trans* form. In turn, photoexcited rhodopsin, termed metarhodopsin II, activates transducin, which is a trimeric G protein composed of alpha, beta and gamma subunits. Activated transducin exchanges its bound GDP for GTP on the alpha subunit and dissociates from the beta and gamma subunits. The third component of the phototransduction cascade is the enzyme phosphodiesterase (PDE) which comprises catalytic alpha and beta subunits and two inhibitory gamma subunits. The alpha subunit of transducin binds to the gamma subunits of PDE thereby releasing their inhibitory constraint. Activated PDE immediately hydrolyses many molecules of cGMP, the nucleotide responsible for maintaining open cGMP-gated cation channels in the dark. The closure of the channels results in a decrease in the free Ca^{2+} concentration in the outer segment because Ca^{2+} can no longer enter the cell but continues to exit through the Na^{+}-Ca^{2+}-K^{+} exchanger.

This process generates a transient electrical hyper-polarisation of the plasma membrane, which produces a neural signal. Rod cells usually have an electrical membrane potential of about -40 mV due to an uneven

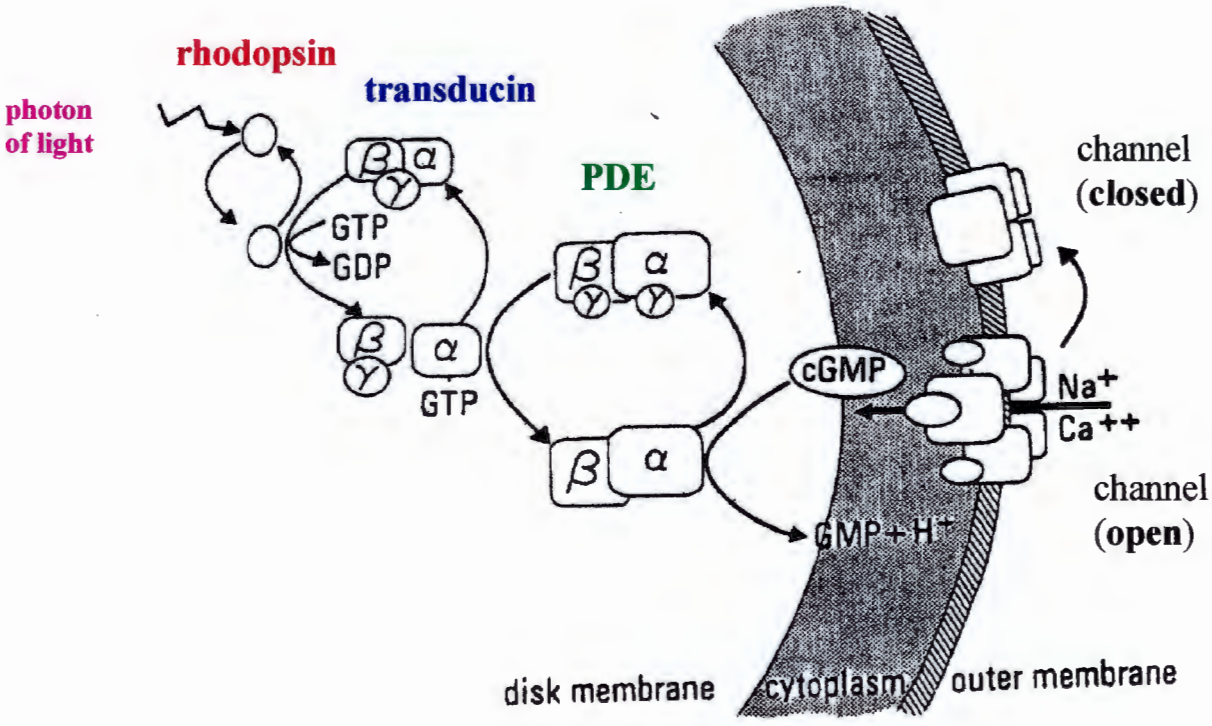


Figure 1.4 Scheme representing the rod phototransduction pathway. Incident light (photon) photo-activates rhodopsin, and initiates a cascade of reactions involving transducin and PDE. This pathway produces a relative decrease in intracellular cGMP leading to closure of the sodium channels which generates the visual signal.

GDP = guanosine diphsphate; GTP = guanosine triphosphate;
PDE = phosphodiesterase; cGMP = cyclic guanosine 3',5'-monophosphate.
(Reproduced from Lolley 1994).

distribution of positive and negative charges inside and outside the plasma membrane. When the channels close, however, the membranes become more negative, about -70 mV, and this gives rise to a brief voltage pulse. Further processing of the neural signal occurs in the inner retinal layers and in specialised regions of the brain. After activation, several different mechanisms are responsible for restoring the cell to the resting (dark) state. Photoexcited rhodopsin is deactivated through phosphorylation by rhodopsin kinase and subsequent binding of arrestin. A similar phototransduction cascade has been proposed for cone photoreceptors, with different visual pigments in each of the three (red, blue and green) cone cell types.

1.2 HEREDITARY RETINAL DYSTROPHIES

Over the last decade, molecular genetics and molecular biology techniques have identified the genetic defects underlying numerous hereditary eye disorders. This knowledge has enabled an improved classification of these disorders, which could not have been elucidated using clinical classification schemes alone.

This dissertation deals with a subgroup of genetic eye conditions in which mainly the retinal tissue is affected. In this regard, a selected list of photoreceptor and retinal pigment epithelium dystrophies, which have been mapped, is presented in Table 1.1. Only a small proportion of the inherited retinal dystrophies is listed since a complete categorisation is beyond the scope of this dissertation. A comprehensive list is, however, available on the World Wide Web at the site http://www.sph.uth.tmc.edu/Retnet/.

Table 1.1 Chromosomal loci for hereditary retinopathies.

CHROMOSOME (GENE)	DISORDER	REFERENCE
1q13-23	adRP	Xu et al. 1996
1p13-21	arRP	Martinez-Mir et al. 1997
1p21-13 (ABCR)	Stargardt macular dystrophy	Allikmets et al. 1997
1q31-32.1	arRP	van Soest et al. 1994
1q41 (USH2A)	Usher syndrome type II	Eudy et al. 1998
1q42-qter	Choroideremia-like dystrophy	van Bokhoven et al. 1994
3p11-13	Bardet-Biedl syndrome type III	Sheffield et al. 1994
3q21 (rhodopsin)	adRP	Dryja et al. 1990
3q21 (rhodopsin)	arRP	Rosenfeld et al. 1992
3q21 (rhodopsin)	Stationary night blindness	Dryja et al. 1993
3q21-25	Usher syndrome type III	Sankila et al. 1995
4p16.3 (PDEB)	arRP	McLaughlin et al. 1993
4p16.3 (PDEB)	Stationary night blindness	Gal et al. 1994
4p14-q13 (CNGC)	arRP	Dryja et al. 1995
5q31-34 (PDEA)	arRP	Huang et al. 1995
6p12 (RDS)	adRP	Farrar et al. 1991
6p12 (RDS)	Vitelliform macular dystrophy	Wells et al. 1993
6p12 (RDS)	Digenic RP	Kajiwara et al. 1994
6p12 (RDS)	Pattern macular dystrophy	Weleber et al. 1993
6p12 (RDS)	Retinitis punctata albescens	Kajiwara et al. 1993
6p12 (RDS)	Fundus flavimaculatus	Weleber et al. 1993
6p12 (RDS)	Cone-rod dystrophy	Nakazawa et al. 1996
6p12 (RDS)	Butterfly-shaped pigment dystrophy	Nichols et al. 1993
6p	arRP	Knowles et al. 1994
6q11-15	Stargardt-like syndrome	Stone et al. 1994

CHROMOSOME (GENE)	DISORDER	REFERENCE
6q16	North Carolina macular dystrophy	Small et al. 1992
6q25-26	Cone dystrophy	Milosevic et al. 1975
7p15-21	Cystoid macular dystrophy	Kremer et al. 1994
7p15-13	adRP	Inglehearn et al. 1993
7q31-35	adRP	Jordan et al. 1993
8q11-21	adRP	Blanton et al. 1991
8q24	Atypical vitelliform dystrophy	Ferrel et al. 1983
10p (PAHX)	Refsum disease	Mihalik et al. 1997
10q26 (OAT)	Gyrate atrophy	Dietz et al. 1993
10q	Usher syndrome type ID	Wayne et al. 1996
11p15	Usher syndrome type IC	Keats et al. 1994
11q13	Exudative vitreoretinopathy	Li et al. 1992
11q13 (bestrophin)	Best macular dystrophy	Petrukhin et al. 1998
11q13 (myosin VIIA)	Usher syndrome type IB	Weil et al. 1995
11q13	Inflammatory vitreoretinopathy	Stone et al. 1992
11q13	Bardet-Biedl syndrome type I	Leppert et al. 1994
11q13 (ROM1)	Digenic RP	Kajiwara et al. 1994
11q14-21 (TYR)	Oculocutaneous albinism	Oetting et al. 1993
13q34	Stargardt macular dystrophy	Zhang et al. 1994
13q14 (Rb)	Retinoblastoma	Sippel et al. 1998
14q32	Usher syndrome type IA	Kaplan et al. 1992b
15q11-12 (P)	Oculocutaneous albinism	Lee et al. 1994
15q22-23	Bardet-Biedl syndrome type IV	Carmi et al. 1995
16q13-22	Bardet-Biedl syndrome type II	Kwitek-Black et al. 1993
17p13.3	adRP	Greenberg et al. 1994a
17p (GUC2D)	Leber's congenital amaurosis	Perrault et al. 1996

CHROMOSOME (GENE)	DISORDER	REFERENCE
17q11	Cone rod dystrophy	Kylstra et al. 1993
17q22	adRP	Bardien et al. 1995
18q21	Cone rod dystrophy	Warburg et al. 1991
19q13	Cone rod dystrophy	Evans et al. 1994
19q	adRP	Al-Maghtheh et al. 1994
21q21	Usher syndrome type IE	Chaib et al. 1997
22q13-qter (TIMP3)	Sorsby fundus dystrophy	Weber et al. 1994
Xp22 (OA1)	Ocular albinism	Bassi et al. 1995
Xp22.2 (XLRS1)	Juvenile retinoschisis	Sauer et al. 1997
Xp21.1 (RPGR)	X-linked RP (RP3)	Meindl et al. 1996
Xp21	Aland Island eye disease	Pillers et al. 1990
Xp21	X-linked RP (RP6)	Ott et al. 1990
Xp11.4	Cone-rod dystrophy	Seymour et al. 1998
Xp11.4 (NDP)	Norrie disease	Meindl et al. 1992
Xp11	Stationary night blindness	Bech-Hansen et al. 1998
Xp11	Exudative vitreoretinopathy	Chen et al. 1993
Xp11.3 (RP2)	X-linked RP (RP2)	Schwahn et al. 1998
Xp11.3	Stationary night blindness	Musarella et al. 1989
Xp11.2	Incontinentia pigmenti	Hodgson et al. 1985
Xq22 (REP1)	Choroideremia	Cremers et al. 1990
Xq26-27	Albinism-deafness syndrome	Shiloh et al. 1990
Xq27-28	Incontinentia pigmenti	Sefiani et al. 1989
Xq28 (red cone opsin)	Cone dystrophy	Bergen et al. 1997
Mitochondria	Kearns-Sayre syndrome	Moraes et al. 1989
Mitochondria	Leber's optic atrophy	Wallace et al. 1988

Where known, the causative gene is given in parenthesis after the chromosomal location.

The hereditary retinal dystrophies are very heterogeneous. The information provided with each disorder in Table 1.1 exemplifies the high level of:

- Genetic or locus heterogeneity (different genes resulting in the same disorder, e.g. nine loci for autosomal dominant retinitis pigmentosa (adRP); three loci for X-linked RP (xlRP); four for Bardet-Biedl syndrome)

- Allelic heterogeneity (different mutations in the same gene causing either the same disorder or distinct conditions, e.g. different mutations in *rhodopsin* responsible for autosomal recessive RP (arRP), adRP and congenital stationary night blindness)

- Clinical heterogeneity (the same mutation causing different manifestations in affected individuals, even within the same family, e.g. a specific mutation in *peripherin/RDS* causing either RP, pattern macular dystrophy or fundus flavimaculatus).

The clinical features of a few of the well-characterised inherited retinopathies listed in Table 1.1 (with the exception of retinitis pigmentosa which is discussed in Section 1.3) are summarised in Table 1.2.

Table 1.2 Clinical phenotype of a selection of the well characterised inherited retinopathies.

DISORDER	CLINICAL FEATURES
Macular dystrophies including, - Stargardt macular dystrophy - Best macular dystrophy - Cone-rod dystrophy - Butterfly-shaped pattern dystrophy - Sorsby fundus dystrophy	A heterogeneous group of disorders with funduscopic changes involving the macula and the RPE. Progressive loss of central vision is a primary feature.
Usher syndrome - Type I Type II Type III	RP, congenital severe-to-profound sensorineural deafness and vestibular ataxia. RP, congenital moderate-to-severe hearing impairment, and normal vestibular function. RP and progressive hearing loss.
Bardet-Biedl syndrome	RP, polydactyly, obesity, kidney disease and mental retardation.
Stationary night blindness	Non-progressive night blindness, reduced visual acuity and myopia.
Choroideremia	Constriction of peripheral vision, reduction of central vision and night blindness.
Gyrate atrophy	Myopia, night blindness, cataracts, and loss of peripheral vision.
Leber's congenital amaurosis	Early age of onset, greatly impaired vision and extinguished electroretinograms.
Ocular albinism	Albinism of the peripheral and central fundus, decreased visual acuity and nystagmus.
Aland island eye disease	Impaired night and colour vision, myopia and albinism of the central fundus.
Hereditary juvenile retinoschisis	Poor visual acuity and cystic degeneration of the peripheral and central retina.
Retinitis punctata albescens	Retinal pigmentation, arteriolar attenuation, night blindness and constriction of visual fields.

1.3 RETINITIS PIGMENTOSA (RP)

1.3.1 Clinical aspects of RP

1.3.1.1 *Delineation of the disorder*

In 1882, J Hutchinson wrote:

"Many years ago a well dressed man who looked in excellent health, came to my desk in the outpatient room, at Moorfields, and in a somewhat excited manner declared 'I am going blind Sir! you can do nothing for me I know! It is in the family, and has been for centuries, and at the present time I know more than thirty who are either blind or on the way to it. When once it begins it always goes on; still I should be glad if you would look at my eyes.' I did so and found, as I expected, that he was the subject of retinitis pigmentosa."

Retinitis pigmentosa (RP) is a generic name for a group of hereditary retinal dystrophies characterised by progressive, symmetrical and bilateral degeneration of retinal tissue. It is a common cause of severe visual handicap and complete blindness may develop in the later stages. The prevalence of RP in Europe and the USA has been estimated to range from 1 in 3000 to 1 in 7000 (Humphries et al. 1990).

The term 'retinitis' implies an inflammatory disease of the retina and 'pigmentosa' refers to the characteristic appearance of abnormal clumps of black, spicular pigment which are present in the retina of affected persons. Since the RP group of conditions refers to hereditary retinal dystrophies and not inflammatory diseases, the 'itis' part of the name is unjustified. Also, it is now known that the presence of the pigmentary patches is a consequence of RP and not its cause. Histological analysis has revealed that the black pigmentation is due to an invasion of the degenerating neural retina by melanin-pigmented cells of the RPE layer (Bird 1975). Many names have been suggested for this group of

disorders, but the misleading term 'retinitis pigmentosa', first coined by Donders (1857), is still used.

1.3.1.2 *Manifestations of RP*

The clinical features of RP are well documented and vary amongst affected persons (Kanski 1994). One of the earliest manifestations is night blindness, also referred to as 'nyctalopia'; another common feature is poor contrast sensitivity. As the peripheral cone photoreceptor cells gradually degenerate, there is a concomitant loss of peripheral vision and eventually, affected persons experience 'funnel vision', retaining only the central portion of their visual fields. In advanced stages of the disorder, poor central vision and an inability to distinguish colours may ensue, usually culminating in severe visual impairment or blindness. A RP fundus (*i.e.* inside of the eyeball as viewed through an ophthalmoscope) usually exhibits three striking features: (i) attenuation of the retinal blood vessels, (ii) intraretinal spicular pigmentation and (iii) waxy pallor of the optic disc. In affected persons, progressive deterioration of retinal function may be demonstrated by electroretinography (ERG), in which the electrical signals generated by the retina in response to flashes of light are reduced in amplitude and delayed in response times (Berson 1993).

1.3.2 Genetics of RP

The monogenic forms of RP can be inherited as autosomal dominant (adRP), autosomal recessive (arRP), X-linked recessive (xlRP), X-linked dominant and syndromic forms (Rosenfeld et al. 1994). There has also been a report of digenic RP in which mutations in two genes, *peripherin/RDS* and *ROM1*, are necessary for manifestation of the disease phenotype (Kajiwara et al. 1994). Affected persons without a positive family history are designated as having 'sporadic' or 'simplex' RP. In general, the age of onset, rate of progression, extent of eventual visual

loss, and the presence or absence of associated ocular features are related to the mode of inheritance of RP. The X-linked recessive form of the disorder usually runs the most severe course and the affected person often has significant visual handicap by the fourth decade (Kanski 1994).

The prevalence of the various genetic forms of RP varies considerably from country to country (Jay 1982; Greenberg et al. 1993). This situation reflects ethnic and socio-demographic factors (i.e. founder effects, consanguinity etc.) which influence the frequency of any genetically determined disease in a given population.

1.4 AUTOSOMAL DOMINANT RP (adRP)

This dissertation is focused upon the autosomal dominant forms of RP, which were originally subdivided into three broad categories (Lyness et al. 1985):

(i) Type 1 or D-type presents with diffuse and severe loss of rod function early in life but cone function is preserved until much later. This form of the disorder is also characterised by an early age of onset and rapid progression.
(ii) Type 2 or R-type results in simultaneous regional or patchy loss of both rod and cone function. This type has a slower progression and a variable age of onset.
(iii) Sectorial or sector RP is rare and consistently involves only one quadrant of the eye.

1.4.1 Molecular background

In autosomal dominant disorders there is direct parent-to-child transmission of the condition and each offspring of an affected parent,

regardless of the sex of the parent or child, is at 50% risk of inheriting the condition. In this mode of inheritance, the abnormality is expressed in the heterozygous state.

An understanding of the aetiology of RP has improved dramatically over the past decade with the discovery of several of the causative genes. In adRP, mutations in two genes viz. *rhodopsin* (Dryja et al. 1990) and *peripherin/RDS* (Farrar et al. 1991; Kajiwara et al. 1991) have been associated with the condition. The disorder may be the result of either a dominant negative effect i.e. where the product of the abnormal gene interferes with cell function, or through the shortage of product from the normal gene (haploinsufficiency) (Strachan and Read 1996). The dominant negative effect was elucidated following the observation that mice transfected with a mutant *rhodopsin* gene develop retinal degeneration, despite having the usual complement of normal genes (Olsson et al. 1992). The second possibility of haploinsufficiency was first demonstrated in the rds mouse, in which transgenic rescue was achieved by insertion of a normal *peripherin/rds* gene (Travis et al. 1992).

During the last decade an additional seven anonymous adRP loci have been mapped to chromosomes:

(i) 8q (RP1 locus, Blanton et al. 1991),
(ii) 7p (RP9 locus, Inglehearn et al. 1993),
(iii) 7q (RP10 locus, Jordan et al. 1993),
(iv) 19q (RP11 locus, Al-Maghtheh et al. 1994),
(v) 17p (RP13 locus, Greenberg et al. 1994a),
(vi) 17q (RP17 locus, Bardien et al. 1995)
(vii) 1q (RP18 locus, Xu et al. 1996).

Positional cloning approaches have been embarked upon in attempts to isolate the above disease-causing genes, but by the end of 1998 none

had been identified (Inglehearn 1998). The numerical designation for each locus (e.g. RP10, RP17), used by McKusick in the catalogue 'Mendelian Inheritance in Man' (McKusick 1994) and the online version (OMIM, http://www.ncbi.nlm.nih.gov/Omim/) is significant only for classification purposes and does not correlate with the chronological order of gene identification or phenotypical characteristics.

1.4.2 *rhodopsin* gene (RP4 locus)

The first gene shown to be causative of adRP was identified through a combination of forward and reverse genetics. The Humphries group in Dublin initially established linkage of an adRP gene to chromosome 3q (McWilliam et al. 1989). Later, Dryja and co-workers in Boston, who were pursuing a candidate gene approach, investigated the *rhodopsin* gene which they knew mapped to this region and found a mutation which was associated with the disorder in a group of RP patients (Dryja et al. 1990). To date, over 100 mutations in *rhodopsin* have been shown to be associated with RP and other forms of retinal degeneration. A comprehensive *rhodopsin* mutation database is available at the World Wide Web site at http://mol.ophth.uiowa.edu/MOL_WWW/Rhotab.html.

Rhodopsin is the visual pigment, which mediates dim light vision in the rod phototransduction cascade (Section 1.1.3). This molecule is formed by a complex of opsin (a protein consisting of seven transmembrane helices) and 11-*cis*-retinal (a Vitamin A-derived chromophore). Opsin and the chromophore are linked covalently via a Schiff base bond. In 1958, the photoisomerisation of retinal from 11-*cis* to the all-*trans* form was identified as the initiating event in the rod visual excitation cascade (Hubbard and Kropf 1958). The product of *rhodopsin* is 348 amino acids in length and it is a member of the ancient superfamily of G-protein coupled receptors. Rhodopsin is rod cell-specific and it is located

exclusively in the disc and plasma membranes of rod outer segments (Fig. 1.3), where it constitutes approximately 90% of the protein component. A human rod contains approximately 5 X 10^7 rhodopsin molecules, which are synthesised throughout life at a rate of 5 X 10^6 molecules/rod/day (Knowles and Dartnall 1977). This protein is produced in rod inner segments and post-translationally modified by the addition of oligosaccharides and fatty acids. It is then assembled into vesicles, transported to the connecting cilium and inserted into nascent discs.

1.4.3 *peripherin/RDS* gene (RP7 locus)

The second gene identified for adRP is *peripherin/RDS* (Farrar et al. 1991; Kajiwara et al. 1991). A mutation in this gene was first found to be responsible for retinal degeneration in the mouse and resulted in the phenotype, 'retinal degeneration slow' (Van Nie et al. 1978). The subsequent investigation of the human homologue has revealed that mutations within the gene are associated with a variety of clinically distinct retinal degenerations, including retinitis pigmentosa, adult vitelliform macular dystrophy (Wells et al. 1993), pattern macular dystrophy (Weleber et al. 1993), fundus flavimaculatus (Weleber et al. 1993), butterfly-shaped pigment dystrophy of the fovea (Nichols et al. 1993) and retinitis punctata albescens (Kajiwara et al. 1993). The comprehensive and current *peripherin/RDS* mutation database can be accessed at the World Wide Web site:
http://mol.ophth.uiowa.edu/MOL_WWW/RDStab.html.

The protein product of *peripherin/RDS* is a 346 amino acid glycoprotein consisting of four transmembrane domains. Unlike rhodopsin, peripherin/RDS is associated with the outer segment disc membranes of both rods and cones. In rods it forms a complex with another membrane-

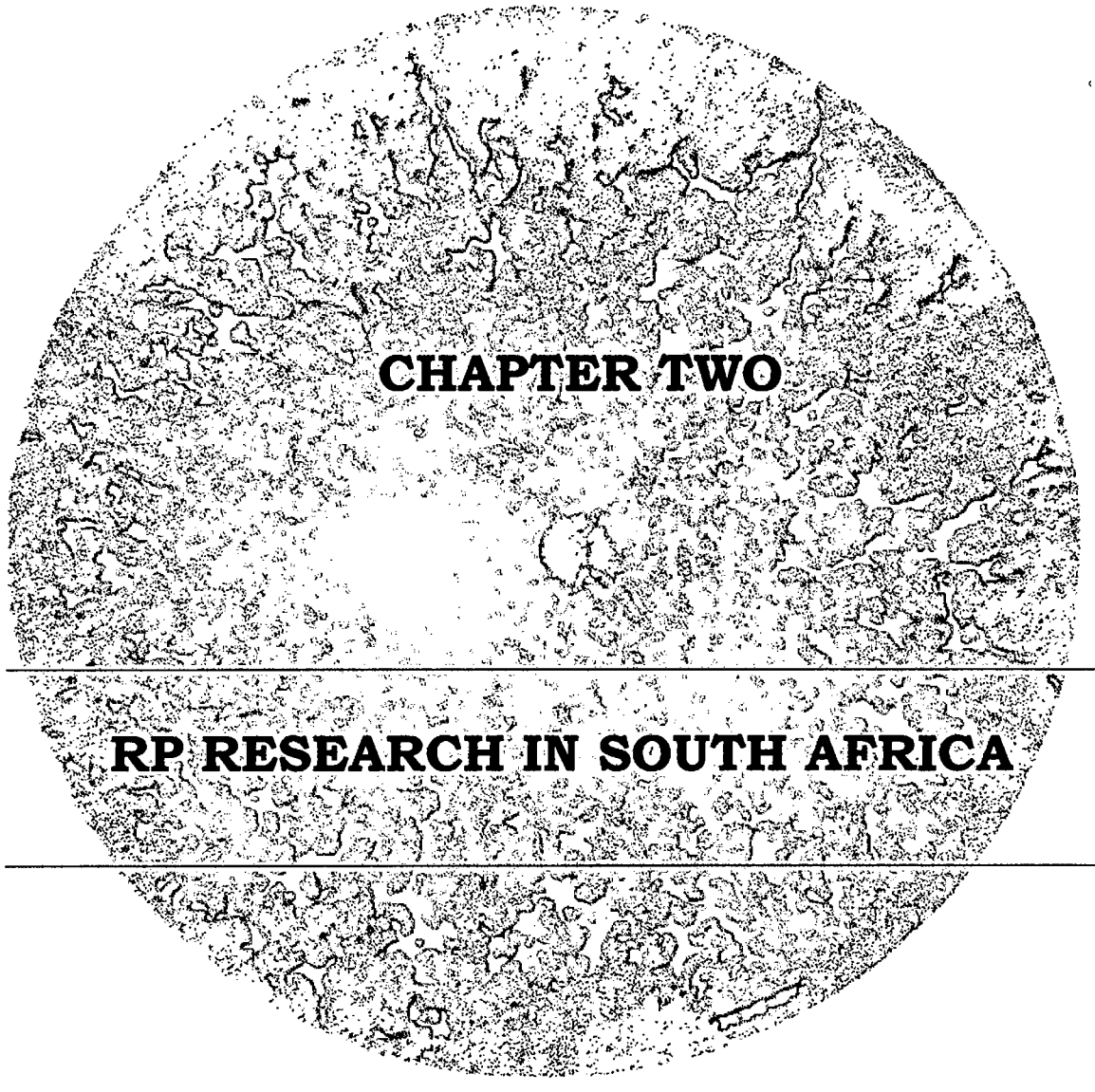
spanning protein, rod outer segment membrane protein 1 (ROM1). The two proteins are structurally similar and share about 35% homology in sequence (Bascom et al. 1992a). Currently, the exact function of peripherin/RDS is unknown but it is speculated, based on the localisation of peripherin/RDS and ROM1 to the disc rims, that these two proteins may function together as an adhesive complex for the stabilisation of the outer segment discs (Bascom et al. 1992a).

An understanding of the aetiology of retinal dystrophies has improved over the last decade with the identification of many of the causative genes, but many questions still remain unanswered eg:

- How does a mutation in the rod-specific proteins, rhodopsin and ABCR lead to the degeneration of cone cells?
- Why does an abnormality of a biochemical pathway not essential for cell survival (such as phototransduction) result in cell death?
- What is the shared pathway that culminates in retinal degeneration for different mutations in different genes?
- What are the genetic and/or environmental factors affecting clinical expression of specific pathogenic mutations.

These are some of the questions that need to be addressed before a complete understanding of the molecular patho-physiology of inherited retinal degenerations is possible.

This dissertation describes an investigation into a form of adRP, which will be discussed in detail over the next five chapters.



CHAPTER TWO

RP RESEARCH IN SOUTH AFRICA

2

RP RESEARCH IN SOUTH AFRICA

RP RESEARCH IN SOUTH AFRICA

Summary:

Chapter two discusses the development of research on RP in South Africa. An introduction is provided to the present study, which is based on the investigation of an adRP gene in a South African kindred. In this study, the experimental approach used included genetic linkage analysis, physical mapping and positional candidate gene screening. An outline to these approaches is discussed at the end of the chapter.

2.1 INTRODUCTORY BACKGROUND

Research into RP in South Africa (SA) was initiated in 1972 in the Department of Human Genetics, University of Cape Town (UCT) [hereafter referred to as the Department]. The Department, in collaboration with the Department of Ophthalmology at Groote Schuur Hospital in Cape Town, documented and maintained records concerning South African RP patients and families. In 1985, a questionnaire survey was undertaken to determine the relative frequencies of the various genetic forms of RP in SA (Oswald et al. 1985). Although preliminary, the clinical and genetic data obtained from a total of 130 affected individuals in 63 families provided a perspective of the overall situation. The proportions of the different genetic subtypes of RP were found to be 14% autosomal dominant, 9.5% autosomal recessive and 6% X-linked recessive.

The identification of the first RP gene, *rhodopsin* (Dryja et al. 1990) prompted investigations into the mapping of genes in SA RP families (Greenberg et al. 1992; Greenberg et al. 1994b). DNA banking was commenced in 1990 in the molecular genetics laboratory in the

Department of Human Genetics, with the support from the Retinal Preservation Foundation of SA (Greenberg et al. 1993). This laboratory now archives patient data and biological material from all forms of inherited retinopathies in the SA population, which comprises approximately 38 million individuals of different ethnic origins.

Approval from the Research Ethics Committee at UCT was obtained prior to commencement of this project. With informed consent, genetic nurses collect 20 to 30 ml of venous blood from potential research subjects throughout the country. The blood specimen, accompanied by a completed 'biological specimens collection form' (Appendix A) containing the relevant pedigree data, is sent to the molecular genetics laboratory in the Department for archiving. As part of the protocol, the blood which is drawn in 10 ml aliquots is processed to DNA, as described in Appendix B. Arrangements are also made for the affected and at-risk family members to have diagnostic ophthalmological investigations at a referral centre situated conveniently for the patient. These guidelines are adhered to because the collection of accurate clinical details and pedigree data is crucial for the mapping of disease-causing genes using genetic linkage analysis.

2.1.1 The current situation

By May 1999, DNA from 380 families with various hereditary retinopathies (including different genetic forms of RP, macular dystrophy, Usher syndrome etc.) had been archived. The number of families with the different genetic categories, from which DNA is currently stored for investigation, is listed in Table 2.1. The term 'sporadic' is used to describe a single RP patient with no family history of the disorder and the term 'indeterminate' was assigned to those families where, although there was more than one affected RP member, the mode of transmission

could not be deduced from the pedigree. It should be emphasised that, due to the Department's research interest in adRP, the incidence of this form (16%) presented in Table 2.1 may not be a true reflection of the frequency in the population. About a third (30%) of the families from which DNA is archived, display some form of macular dystrophy; the majority of these kindreds belong to the Afrikaner population group of SA and have an autosomal recessive form of Stargardt macular dystrophy. Genealogical information suggests that the high incidence of this type of inherited retinal degeneration may be due, in part, to a founder effect in the Afrikaner population.

Unpublished data obtained by researchers in the Department suggest that the gene pool for RP in SA is likely to vary from that in other parts of the world, which may be partly due to the Black African ethnic group in this country. This contention is supported by the fact that only 4.5% of the families listed in Table 2.1 demonstrate an X-linked mode of inheritance, as opposed to the value of 25% reported in the UK (Jay 1982). Oswald et al. (1985) postulated that a 'reversed founder effect' mechanism might be responsible for this difference. Additional evidence includes the relative low frequency of *rhodopsin* mutations in SA families when compared with the frequencies reported in overseas studies of disease gene demographics, the mapping of two novel SA adRP loci and the as yet unmapped loci in other SA adRP families.

In SA, individuals with retinal degenerative disorders can be divided into four different ethnic groups:

- Black - people of indigenous Black African origin.
- Mixed ancestry - representing an admixture of San, Khoi-Khoi, West African, Madagascan, Javanese and Western European groups.
- Caucasian - of Western European origin, mainly from Dutch, French, German and British stock
- Asian - of Asian Indian ancestry

Table 2.1a Genetic categorisation of 380 South African families with inherited retinopathies from which DNA is archived in the molecular genetics laboratory at UCT- May 1999.

Mode of inheritance	Number of families	Proportion of families (%)	Number of individuals
adRP	61	16	590
arRP	48	12	160
xlRP	17	4.5	69
Sporadic or Indeterminate	90	24	194
Usher syndrome	37	10	120
Macular dystrophies	113	30	291
Other retinopathies	14	3.5	30
TOTAL	380	100	1454

Table 2.1b Racial breakdown of the 380 South African families.

Category	No. of families	Black	Caucasian	Mixed ancestry	Asian	Unknown
adRP	61	7	43	7	1	3
arRP	48	3	28	0	4	13
xlRP	17	2	8	1	1	5
Sporadic	90	19	52	5	0	14
Usher syndrome	37	4	29	0	3	1
Macular dystrophies	113	4	90	7	0	12
Other retinopathies	14	0	11	0	0	3

2.2 THIS STUDY

2.2.1 Aim and objectives

The aim of this study is to investigate the molecular basis of adRP in a SA family of Western European descent. This family is one of the largest pedigrees from which DNA is archived in the Department, and the pedigree structure and adRP phenotype will be discussed in detail in Chapter Three.

The objectives of this study are as follows:

i) Short term application

The identification of the disease-causing gene is important, as it will allow for pre-symptomatic testing and informed genetic counselling for at-risk members from this large family with late-onset adRP; as well as other adRP families linked to this gene.

ii) Long-term application

The study could lead to an improved understanding of the pathogenic pathways or mechanisms causative of adRP and ultimately, an improved understanding of the process of vision. In addition, information gleaned about the determinant genes is necessary for the development of appropriate therapeutic modalities for the affected individuals.

It is vital to establish which genes are involved in RP in SA as this is a highly heterogeneous group of disorders and it is possible that the genes detected in local families will be different to those defined in other international studies. It therefore follows that gene-specific or mutation-specific therapeutic modalities developed in other countries may not be applicable to SA RP families.

2.2.2 The experimental approach used

There are currently two main methods for the identification of defective genes underlying hereditary disorders:

(i) Genetic linkage analysis and physical mapping (positional cloning approach)
(ii) Positional candidate gene approach.

2.2.2.1 *Genetic linkage analysis*

In linkage analysis, defective genes are located by searching for the co-inheritance of a specific DNA marker and the disease phenotype. This technique is based upon the observation that alleles of two genes or loci situated very close to each other on the same chromosome tend to co-segregate more often than would be expected by chance (Terwilliger and Ott 1994). With the discovery of highly polymorphic microsatellite DNA markers, an exhaustive investigation of the entire genome is possible using this technique.

2.2.2.2 *Physical mapping*

Physical mapping frequently follows genetic linkage analysis as the second step towards the identification of the defective gene. Linkage studies usually localise the gene to a chromosomal region, which may contain several million base pairs and it is therefore necessary to create a library of smaller overlapping DNA fragments (a contig), which collectively provides complete representation of the region of interest.

The DNA sequence in each of these fragments could then be assayed for gene transcription by a variety of techniques. A search for exon/intron boundaries or hypomethylated sites (CpG islands) may indicate the presence of a coding sequence. Alternatively, the sequences can be

compared to those of known evolutionarily conserved genes in other species. Irrelevant genes may be found during this procedure and it is necessary to determine which of the potential genes exhibit the tissue expression pattern consistent with the disease phenotype, through techniques such as Northern hybridisation. Transcripts of these genes are more likely to be derived from the mutant gene in question. Finally, it is important to identify a mutation in the gene that is present in all the affected individuals in the family. The putative pathogenic mutation should not be found in s significant sample of the relevant normal population in order to rule out a polymorphism.

While genetic linkage analysis and physical mapping used in concert have been successfully employed to clone genes, the latter approach is laborious and time consuming. Many factors may hinder the isolation of the defective gene from a genetically defined region e.g. the complexity of the disease phenotype, the frequency of the disorder in the general population and the structure of the gene. The problems in implementing this method are highlighted by the fact that it has not been completed successfully for any of the adRP genes assigned by linkage thus far. Despite the difficulties associated with the positional cloning approach, it is inherently valuable because it makes no prior assumptions about the genes which are involved. For this reason, this strategy could identify genes encoding proteins in novel biochemical pathways that may otherwise never be discovered using the candidate gene approach.

2.2.2.3 *Positional candidate gene approach*

This strategy has the potential to reveal the defective gene without exhaustive cloning. In this approach, genetic databases and the literature are surveyed for previously characterised genes and candidates are selected, based on the following guidelines:

- The biological function of the product of the candidate gene should be logically related to the disease process.

- The candidate should reside in the particular chromosomal region to which the defective gene has been mapped.
- Markers situated within or close to the gene should exhibit tight linkage to the disease phenotype with no recombinations.
- The candidate gene product should be expressed in the appropriate cells and tissues related to the disease phenotype.

Affected persons and their families could then be studied for genetic linkage to the candidate gene(s), or the genes could be directly sequenced from the affected individual's DNA. Currently, the small number of known genes in the candidate chromosomal regions limits this strategy. Nevertheless, the Human Genome Project (an international collaboration aimed at mapping genes and sequencing the entire human genome) should increase the number of mapped genes thereby facilitating this approach.

Both these approaches have been relatively successful in the cloning of genes e.g. the dystrophin gene causative of Duchenne muscular dystrophy was isolated using positional cloning (Kunkel 1986) and the fibrillin gene responsible for Marfan syndrome was identified using the candidate gene method (Peltonen and Kainulainen 1992). In the present investigation, both strategies were utilised in an attempt to optimise efforts to identify the adRP gene.

3

MAPPING OF THE GENE UNDERLYING adRP IN A LARGE SOUTH AFRICAN KINDRED

MAPPING OF THE GENE UNDERLYING adRP IN A LARGE SOUTH AFRICAN KINDRED

Summary:

The adRP phenotype in the family on which this study is based is presented in this chapter. Genetic linkage analysis was used to map the adRP gene in this kindred to chromosome 17q22. Further investigations revealed that the disease phenotype in a second SA adRP family was linked to the same locus. The construction of haplotypes in conjunction with multipoint linkage analyses fine-mapped the locus to a 1 cM region. This critical interval resides between the anonymous microsatellite markers D17S948 and D17S1604.

3.1 INTRODUCTION

The assumption that only retina-specific genes would be responsible for retina-specific disorders has now been discarded with the discovery of some of the causative genes. One example is the product of the *TIMP3* gene, causative of Sorsby fundus dystrophy, which is expressed not only in the retina but also in many other tissues and organs (Weber et al. 1994). The *RPGR* gene product associated with xlRP is also ubiquitously expressed and is found at surprisingly low levels in retina and RPE cDNA libraries (Meindl et al. 1996). Another example is the *ABCR* gene product, causative of Stargardt macular dystrophy, which is mainly found in rod cells even though the disorder primarily affects cone cells (Allikmets et al. 1997). These observations indicate that future research efforts into the identification of genes causative of inherited retinopathies need to be broad-based and with as few assumptions about the patho-physiology as possible.

Many of the genes causative of genetic disorders have been identified with the classical positional approach which involves isolation of the gene using only knowledge about its sub-chromosomal location. Following the cloning of the gene, further studies can be undertaken to determine its function. This process was originally referred to as 'reverse genetics' (Ruddle 1984). Usually, the first phase in this strategy is to enlist large affected pedigrees with multiple meioses which are then examined using genetic linkage analysis.

3.1.1 Genetic linkage analysis

This technique facilitates the identification of the chromosomal location of a gene without any prior knowledge of its function or any hypothesis concerning its role in the pathogenesis of the disorder. In order to map the defective gene, a search is undertaken for the co-inheritance of a specific genetic marker with the clinical phenotype of the condition within a family.

3.1.1.1 *Microsatellite DNA markers*

In the past, human genetic markers included:

- blood groups (about 20 loci)
- electrophoretic mobility variants of serum proteins (about 30 loci)
- human leukocyte antigen (HLA) tissue types (one linked set)
- DNA restriction fragment length polymorphisms (RFLPs, > 10^5)
- DNA mini and microsatellites (> 10^4) [Strachan and Read 1996].

The relatively recent discovery of the highly polymorphic microsatellite DNA families (Weber and May 1989) has made genetic linkage analysis more powerful and informative than was previously possible with RFLPs.

The microsatellite DNA families consist of small arrays of tandem repeats which are simple in sequence (usually 1 - 4 bp) and are widely dispersed throughout the genome. Of the mononucleotide repeats, runs of A and of T are very common. A class of dinucleotide repeats designated $(CA)_n$ which constitutes one of the most abundant families of human repetitive DNA (approximately 50 000 to 100 000 repeats blocks) was identified in the 1980s. Uniform spacing of these repeats would place them roughly every 30 - 60 kb in the genome. The *Alu* repeat is the most abundant sequence in the human genome, with a copy number of 750 000. The full-length *Alu* repeat is roughly 280 bp long and is usually flanked by short direct repeats.

The function of microsatellite DNA families is currently unknown, but they may play a role in gene regulation or act as hot spots for recombination (Slightom et al. 1980; Hamada et al. 1984). The $(CA)_n$ repeats vary in length among individuals, but are inherited in a mendelian manner and can therefore be used as markers in exhaustive searches of the entire genome to localise disease-causing genes.

3.1.1.2 *Recombination events*

The basis of linkage analysis is that recombination events occur between two genetic loci (a DNA marker and a phenotypic trait) at a rate proportional to the distance between them (Terwilliger and Ott 1994). Two loci that are situated physically very close to each other tend to be co-inherited but as the distance between them increases, the creation of new combinations of alleles by recombination becomes more likely. Recombination occurs during meiosis when two homologous chromosomes line up on the spindle and exchange DNA segments by a process termed "crossing over". This event occurs between precisely corresponding sequences, so that no base pairs are added to or lost from the recombinant chromosome.

The probability of a recombination event occurring between two loci is termed the "recombination fraction" (denoted θ). This expression ranges

in value from 0.00 (for loci next to each other) to 0.5 (for unlinked loci situated either far apart or on different chromosomes). A genetic map distance of 1 centiMorgan (cM) corresponds to a recombination fraction of 0.01. At low values of the recombination fraction, the correlation between cM and θ is close. At higher values, however, this agreement is no longer valid because double crossovers are more likely to occur at larger map distances, thereby negating the effects of the initial crossover event. The Haldane or Kosambi map functions may then be used to transform the larger recombination fraction values into the equivalent cM value (Ott 1991). Haldane takes account of double crossovers but does not allow for crossover suppression, Kosambi does both.

3.1.1.3 *Calculation of lod scores*

In linkage analysis a calculation is made of the **probability** of an observed association between the inheritance of a specific DNA marker allele and the presence of a phenotypic trait. In essence, a comparison is made of the probability that the observed distribution of alleles would arise under the hypothesis of linkage (i.e. $0.00 < \theta < 0.5$) to the probability that this distribution would occur randomly (i.e. $\theta = 0.5$). The ratio of these two possibilities is the odds ratio (L). For convenience, L is converted to a decimal logarithm termed a lod score ("log of the odds"). An odds ratio of 100:1 is therefore equivalent to a lod score of 2.

The formula for the lod score (Z) is as follows:

$$Z(\theta) = \log_{10} \frac{L(0.00 < \theta < 0.5)}{L(\theta = 0.5)}$$

Lod scores are a function of the recombination fraction and are calculated for a range of θ ranging from 0.00 to 0.5. All lod scores are zero at $\theta = 0.5$, since they are then measuring the ratio of two identical probabilities, and $\log_{10}(1) = 0$.

Computer programs such as LINKAGE which implement the Elston-Stewart algorithm (Terwilliger and Ott 1994) can be used to evaluate the likelihood of linkage of a given pedigree under different assumptions about the recombination fraction between the two loci. By convention, odds of more than 1000 to 1 (lod score of > 3) are necessary to prove linkage and odds of less than 1 to 100 (lod score of < -2.00) are sufficient to reject the hypothesis of linkage. Values of Z between -2 and +3 are inconclusive. Once a disease gene is localised to a specific chromosomal region with sufficient precision, the application of other techniques such as positional cloning or the candidate gene approach can be used to lead to its eventual identification.

3.1.2 This study

3.1.2.1 *The South African adRP families*

In this investigation, a genetic linkage analysis was utilised in an attempt to localise the gene responsible for the adRP phenotype in a large South African kindred. This family, designated RPD8, was chosen for study since it is one of the largest families from which DNA is archived in the molecular genetics laboratory of the Department of Human Genetics, UCT. Family RPD8 is thought to have descended from a single ancestor from Hesse (a state in Western Europe, now Germany) who settled in South Africa in 1762. Most of the SA descendants live in the Gauteng and Free State Provinces of SA.

Following the initial localisation of the adRP locus in family RPD8, the other large SA adRP families from which DNA had been banked, were investigated and a second family, designated RPD19, was linked to the same locus. This family is from the mixed ancestry population group and the majority of family members are resident in Cape Town in the Western Cape Province of SA. The pedigree structure and ophthalmological

phenotype of families RPD8 and RPD19 will be discussed in Section 3.2.1.

3.1.2.2 *Experimental strategy*

Random genome-wide screens using genetic linkage analysis require the use of DNA markers spaced at intervals not greater than 20 cM across the genome. A minimum of 200 markers is needed for the first line or initial screening which implies that this approach is rather time-consuming and laborious. In order to optimise efforts to map the adRP gene with the laboratory's limited resources, a combination of genetic linkage analysis and candidate gene screening was embarked upon in this study. For retinal degenerative disorders, candidate genes include those which code for components involved in the light-activated rod phototransduction cascade, vitamin A (retinol) metabolism and the structure of the disc membrane. Candidate genes were chosen based on these criteria, and microsatellite markers situated in close proximity to the candidates were investigated for linkage to the adRP phenotype in family RPD8.

3.2 PATIENTS, MATERIALS AND METHODS

3.2.1 Patient recruitment

All procedures used were reviewed and approved by the Research Ethics Committee at UCT (REC reference 044/95, March 1995). At the start of this investigation, the DNA of 29 members from family RPD8, including 18 adRP affected individuals, was collected and available for study (Fig. 3.1). Later in the investigation, an additional 15 family members were recruited and genotyped for inclusion in the analysis. The phenotypic expression of the disorder is consistent in the kindred and there is no evidence of non-penetrance. In most family members, the age of onset of

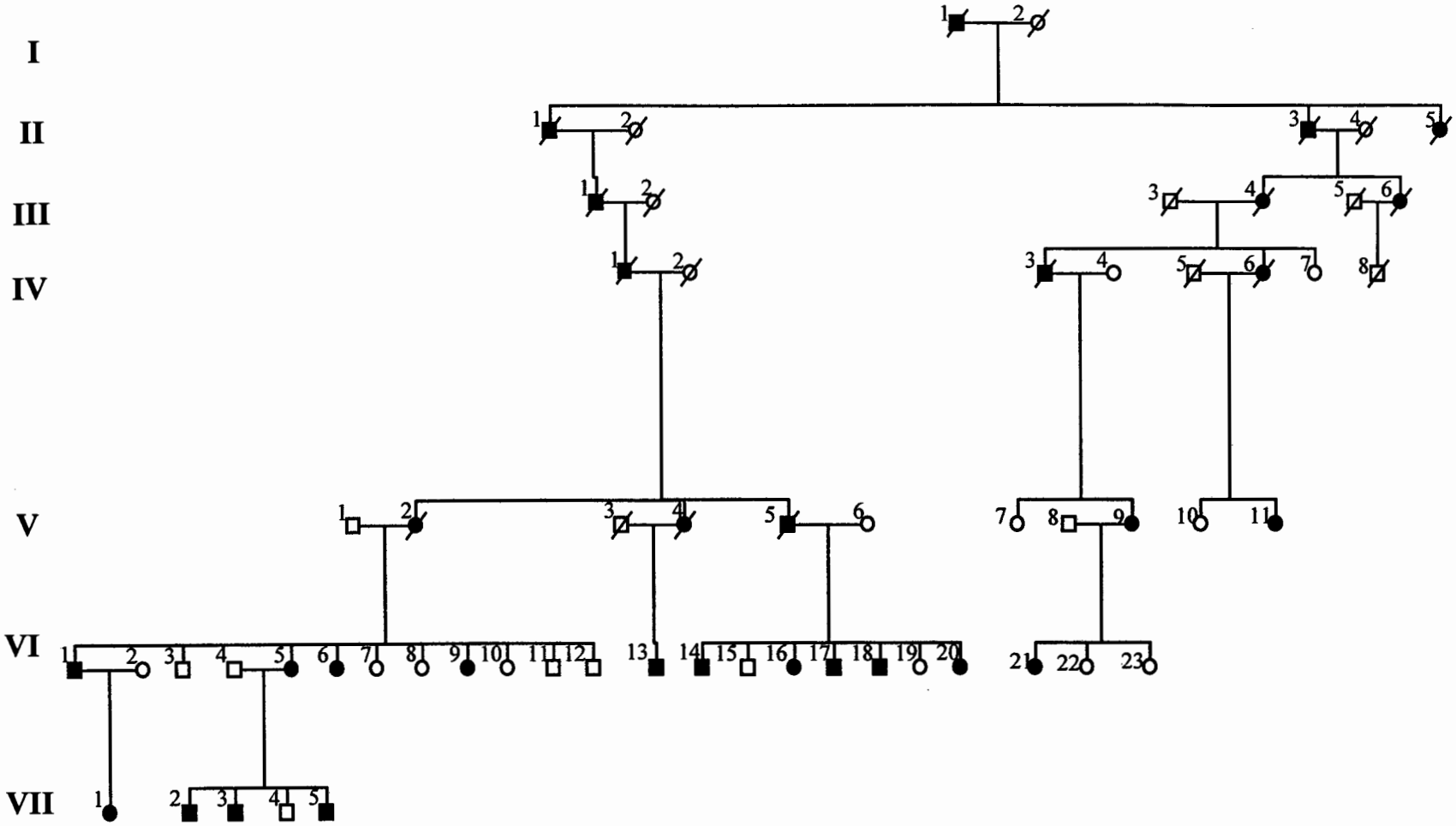


Figure 3.1 Abridged pedigree of a South African adRP family designated RPD8.

Legend: □ - male,
○ - female
■ ● - affected adRP individual
⊘ ∅ - deceased individual

visual disturbance is between 20 and 30 years of age with night blindness often being the first manifestation. Family RPD19 was later included in the linkage analysis and comprised 25 members, including nine affected individuals.

With informed consent, 20 ml of venous blood was collected from every individual who participated in the study. Genomic DNA was extracted from peripheral blood lymphocytes by standard techniques as described in Appendix B. The pedigree relationships in both families were confirmed by the appropriate segregation of all the tested microsatellite markers.

Ophthalmological phenotype

In order to emphasise the diagnostic status of the disorder in families RPD8 and RPD19, ophthalmological reports concerning two adRP affected members from each family are presented below.

i) Family RPD8: a 71-year-old female (individual IV-6, Fig. 3.1, page 41).

[Report dated 1986; Dept. of Ophthalmology, University of Witwatersrand]

This patient suffered from poor night vision from the age of 20 years. When examined in late adulthood, her visual fields were extinguished. Her fundi showed pigmentary changes, very atrophic retina, pale optic discs and narrow blood vessels. Electroretinogram (ERG) studies showed absent blue, red and white photopic and scotopic responses as well as an absent flicker. These ERG findings were consistent with the diagnosis of retinitis pigmentosa.

Fundus photographs of this individual are presented in Fig. 3.2.

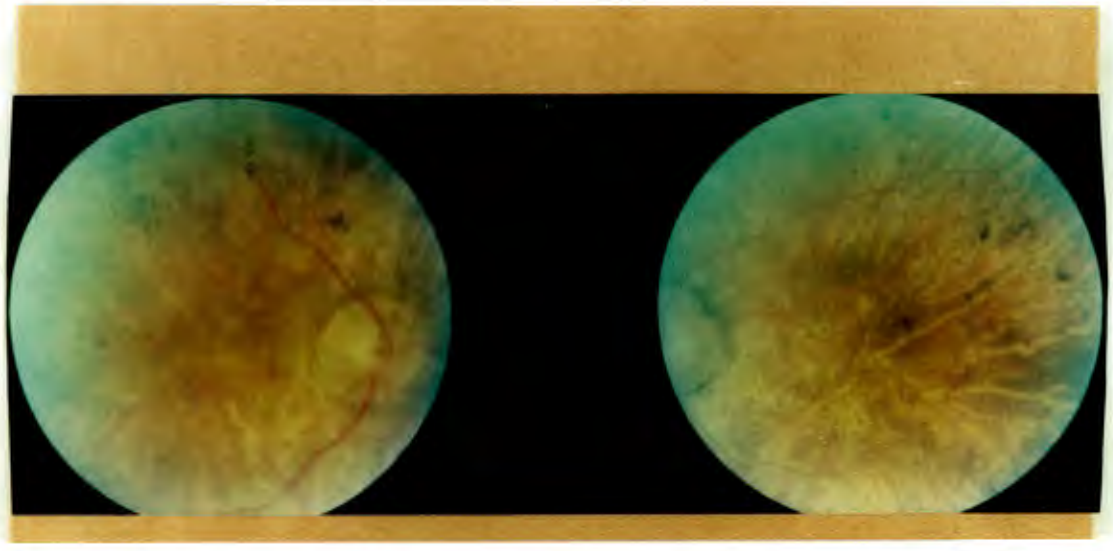


Figure 3.2 Fundus photographs of an RP affected individual from family RPD8.

ii) Family RPD8: a 53-year-old male (individual V-15, Fig. 3.8, page 63).

[Report dated 30 November 1995; Retinitis pigmentosa clinic, Groote Schuur Hospital, Cape Town]

"He has a well documented history of autosomal dominant retinitis pigmentosa with visual loss commencing in the 20's - 30's. He is at present aged 53 years and has noticed reduced visual fields and some reduction in central vision. On examination his corrected visual acuity measured 6/24 on the right and 6/18 on the left. No abnormalities of the anterior segments were noted including no cataract formation. On dilated fundal examination the typical features of retinitis pigmentosa were noted, (viz. waxy pallor of the optic nerve heads, retinal vascular attenuation, moderate clumping of the retinal pigment epithelium in the mid-periphery extending to the equator, some retinal pigment epithelial atrophy underlying the macular and the retinal periphery). There is an early epiretinal membrane overlying both foveas. In summary [this patient] has an autosomal dominant diffuse retinitis pigmentosa."

iii) Family RPD19: a 44-year-old female (individual IV-2, Fig. 3.7, page 62).

[Report dated 7 November 1997; Retinitis pigmentosa clinic, Groote Schuur Hospital, Cape Town]

"She has a family history of autosomal dominant retinitis pigmentosa. She first noticed decreased visual acuity at the age of 28 with constriction of the peripheral fields and night blindness. On examination her best corrected visual acuities were 6/12 that improved to 6/9 with pinhole on the right and 6/9 on the left. Ishihara colour vision charts were 4/14 on the right and 6/14 on the left. She had macular threshold fields done to both eyes, which showed severe constriction of both visual fields, more so on the right. A dilated fundal examination revealed the typical

changes of retinitis pigmentosa. She has waxy pallor optic discs, and diffuse equatorial bone spicules. Her peripheral retina were flat and featureless, with areas of atrophy. She has perifoveal RPE atrophy. [This patient] has autosomal dominant retinitis pigmentosa with good central vision, but severely restricted, peripheral vision."

iv) Family RPD19: a 37-year-old female (individual IV-14, Fig. 3.7, page 62).
[Report dated 15 September 1995; Retinitis pigmentosa clinic, Groote Schuur Hospital, Cape Town]
"She has a family history of retinitis pigmentosa in that 2 of her brothers, her father and her paternal grandmother were affected by the disorder. She complains of poor nocturnal vision but is managing well by day. She noticed change in her vision from the age of 7 but was diagnosed with retinitis pigmentosa at the age of 22. On examination her best corrected visual acuities measured 6/36 on the right and 6/24 on the left. No cataracts were present. On dilated fundal examination she was noted to have the typical features of retinitis pigmentosa including waxy pallor of the optic nerve heads, attenuation of the retinal vasculature, diffuse retinal pigment epithelial atrophy, bone spicule pigmentary deposits in all 4 quadrants of the mid peripheral retina, and some slight retinal pigment epithelial atrophy underlying both maculae. On the left side she also had some foveal scarring."

3.2.2 Genetic linkage analysis

3.2.2.1 *Candidate gene loci*

A screen of the seven adRP loci known at the time (viz. *rhodopsin*, *peripherin/RDS* and the anonymous loci on 8q, 7p, 7q, 19q and 17p) were the obvious candidate loci at which to initiate the genetic linkage

analysis. Subsequently, seven additional candidate gene loci were investigated for linkage (Table 3.1). These loci included components of the rod phototransduction cascade viz. the alpha (*PDEA*; Pittler et al. 1990), beta (*PDEB*; Altherr et al. 1992) and gamma (*PDEG*; Dollfus et al. 1993) subunits of *PDE*. To date, several members of this pathway, including *rhodopsin*, *PDEA*, *PDEB* and the alpha subunit of the rod cGMP-gated channel gene are known to be causative of various retinal dystrophies (Gregory-Evans and Bhattacharya 1998). Also chosen for study were the locus for Best macular dystrophy (Nichols et al. 1994), the locus for Usher syndrome Type IC (Keats et al. 1994), the neural retina-specific leucine zipper gene (*NRL*; Yang-Feng and Swaroop 1992) and the rod outer segment membrane protein 1 (*ROM1*) gene (Bascom et al. 1992b).

All the available family members of RPD8 were genotyped with microsatellite DNA markers situated in close proximity to the 14 selected candidate gene loci. Two to three markers were chosen per locus (Table 3.1) from the Genethon human genetic linkage map which had been constructed using the Centre d'Etudes du Polymorphisme Humain (CEPH) reference families. The RHO-CA marker is an intragenic CA repeat within *rhodopsin* (Weber and May 1989) and a set of primers for this marker were synthesised at the Department of Biochemistry, UCT. All the other microsatellite markers listed in Table 3.1 were obtained from Research Genetics, Inc (USA).

The *rhodopsin* locus was further investigated in family RPD8 using standard single strand conformation polymorphism (SSCP) techniques according to the method of Orita et al. 1989 (Appendix B). The primers used to amplify exon 3 of *rhodopsin* are: forward 5'-TTg gCT gTT CCC AAg TCC CT-3' and reverse 5'-TCC AgA CCA Tgg CTC CTC CA-3' (Inglehearn et al. 1992).

Table 3.1 Markers used to test linkage of adRP in family RPD8.

Candidate	Chromosome	DNA marker	Heterozygosity
rhodopsin	3q	D3S621 RHO-CA	0.79 0.34
peripherin/RDS	6p	D6S260 D6S291 D6S257	0.84 0.70 0.86
RP1 locus	8q	D8S166 D8S165 D8S285	0.88 0.54 0.79
RP9 locus	7p	D7S460 D7S484 D7S435	0.95 0.75 0.59
RP10 locus	7q	D7S480 D7S496 D7S487	0.87 0.76 0.75
RP11 locus	19q	D19S180 D19S214 D19S254	0.72 0.64 0.78
RP13 locus	17p	D17S849 D17S796 D17S938	0.68 0.82 0.90
Best disease locus	11q	D11S937 D11S911 INT2	0.88 0.86 0.85
Usher syndrome type IC	11p	D11S902 D11S904	0.81 0.83
NRL locus	14q	D14S64 D14S50 D14S70	0.77 0.77 0.77
ROM1 locus	11q	D11S956 D11S916	0.88 0.74
PDEA locus	5q	D5S403 D5S393 D5S210	0.65 0.84 0.75
PDEB locus	4p	D4S227 D4S403	0.73 0.78
PDEG locus	17q	D17S798 D17S800 D17S809	0.80 0.74 0.72

A total of 29 highly polymorphic markers was used to investigate linkage to chromosome 17q in families RPD8 and RPD19 (Table 3.2). The Genethon chromosome 17 genetic linkage map (Fig. 3.3) illustrates the most likely order and the sex average recombination fractions calculated between adjacent markers.

3.2.2.2 *Polymerase chain reaction*

Genotypes were interpreted from the polymerase chain reaction (PCR) amplification of alleles from the microsatellite repeat polymorphisms. Detection of PCR products was achieved by two methods:

i) *Radioactive labelling*. The forward primer was first end-labelled using T4 polynucleotide kinase and [χ^{32}P]ATP before being added to the PCR reaction. The standard PCR reaction was performed in a 10 µl volume containing 200 ng genomic template DNA, 2.5 pmol of each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 250 µM of each dNTP and 0.6 units (U) of *Taq* DNA polymerase (Promega, USA or GIBCO/BRL, USA). Amplification was carried out by denaturing at 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, 55°C (50°C for some primers) for 1 min and a final extension of 7 min at 72°C on an Omnigene Thermal Cycler (Hybaid, USA). An overlay of liquid paraffin was added to each sample to prevent evaporation during the PCR reaction.

Three microlitres of formamide loading dye (Appendix C) was added to 3 µl of the PCR product and denatured on a heating block at 95°C for 5 min. The samples were placed on ice before loading on 6% denaturing polyacrylamide gels (Appendix C). The gels were electrophoresed at 60 - 70 watts (W) in a 1X TBE running buffer, using the marker dyes in the formamide loading dye to determine at which point the repeat fragments

Table 3.2 The 29 polymorphic microsatellite markers used to test linkage of adRP to chromosome 17q.

cen

DNA marker	Heterozygosity
D17S798	0.80
D17S800	0.73
D17S934	0.84
D17S809	0.70
D17S790	0.77
D17S787	0.81
D17S1607	0.66
D17S1606	0.88
D17S957	0.44
D17S1604	0.89
D17S923	0.45
D17S792	0.42
D17S948	0.82
D17S944	0.75
D17S1835	0.70
D17S808	0.67
D17S924	0.63
D17S794	0.74
D17S1874	0.75
D17S807	0.85
D17S942	0.66
D17S795	0.80
D17S789	0.82
D17S840	0.63
D17S949	0.80
D17S929	0.52
D17S785	0.83
D17S939	0.83
D17S784	0.77

tel

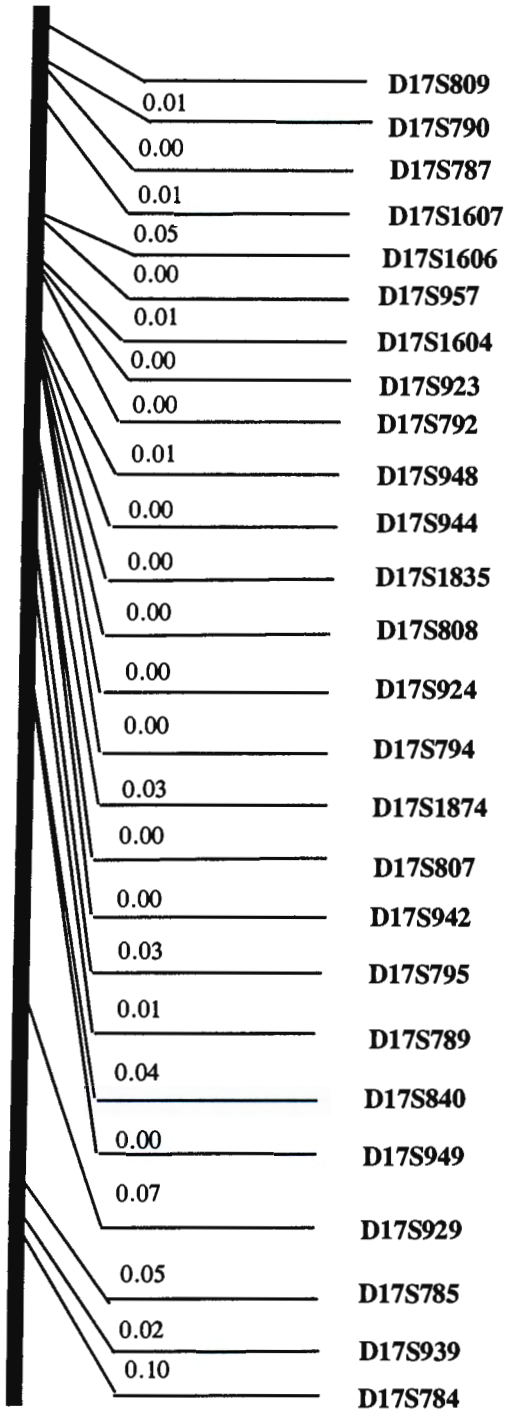


Figure 3.3 Abridged diagram of the CEPH/Genethon human genetic linkage map on part of chromosome 17q (January 1996).

would be fully resolved. At the end of the run, the gels were transferred onto Whatmann 3MM paper, dried on a Hoeffer Slab Gel Dryer at 80°C for 1 hour and exposed to X-ray film (Agfa Curix, Germany) overnight (O/N) at -70°C, with intensifying screens. The following day, the X-ray film was developed in Cronex developer and fix solutions (Protea, South Africa) and the genotypes ascertained.

ii) Fluorescent labelling. Genomic template DNA was amplified in 10 µl reactions containing 3.5 ng of each primer (the 5' end of one primer was labelled with a fluorescent dye), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 µM of each dNTP and 0.6 U of *Taq* DNA polymerase (Promega, USA). Amplification conditions were the same as for radioactively labelled PCR. The amplification products were separated on 6% polyacrylamide gels on an Applied Biosystems Inc. Model 373 DNA Sequencing System (USA) according to the manufacturer's instructions. GENESCAN 672 software allowed automated laser scanning and detection of fluorescent PCR products and eliminated the need for post-run gel handling.

3.2.2.3 *Two point lod scores*

Two-point lod scores were calculated using the LINKAGE package of programs, version 5.1 and the MLINK and ILINK core programs (Lathrop et al. 1984), from data prepared by the LINKSYS data management package (Attwood and Bryant 1988). Male and female recombination rates were taken to be equal. The oldest age of onset of the disorder in family RPD8 was 40 years and for this reason, asymptomatic individuals younger than 40 years were omitted from the analysis. A full penetrance and a gene frequency of 0.0001 for the disease locus were assumed for the computation of all lod scores.

3.2.2.4 *Multipoint linkage analysis*

Multipoint linkage is usually performed to locate a disease locus on a fixed framework of markers. This analysis was attempted because linkage analysis can be more efficient if data for more than two loci are analysed simultaneously. Several overlapping four-point analyses (three markers and the disease locus) were calculated, computing the lod score at each map point within each interval. This multipoint analysis was performed using LINKMAP (Lathrop et al. 1984) and run at the Human Genome Mapping Project Resource Centre's computing facilities in London, UK and the VAX at the University of Cape Town, SA.

3.2.3 Construction of haplotypes

Recombination events rarely separate loci which lie very close together, therefore sets of alleles on the same small chromosomal segment tend to be transmitted as a block (or haplotype) through a pedigree. Haplotypes can be treated for mapping purposes as alleles at a single highly polymorphic locus. A subset of polymorphic microsatellite markers situated close together on chromosome 17q were used for the construction of haplotypes for members of families RPD8 and RPD19.

3.3 RESULTS

3.3.1. Exclusion of 14 candidate loci

All the available family members of family RPD8 were genotyped with 38 polymorphic markers from a total of 14 candidate gene loci. Two point lod score analyses between each marker and the disease phenotype are presented in Table 3.3. Significantly negative lod scores of Z less than -2.00, providing evidence for exclusion, were obtained for all the candidate loci except *rhodopsin*. The family was uninformative for the two markers chosen at this locus viz. RHO-CA and D3S621. Further

Table 3.3 Two point lod scores (Z) calculated between adRP in family RPD8 and microsatellite markers situated close to candidate gene loci.

		RECOMBINATION FRACTION (θ)					
Gene	**Marker**	**0.001**	**0.05**	**0.10**	**0.15**	**0.20**	**0.25**
rhodopsin	D3S621	-0.97	-0.60	-0.38	-0.25	-0.15	-0.09
	RHO-CA	0.18	0.15	0.12	0.10	0.07	0.05
peripherin/RDS	D6S260	**-16.3**	**-5.16**	**-3.11**	**-1.99**	-1.28	-0.82
	D6S291	**-15.63**	**-4.44**	**-2.59**	-1.62	-1.01	-0.61
	D6S257	**-16.70**	**-2.58**	-1.20	-0.56	-0.23	-0.09
RP1 locus	D8S166	**-12.46**	-1.90	-1.11	-0.78	-0.62	-0.52
	D8S165	0.49	0.50	0.47	0.42	0.36	0.30
	D8S285	**-6.77**	-1.40	-0.51	-0.13	0.04	0.10
RP9 locus	D7S460	0.49	0.50	0.47	0.42	0.36	0.30
	D7S484	**-14.53**	**-3.95**	**-2.53**	-1.71	-1.16	-0.78
	D7S435	**-19.00**	**-3.87**	**-2.30**	-1.43	-0.89	-0.54
RP10 locus	D7S480	**-2.30**	**-2.30**	0.59	0.62	0.59	0.54
	D7S496	**-11.93**	-1.42	-0.39	0.05	0.23	0.29
	D7S487	**-10.85**	-1.58	-0.54	-0.07	0.16	0.26
RP11 locus	D19S180	**-14.24**	**-5.15**	**-2.90**	-1.74	-1.04	-0.60
	D19S214	**-6.16**	-1.62	-1.07	-0.79	-0.60	-0.46
	D19S254	**-14.35**	**-3.31**	-1.76	-0.99	-0.55	-0.29
RP13 locus	D17S849	**-15.20**	**-3.78**	**-2.42**	-1.67	-1.19	-0.84
	D17S796	-∞	-0.47	-0.03	0.14	0.22	0.24
	D17S938	-∞	0.31	0.66	0.75	0.74	0.66
Best macular dystrophy	D11S937	**-15,15**	**-3.84**	**-2.15**	-1.26	-0.72	-0.38
	D11S911	**-17.70**	**-3.42**	-1.73	-0.87	-0.38	-0.10
	INT2	**-21.86**	**-4.83**	**-2.88**	-1.84	-1.20	-0.77
Usher Type 1C	D11S902	**-13.20**	**-4.08**	**-2.47**	-1.56	-0.99	-0.61
	D11S904	**-17,89**	**-4.45**	**-2.71**	-1.76	-1.15	-0.73
NRL	D14S64	**-14.20**	-1.35	-0.12	0.41	0.64	0.70
	D14S50	**-17.82**	**-2.65**	-1.16	-0.44	-0.05	0.17
	D14S70	**-12.39**	**-2.15**	-0.95	-0.38	-0.09	0.06
ROM1	D11S956	**-18.88**	**-4.94**	**-3.04**	**-2.06**	-1.46	-1.06
	D11S916	**-17.82**	**-2.40**	-0.86	-0.14	0.28	0.38
PDEA	D5S403	**-21.30**	**-7.32**	**-4.84**	**-3.45**	**-2.51**	-1.81
	D5S393	**-20.90**	**-6.53**	**-4.20**	**-2.93**	**-2.09**	-1.49
	D5S210	**-10.70**	-1.01	-0.27	0.06	0.20	0.23
PDEB	D4S227	**-13.16**	**-3.78**	-1.89	-0.94	-0.39	-0.08
	D4S403	**-17.28**	**-5.15**	**-2.95**	-1.81	-1.11	-0.66
PDEG	D17S798	**-10.24**	**-3.32**	-1.89	-1.14	-0.67	-0.36
	D17S800	**-8.68**	-1.77	-0.75	-0.26	-0.00	0.11

Lod scores < -2.00 which are indicative of exclusion, are shown in bold type.

investigation of *rhodopsin*, using SSCP analysis revealed a neutral polymorphism in exon 3 of the gene, which was informative in a nuclear section of family RPD8 (data not shown). A recombination event was detected thereby excluding *rhodopsin* from being the gene causative of the disorder.

The results presented in Table 3.3 provide evidence for the exclusion of the involvement of all seven known adRP loci in this family. Hence, the existence of a novel adRP locus for family RPD8 was firmly established.

3.3.2 Linkage to chromosome 17q

Evidence of linkage in family RPD8 was first detected when a marker on chromosome 17q, situated in close proximity to the *PDEG* gene, was genotyped in the family. A promising lod score of $Z = 1.91$ was obtained at $\theta = 0.15$ for the marker D17S809. In order to further investigate this tentative linkage result, the kindred was genotyped with additional markers situated approximately 15 cM distal and proximal to D17S809.

Significantly positive lod scores indicative of linkage were obtained and no recombination events were observed between the disorder and the following markers: D17S790, D17S787, D17S808 and D17S807 (Table 3.4). Flanking recombinants were detected by D17S809 and D17S942, which provisionally places the adRP locus in this interval. In order to locate the disease locus on the framework of microsatellite markers, multipoint analysis was performed using D17S808, D17S807, D17S942 and D17S789; this yielded a maximum lod score of 8.28 for the adRP locus between D17S808 (proximal) and D17S807 (distal) as shown in Fig. 3.4. This was the eighth locus identified for adRP (Bardien et al. 1995) and has been designated RP17 (MIM 600852).

Table 3.4 Two point lod scores (Z) between adRP and markers on chromosome 17q. Maximum lod scores at corresponding recombination fractions are shown in bold type.

	RECOMBINATION FRACTION (θ)								
DNA marker	**0.00**	**0.01**	**0.05**	**0.10**	**0.20**	**0.30**	**0.40**	Z_{max}	θ_{max}
cen									
D17S809	-∞	-0.31	1.42	1.85	1.76	1.23	0.52	**1.90**	0.13
D17S790	0.32	0.31	0.28	0.23	0.15	0.09	0.04	**0.32**	0.00
D17S787	1.23	1.20	1.08	0.97	0.77	0.55	0.30	**1.23**	0.00
D17S808	4.63	4.53	4.14	3.63	2.56	1.46	0.44	**4.63**	0.00
D17S807	5.69	5.62	5.26	4.75	3.61	2.38	1.09	**5.69**	0.00
D17S942	-∞	4.07	4.38	4.17	3.39	2.36	1.14	**4.38**	0.04
D17S795	-∞	2.98	3.36	3.23	2.61	1.78	0.80	**3.36**	0.05
D17S789	-∞	5.31	5.47	5.09	3.96	2.65	1.24	**5.52**	0.03
D17S929	-∞	-4.39	-1.77	-0.79	-0.05	0.17	0.16	**0.19**	0.34
D17S785	-∞	-1.68	-0.41	0.03	0.31	0.31	0.20	**0.33**	0.25
tel									

Asymptomatic individuals below 40 years of age were omitted from the lod score analysis.

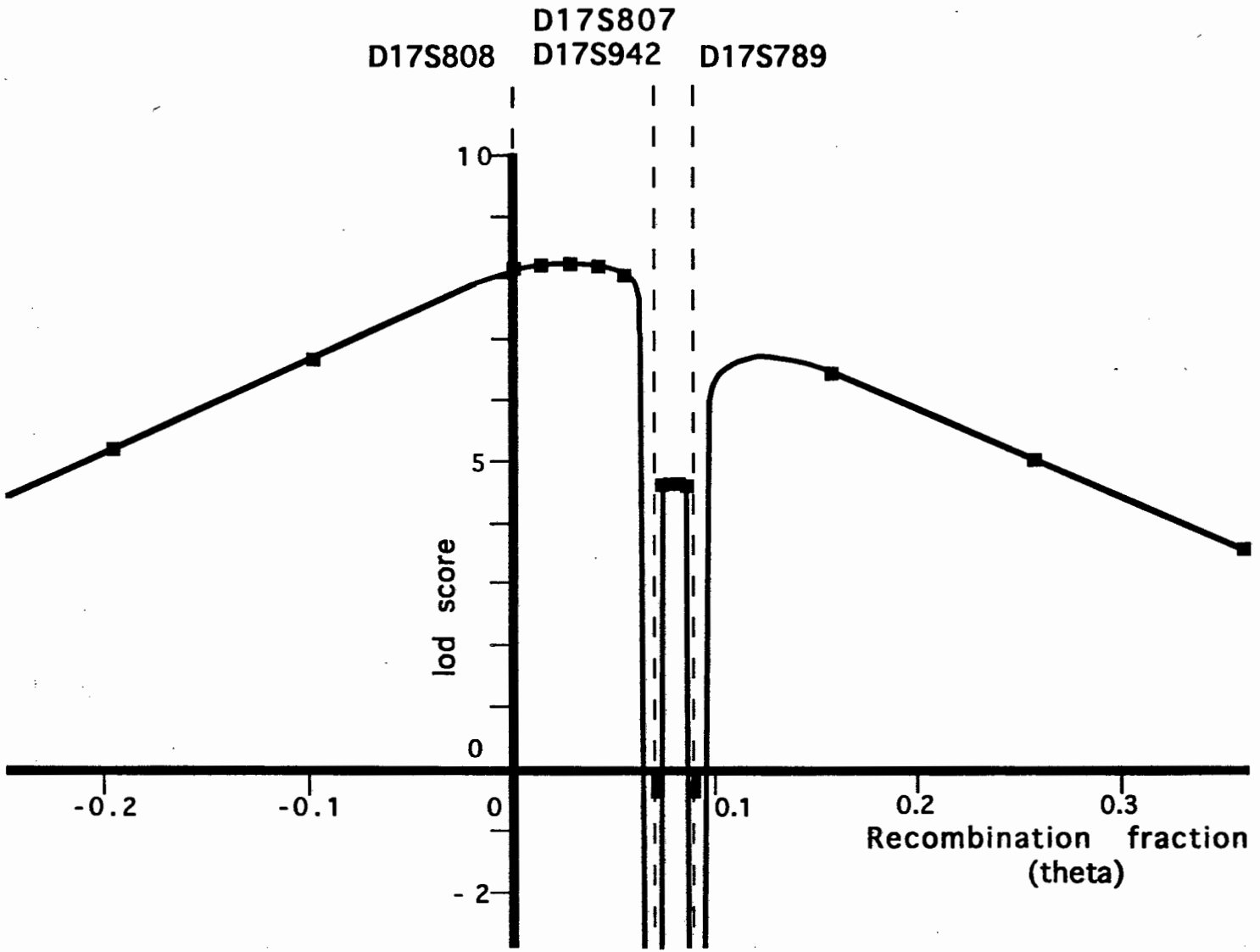


Figure 3.4 Multipoint linkage analysis between adRP and markers D17S808, D17S807, D17S942 and D17S789.

3.3.3 Fine mapping of the adRP locus

To facilitate the fine mapping of the RP17 locus, family RPD8 was genotyped with a further 17 microsatellite markers on chromosome 17q. In addition, DNA of three more family members was collected for study (individuals VI-2, VI-7 and VI-11; Fig. 3.5) and their genotypes were included in the linkage analysis.

Two point lod scores obtained between the adRP locus and the series of markers are presented in Table 3.5. These data confirm the initial linkage results and indicate that the RP17 locus maps to the 11 cM interval (converted by the Haldane map function) between D17S1607 and D17S1874. In order to identify the most likely location of the adRP gene relative to the new series of markers, a subset of the loci from Table 3.5 was used to generate a multipoint map of the region (Fig. 3.6). The adRP locus was moved up to and in between the eight fixed loci: D17S809, D17S787, D17S1607, D17S957, D17S792, D17S944, D17S1874 ending at D17S795 which spans a total genetic distance of approximately 18 cM (Haldane map function). The multipoint graph peaked at a lod score of 9.15 at D17S944 and the 3-unit-of lod-score support interval (a more meaningful support interval in multipoint analysis; Terwilliger and Ott 1994) encompasses a region between D17S1607 and D17S1874, indicating the most likely interval containing the disease gene. Further evidence for this localisation is provided by the construction of disease-associated haplotypes, which illustrate meiotic recombination events between the disease phenotype and D17S1607 and D17S1874 in family RPD8 (Fig. 3.5). Individual IV-4 is recombinant for markers D17S1874, D17S807 and D17S942; and individual VI-1 is recombinant for D17S790, D17S787 and D17S1607.

The two-point, multipoint and haplotype data provided here together confirm and refine the localisation of RP17 to an 11 cM interval on chromosome Giemsa (G) band 17q22.

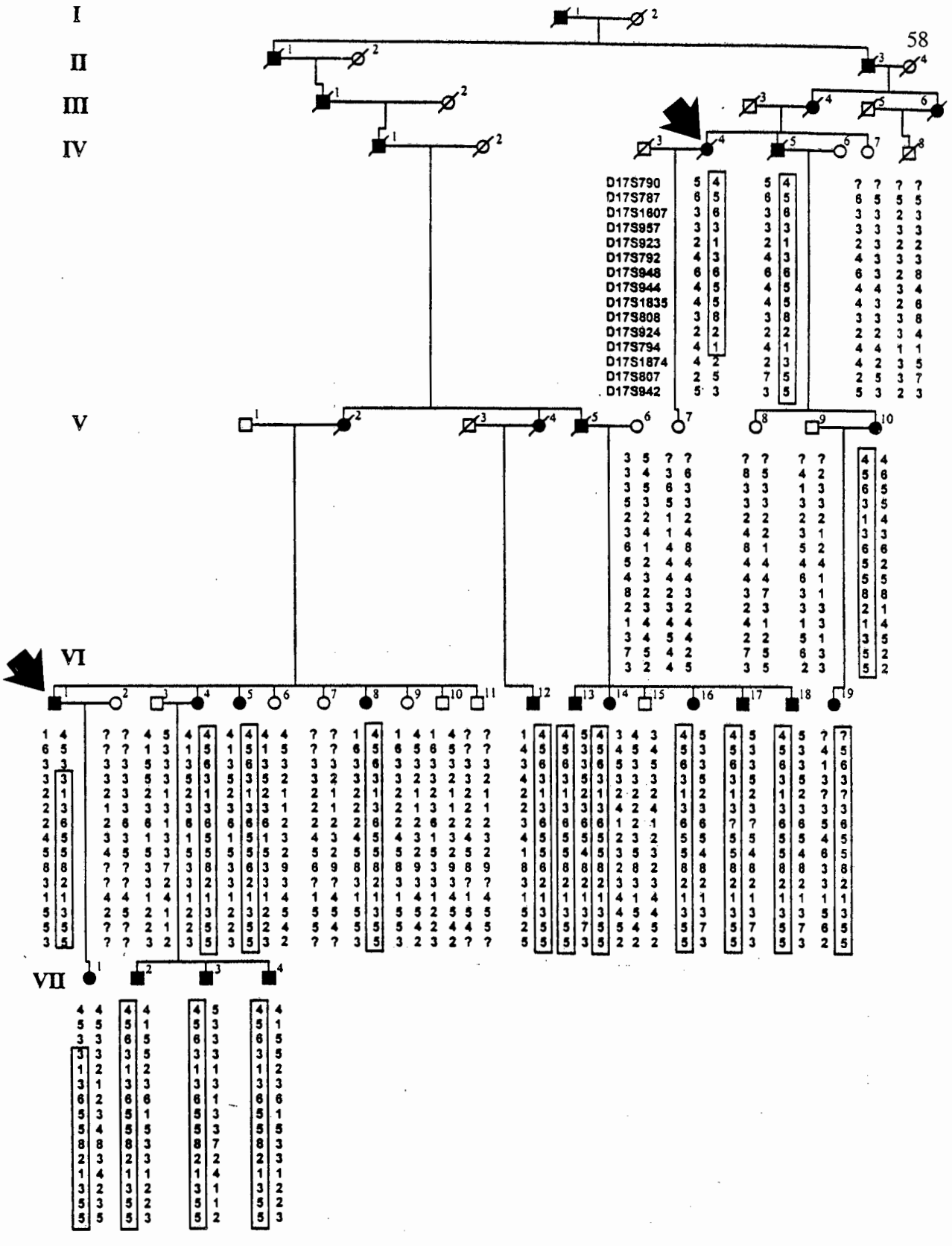


Figure 3.5 Haplotypes were constructed for members of family RPD8 at 15 chromosome 17q loci and are shown under each individual.

Table 3.5 Pairwise lod scores between adRP and anonymous markers on chromosome 17q.

	RECOMBINATION FRACTION (θ)							
DNA Marker	**0.00**	**0.01**	**0.05**	**0.10**	**0.20**	**0.30**	**0.40**	**0.50**
cen								
D17S798	-10.24	-	-3.32	-1.89	-0.67	-0.16	0.02	0.00
D17S800	-20.02	-5.41	-2.42	-1.27	-0.39	-0.09	-0.01	0.00
D17S934	-4.22	-0.72	-0.10	0.09	0.18	0.15	0.07	0.00
D17S809	-10.77	0.52	2.18	2.52	2.25	1.54	0.66	0.00
D17S790	0.41	0.40	0.37	0.32	0.22	0.13	0.06	0.00
D17S787	1.68	1.65	1.55	1.41	1.09	0.75	0.38	0.00
D17S1607	-∞	5.04	5.21	4.83	3.69	2.35	0.95	0.00
D17S957	3.32	3.29	3.09	2.78	2.04	1.21	0.40	0.00
D17S923	2.98	2.89	2.57	2.15	1.33	0.59	0.10	0.00
D17S792	5.04	4.93	4.51	3.97	2.84	1.65	0.58	0.00
D17S948	4.66	4.57	4.19	3.71	2.73	1.74	0.75	0.00
D17S944	8.32	8.17	7.56	6.77	5.11	3.33	1.49	0.00
D17S1835	3.98	3.89	3.55	3.09	2.10	1.10	0.30	0.00
D17S808	5.85	5.76	5.37	4.81	3.55	2.15	0.75	0.00
D17S924	3.90	3.84	3.58	3.22	2.41	1.54	0.64	0.00
D17S794	3.09	3.03	2.75	2.37	1.53	0.74	0.18	0.00
D17S1874	-∞	4.29	4.63	4.43	3.60	2.50	1.20	0.00
D17S807	6.39	6.32	5.95	5.40	4.11	2.71	1.23	0.00
D17S942	-∞	4.25	4.55	4.33	3.50	2.43	1.17	0.00
D17S795	-∞	3.00	3.43	3.34	2.72	1.84	0.82	0.00
D17S789	-∞	5.03	5.19	4.80	3.68	2.43	1.11	0.00
D17S840	-4.72	-0.69	-0.04	0.14	0.17	0.10	0.04	0.00
D17S949	-∞	4.82	5.07	4.79	3.84	2.65	1.28	0.00
D17S929	-17.98	-4.07	-1.47	-0.51	0.14	0.28	0.20	0.00
D17S785	-7.59	-1.55	-0.28	0.15	0.39	0.36	0.21	0.00
D17S939	-8.18	-2.14	-0.86	-0.40	-0.08	-0.01	-0.00	0.00
D17S784	-8.86	-3.92	-1.90	-1.07	-0.38	-0.13	-0.04	0.00
tel								

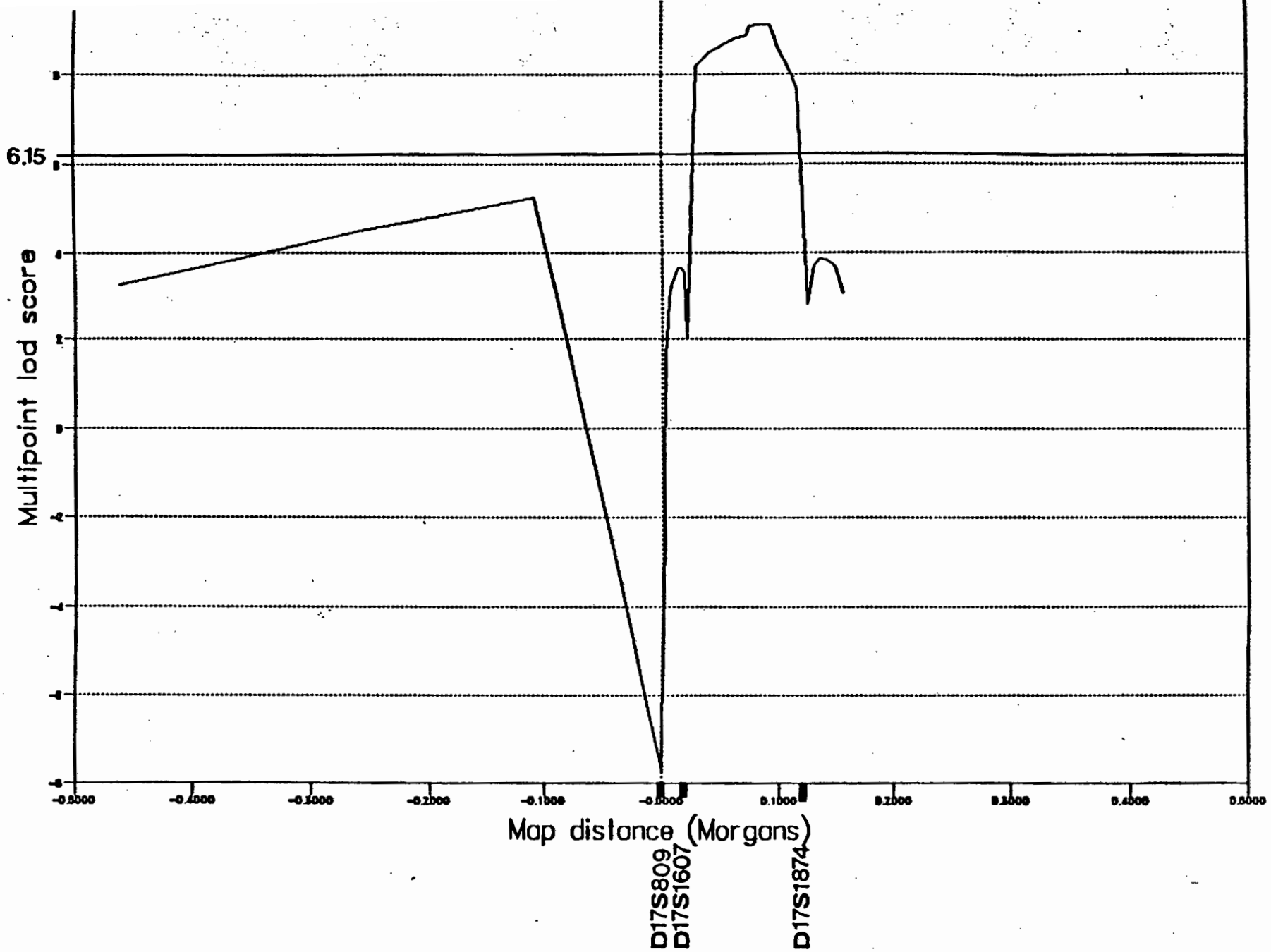


Figure 3.6 Multipoint linkage analysis between adRP and D17S809, D17S787, D17S1607, D17S957, D17S792, D17S944, D17S1874 & D17S795.

3.3.4 Linkage of a second adRP family to 17q

Subsequent to the mapping of RP17, all large unlinked South African adRP families from which DNA is archived in the molecular genetics laboratory at UCT were investigated for linkage to 17q22. A second family, designated RPD19 (Fig. 3.7) was shown to be linked to this locus (D17S948, $Z = 5.45$; D17S944, $Z = 5.40$; D17S808, $Z = 4.31$, all at $\theta = 0.00$; Bardien et al. 1997).

3.3.5 Further refinement of RP17 to a 1 cM interval

The 11 cM interval within which the adRP gene was mapped was not yet sufficiently small to consider physical mapping and positional cloning approaches in efforts to identify the gene. Additional linkage analyses were embarked upon in an attempt to further refine the RP17 critical interval and after intensive investigations, DNA from an additional 12 members (including six affected persons) from family RPD8 was collected and genotyped. Haplotypes were constructed for family RPD19 (Fig. 3.7) and for a total of 44 members of family RPD8 (Fig. 3.8) using a selected subset of markers on 17q22. Although the two kindreds are of different population groups, the affected individuals from the two families exhibit the same disease-associated alleles and haplotype, comprising 17 markers spanning an interval of 12 cM. This observation indicates that the two families share a common ancestor.

The observation of recombination events in informative meioses facilitated the refinement of the RP17 locus to a 1 cM critical interval. One individual, V-15 (Fig. 3.8) is recombinant for the markers D17S790 through to D17S1604 and it is evident that in this person, the maternally inherited chromosome had recombined. Individual V-15 is an unequivocally adRP affected male aged 55 years and on dilated fundal examination, was found to have typical features of RP (Ophthalmologist's

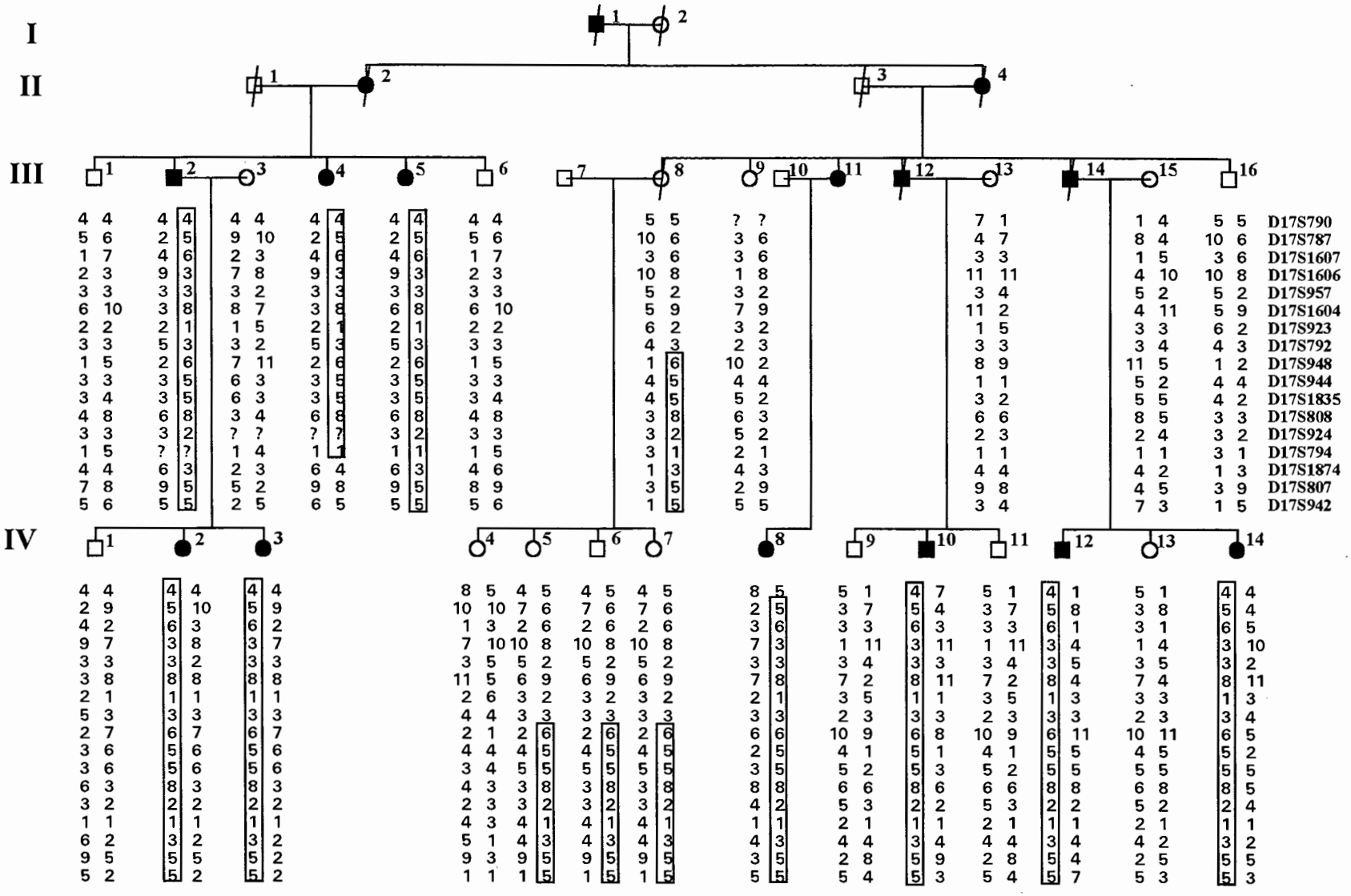


Figure 3.7 Pedigree of family RPD19. The disease-associated haplotype is shown in a box beneath each individual. Individuals III-8, IV-5, IV-6 and IV-7 have the haplotype even though they do not exhibit any signs of the disorder (see text).

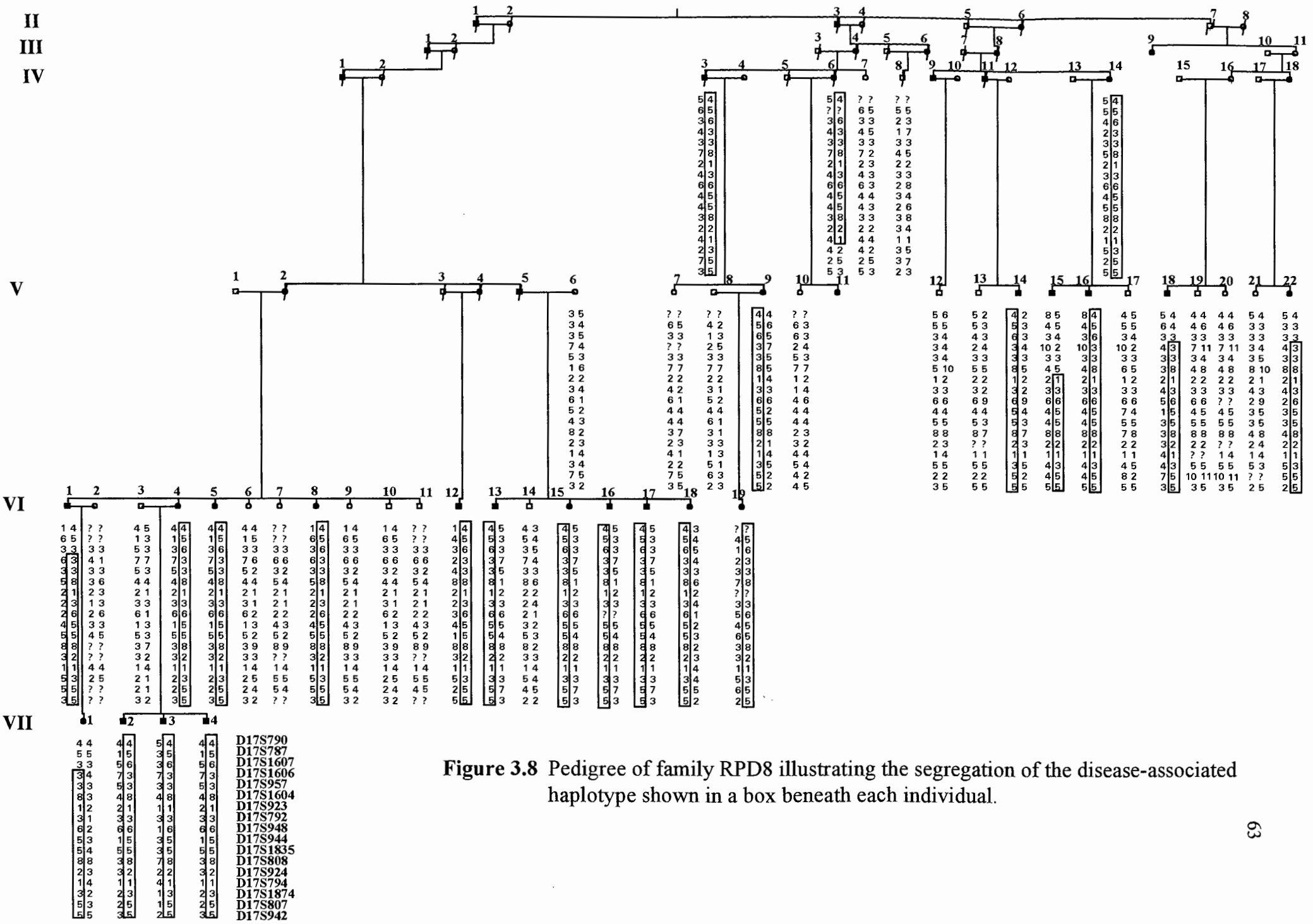


Figure 3.8 Pedigree of family RPD8 illustrating the segregation of the disease-associated haplotype shown in a box beneath each individual.

report in Section 3.2.1, page 44). This recombinant provides conclusive evidence that the adRP gene maps distal to D17S1604.

Unaffected individual III-8 (Fig. 3.7) is recombinant for the markers D17S948 through to D17S942 and it is her maternally inherited chromosome which had recombined. Individual III-8 had died at age 73 and had not shown any symptoms of visual problems up to her death, except for the presence of cataracts. The construction of haplotypes reveals that three of her four offspring (individuals IV-5, IV-6 and IV-7) had inherited the recombined chromosome from their mother. They are all over 50 years of age and on ophthalmologic examination did not exhibit any manifestations indicative of RP. This recombinant provides evidence that the adRP gene maps proximal to D17S948.

In conclusion, by combining the haplotype data obtained from the two families, it was possible to further delimit the critical interval for RP17 from a 11 cM to a 1 cM interval between D17S1604 and D17S948 (Fig. 3.9) [Bardien-Kruger et al. 1999]. This refined region is sufficiently small to facilitate positional cloning techniques in an attempt to identify the defective gene.

3.4 DISCUSSION

As discussed in Chapter One, there is a high level of genetic heterogeneity, both allelic and non-allelic, within RP. An inevitable benefit of this heterogeneity would be the characterisation of a large number of biologically important retina expressed genes and an increase in the understanding of the functioning of the healthy retina. This dissertation reports the mapping of a novel adRP locus (designated RP17) on chromosome 17q22, which is the eighth locus mapped for adRP

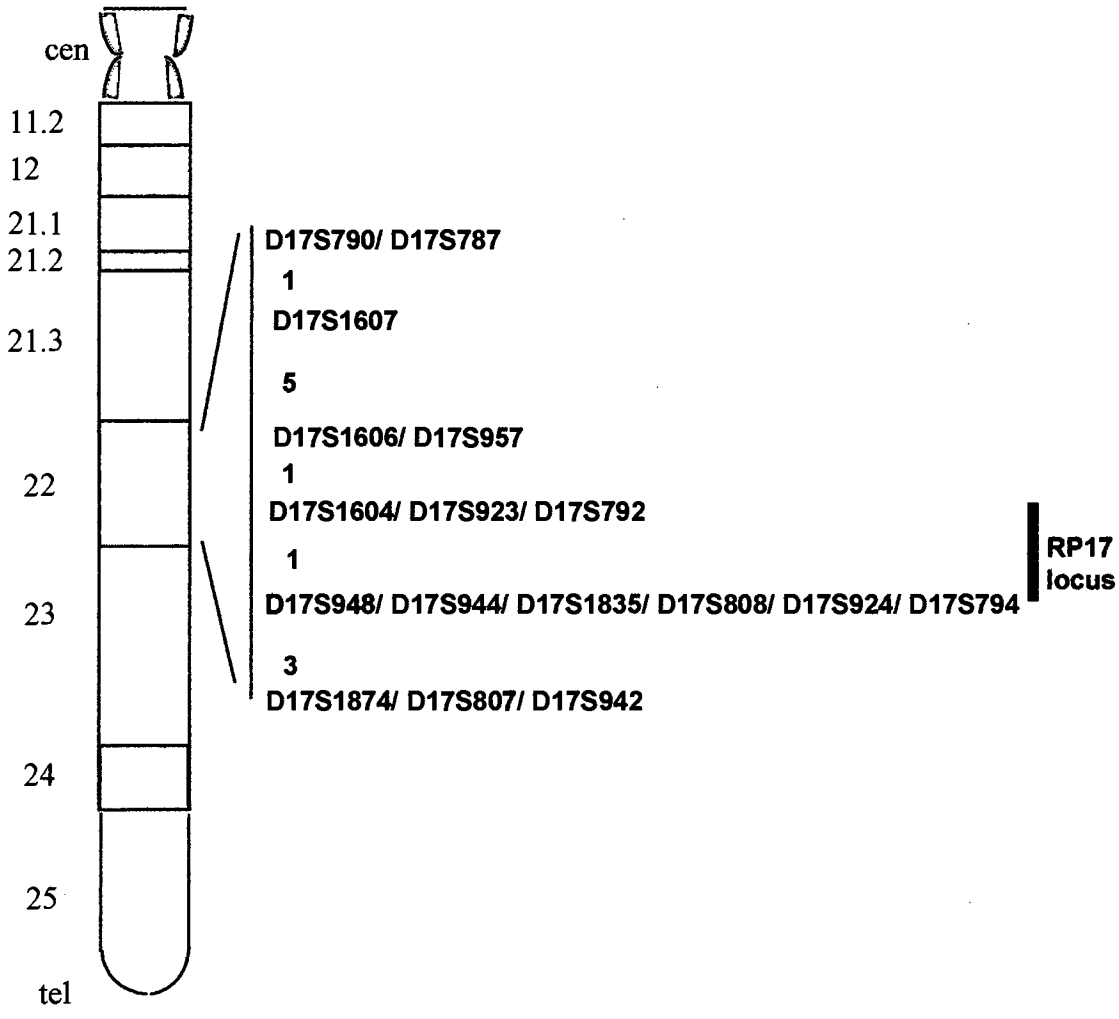


Figure 3.9 An ideogram of chromosome 17q depicting the position of the RP17 locus relative to microsatellite markers on 17q22.

following *rhodopsin, peripherin/RDS* and anonymous loci on 8q, 7p, 7q, 19q and 17p. Subsequently, a ninth locus for adRP has been mapped to chromosome 1q (Xu et al. 1996).

The two 17q-linked adRP families are members of different South African population groups (Afrikaner and mixed ancestry) and according to genealogical data, are apparently unrelated. It is noteworthy, however, that they share a common disease-associated haplotype, which indicates that the same mutation of the same gene accounts for adRP in both families.

The refined localisation of RP17 by genetic mapping provides a step towards the identification of the adRP gene; and delineation of a 1 cM region defines a realistic target for subsequent isolation of the gene by positional cloning techniques. The 1 cM critical interval mapped for RP17 may be considered small by genetic standards but relates to about 1 Mb of DNA. Given an average gene density of 5 genes per 100 kb for the human genome (Strachan and Read 1996), it is possible that more than 50 separate genes may reside in this interval. The next step towards isolating the RP17 gene would be the identification and subsequent characterisation of each of these genes, which would then have to be tested individually for evidence linking them to the adRP phenotype.

Several different techniques may be used to isolate genes from a specific region of mammalian DNA, and they generally fall into two basic categories i.e. cDNA-based approaches and genome-based approaches. The degree of success achieved with each of these methods is variable due to their inherent strengths and weaknesses. It is estimated that only about 3% of the mammalian genome codes for genes which are often separated by large intergenic (non-coding) DNA tracts. One way to tackle the problem of identifying genes in genomic DNA has been to find CpG

islands. In a study using fluorescent *in situ* suppression hybridisation to metaphase chromosomes, chromosome 17 was found to have a high CpG island density, thereby implying that it is a gene-rich chromosome (Craig and Bickmore 1994).

Other retinopathies that have been mapped to human chromosome 17 include:

i) an adRP locus (RP13) on 17p13.3 (Greenberg et al. 1994a),
ii) Leber's congenital amaurosis Type I (LCA1) on 17p13.1 associated with mutations in the gene for retinal guanylate cyclase (Perrault et al. 1996),
iii) progressive cone dystrophy (CORD5) on 17p13-12 (Balciuniene et al. 1995),
iv) cone-rod dystrophy (CORD6) on 17p13-12 associated with mutations in the gene for retinal guanylate cyclase (Kelsell et al. 1998),
v) Sjogren-Larsson syndrome on 17p11.2 caused by mutations in the fatty aldehyde dehydrogenase gene (De Laurenzi et al. 1996),
vi) central areolar choroidal dystrophy (CACD) on 17p (Lotery et al. 1996) ,
vii) cone-rod dystrophy locus (CORD4) on 17q11 (Kylstra and Aylsworth 1993).

From this list, it is evident that chromosome 17p is a hot spot for several phenotypically distinct retinal disorders. These differences in phenotypic manifestations may be due to genetic heterogeneity, allelic heterogeneity or the presence of epistasis (Joshi et al. 1997). Several retinally expressed genes have been assigned to chromosome 17. On the short arm these include the genes coding for: recoverin (*RCV1*, Murakami et al. 1992), retinal guanylate cyclase 2D (*GUC2D*, Oliveira et al. 1994),

pigment epithelium-derived factor (*PEDF*, Tombran-Tink et al. 1994), phosphatidylinositol transfer protein (*PITPN*, Fitzgibbon et al. 1994) and beta-arrestin 2 (*ARRB2*, Calabrese et al. 1994). On the long arm, the *retinal fascin* gene, which may play a role in photoreceptor cell disc morphogenesis, has been localised to 17qter by fluorescent *in situ* hybridisation (FISH) techniques (Saishin et al. 1997). In addition, the *PDEG* gene, one of the components of the rod phototransduction pathway, has been genetically linked to 17q25 (Dollfus et al. 1993). *Retinal fascin* and *PDEG* are both attractive candidates for RP17 and it would be appropriate for them to be investigated for pathogenic mutations in the two 17q-linked adRP families.

At the level of comparative gene mapping, human chromosome 17 is evolutionarily conserved with chromosomes from other animal species. This chromosome displays considerable synteny with mouse chromosome 11 (O'Brien et al. 1993) and according to the NCBI Genbank Human to Mouse Homology Maps (http://www.ncbi.nlm.nih.gov/Omim/Homology/human17.html) more than 100 gene loci from human chromosome 17 are represented on mouse chromosome 11. To date, there are, however, no obvious candidates for RP in the region corresponding to RP17 in mice. Furthermore, bovine chromosome 19 is thought to be entirely and exclusively conserved with chromosome 17, nevertheless, rearrangements in the linear order of homologous genes are apparent between the two chromosomes (Yang et al. 1998). Canine chromosome 9 shares synteny with the long arm of chromosome 17, using FISH analysis and linkage mapping but the gene order in the dog is inverted with respect to the centromere (Werner et al. 1997, Acland et al. 1998).

A locus for progressive rod-cone degeneration (prcd) has been mapped to dog chromosome 9 and based on the phenotype and the synteny between the two chromosomes it is proposed that this is the canine homologue of RP17 (Acland et al. 1998). Further investigations are required to determine if these two loci are orthologous. Prcd is inherited as an autosomal recessive trait and is present as an allelic condition in several breeds (miniature and toy poodle, English and American cocker spaniel, Labrador retriever and Portuguese water dog). In this condition, first the rods and then the cones degenerate both structurally and functionally after apparently normal postnatal development. The prcd gene has not yet been identified.

Construction of a high-resolution physical map of chromosome 17 has made extensive use of somatic cell hybrids carrying various deletions and translocations of this chromosome. These hybrid mapping panels have played an important role in the regional localisation of numerous anonymous probes and cloned genes. Other mapping reagents or resources currently available include a *Not*I linking library constructed for 17q (Borrow et al. 1991), a high-density genetic map of 17q12-21 (Anderson et al. 1993), somatic cell hybrid mapping panels spanning the 17q22-24 region (Flejter et al. 1993), and a high-resolution radiation hybrid map of 17q22-25.3 covering the region from the growth hormone gene (*GH*) to the thymidine kinase gene (*TK*) (Foster et al. 1996). These reagents could prove useful in attempts to clone the RP17 gene.

CHAPTER FOUR

CONSTRUCTION OF A PHYSICAL MAP FOR THE REGION HARBOURING THE RP17 GENE

4

CONSTRUCTION OF A PHYSICAL MAP FOR THE REGION HARBOURING THE RP17 GENE

CONSTRUCTION OF A PHYSICAL MAP FOR THE REGION HARBOURING THE RP17 GENE

Summary:

This chapter describes the construction of a physical map for the RP17 locus using sequence-tagged-site content mapping. The completed contig comprises seven YACs and two BACs which span the 1 cM critical interval. In an attempt to construct a transcription map of the region, the cDNA selection technique was applied to one of the YACs from the contig using a foetal brain cDNA library. Two rounds of selection were performed and the technique was accomplished successfully as indicated by the positive reporter gene. The sub-library created for enriched cDNAs derived from YAC 751c12 consists of a total of 480 selected transformants. This library was subsequently screened for the presence of contaminating sequences i.e. human COT-1 DNA and total yeast AB1380 DNA and 7% of the clones were found to contain repetitive sequences. Ten randomly chosen transformants were sequenced and subjected to BLASTN homology searches.

4.1 INTRODUCTION

The construction of detailed physical maps of the human genome is an important tool for the identification and cloning of genes causative of inherited disorders. Over the last decade, significant time and effort has been invested in establishing genomic libraries that cover the entire genome in relatively small overlapping fragments.

4.1.1 Yeast artificial chromosomes

Yeast artificial chromosomes (YACs; Burke et al. 1987) have led the way in mapping complex genomes and YAC libraries now cover a large part of the human genome. The various components of a typical YAC are presented in Fig. 4.1. One of the major advantages of these vectors is their ability to contain the complete genomic region of large genes, including the promoter sequences and upstream control elements. There are several major problems, however, that need to be considered when working with YAC clones. One disadvantage is the high number of YACs (40-60%) that are chimaeric i.e. containing two or more fragments derived from non-contiguous segments of human DNA. A second problem is that some clones are unstable and tend to delete internal regions from their inserts which could be deleterious in gene isolation experiments or when constructing physical maps of specific chromosomal regions. A third major disadvantage is that the YAC DNA cannot be isolated from the 15 Mb yeast host chromosome background by simple methods. In order to overcome some of these problems, several alternative cloning systems have been developed e.g. bacterial artificial chromosomes (BACs), P1-derived artificial chromosomes (PACs) and cosmids (which contain a bacteriophage lambda cos site) and these are summarised in Table 4.1.

4.1.2 Construction of contigs

To ensure that that there is complete representation of the genomic region of interest, and no gaps, the series of clones should contain overlapping inserts forming a comprehensive clone *contig*. In principle, contig assembly is facilitated by the way in which genomic DNA libraries are constructed. As part of the protocol, the genomic DNA is deliberately subjected to partial digestion with a restriction endonuclease e.g. *Eco*R1 before being cloned into the vector. As a result, individual genomic DNA

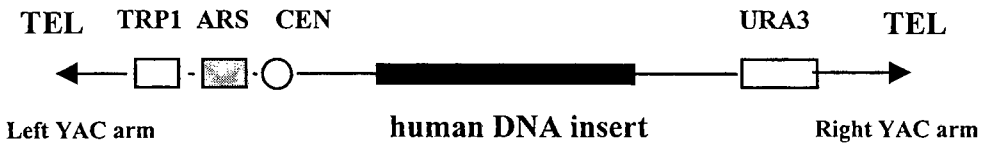


Figure 4.1 Schematic diagram of a YAC cloning vector, illustrating the telomeres (TEL) at either end, yeast selectable markers TRP1 and URA3, an autonomously replicating sequence (ARS), a yeast centromere (CEN), and a large human DNA insert.
Telomeres confer structural integrity upon linear DNA molecules and centromeres confer the property of accurate mitotic and meiotic segregation upon either circular or linear plasmid DNA. Autonomously replicating sequences are required for the plasmid to replicate autonomously inside the yeast cell.

Table 4.1 Cloning vectors used in the positional cloning strategy.

VECTOR	HOST	INSERT SIZE
YAC	*S.cerevisiae*	100-2000 kb
PAC	*E.coli*	100-300 kb
BAC	*E.coli*	130-300 kb
P1 clone	*E.coli*	70-100 kb
Cosmid	*E.coli*	35-45 kb

clones usually contain DNA sequences that partially overlap with other clones in the library.

One widely used technique for identifying clones with overlapping inserts is using a specific DNA probe from one clone to screen the rest of the library. This involves the purification and labelling of terminal sequences from the insert DNA of the starting clone i.e. an end-probe. Positively hybridising clones can then be purified and new, distal end-probes can be prepared for further rounds of hybridisation screening of the library. A popular method for generating end-probes is vectorette PCR (or bubble linker PCR; Riley et al. 1990). In this technique, the clone DNA is digested with a restriction endonuclease and the digestion products are ligated to a double-stranded oligonucleotide linker designed to have non-complementary oligonucleotides in the middle, forming a "bubble"-shape. Using these vectorette libraries as templates, a PCR reaction is performed with a linker-specific primer and a vector-specific primer, which permits amplification of the uncharacterised insert sequence flanking the vector arm. In this way, the clone insert terminal sequences can be isolated and made available for further manipulation.

Vectorette PCR and other such techniques e.g. inverse PCR (Ochman et al. 1988), permits the assembly of a clone contig by bi-directional chromosome walking from a fixed starting point. Since YAC clones often have chimaeric inserts, however, the chromosomal location of any new YACs identified in a YAC walk always ought to be verified by FISH analysis.

Chromosome walking is a highly directional and location-restricted procedure for generating clone contigs. In order to assemble contigs rapidly over large fractions of the genome, random clone fingerprinting

methods are preferred. Some examples of these methods are:

i) Repetitive DNA fingerprinting

In this technique, the large component of highly repetitive interspersed DNA sequences e.g. human *Alu* and LINE-1 repeats are used to generate repetitive DNA fingerprinting patterns of YACs. LINE-1 repeats occur about once every 50 kb and the full-length consensus sequence is 6.1 kb long. Clones with overlapping inserts are identified by similar patterns obtained, following digestion with a suitable restriction endonuclease and hybridisation with repetitive DNA probes.

ii) Sequence-tagged-site content mapping

Sequence-tagged-sites (STSs) are PCR-based markers that are unique for a specific known sequence of DNA. Microsatellite markers (described in Section 3.1.1.1) are a subset of STSs and they represent a critical link between genetic and physical maps. If numerous individual STS sequences have been positioned on a chromosome and YACs have been mapped to the same general area (e.g. by FISH) then the ability to type for STSs, allows a rapid and convenient method for identifying YACs with overlapping sequences i.e. STS-content mapping. For a typical STS, primers of approximately 20 bp are designed for use in a standard PCR reaction. Even for a complex genome, the probability of both primers binding non-specifically to sequences in close proximity and in the correct orientation is extremely low. By size-fractionation of the PCR products on an agarose gel and obtaining a single DNA band of the expected size, there is a good chance that the assay is specific for the intended target sequence.

iii) Hybridisation with interspersed repeat elements (IRE)-PCR

Hybridisation using IRE-PCR is a PCR reaction in which only a single primer is used, corresponding to a common repetitive element. An example is *Alu*-PCR (or inter *Alu*-PCR) in which the primer is derived

from *Alu* sequences, which occur on average every 4 kb in the human genome. The single primer binds to the repetitive elements and if they are in close enough proximity, allows amplification of the sequence between them. These products can then be used as probes for screening other YACs.

4.1.3 Isolation of gene transcription units

Once a continuous, overlapping clone contig has been assembled for the region of interest, the individual clone insert DNA can be examined for gene transcription units. As mentioned previously, purification of YAC DNA is not a simple task because YACs have a very similar structure to the natural yeast chromosomes. Techniques such as separation by pulsed-field gel electrophoresis (PFGE) or direct subcloning of the entire yeast genome into bacteriophage vectors, followed by identification of human-specific clones derived from the YAC, are usually required. During a PFGE run, the relative orientation of the gel and the electric field is periodically altered; and the DNA molecules are intermittently forced to change their conformation and direction of migration. The time taken for a DNA molecule to alter it conformation and re-orient itself in the direction of the new electric field is strictly size dependant, and as a result, DNA fragments of up to several megabases in size can be fractionated efficiently using this method.

The rate limiting steps in the positional cloning of disease-causing genes are the identification of discrete genes within a large genomic region and the analysis of mutations within these genes. A number of strategies, summarised in Table 4.2, have been developed to isolate transcription units in complex genomes, which by definition contain a small percentage of coding sequences amidst a sea of non-coding sequences. Some of these methods are relatively straightforward and involve using

Table 4.2 Strategies for identifying genes in large genomic regions.

	METHOD	REFERENCE
I.	**Hybridisation-based**	
	a. Hybridisation to RNA/cDNA	-
	b. Evolutionary conservation (zoo blots)	Claudio et al. 1994
	c. Identification of CpG islands	Larsen et al. 1992
	d. Direct cDNA library screening	Elvin et al. 1990
	e. cDNA selection	Parimoo et al. 1991/Lovett et al. 1991
II.	**Function-based**	
	a. Splice-junctions - exon trapping	Duyk et al. 1990/ Buckler et al. 1991
	b. Promoter trapping	-
	c. Poly A signal trapping	-
III.	**Sequence-based**	
	a. Exon prediction	Uberbacher and Mural 1991
	b. Species comparison/homology searches	-

the genomic clone as a hybridisation probe. In this way, genes can be identified by hybridising DNA clones against Northern blots, cDNA libraries, zoo blots and Southern blots of genomic DNA digested with rare-cutter restriction endonucleases.

4.1.3.1 *The cDNA selection technique*

In the case of identification of human genes, perhaps the biggest limitation is the abundance problem - some rare genes may be expressed at levels as low as 1 in 10^6, making identification of these transcripts difficult and unlikely by conventional screening of a cloned cDNA library. For many of the techniques mentioned in Table 4.2, finding all the genes in even a single megabase of the human genome is labour intensive and technically complex; and some methods also suffer from being low in sensitivity. One strategy that may be used to avoid some of these problems is cDNA selection (also called direct selection, direct cDNA selection and hybrid selection; Lovett 1994).

cDNA selection is a PCR-based hybridisation method developed to enrich for coding sequences. It is particularly useful in the positional cloning of loci associated with genetic disorders and for searching large genomic regions (> 2 Mb) for transcriptional units that are expressed at low levels. The concept underlying this technique is that a large genomic region or clone (e.g. YAC or cosmid) may be used to 'fish out' its cognate cDNAs from a starting mixture of cDNAs. Several rounds of hybridisation should lead to a huge enrichment of the desired cDNA sequences, enabling identification of the corresponding genes. Enrichments of approximately 10 000 fold can be obtained after two rounds of selection when a region of about 1 Mb is targeted. In general, a third round of selection does not increase the yield substantially.

In this technique, the requirement for high quality starting cDNAs is very important, preferably derived from sources other than conventional cloned libraries. These libraries often contain contaminants and the representation of sequences may be skewed. Furthermore, when derived from complex tissues they are unlikely to contain cDNAs that represent rare transcripts. Most cDNA libraries consist of about 10^6 recombinant clones which is probably adequate sequence representation from transcripts expressed in one cell type. If the library is, however, derived from a complex tissue such as total brain and the transcript of interest is expressed at a low level in a sub-region of the brain, then 10^6 clones will probably not contain the cDNA of interest. It is therefore preferable to use complex sets of uncloned cDNAs that better represent the complexity of the starting mRNA. In addition, the choice of tissue or cell type from which to generate a set of cDNAs is an important first decision for this method.

To efficiently identify new genes within selected cDNAs, it is crucial to either block or screen out common contaminants. In selections where YAC DNA is used as a target, ribosomal cDNA represents a major contaminant because YACs purified by PFGE are inevitably slightly contaminated with all the yeast chromosomes, including chromosome XII which carries >100 copies of the yeast ribosomal locus. In addition, ribosomal RNAs are represented in most, if not all, libraries or pools of cDNAs, so in selections where ribosomal sequences are not pre-blocked, ribosomal cDNAs can comprise up to 70% of selected material. Fortunately, these sequences are easily blocked and/or screened out after selection. The yeast 2μ plasmid is another DNA sequence that contaminates YAC preparations and can be avoided by pre-electrophoresing YAC preparations before purification. Intermediate

repetitive sequences such as the *Alu* repeats, can be blocked efficiently and are not usually a major problem.

One disadvantage with cDNA selection is that genes containing very short exons may be missed because the heteroduplexes formed with cognate cDNAs may not be sufficiently stable. A second problem is that cDNAs may hybridise to pseudogenes showing a high degree of homology to the cognate functional genes or to related members of a gene family.

4.1.4 This study

Following the mapping of the RP17 locus to chromosome 17q22, the positional cloning approach was attempted in an effort to identify the defective gene. An initial step in this approach involved the construction of a physical map for the 1 cM critical interval. A large scale sequencing effort on chromosome 17 is currently underway at the Center for Genome Research at the Whitehead Institute for Biomedical Research, USA (http://www-genome.wi.mit.edu/) and this database was used as a starting point for the assembly of a contig spanning the RP17 locus. STS content mapping was used to identify overlapping YAC clones.

The cDNA selection method was employed for the identification of gene transcription units in the RP17 YAC/BAC contig. This technique was chosen due to its relatively high success rate in other positional cloning projects and because it is amenable to the use of large genomic clones i.e. YACs. A foetal brain cDNA library was used in the selections since a suitable uncloned retina-specific cDNA library was unobtainable at the time.

4.2 MATERIALS AND METHODS

4.2.1 STS content mapping of YACs

The RP17 physical map was constructed using YAC clones selected from the Whitehead/MIT genome Center physical map of YAC contig WC 17.8 (Hudson et al. 1995). For analysis of STS content, the seven CEPH mega YACs 913d6, 751c12, 948c8, 761e2, 961f1, 813b7 and 926c12 were streaked onto casein (Ura-, Trp-) media plates (Appendix C) and incubated at 30°C for two days. Single colonies of each YAC were screened directly using PCR by dipping a sterile toothpick into the colony and immediately mixing it into the PCR cocktail. The YACs were screened for the following nine STSs: D17S1604, D17S792, D17S923, D17S1855, CHLC.GATA11C11, CHLC.GATA6G10, D17S808, D17S948 and D17S794; and the three ESTs WI-6034, WI-6805 and FB10A2 (purchased either from Research Genetics Inc., USA or GIBCO/BRL, USA).

PCR conditions were 95°C for 5 min (to lyse the colonies and denature the DNA) followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. A final extension step of 72°C for 5 min was also performed. The PCR reactions contained 5 pmol of each primer, 250 μM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), and 0.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, USA) in a total reaction volume of 10 μl. The PCR was performed on a Perkin-Elmer Thermal Cycler and the products were fractionated on 2% ethidium bromide-stained agarose gels. The presence or absence of a single PCR product of the appropriate size was tested at least twice.

4.2.2 BAC library screening

To cover a gap detected in the YAC contig, the Human Bacterial Artificial Chromosome DNA pools, Release III (Research Genetics Inc., USA) was

investigated for the identification of clones positive for the markers WI-6805 and CHLC.GATA11C11. The BAC library is provided in a pooled format in 96-well microtitre plates and was screened according to the manufacturer's instructions.

4.2.3 Isolation of BAC and YAC DNA

Purified BAC and YAC DNA was required for the vectorette PCR and cDNA selection protocols, respectively. BAC DNA was isolated from the bacterial host genome using a standard alkaline lysis miniprep method as described in Sambrook et al. (1989).

YAC DNA from each clone was prepared in agarose plugs and subjected to pulsed-field gel electrophoresis in 1% agarose gels (Appendix B). These gels were run in 0.5X TBE at 14°C. A contour-clamped homogeneous electric field (CHEF) apparatus (BIORAD-CHEF DR II System) was used and the running conditions used for each of the YACs are summarised in Table 4.3. After ethidium bromide staining for visualisation, the gels were Southern blotted onto Hybond N^+ membranes (Amersham, USA) and hybridised with [α^{32}P]dCTP-labelled human COT-1 DNA in order to identify the YAC band. A Prep-A-Gene DNA isolation kit (BIORAD, USA) was used for the gel extraction of YAC DNA. The purification of YACs on PFGE is time-consuming but effectively eliminates the high background levels that result due to the presence of the total yeast genome.

4.2.4 Vectorette PCR

End probes of BACs were generated by the vectorette end fragment isolation method adapted from Riley et al. (1990). Briefly, the clone DNA was digested with a range of restriction enzymes (*Alu*I, *Eco*RV, *Pvu*II and *Rsa*I) and ligated with the blunt-end vectorette cassette as described. These four vectorette libraries were used as templates and PCR was

performed with a universal vectorette primer and either the SP6 or T7 primers to amplify the terminal sequences.

The oligonucleotides and primers used in this method are as follows:
Top strand blunt cassette 5'-AAg gAg Agg ACg CTg TCT gTC gAA ggT AAg gAA Cgg ACg AgA gAA ggg AgA g-3'
Universal bottom strand cassette 5'-CTC TCC CTT CTC gAA TCg TAA CCg TTC gTA CgA gAA TCg CTg TCC TCT CCT T–3'
Vectorette primer 5'-TCT CCC TTC TCg AAT CgT AAC CgT TCg TAC-3'
T7 primer 5'-AAT ACg ACT CAC TAT Ag-3'
SP6 primer 5'-gAT TTA ggT gAC ACT ATA g-3'

4.2.5 cDNA selection

The cDNA selection protocol was employed to isolate coding sequences in the newly constructed RP17 critical interval. Purified YAC 751c12 DNA was labelled with biotin using a Nick Translation Kit (Boehringer Mannheim, Germany) and hybridised to a pre-blocked foetal brain cDNA library (obtained from Dr. M. Lovett, UT Southwestern Medical Center, Dallas, USA). cDNA/YAC hybrids were captured using streptavidin coated magnetic beads (DYNAL, USA). Following the post-hybridisation washes, the primary selected cDNAs were subjected to a second round of selection. In order to monitor the level of enrichment obtained for the primary and secondary selected cDNAs, Southern blot analysis was performed using a reporter gene, the EST WI-6034, as a probe. The cDNA selection protocol is depicted in Fig. 4.2 and outlined in Appendix B.

4.2.6 Cloning of secondary selected cDNA

Secondary selected cDNAs were cloned into *E-coli* cloning vector pAMP10 using the CLONEAMP pAMP10 System [GIBCO/BRL, USA]. Ligation

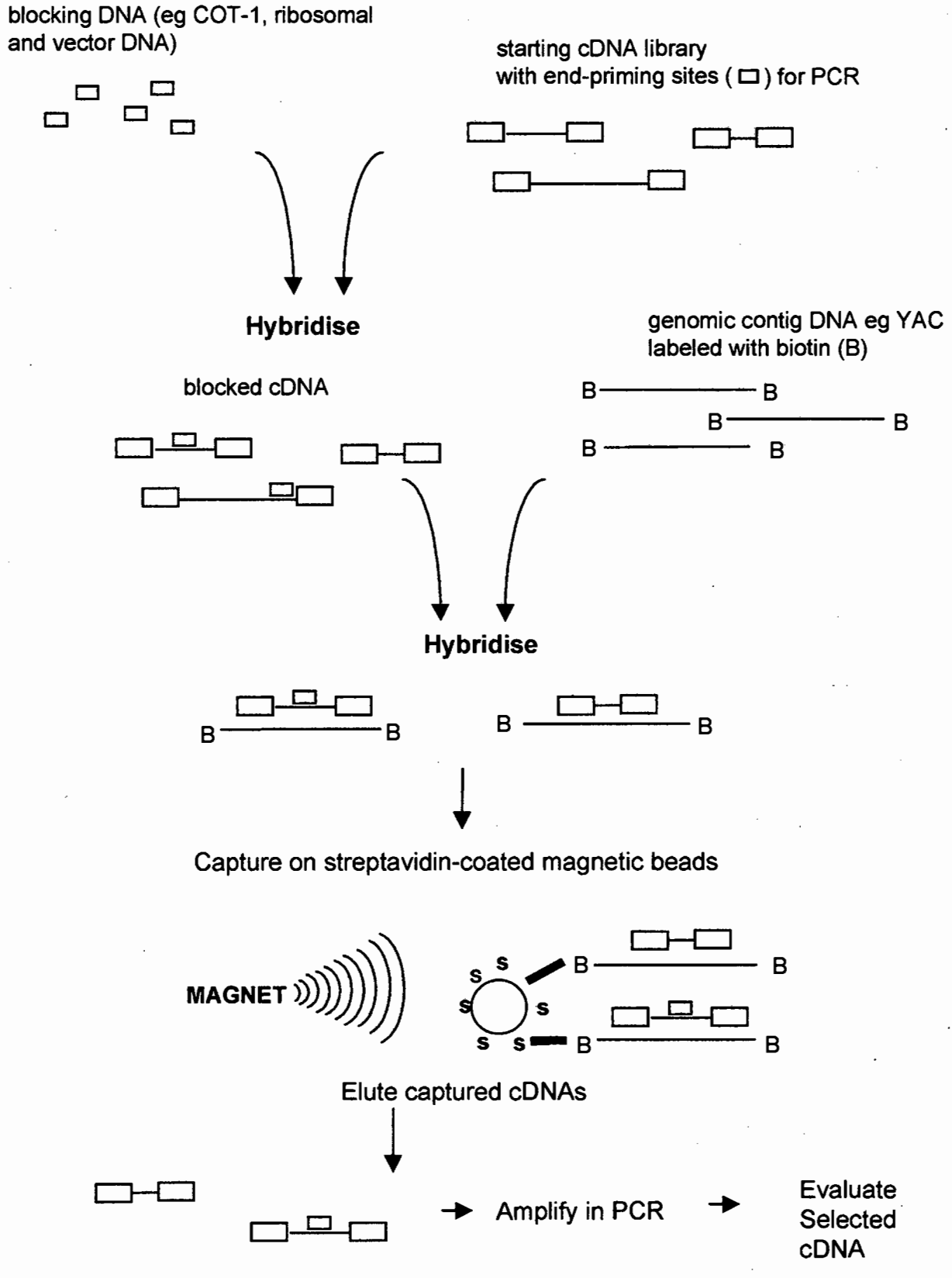


Figure 4.2 Flow diagram of the cDNA selection protocol using magnetic bead capture.

reactions were transformed into Library Efficiency DH5α Competent Cells (GIBCO/BRL, USA) according to the recommended instructions and transformants were plated on Luria-Bertani agar (LA) containing Ampicillin (200 μg/ml) and grown at 37°C O/N.

Single colonies were picked and arrayed using sterile toothpicks, into five 96-well microtitre plates containing Luria-Bertani broth (LB) with 200 μg/ml Ampicillin. The plates were incubated at 37°C O/N, after which LB containing 30% glycerol and 100 μg/ml Ampicillin was added. These five plates constitute the master set (maintained at -70°C), and a duplicate working copy of plates were made to prevent contamination.

4.2.7 Evaluation of clones

The lids of ten 96-well microtitre plates were filled with LA containing 200 μg/ml Ampicillin and overlaid with Hybond N^+ nylon membranes (Amersham, USA). A 96-well replica plater (robotic workstation) was used to stamp the arrayed clones from the five 96-well plates onto the membranes in duplicate in a high-density format. The lids were incubated at 37°C O/N to allow the colonies to grow onto the filters. The following day, the filters were fixed in two solutions: 1.5 M NaCl/ 0.5 M NaOH and 1.5 M NaCl/ 0.5 M Tris-HCl (pH 7.4) at 65°C and pre-hybridised with denatured herring sperm. One filter was probed with [α^{32}P]dCTP-labelled human COT-1 (GIBCO/BRL, USA) and a second filter was probed with yeast strain AB1380 DNA at 65°C O/N. Following hybridisation, the blots were exposed to autoradiography at -70°C O/N.

4.2.8 Sequencing of cDNA inserts

The plasmid DNA of ten colonies were isolated using a QIAprep Spin Miniprep Kit (QIAGEN, USA) according to the manufacturer's instructions. The cDNA inserts of these colonies were sequenced using

an ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, USA). Sequencing reactions were performed with the M13 primer and AmpliTaq DNA Polymerase, FS and analysed by an ABI PRISM 377 DNA Sequencer (Perkin-Elmer, USA).

4.3 RESULTS

4.3.1 Construction of a YAC/BAC contig for RP17

To facilitate the identification of the RP17 adRP gene, an attempt was made to construct a YAC contig spanning the 1 cM critical interval between the markers D17S1604 and D17S948. A contig consisting of seven overlapping CEPH mega YACs was assembled according to their marker content, and the results are summarised in Fig. 4.3.

STS content mapping revealed a gap in the YAC contig between markers WI-6805 and CHLC.GATA11C11. In an effort to cover this gap, a BAC library was screened and two positive clones were identified: BAC 100E23 (positive for WI-6805) and BAC 229L19 (positive for CHLC.GATA11C11). Since each BAC was positive for one marker only, the vectorette PCR method was used to determine whether the inserts of the two BACs overlapped. An end-probe derived from the T7 terminus of BAC 100E23 was shown to hybridise to BAC 229L19 (Fig. 4.4a). Similarly, an end-probe derived from the SP6 terminus of BAC 229L19 hybridised to BAC 100E23 (Fig. 4.4b). The specificity of these hybridisation experiments was verified by the observation that an SP6 terminal sequence generated from BAC 100E23 did not bind to BAC 229L19 (Fig. 4.4c). These findings indicate that the BAC vector arm sequences are not contributing to the hybridisation results and provide evidence which suggests that the two BAC inserts overlap. Therefore, assuming that none of the YACs have an internal deletion, continuous

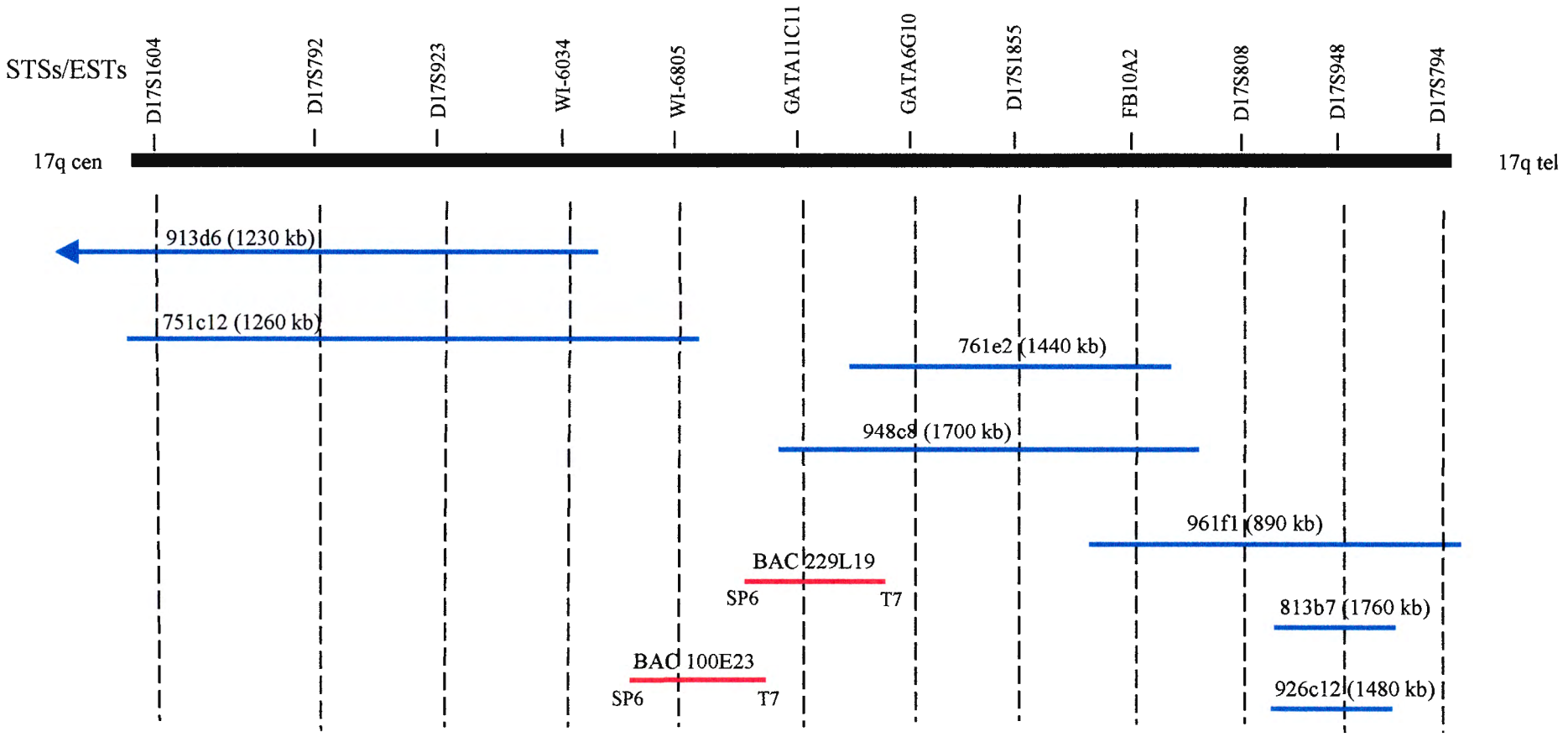


Figure 4.3 Schematic diagram of a YAC/BAC contig constructed for the RP17 critical interval. The bold upper line indicates the chromosome with the centromere to the left and the telomere to the right. STS locations are indicated by dashed lines.

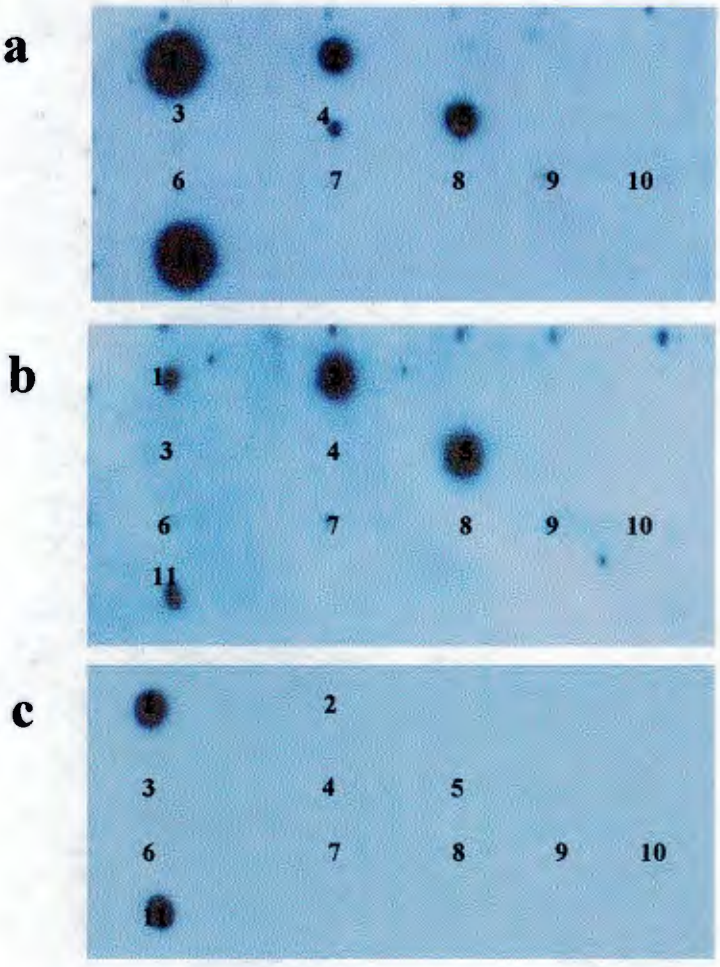


Figure 4.4 Dot blot analysis of the seven YACs and two BACs encompassing the RP17 contig, which were hybridised to end-probes generated from:
(a) BAC 100E23 using primers 224 & T7;
(b) BAC 229L19 using primers 224 & SP6;
(c) BAC 100E23 using primers 224 & SP6.

Key to Figure 4.4

1 = BAC 100E23	2 = BAC 229L19			
3 = 913d6	4 = 751c12	5 = BAC 229L19		
6 = 761e2	7 = 948c8	8 = 961f1	9 = 813b7	10 = 926c12
11 = BAC 100E23				

and overlapping clone coverage of the RP17 critical interval has been assembled.

4.3.2 YAC DNA isolation

High molecular weight DNA from each of the seven YAC clones were prepared in agarose blocks and subjected to pulsed-field gel electrophoresis in order to separate the YAC DNA from the yeast host chromosomes. Southern blot analysis was performed to identify the YAC band on the pulsed-field gel and the YAC DNA was subsequently gel-purified for use in subsequent gene identification experiments.

4.3.3 cDNA selection

The cDNA selection protocol was employed in order to search for candidate genes residing in the RP17 critical interval. After two rounds of selection using YAC 751c12, significant enrichment of the reporter gene (WI-6034) was obtained which indicates that the selections had been successful (Fig. 4.5). The secondary selected cDNAs were cloned into the *E-coli* cloning vector pAMP10 and a total of 480 transformants were selected and patched onto five 96-well microtitre plates.

In order to evaluate the clones, the colonies were probed with human COT-1 DNA and yeast strain AB1380 total DNA to eliminate or screen out contaminating repetitive DNA elements and yeast sequences. The hybridisation experiments to COT-1 DNA revealed 32 out of 480 (i.e. 7%) positive colonies (data not shown). This figure correlates well with the roughly 10% of colonies routinely found to contain repetitive sequences in most cDNA selection experiments (Lovett 1994). None of the colonies hybridised to the yeast AB1380 probe (data not shown) which may indicate that there were no contaminating yeast DNA present in the selected clones. The plasmid DNA of ten randomly chosen clones were

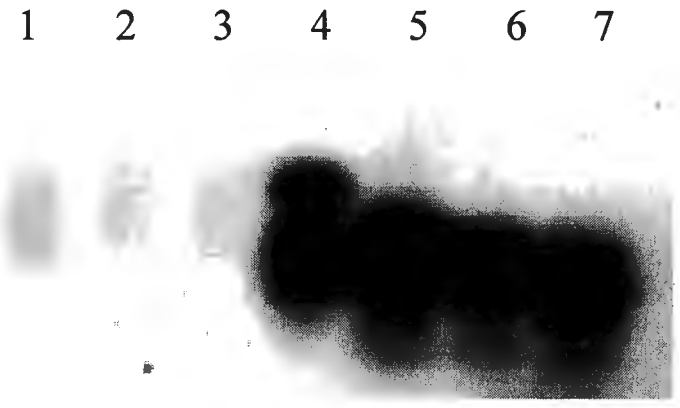


Figure 4.5 A Southern blot of the cDNA selections using YAC 751c12, which was probed with the reporter gene WI-6034. Lanes 1, 2 & 3 contain 1, 0.2 & 0.1 ng of starting cDNA, respectively; lane 4 contains 2 µl of 1° selected cDNA and lanes 5, 6 & 7 contain 1, 2 & 5 µl of 2° selected cDNA, respectively.

sequenced and subjected to BLASTN analysis (Table 4.4). Two of the selected cDNAs were highly homologous to the human putative cerebral cortex transcriptional regulator T-Brain-1 mRNA and another two exhibited significant similarity to human 18S ribosomal RNA. The presence of ribosomal sequences may be due either to ribosomal genes residing on YAC 751c12 or to contamination with yeast chromosome XII which carries the yeast ribosomal locus. A further two clones exhibited homology to the chromosome 17-specific clones hRPC.18_F_17 and hCIT.91_J_4 thereby implying that the selections were specific for the correct chromosome. One of the selected mRNAs is part of the chromosome 16 P1 clone 94-10H, which indicates that the YAC 751c12 may be chimaeric. This observation should be confirmed with FISH experiments using this YAC as a probe. Another two of the selected mRNAs exhibited weak similarity to the Homo sapiens retroviral-like sequence S71. The last clone to be sequenced contained only a 23 bp insert that, upon inspection, proved to be the linker oligonucleotide which had been attached to the starting pool of cDNAs as a priming site for PCR. It is imperative that the prevalence of this contaminant in the selected material be assessed and screened out. This sequence cannot, however, be screened by hybridisation experiments since all the cloned cDNAs contain this oligonucleotide and further investigations to resolve this problem are necessary.

4.4 DISCUSSION

A physical map has been constructed that encompasses the adRP locus on 17q22. Evidence is provided which indicates that an overlapping array comprising of seven mega YACs and two BACs has been assembled spanning the critical region. This YAC/BAC contig also contains the respective adRP-flanking recombinant markers (D17S1604 and

Table 4.4 Sequencing results of ten secondary selected cDNAs.

Clone	Insert length (bp)	Homologous to:	BLASTN e value
1	416	Human putative cerebral cortex transcriptional regulator	e-171
2	162	Homo sapiens retroviral-like sequence S71	0.002
3	424	Human putative cerebral cortex transcriptional regulator	4e-77
4	411	Homo sapiens chromosome 16, P1 clone	0.0
5	485	Homo sapiens chromosome 17 clone	5e-74
6	380	Homo sapiens chromosome 17 clone	e-171
7	232	Human 18S ribosomal RNA	2e-99
8	162	Homo sapiens retroviral-like sequence S71	0.002
9	232	Human 18S ribosomal RNA	5e-97
10	23	Linker sequence only	0.0

D17S948) thereby ensuring that the RP17 gene is present. The high density of PCR-based genetic markers currently available in this region, which allows for contig assembly without the need for extensive YAC walking or restriction mapping to establish overlaps, facilitated the construction of this map.

Refined localisation of the RP17 critical region by both genetic and physical mapping provides the next step towards the eventual identification of the adRP gene. The YAC/BAC contig described here lays the groundwork for the construction of a transcriptional map for this 1 cM interval. A gene map should facilitate the identification of the adRP gene and also provide candidate genes for other genetic disorders that map to this region of chromosome 17q22.

There is an excess of genes on chromosomes 1, 17 and 19, based on a comparison of the number of mapped ESTs to the cytogenetic lengths of the various chromosomes (Schuler et al. 1996). Genes that are known to map to the RP17 critical interval include the three ESTs used in the physical mapping viz. WI-6034, WI-6805 and FB10A2. WI-6034 is the p70 ribosomal protein S6 kinase and is expressed in eye, foreskin, lung, placenta and testis; and WI-6805 is an unidentified transcript found in adrenal gland, brain, lung and uterus. FB10A2 is another unidentified transcript, which had been isolated from a foetal brain cDNA library. According to the Human Gene Map database (http://www.ncbi.nlm.nih.gov/cgi-bin/SCIENCE96/) from the National Center for Biotechnology Information, other genes residing in the region of the RP17 contig include the T-box 2 gene, human platelet endothelial cell adhesion molecule, p80-coilin and myeloperoxidase precursor. None of these genes, however, is a strong candidate at this stage.

Tremendous time and effort are required to complete a positional cloning project successfully. This implies that the candidate gene isolation techniques used must be both rapid and thorough. In the present study, the cDNA selection technique was applied to a YAC from the RP17 contig. This novel approach was utilised because the cDNA selection protocol is less labour-intensive and less technically demanding than many of the other gene identification methods currently available. Two rounds of selection were performed and ten clones were sequenced in order to monitor the success of the method. The presence of a 23 bp contaminant was detected in one of the clones sequenced. As the cDNA selection method is PCR-based, it is likely that the cloning of this short oligonucleotide may be due to a sequence and length bias of the PCR technique. Usually shorter fragments tend to be selected at each successive round of selection at the expense of the longer sequences.

Future prospects for this project include the further evaluation of the secondary selected material as well as the screening out of contaminants e.g. the 18S ribosomal mRNA and the linker oligonucleotide (Table 4.4). Additional cDNA selections should also be performed using an uncloned retina-specific pool of cDNAs, rather than a conventional commercially-available library. The construction of a high-quality retina cDNA library would be a valuable resource for the cloning of genes underlying various retinal degenerative disorders. It is also imperative that FISH analysis be performed on the YACs and BACs comprising the RP17 contig in order to determine if any of the clones are chimaeric. This information would prove useful when interpreting results obtained from cDNA selection experiments.

In conclusion, in order to increase the chances of identifying the RP17 gene, a number of positional cloning techniques need to be employed simultaneously. In one study, the cloning of the breast cancer

susceptibility gene *BRCA1* was achieved by a combination of three methods: (i) hybrid selection (ii) direct screening of cDNA libraries and (iii) random sequencing (Harshman et al. 1995). This group found that by using a range of gene isolation techniques they identified a large number of candidate gene fragments, which subsequently facilitated the isolation of the causative gene.

CHAPTER FIVE

SCREENING OF POSITIONAL CANDIDATE GENES FOR RP17

5

SCREENING OF POSITIONAL CANDIDATE GENES FOR RP17

SCREENING OF POSITIONAL CANDIDATE GENES FOR RP17

Summary:

Four positional candidate genes for the RP17 locus viz. gamma subunit of phosphodiesterase (*PDEG*), tissue inhibitor of metalloproteinases-2 (*TIMP2*), protein kinase C alpha (*PKCA*) and retinal fascin, were examined in the two RP17-linked families. Recombination events between the adRP locus and (i) single-stranded conformation polymorphisms in *PDEG* and retinal fascin; and (ii) a restriction fragment length polymorphism in *TIMP2* provided evidence for the exclusion of these three candidate genes. The PKCA gene was excluded on the basis of physical map data. Also, 1727 bp of the 2245 bp PKCA cDNA was sequenced and no mutations detected. In addition, direct DNA sequencing revealed three novel polymorphisms in the retinal fascin gene.

5.1 INTRODUCTION

5.1.1 The positional candidate gene approach

The choice of strategy used to identify genes causative of inherited disorders depends on what is known about the pathogenesis of the condition and what resources are available i.e. animal models, clone libraries, chromosomal abnormalities etc. Ultimately, several of these strategies generate candidate genes which are then investigated individually for any evidence that implicates them as the defective gene.

For the positional candidate gene approach, once a disorder has been mapped to a specific sub-chromosomal localisation, database searches can be made to identify candidate genes; and with more and more

human genes being mapped to specific chromosomal regions, this approach is set to dominate the field. Some of the notable successes of inherited disease genes identified by positional candidate methods are the fibrillin gene for Marfan syndrome (Peltonen and Kainulainen 1992), paired box gene *PAX3* for Waardenburg syndrome type 1 (Baldwin et al. 1992) and β-Amyloid precursor for Alzheimer disease (Goate et al. 1991). In addition, with this method both *rhodopsin* and *peripherin/RDS* were successfully identified as adRP genes (Dryja et al. 1990, Farrar et al. 1991, Kajiwara et al. 1991).

Once a disorder has been localised, a few selection criteria may be used to choose potential candidate genes for further screening:

- **The candidate gene might exhibit the appropriate expression pattern or function.**
  For some disorders, observations on the pathogenesis may immediately suggest candidate genes with an appropriate expression pattern or function e.g. for RP, potential candidates are genes expressed in the photoreceptors or genes coding for components of the phototransduction pathway etc.

- **The candidate gene might show homology to a gene implicated in an animal model of the disorder.**
  If an animal phenotype shows a striking similarity to a human disorder, it may result from mutations in the animal ortholog of the human disease-causing gene. In cases where the human gene is unknown, knowledge of the animal ortholog could be used to help identify and characterise the human gene. Such an animal model may have originated spontaneously or have been created artificially by X-rays, chemical mutagenesis or even by a gene targeting approach.

- **The candidate gene might show homology or functional relatedness to a gene implicated in a similar human disease phenotype.**
  If a gene shown to be responsible for a particular condition is a member of a multigene family, then other members of the family would be candidates for phenotypically similar disorders. A candidate gene may also be suggested on the basis of a close functional relationship to a known disease-causing gene i.e. both genes are part of the same metabolic or developmental pathway.

5.1.2 Introductory background to candidate genes for RP17

Using the above selection criteria, four candidate genes were investigated for their possible involvement in the pathogenesis of adRP in families RPD8 and RPD19. The positional candidates chosen for study were:

i) Gamma subunit of phosphodiesterase,
ii) Tissue inhibitor of metalloproteinases-2,
iii) Protein kinase C alpha and
iv) Retinal fascin.

5.1.2.1 *Gamma subunit of rod phosphodiesterase (PDEG)*

cGMP-phosphodiesterase (PDE) is a key component of the phototransduction pathway in retinal rod cells and is composed of alpha, beta and two identical gamma subunits (Farber 1995). The alpha and beta subunits together contain the catalytic site involved in the hydrolysis of cyclic GMP to 5' GMP and the enzyme becomes active only when the gamma subunits are removed by activated transducin (refer to phototransduction cascade, Section 1.1.3).

In 1990, a 1012 bp human cDNA encoding the gamma subunit of PDE (*PDEG*; MIM 180073) was cloned and sequenced (Tuteja et al. 1990). The

coding region of 261 bp was found to be highly homologous to the corresponding cDNAs from bovine and mouse retinas; and a comparison of the deduced amino acid sequences of the proteins indicated that this gene has been well conserved through evolution (97.7% identity between human and bovine; and 96.6% identity between human and mouse). The human *PDEG* gene was originally assigned to chromosome 17q21.1 using *in situ* hybridisation techniques (Tuteja et al. 1990). It was subsequently mapped to a more distal location on 17q25 by a combination of *in situ* hybridisation and genetic linkage analysis (Dollfus et al. 1993).

Mutations in genes encoding the alpha (*PDEA*) and beta (*PDEB*) subunits of PDE have been implicated in various hereditary retinal degenerations. Pathogenic point mutations in *PDEA* have been associated with arRP in two pedigrees (Huang et al. 1995). Mutations in *PDEB* have been shown to co-segregate with some forms of arRP (McLaughlin et al. 1993) and in one family with autosomal dominant congenital stationary night blindness (Gal et al. 1994).

To date, the *PDEG* gene has not been associated with any human retinal degeneration. A cloned fragment of the gene was used as a probe to search for submicroscopic deletions or rearrangements of *PDEG* in patients with RP and Usher syndrome type 1, but no altered size or homozygous deletions were detected (Cotran et al. 1991). In another study, an extensive screen was undertaken using SSCP analysis of the coding region of *PDEG* in 704 unrelated patients with RP or allied disorders (Hahn et al. 1994). No pathogenic mutations were revealed, however, two frequent polymorphisms were detected in the 5' and 3' untranslated regions of the gene. Disruption of the mouse *PDEG* gene using a gene targeting approach resulted in a rapid retinal degeneration resembling human RP (Tsang et al. 1996). This finding indicates that the

inhibitory gamma subunits appear to be essential for the integrity of the photoreceptors and the expression of PDE activity *in vivo*.

PDEG was thought to be a likely candidate for the RP17 locus on the basis of its chromosomal location, photoreceptor-specific expression pattern and known involvement in the rod phototransduction cascade. The possible association of this gene with adRP in the two 17q-linked adRP families was therefore investigated in this study.

5.1.2.2 *Tissue inhibitor of metalloproteinases-2 (TIMP2)*

The matrix metalloproteinases are a family of enzymes involved in the degradation of components of the extracellular matrix. The unrestrained activity of these enzymes may result in extensive tissue damage and they have been implicated in several different pathological conditions, including tumour cell invasion and rheumatoid arthritis (Matrisian 1990). The natural inhibitors of metalloproteinases are the tissue inhibitors of metalloproteinases (TIMP) protein family, and individual members of this family may possess selective affinities for different matrix metalloproteinases (Matrisian 1990).

TIMP3, a member of the TIMP gene family, has been shown to be causative of Sorsby fundus dystrophy (Weber et al. 1994). This rare type of retinal degeneration manifests with loss of central vision due to macular degeneration and progressive atrophy of the retina and choroid. Furthermore, increased expression of *TIMP3* mRNA has been reported in retinas from two individuals with simplex RP (Jomary et al. 1995).

TIMP2, identified in 1989 (Stetler-Stevenson et al. 1989), has been mapped to 17q25 by analysis of somatic cell hybrids and fluorescent *in situ* hybridisation (De Clerck et al. 1992). This gene comprises 5 exons

and the complete genomic nucleotide sequence spans 83 kb (Hammani et al. 1996).

Although not previously associated with retinal dystrophies, *TIMP2* (MIM 188825) was investigated as a positional candidate for RP17 in family RPD8. An intragenic restriction fragment length polymorphism (RFLP) was used as a marker for linkage studies in the adRP kindred.

5.1.2.3 *Protein kinase C alpha (PKCA)*

Protein kinase C alpha (*PKCA*, MIM 176960) is a candidate for the RP17 locus because a photoreceptor-specific protein kinase C was shown to be required for the deactivation and rapid desensitisation of the phototransduction cascade in Drosophila (Schaeffer et al. 1989; Smith et al. 1991). In addition, PKC has been shown to be involved in the *in vitro* phosphorylation of rhodopsin in the outer segments of rod cells (Newton and Williams 1993).

Three members of the PKC gene family viz. PKC alpha (*PKCA*), PKC beta (*PKCB*) and PKC gamma (*PKCG*) are highly homologous to each other (Coussens et al. 1986). A mutation in *PKCG* has been shown to segregate with RP in two families linked to the adRP locus on 19q (Al-Maghtheh et al. 1998). This change (Arg659Ser) may, however, be a rare polymorphism since no pathogenic mutations were detected in three other 19q-linked kindreds. *PKCA* was originally isolated from bovine brain (Parker et al. 1986) and the human homologue has been assigned to human chromosome 17q22-24 using *in situ* hybridisation (Coussens et al. 1986). Subsequently, a high-resolution radiation hybrid map was used to refine the position of *PKCA* relative to microsatellite markers on 17q22 (Foster et al. 1996).

The involvement of the *PKCA* gene in adRP in family RPD8 was investigated using three intragenic RFLPs, as well as sequencing of the cDNA in a RP affected family member. In addition, the presence of a *PKCA*-specific expressed sequence tag (EST) was screened for in the RP17 critical interval.

5.1.2.4 *Retinal fascin*

Fascins are an evolutionarily conserved family of proteins that organise filamentous actin into bundles (Edwards and Bryan 1995). Due to their wide distribution, they are thought to play important roles in the morphogenesis of diverse intracellular structures. Mutations in the Drosophila homologue, the *singed* gene product, result in malformed bristles and sterile females (Edwards and Bryan 1995).

The recently identified *retinal fascin* gene, isolated from a bovine retina cDNA library, was shown to be retina-specific and expressed exclusively in the inner segment of photoreceptor cells (Saishin et al. 1997). Sequence analysis of the deduced protein indicates that it has 55% amino acid identity with murine fascin, 57% with human fascin and 52% with that of Xenopus fascin, thereby providing evidence that retinal fascin is a novel member of the fascin gene family. The exact function of *retinal fascin* is unknown, but it is thought to play a role in photoreceptor cell-specific events, such as disc morphogenesis (Saishin et al. 1997). The gene was recently mapped to chromosome 17q by fluorescent *in situ* hybridisation analysis (B. Tubb, personal communication)

Retinal fascin was examined as a candidate for RP17 on the basis of both its localisation to chromosome 17q and its photoreceptor cell-specific expression pattern.

5.2 MATERIALS AND METHODS

5.2.1 Single strand conformation polymorphism (SSCP) analysis of *PDEG*

Two intragenic polymorphisms have been reported in the 5' and 3' untranslated regions (UTRs) of the *PDEG* gene (Hahn et al. 1994). One polymorphism is the deletion of a cytosine in the 5' UTR 14 bp upstream of the initiation codon. The allele frequencies are calculated to be 0.11 and 0.89 based on 112 individuals of mixed North American ancestry. The second polymorphism is an A1447G transversion in the 3' UTR 45 bp after the termination codon, with allele frequencies 0.36 and 0.64. In order to ascertain whether the *PDEG* gene segregates with the adRP disease phenotype, the two SSCP-detectable polymorphisms were analysed in families RPD8 and RPD19 using standard SSCP techniques (Appendix B, Orita et al. 1989).

The following primer sets were used:
for the 5' polymorphism, forward: 5'-TgC ACT TgA CCg CAg CAg gA-3', reverse: 5'-CCC ATC CCC CAg CTC TgC TT-3';
and for the 3' polymorphism, forward: 5'-CTC TgA TCC gTg gCC CgT TT-3', reverse: 5'-CTC AAC Agg AAT CCT gAg CA-3'.
PCR conditions were as described in Chapter 3 except the [α^{32}P]dCTP was omitted from the PCR reaction and the PCR products were detected by the silver-staining technique (Appendix B).

Two point lod scores were calculated using the MLINK option of the LINKAGE package of programs (Version 5.1; Lathrop et al. 1984). The penetrance was set at 100% with a gene frequency of 0.0001.

5.2.2 Restriction fragment length polymorphism (RFLP) analysis of *TIMP2*

A *Taq*I RFLP in the *TIMP2* gene has previously been reported with polymorphic bands at 3.0 kb and 1.0 kb, and three additional constant bands at 2.5 kb, 1.4 kb, and 0.9 kb (Jares et al. 1994). The frequencies of the 3.0 kb and 1.0 kb alleles, as calculated in Caucasian individuals, are 0.76 and 0.24, respectively, with an observed heterozygosity of 0.28 (Jares et al. 1994).

The *Taq*I RFLP was genotyped in family RPD8 using Southern blot hybridisation and a 791 bp *Eco*RI-*Xba*I fragment of *TIMP2* cDNA (provided by WG Stetler-Stevenson, NIH, USA; Stetler-Stevenson et al. 1990) as a probe. Southern blots were prepared, according to protocols in Appendix B, from *Taq*I-digested genomic DNA (Vandenplas et al. 1984). The membranes were hybridised overnight at 65°C with the [α^{32}P]dCTP-labelled probe (Appendix B), washed at a final stringency of 0.6X SSC; 0.3% SDS at 65°C and exposed to Agfa Curix X-ray film at -70°C for one week. Using this two-allele polymorphism, each family member tested could be assigned a [1/1], [1/2] or [2/2] genotype.

Two point lod scores were calculated, as described in Section 5.2.1, between the adRP locus and the *Taq*I RFLP in the *TIMP2* gene.

5.2.3 Investigation of *PKCA*

i) RFLP analysis

Three RFLPs in the *PKCA* gene have been reported and are detected with the restriction enzymes: *Rsa*I, *Kpn*I and *Dra*I (Summar et al. 1989).

Polymorphic bands are detected with *Rsa*I at 4.3 kb and 2.5 kb; with *Kpn*I at 6.8 kb and 5.7 kb and with *Dra*I at 2.6 kb and 1.6 kb. All three RFLPs were genotyped in family RPD8 using Southern blot hybridisation and a 1.29 kb fragment of *PKCA* cDNA from plasmid phPKC-alpha7 (American Type Culture Collection, USA) as a probe. The plasmid, obtained as a stab culture in *E-coli*, was purified according to standard protocols (Sambrook et al. 1989) and digested with *Eco*RI at 37°C overnight, to excise the insert. Southern blots were prepared from genomic DNA digested with the appropriate restriction enzyme and subsequently hybridised, overnight, with [α^{32}P]dCTP-labelled probe (Appendix B). After the post-hybridisation washes, the blots were exposed to autoradiography, overnight.

ii) Sequencing of *PKCA* cDNA

In order to screen for pathogenic mutations in the *PKCA* gene, seven sets of primers (Table 5.1) were designed to amplify and sequence the 2245 bp of *PKCA* cDNA (Finkenzeller et al. 1990; EMBL accession number X52479). The primers A to E were originally designed to amplify the *PKCA* cDNA in five overlapping fragments of approximately 500 bp each (Fig. 5.1), however, primers A, D and E either did not produce a PCR product (set A) or multiple non-specific PCR products were evident (sets D and E). In order to resolve these problems, primers F and G (Fig. 5.1) which amplify the first 985 bp of the 5' end and the last 945 bp of the 3' end of the cDNA, respectively were used (Alvaro et al. 1993).

Total RNA was extracted from Epstein-Barr virus-transformed lymphoblasts (Appendix B) of both an adRP affected member and an unaffected member from family RPD8 by a method derived from Chomczynski and Sacchi (1987). The RNA was reverse transcribed with a reverse transcriptase and PKCA cDNA was amplified by PCR and directly sequenced in order to screen for potentially pathogenic mutations (Appendix B).

iii) Screening for *PKCA* 5' UTR expressed sequence tag

Primers were designed which amplify a 3'UTR expressed sequence tag (EST) specific for the *PKCA* gene (Fig. 5.1). The sequences are as follows: forward, 5'-AgT ggA AgT gAA TCC TTA AC-3', reverse, 5'-CTT CCA CTA AGA TAA TgT TC-3'.

The seven YACs and two BACs spanning the RP17 critical interval (Chapter 4) were screened for the presence of the PKCA EST by PCR. The reactions were performed with approximately 20 ng of YAC or BAC DNA in 10 µl reaction volumes containing 1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl (pH 8.4), 250 µM of each dNTP, 10 pmol of each primer and 0.5 U of *Taq* DNA polymerase (GIBCO/BRL, USA). The PCR reactions were amplified at 94°C for 3 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, with a final extension of 72°C for 5 min on an Omnigene Thermal Cycler (Hybaid, USA). PCR products were visualised on ethidium bromide-stained 1% agarose gels. The clones from which the EST primers amplified either an appropriately sized product or no product were recorded as positive or negative, respectively.

Table 5.1 List of primers used to sequence *PKCA* cDNA.

Primer Set	Annealing temperature	Forward	Reverse
Set A	-	5'-ggA gCA AgA ggT ggT Tgg gg-3'	5'-CTC AgT gTg ATC CAT TCC gC-3'
Set B	55°C	5'-Tgg gTC ACT gCT CTA Tgg AC-3'	5'-gCC AAg TTT ggC TTT CTC gA-3'
Set C	55°C	5'-ggA gTT TCg gAg CTg ATg AA-3'	5'-ggT CCC CAC CgT TgA CAT AT-3'
Set D	60°C	5'-TgA TgA CgT ggA gTg CAC CA-3'	5'-CgT TgT gCT CCA TgA TAg AC-3'
Set E	60°C	5'-CTA Tgg CgT CCT gTT gTA Tg-3'	5'-Agg ATT CAC TTC CAC TgC gg-3'
Set F	55°C	5'-ggA gCA AgA ggT ggT Tgg-3'	5'-CTT CAg Agg gAC TgA TgA CT-3'
Set G	60°C	5'-gAC CTC ATg TAC CAC ATT CA-3'	5'-CTT CCA CTA AgA TAA TgT TC-3'

primer F_{for}/ primer A_{for}

1 GGAGCAAGAG GTGGTTGG/GG GGGGACCATG GCTGACGTTT TCCCGGGCAA

51 CGACTCCACG GCGTCTCAGG ACGTGGCCAA CCGCTTCGCC CGCAAAGGGG

101 CGCTGAGGCA GAAGAACGTG CACGAGGTGA AGGACCACAA ATTCATCGCG

151 CGCTTCTTCA AGCAGCCCAC CTTCTGCAGC CACTGCACCG ACTTCATCTG

201 GGGGTTTGGG AAACAAGGCT TCCAGTGCCA AGTTTGCTGT TTTGTGGTCC

251 ACAAGAGGTG CCATGAATTT GTTACTTTTT CTTGTCCGGG TGCGGATAAG

301 GGACCCGACA CTGATGACCC CAGGAGCAAG CACAAGTTCA AAATCCACAC

primer B_{for}

351 TTACGGAAGC CCCACCTTCT GCGATCACTG TGGGTCACTG CTCTATGGAC

401 TTATCCATCA AGGGATGAAA TGTGACACCT GCGATATGAA CGTTCACAAG

451 CAATGCGTCA TCAATGTCCC CAGCCTCTGC GGAATGGATC ACACTGAGAA

primer A_{rev}

501 GAGGGGGCGG ATTTACCTAA AGGCTGAGGT TGCTGATGAA AAGCTCCATG

551 TCACAGTACG AGATGCAAAA AATCTAATCC CTATGGATCC AAACGGGCTT

601 TCAGATCCTT ATGTGAAGCT GAAACTTATT CCTGATCCCA AGAATGAAAG

651 CAAGCAAAAA ACCAAAACCA TCCGCTCCAC ACTAAATCCG CAGTGGAATG

701 AGTCCTTTAC ATTCAAATTG AAACCTTCAG ACAAAGACCG ACGACTGTCT

751 GTAGAAATCT GGGACTGGGA TCGAACAACA AGGAATGACT TCATGGGATC

primer C_{for}

801 CCTTTCCTTT GGAGTTTCGG AGCTGATGAA GATGCCGGCC AGTGGATGGT

851 ACAAGTTGCT TAACCAAGAA GAAGGTGAGT ACTACAACGT ACCCATTCCG

901 GAAGGGGACG AGGAAGGAAA CATGGAACTC AGGCAGAAAT TCGAGAAAGC

primer B_{rev}

951 CAAACTTGGC CCTGCTGGCA ACAAAGTCAT CAGTCCCTCT GAAGACAGGA

primer F_{rev}

1001 AACAACCTTC CAACAACCTT GACCGAGTGA AACTCACGGA CTTCAATTTC

1051 CTCATGGTGT TGGGAAAGGG GAGTTTTGGA AAGGTGATGC TTGCCGACAG

1101 GAAGGGCACA GAAGAACTGT ATGCAATCAA AATCCTGAAG AAGGATGTGG

primer D_{for}

1151 TGATTCAGGA TGATGACGTG GAGTGCACCA TGGTAGAAAA GCGAGTCTTG

1201 GCCCTGCTTG ACAAACCCCC GTTCTTGACG CAGCTGCACT CCTGCTTCCA

Fig. 5.1 continued overleaf

1251 GACAGTGGAT CGGCTGTACT TCGTCATGGA ATATGTCAAC GGTGGG/GACC
primer G_{for} *primer C_{rev}*
1301 TCATGTACCA CATTCAGCAA GTAGGAAAAT TTAAGGAACC ACAAGCAGTA

1351 TTCTATGCGG CAGAGATTTC CATCGGATTG TTCTTTCTTC ATAAAAGAGG

1401 AATCATTTAT AGGGATCTGA AGTTAGATAA CGTCATGTTG GATTCAGAAG

1451 GACATATCAA AATTGCTGAC TTTGGGATGT GCAAGGAACA CATGATGGAT

1501 GGAGTCACGA CCAGGACCTT CTGTGGGACT CCAGATTATA TCGCCCCAGA

1551 GATAATCGCT TATCAGCCGT ATGGAAAATC TGTGGACTGG TGGGCCTATG
primer E_{for}
1601 GCGTCCTGTT GTATGAAATG CTTGCCGGGC AGCCTCCATT TGATGGTGAA

1651 GATGAAGACG AGCTATTTCA GTCTATCATG GAGCACAACG TTTCCTATCC
primer D_{rev}
1701 AAAATCCTTG TCCAAGGAGG CTGTTTCTAT CTGCAAAGGA CTGATGACCA

1751 AACACCCAGC CAAGCGGCTG GGCTGTGGGC CTGAGGGGGA GAGGGACGTG

1801 AGAGAGCATG CCTTCTTCCG GAGGATCGAG TGGGAAAAAC TGGAGAACAG

1851 GGAGATCCAG CCACCATTCA AGCCCAAAGT GTGTGGCAAA GGAGCAGAGA

1901 ACTTTGACAA GTTCTTCACA CGAGGACAGC CCGTCTTAAC ACCACCTGAT

1951 CAGCTGGTTA TTGCTAACAT AGACCAGTCT GATTTTGAAG GGTTCTCGTA

2001 TGTCAACCCC CAGTTTGTGC ACCCCATCTT ACAGAGTGCA GTATGAAACT

2051 CACCAGCGAG AACAAACACC TCCCCAGCCC CCAGCCCTCC CCGCAGTGGA
EST $primer_{for}$ *primer E_{rev}*
2101 AGTGAATCCT/TAACCCTAAA ATTTTAAGGC CACGGCTTGT GTCTGATTCC

2151 GGAGGCCTGA AAATTGTAGG GTTATTAGTC CAAATGTGAT CAACTGGGTC

2201 TCTCTCTTAC AACCAAGAAC ATTATCTTAG TGGAAG
primer G_{rev}/ EST $primer_{rev}$

Figure 5.1 cDNA sequence of the protein kinase C alpha gene (length 2245 bp; Genome Database accession number X52479). The relative positions of the various primers used to amplify the gene are depicted in blue.

5.2.4 DNA sequencing and SSCP analysis of *retinal fascin*

i) Direct DNA sequencing

The 5 exons and the putative promoter region of the *retinal fascin* gene were PCR amplified in an affected and an unaffected family member from each of the two adRP families. The 11 pairs of primers that were used to amplify the exons are listed in Table 5.2 (B. Tubb, personal communication). Due to their large size, the promoter region and exon 1 were amplified in two and five overlapping fragments, respectively. PCR products were purified in QIAquick spin columns (Qiagen, Germany) according to the manufacturers' instructions and sequenced with a *Taq* Dideoxy Terminator Cycle sequencing kit (Perkin Elmer, USA). The sequencing reactions were separated by automated capillary electrophoresis in an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, USA). For confirmation of results, DNA sequencing was performed on both stands.

ii) SSCP analysis

Direct DNA sequencing revealed three novel intragenic polymorphisms in *retinal fascin*: two in the 5' putative promoter region and one in exon 1. All three polymorphisms were genotyped in families RPD8 and RPD19 using standard SSCP techniques in order to determine whether an allele of *retinal fascin* segregates with the disease phenotype.

Allele frequencies of the three polymorphisms were calculated in 88 chromosomes obtained from individuals of diverse ethnic origin.
The frequencies observed for the two 5' polymorphisms are:
0.84 and 0.16 for an A to G transversion located 707 bp upstream of the initiation codon; and 0.96 and 0.04 for a C to G transition located 622 bp upstream of the initiation codon. Both polymorphisms were amplified

Table 5.2 List of primers used to sequence the promoter and coding regions of the retinal fascin gene.

Region of gene	Forward primer	Reverse primer
promoter	5'-gAC gTC gAC Agg CTg CAC ggC CAC TgT gT-3'	5'-TgA TCC CCT TTT CCT CAT AgC-3'
promoter	5'-CCA CTg gAC ggC TTC TAC AT-3'	5'-CCC AgA CCC AgC Cgg CgT CA-3'
exon 1	5'-TAA gAg CTg CCC Agg CTg CT-3'	5'-CAg gTA gCg gTC AgT gTC gT-3'
exon 1	5'-gCA TgA ATT CgA ACg gCC TgC ACC Agg TgC TgA Ag-3'	5'-ggA CCA ggA AgC ggC AgT CA-3'
exon 1	5'-TAC CTg TCg gCA gAA gAg gA-3'	5'-CgT AgT CgA Cgg TAg Cgg CTg TCA CAg gAC TTg Ag-3'
exon 1	5'-gAC AAg CCC Tgg ggC gTg gA-3'	5'-CgT AgT CgA CCT TgC CgC ACA gAg ACg TAg Cgg Tg-3'
exon 1	5'-gCA TgA ATT CgA gCC gAC ggT ACT gCC TCA AgT CC-3'	5'-CCA Cgg CCT CCT CCC TgA Ag-3'
exon 2	5'-CTA gAT gAg ACC CAC CTC Tg-3'	5'-Tgg Tgg ggA Tgg TgA TgA Tg-3'
exon 3	5'-CCA TCT CCT gCT gTC CTg Ag-3'	5'-gAg ACA gCC CCT gCC CCA gTA-3'
exon 4	5'-gTC CCT AAg TCC CAg gTg Tg-3'	5'-CgA AgT CCT Cgg CgC gTT C-3'
exon 5	5'-gCC Cgg AgC ggC AAg TAC CT-3'	5'-TCC CCA TgT CCT TgA gAC CT-3'

and detected in a single PCR product using the following primers, forward 5'-gAC gTC gAC Agg CTg CAC ggC CAC TgT gT-3', and reverse 5'-TgA TCC CCT TTT CCT CAT AgC-3'.

The polymorphism in exon 1 is a T to C transition positioned 738 bp 3' of the initiation codon. The PCR primers used to amplify this polymorphism are: forward 5'-gCA TgA ATT CgA gCC gAC ggT ACT gCC TCA AgT CC-3' and reverse 5'-CgT AgT CgA CCT TgC CgC ACA gAg ACg TAg Cgg Tg-3'.

Two point lod scores were calculated, as described previously, for the intragenic polymorphism in *retinal fascin* versus the adRP phenotype in the two 17q-linked families.

5.3 RESULTS

5.3.1 Exclusion of *PDEG*

An A1447G polymorphism in the 3' UTR of *PDEG* was genotyped in family RPD8 (Fig. 5.2a) and proved to be informative in this family. The segregation of alleles in the pedigree are presented in Fig. 5.2b. This polymorphism showed a lack of co-segregation with the disease locus, and significantly negative lod scores (Z) of less than -2.00 at theta = 0.045 were generated (Table 5.3). These results indicate the RP17 locus can be excluded from a 4.7 cM genetic distance (using the Haldane map function) on either side of the *PDEG* gene. The other polymorphism at the 5' end of the gene was largely uninformative in this kindred (Fig. 5.2b).

Family RPD19 was also genotyped with the informative A1447G polymorphism (Fig. 5.3a and Fig. 5.3b). Negative lod scores for significant exclusion of the *PDEG* gene were also obtained for this family (Table 5.3).

Table 5.3 Pairwise lod scores between adRP and intragenic markers within the positional candidate genes.

Family	DNA marker	RECOMBINATION FRACTION (θ)						
		0.00	0.05	0.1	0.2	0.3	0.4	θ where Z = -2.00
RPD8	PDEG	-10.22	-1.89	-1.22	-0.58	-0.27	-0.10	**0.045**
RPD19	PDEG	-2.98	-0.14	0.02	0.07	0.03	-0.00	**0.001**
RPD8	TIMP2	-20.27	-0.71	0.37	0.96	0.84	0.38	**0.025**
RPD8	retinal fascin	-14.05	-1.64	-0.87	-0.08	0.19	0.12	-
RPD19	retinal fascin	-8.26	-0.95	-0.48	-0.14	-0.05	-0.03	-

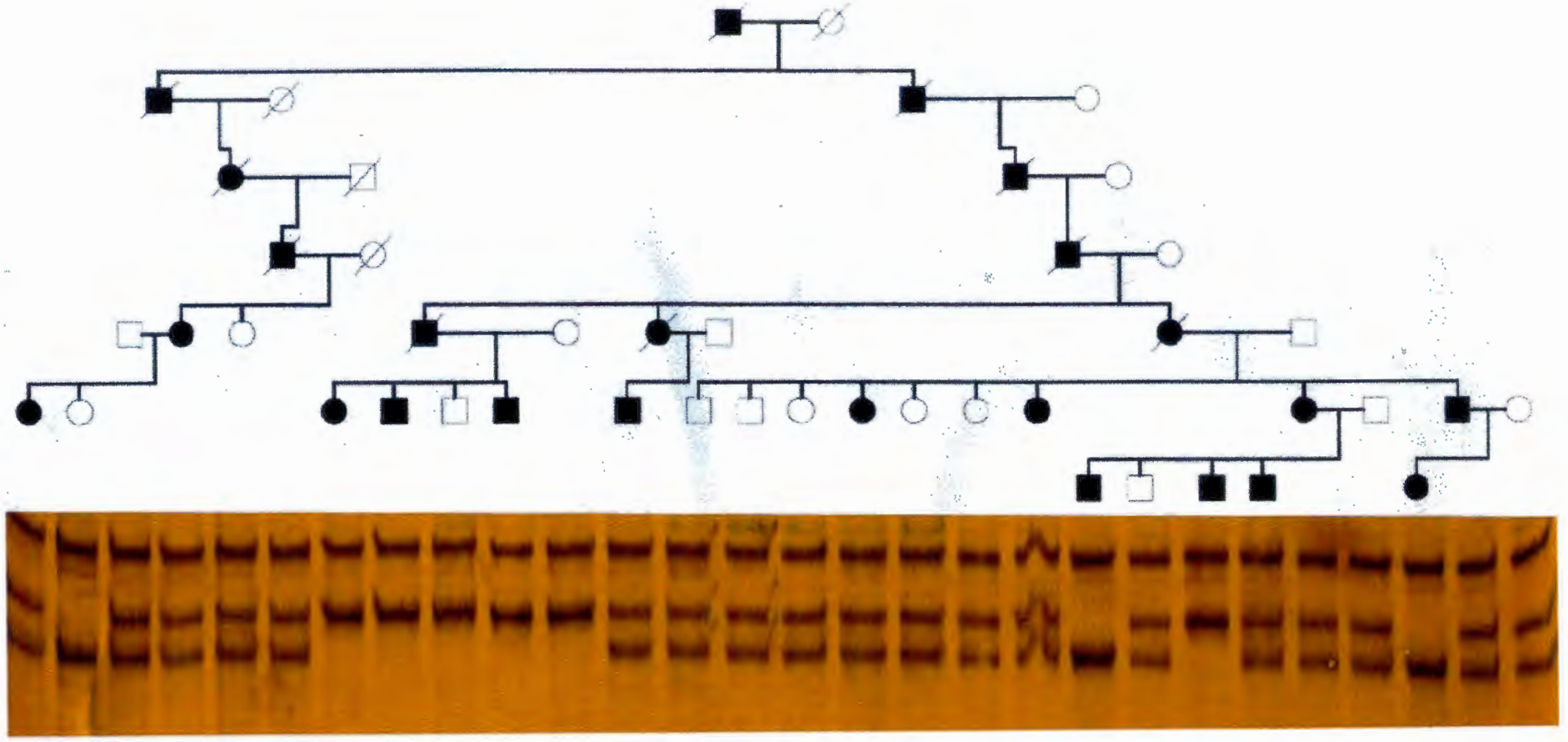


Figure 5.2a SSCP gel illustrating the segregation of the A1447G polymorphism in the *PDEG* gene in family RPD8.

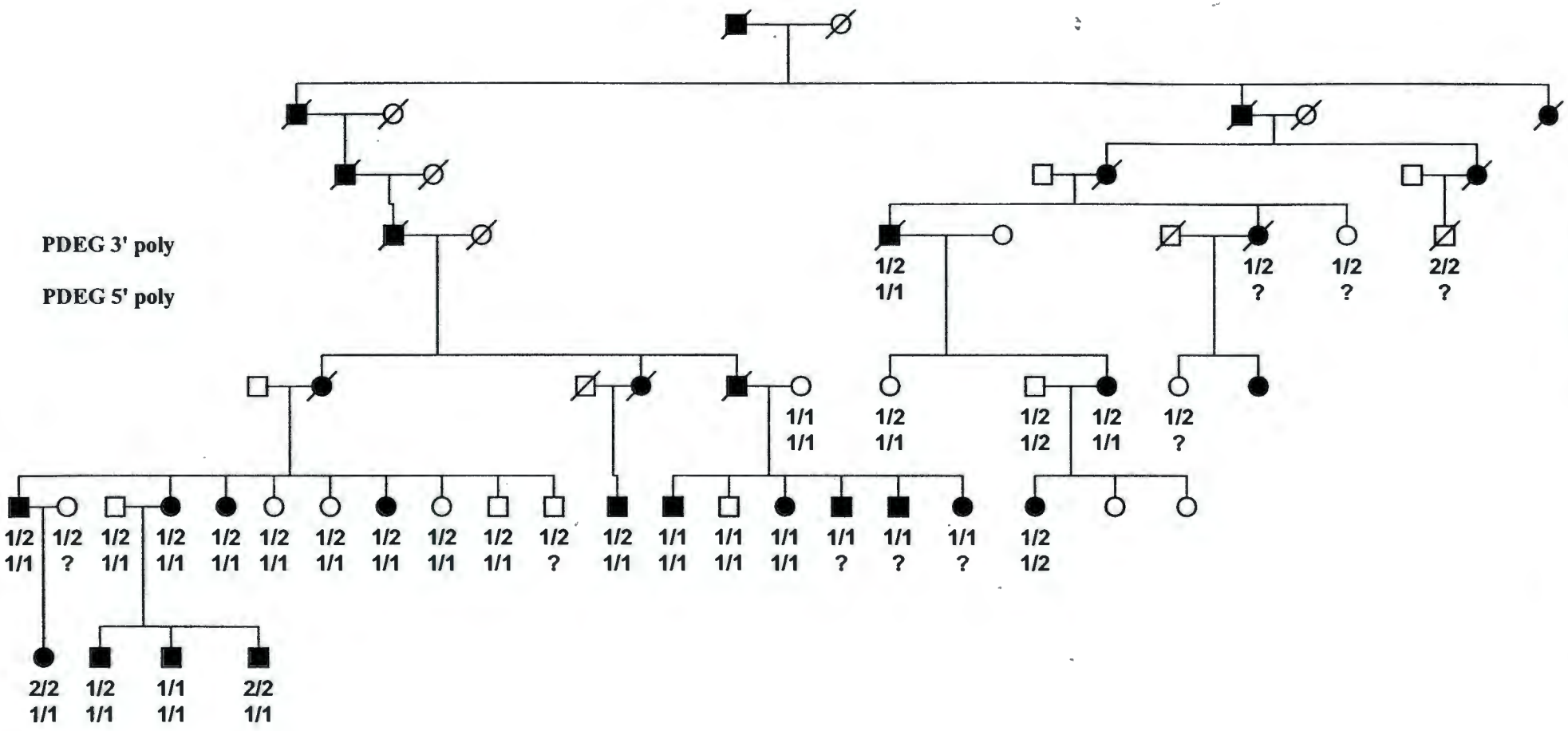


Figure 5.2b Pedigree of family RPD8 illustrating the genotypes of the two polymorphisms in the PDEG gene.

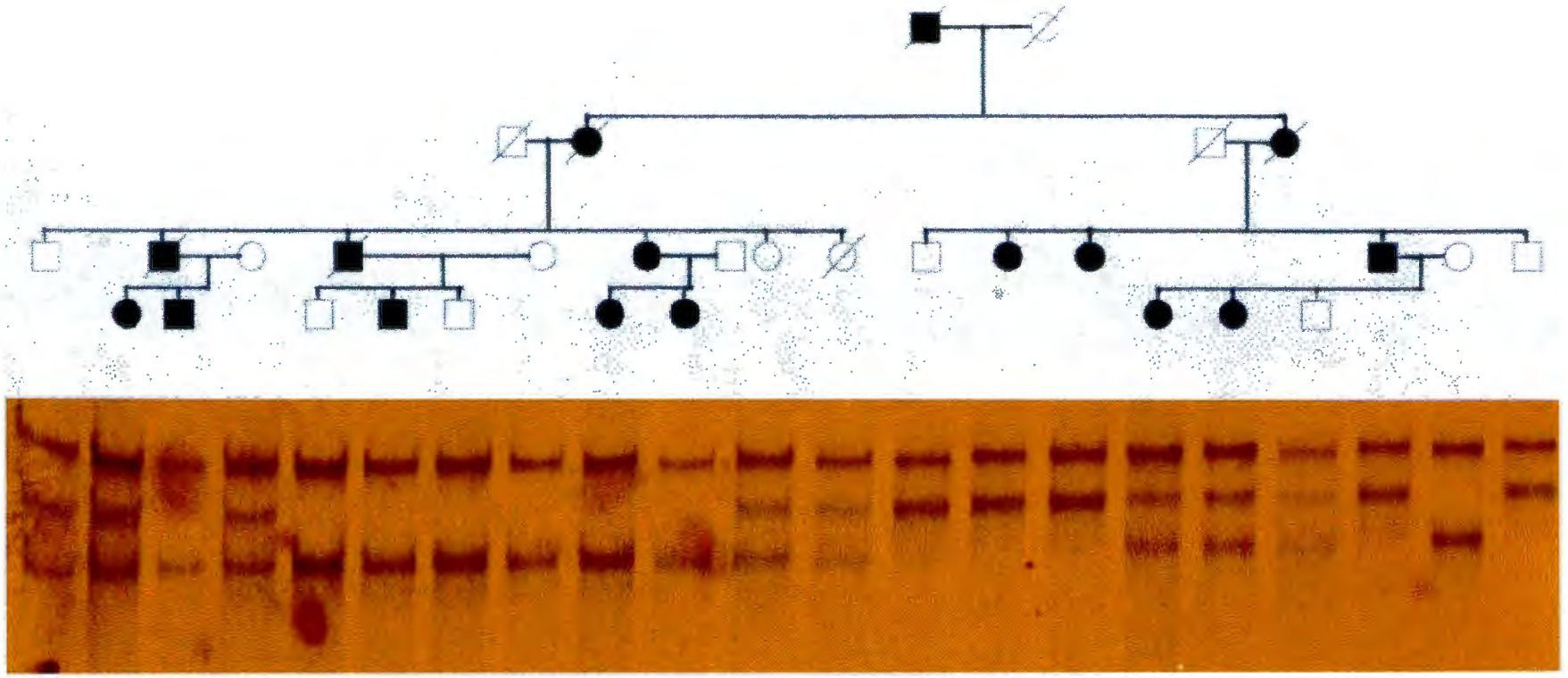


Figure 5.3a Segregation of the A1447G polymorphism in the *PDEG* gene in family RPD19.

PDEG 3' poly

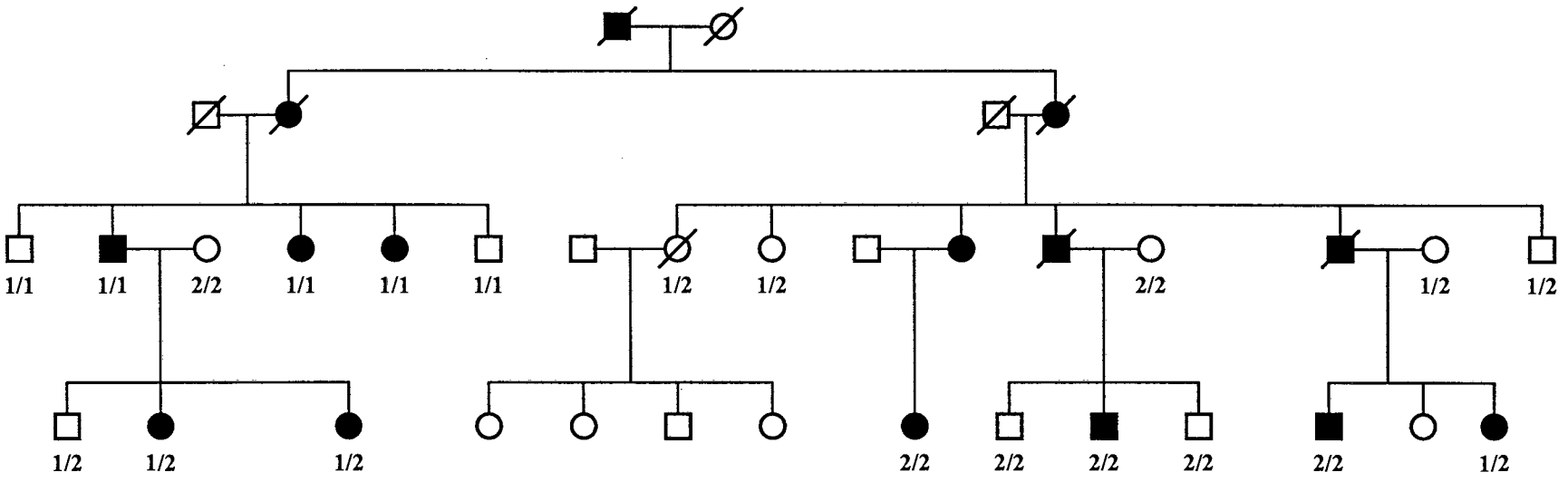


Figure 5.3b Pedigree of family RPD19 depicting the genotypes for the A1447G polymorphism in the PDEG gene.

5.3.2 Exclusion of *TIMP2*

The *Taq*I RFLP in the *TIMP2* gene was genotyped in family RPD8 and a number of recombination events were evident between the gene and the adRP locus (Fig. 5.4). It is apparent that some of the adRP affected family members have the genotype [1/1], whereas others have the genotype [2/2], indicating that these individuals do not share a common allele. This suggests that the *TIMP2* gene is not linked to the adRP locus in this family. Significantly negative two point lod scores between the disease locus and the *TIMP2* gene were obtained, providing evidence for exclusion of this candidate gene (Table 5.3, page 118). Lod scores of -2.00 were obtained at theta = 0.025, which excludes a 2.6 cM genetic distance on either side of the *TIMP2* gene.

5.3.3 Exclusion of *PKCA*

All three RFLPs in the PKCA gene, which had been genotyped in family RPD8 were uninformative in the family. For the *Rsa*I and *Kpn*I RFLPs, all the family members were homozygous at these two loci. For the *Dra*I RFLP, eight individuals were heterozygous at this locus, however, the marker was effectively uninformative in the family. These results may be due to the low level of heterozygosity reported for these intragenic markers (Summar et al. 1989). The lack of informativity means that no conclusions could be drawn as to whether this gene may be included or excluded as a candidate for the RP17 locus and further investigations involving this gene were therefore necessary.

Since the intron-exon boundaries of the *PKCA* have yet to be established, the cDNA was sequenced in an adRP patient and an unaffected family member from family RPD8 in order to screen for pathogenic mutations.

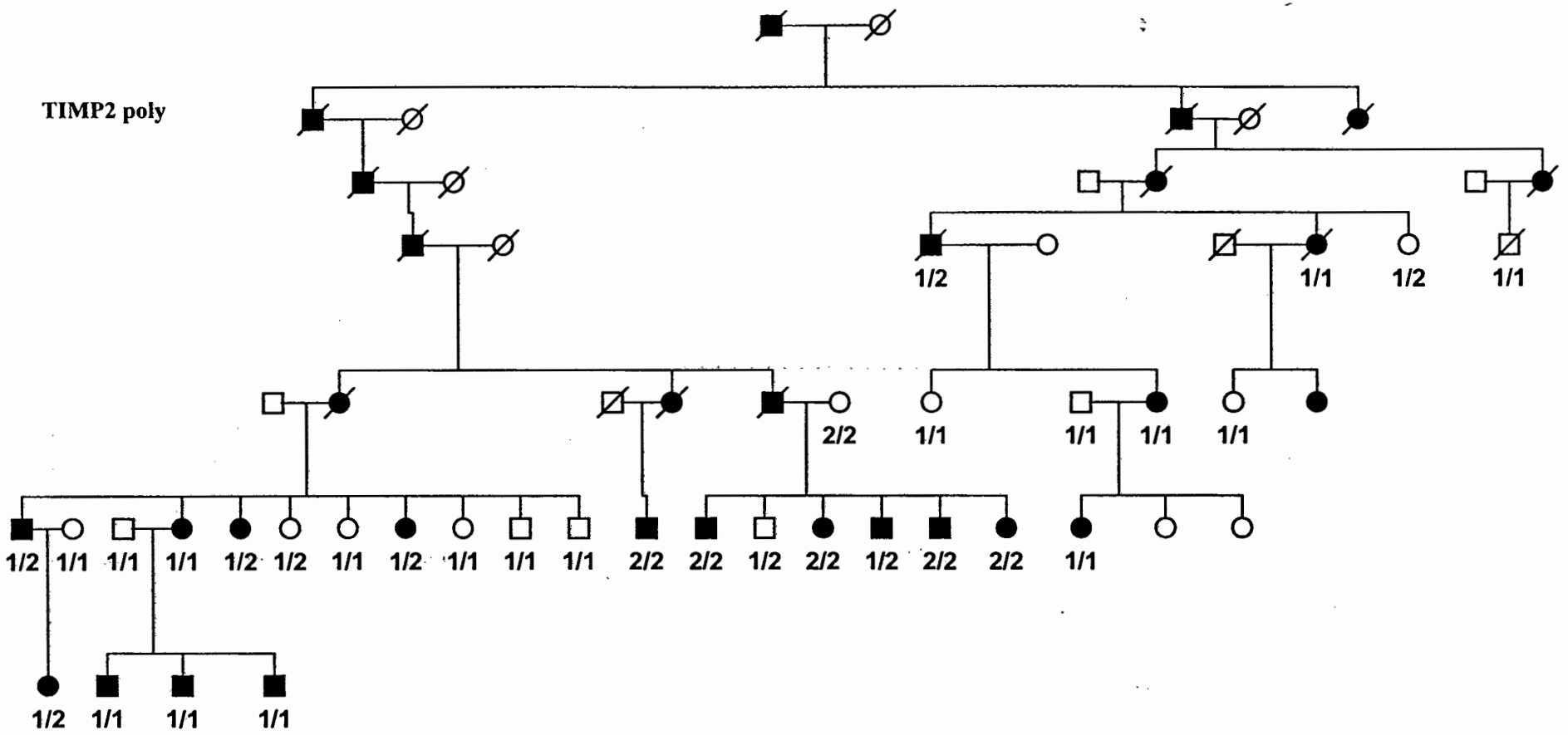


Figure 5.4 Pedigree of family RPD8 illustrating the genotypes of the polymorphism in the TIMP2 gene.

In total, 1727 bp of the 2245 bp *PKCA* cDNA was sequenced in an adRP affected individual but no disease-causing mutations were detected.

During the sequencing of *PKCA* cDNA, a YAC/BAC contig was constructed for the RP17 critical interval, as discussed in Chapter 4. The YACs and BACs comprising the contig were screened for the presence of a *PKCA* EST. The lack of a PCR product of the appropriate size in any of the clones, except in the lane for the positive control, suggest that *PKCA* may not reside in the RP17 critical interval. Furthermore, according to a radiation hybrid map of 17q22-25.3, PKCA was shown to map between the microsatellite markers D17S942 and D17S807 which lie distal to the refined RP17 locus.

5.3.4 Exclusion of retinal fascin

The entire coding region and the putative promoter region of *retinal fascin* were screened for mutations in families RPD8 and RPD19 by direct DNA sequencing. Two different intragenic single nucleotide polymorphisms (SNPs) in the putative promoter region and an intragenic SNP in exon 1 were identified. These SSCP-detectable polymorphisms were genotyped in the two adRP kindreds (Fig. 5.5 and Fig. 5.6) Negative lod scores of Z= <-2.00 were obtained for both families (Table 5.3, page 118), thereby providing evidence for exclusion of this candidate gene. Moreover, DNA sequencing of the five exons of the gene in an affected member from each of the families did not reveal any pathogenic mutations, thereby corroborating the negative linkage results.

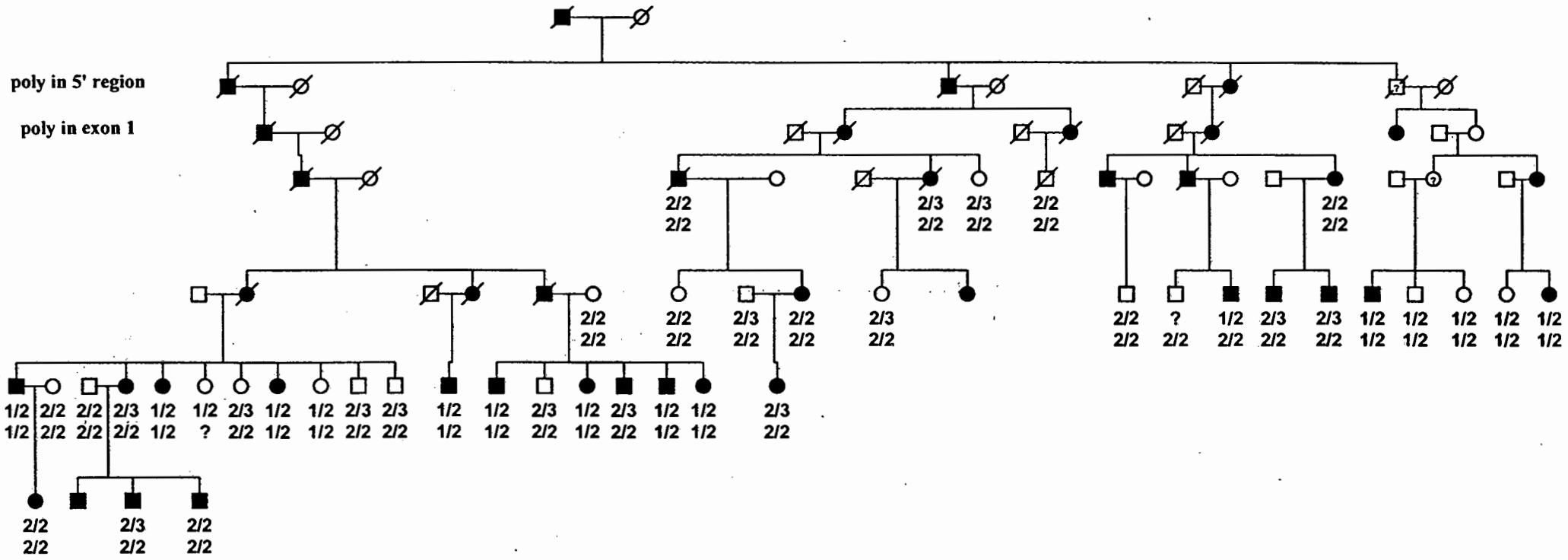


Figure 5.5 Pedigree of family RPD8 illustrating the segregation of the two polymorphisms in the retinal fascin gene.

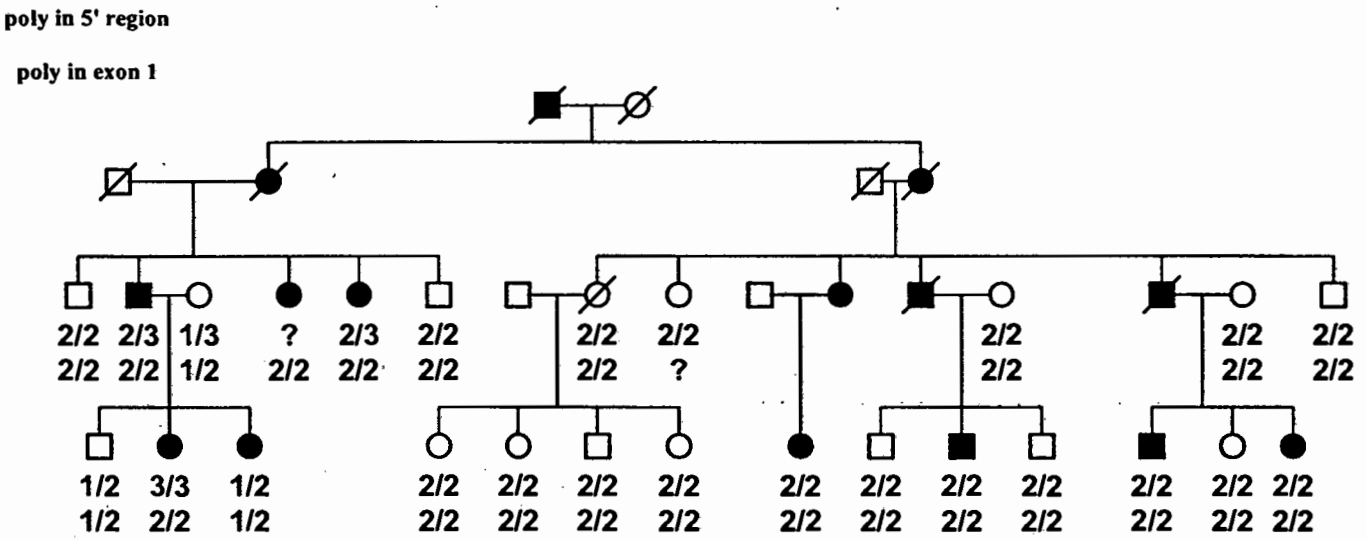


Figure 5.6 Pedigree of RPD19 illustrating the segregation of alleles in the two polymorphisms within the retinal fascin gene.

5.4 DISCUSSION

The search for the complete set of genes causative of retinitis pigmentosa consumes the efforts of numerous laboratories worldwide. Linkage analysis has sometimes pointed the way but the candidate gene approach has been responsible for many successful identifications of defective genes.

However promising a candidate gene appears for a disorder, it must be shown to be mutated in family members affected with the condition. Initially, the candidate gene is examined for co-segregation with the disease phenotype using a closely linked or intragenic polymorphism. In the event that the marker co-segregates, is uninformative in the family, or if no neutral intragenic polymorphisms are available, the next step involves mutation detection methods which are numerous and vary in sensitivity and technical complexity. The most popular of these techniques are SSCP, chemical mismatch cleavage, denaturing gradient gel electrophoresis (DGGE), protein truncation test (PTT), cleavase fragment length polymorphism (CFLP) and direct sequencing of the exons or cDNA. Ultimately, the choice of method depends on a variety of factors including the size of the gene, the level of expertise for a particular procedure and the availability of equipment.

The positional candidate gene approach was adopted in this study in an attempt to identify the RP17 gene. Using a variety of experimental techniques, evidence is provided which suggests that the candidates *PDEG*, *TIMP2*, *PKCA* and *retinal fascin* are not likely to be responsible for the adRP phenotype in the RP17-linked families.

It should be noted that *PDEG* has not yet been implicated in a human hereditary retinal degeneration. Despite its key role in the rod

phototransduction pathway and the fact that other members of this pathway have been associated with retinopathies, *PDEG* has been excluded as a candidate for adRP in families RPD8 and RPD19 (this study) as well as 704 unrelated patients with RP, Ushers syndrome, Leber's congenital amaurosis and Lawrence Moon Bardet-Biedl syndrome (Hahn et al. 1994). There are a number of possible explanations for this finding. One theory is that the protein product of *PDEG* may play a vital role in the cell and deleterious mutations could result in embryonic lethality. A second possibility is that of 'functional redundancy' where another protein may serve the same function as the product of *PDEG*, thereby ensuring that individuals with *PDEG* mutations do not exhibit a recognisable phenotype. A third scenario is that *PDEG* may have such a low mutation rate that an inherited retinal degenerative disorder due to a defect in this gene would occur very rarely. Mutation rates of various genes vary greatly and are dependent on differences in GC content and the timing of DNA replication (Matassi et al. 1999).

Since the *PKCA* gene had not been fully characterised, an attempt was made to sequence the cDNA of an affected adRP family member in order to screen for pathogenic mutations. PKCA cDNA was isolated from illegitimate transcripts in immortalised lymphoblasts obtained from members of family RPD8. The mechanism and biological significance of illegitimate transcription are still obscure, but since these transcripts exhibit the same pathology as legitimate transcripts, they represent a useful tool in the study of genetic diseases (Kaplan et al. 1992a). In autosomal dominant disorders, the pathogenic mutation may result in a null allele, and therefore sequencing of the cDNA to screen for mutations could be misleading since only the non-mutated allele will be sequenced. This approach therefore has serious limitations. Although no mutations were detected, *PKCA* was subsequently shown to not reside on any of the

mega clones spanning the RP17 critical interval, providing supportive evidence for its exclusion.

The photoreceptor-specific expression pattern of *retinal fascin* implicates it as a strong candidate for retinal dystrophies. This gene was excluded, however, from the form of RP segregating in families RPD8 and RPD19. The three intragenic polymorphisms in *retinal fascin* identified in this study may facilitate linkage analysis in other families with hereditary retinopathies linked to chromosome 17q.

In addition to the Human Genome Project's sequencing efforts, gene identification methods are being developed which should generate additional candidates for future studies on RP17. Some strategies have evolved which specifically enrich for and isolate chromosome-specific or tissue-specific genes. One such technique is cDNA representational difference analysis (RDA) which has been employed with some success to isolate chromosome 17-specific cDNAs (Tajima et al. 1997). This DNA subtraction technique was used with a mouse-human chromosome 17 hybrid cell line as the tester and a mouse cell line as a driver and identified two chromosome 17-specific clones. One is a partial cDNA clone containing an open reading frame (ORF) of 687 bp and the second clone is thought to be a full length cDNA containing a single ORF of 1206 bp flanked by 120 and 297 bp UTRs at the 5' and 3' ends, respectively. Both clones are potential candidates for RP17 and should therefore be examined using Northern blot analysis to determine if they are expressed in the retina.

Another successfully employed technique utilises subtractive and differential hybridisation to enrich for retina-specific cDNAs (Imamura et al. 1997). In this method, inserts in human retina cDNA libraries are mixed with brain cDNA inserts. The resulting subtracted retina cDNAs

are hybridised with cDNAs from five different human cDNA libraries including retina, brain and fetal liver. A novel retina-specific protein, designated retina-specific amine oxidase (RAO) was identified using this technique. RAO has an ORF of 2 187 bp and the gene (AOC2) was subsequently mapped by FISH to 17q21. This gene is an appealing candidate for RP17 and should be characterised further for investigation in the RP17-linked families.

In conclusion, it is predicted that over 90% of future studies on human disease genes will employ the positional candidate gene approach due to its relatively high success rate (Strachan and Read 1996). For this reason, it is suggested that investigations of positional candidates for RP17 be continued in conjunction with the positional cloning strategy.

CHAPTER SIX

CONCLUDING REMARKS

6

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6.1 GENERAL DISCUSSION AND FUTURE PROSPECTS

This dissertation describes the progress made towards elucidation of the molecular basis of adRP in a large SA family. Genetic linkage analysis was used to establish linkage of the adRP phenotype in this family to a region on chromosome 17q22 (designated the RP17 locus; Bardien et al. 1995). The disorder in a second SA adRP family was subsequently linked to the same locus (Bardien et al. 1997).

The identification of RP17 illustrates that genetic linkage analysis using lod scores is a powerful and efficient tool for mapping disorders in large families exhibiting multiple meioses. Before this method can be implemented, however, prior knowledge is required concerning the mode of inheritance, gene frequencies and the penetrance of the disorder. The identification of this novel locus, the eighth for adRP, further demonstrates the genetic heterogeneity of this group of conditions.

Haplotype analyses suggested that the disorder in the two unrelated SA kindreds might be as a result of the same pathogenic mutation in the same gene. The two families share a common disease-associated haplotype, which spans 12 cM, even though they are from ethnically diverse origins and reside in different geographical locations in SA. This finding may indicate that the families have a common ancestor and that they have diverged relatively recently. The eventual identification of the disease gene and the pathogenic mutation should resolve this debate. Recently, den Hollander et al. (1999) demonstrated linkage in a large Dutch adRP family to the RP17 locus. It would be interesting to compare the disease-associated haplotype in the Dutch family with that of the SA families. The Dutch group also excluded two additional candidate genes

for RP17 viz. the retina-specific amine oxidase gene (*AOC2*) and the cone transducin gamma gene (*GNGT2*). *GNGT2* is, however, not a likely candidate for RP since it is expressed exclusively in cone cells.

The identification of the disease-associated haplotype has important implications for pre-symptomatic members of the two kindreds. However, the potential advent of a predictive test based on genetic linkage using polymorphic markers raises ethical, moral and psychological issues. Pre-symptomatic testing in the RP17-linked families will therefore only be implemented with appropriate genetic counselling and guidelines (Greenberg et al. 1996).

RP17 was refined to a 1 cM region (Bardien-Kruger et al. 1999) which is sufficiently small to facilitate positional cloning techniques in an attempt to identify the adRP gene. In this regard, a physical map comprising of YACs and BACs, which span the RP17 region, was constructed. This contig consists of overlapping clones and contains both flanking recombinant markers thereby ensuring that the RP17 gene is present. The use of fluorescent *in-situ* hybridisation analysis in order to verify that the clones are not chimaeric would be a logical next step in this study. Moreover, it may prove useful to construct a cosmid or P1 contig for the RP17 region because these clones tend to retain their human DNA inserts, which is crucial for gene isolation experiments. However, more of these smaller clones are required than YACs in order to provide full overlapping coverage of the critical interval.

The cDNA selection technique was utilised in an attempt to generate a transcription map of the RP17 critical interval. This approach was applied to one of the YACs from the RP17 contig with the use of a foetal brain cDNA library. The presence of a 23 bp contaminant was detected and it is essential that this sequence be screened out before further

analyses of the transformants can be undertaken. It would also be advisable to repeat the selections with a retina-specific cDNA library, which should generate fewer candidate genes, thereby increasing the chances of identifying the adRP gene. Furthermore, it would be appropriate to subject the other six YACs and two BACs to cDNA selection and other gene isolation experiments in order to identify all the transcription units in the region. Genes identified in this proposed extensive study would first need to be fully characterised in terms of their expression-pattern, exon-intron boundaries, promoter regions etc. Each of the genes would then have to be investigated in turn for evidence implicating them as the adRP gene. An important observation is that the cloning of genes from the RP17 contig would also provide candidates for other disorders that map to 17q22.

Four positional candidate genes residing on 17q (viz. *PDEG*, *TIMP2*, *PKCA* and *retinal fascin*) were excluded from being associated with the adRP phenotype in the RP17-linked families. Candidate gene screening is set to dominate the field of disease-gene isolation and as additional candidates for RP17 are identified it is advisable that they be investigated in the two SA families. Other potential candidates include the *TBX2* gene which was shown to reside on one of the clones in the RP17 contig viz. YAC 961f1 (Law et al. 1995). It is imperative that the computerised databases and the current literature be scrutinised on a regular basis to reveal additional positional candidates for study.

An important future extension of this study would be to further investigate the canine prcd locus, postulated to be the canine homologue of RP17 (Acland et al. 1998). Genetic linkage analysis facilitated the mapping of the prcd locus to an approximately 5 cM region between the TK1 and GALK1 genes, thereby excluding the RARA [retinoic acid receptor alpha gene] (Acland et al. 1998). RARA had been considered to

be a potential candidate for RP17 due to its role in retinol (vitamin A) metabolism (Petkovich et al. 1987).

One of the two strategies used in the present study, positional cloning, is an arduous and time-consuming approach for the identification of disease-causing genes. This is illustrated by the fact that *RPGR* and *RP2* are the only two RP genes that have been isolated using this method (Table 6.1). The first RP gene to be identified using positional cloning was *RPGR*. This gene was mapped in 1988 to chromosome Xp21.1 and cloned in 1996 (Musarella et al. 1988; Meindl et al. 1996). In the case of RP2, the gene was mapped in 1984 to Xp11.3 by linkage analysis with a DNA marker L1.28 (Bhattacharya et al. 1984). The causative gene was isolated using positional cloning more than a decade later in 1998 (Schwahn et al. 1998). The candidate gene approach has been more successful with the identification of a number of RP genes including *rhodopsin*, *PDEA* and *PDEB* (Table 6.1). In this technique the DNA sequence of the candidate gene is examined for mutations in affected individuals, which can be rather labour-intensive when the genes are large and consist of many exons. Currently, these are the only strategies available for studies on RP since the primary biochemical defects in this group of disorders are still unknown. The conventional approach used to clone genes, functional cloning, requires pre-existing knowledge about the pathogenesis of the disorder. A systematic study of tissues from individuals affected with RP may lead to a consistent biochemical clue, thereby making this approach plausible in the future.

Table 6.1 Strategies used for identification of the genes implicated in retinitis pigmentosa.

Disease-causing gene	Disorder	Strategy used	Reference
Rhodopsin	adRP	Linkage + candidate gene approach	Dryja et al. 1990
Peripherin/RDS	adRP	Linkage + candidate gene approach	Farrar et al. 1991
PDEB	arRP	Candidate gene approach	McLaughlin et al. 1993
ROM1	Digenic RP	Candidate gene approach	Kajiwara et al. 1994
PDEA	arRP	Candidate gene approach	Huang et al. 1995
CNGC	arRP	Candidate gene approach	Dryja et al. 1995
Myosin VIIA	Usher syndrome type 1B	Linkage + candidate gene approach	Weil et al. 1995
RPGR	xlRP	Positional cloning	Meindl et al. 1996
RLBP1	arRP	Linkage + candidate gene approach	Maw et al. 1997
RP2	xlRP	Positional cloning	Schwahn et al. 1998

Reproduced in part from Gonzalez-Duarte et al. 1997.

6.2 MODELS FOR PATHOGENESIS OF RP

6.2.1 Apoptosis

Molecular genetic investigations such as the present study are important to elucidate the molecular patho-physiology of inherited retinal disorders. There are currently numerous hypotheses regarding the pathogenic mechanism responsible for the death of rod and cone cells in RP. One theory implicates apoptosis, a form of programmed cell death, for the loss of photoreceptors (Lolley et al. 1994; Gregory and Bird 1995). In apoptosis, genetic switches in the cell initiate the turning off of normal cellular activity, resulting in DNA fragmentation, condensation of nuclei and cell death. This process is a genetically encoded potential of all cells and it plays an essential part in embryonic development. Apoptosis has been shown to be the cause of photoreceptor cell death in mouse models for RP involving mutations in three different genes viz. rhodopsin, peripherin/rds and the β subunit of phosphodiesterase (Chang et al. 1993). In addition, in the Royal College of Surgeons (RCS) rat model, where expression of the primary genetic defect is in the RPE, the biochemical hallmarks of apoptosis were observed in the photoreceptor cells (Mullen and Lavail 1976). Cell death in some forms of retinal degeneration may therefore not be a direct consequence of the intrinsic genetic defect but may represent a reaction to a change in the metabolic environment of the tissue. It would be interesting to establish whether apoptosis is also responsible for rod and cone cell death in the RP17-linked families.

6.2.2 Constant equivalent light model

Another proposed pathogenic mechanism is the 'constant equivalent light' model (Lisman and Fain 1995). Prolonged exposure of the retina to constant illumination is known to produce photoreceptor cell death in rodents (Noell et al. 1966). Some of the molecular defects associated with

RP are thought to produce electrical signals 'equivalent' to those produced by real light and they thereby trigger degeneration by the same mechanism. Strong support for this hypothesis are the observations that mutations in both the cGMP-gated ion channel gene and the chromophore-opsin attachment site (codon 296) of rhodopsin are causative of RP (Dryja et al. 1995; Lisman and Fain 1995). These mutations are thought to lead to over-stimulation of the phototransduction cascade, a situation which is similar to constant light exposure.

6.3 CONCLUSION

Once the genetic defect responsible for RP17 has been identified, a future extension of the research would be to produce an animal model to determine the exact mechanism of cell death. Rodent models of RP have a rapid rate of photoreceptor cell degeneration, which makes a study of the protracted phase of cone degeneration not feasible. Larger animal models such as the rhodopsin transgenic pig (Petters et al. 1997) have a slower rate of cell death and the size and structure of the porcine eye is roughly similar to that of humans. These features make this species more suitable for the study of human retinal degenerative disorders.

In the long term, the question of treatment for the RP17-linked families could be addressed, once an animal model and the appropriate gene-therapy vectors have been established. The work presented in this dissertation provides the basis for future research, which will have the potential to ultimately contribute towards development of effective therapy for this currently untreatable disorder.

The mapping of RP17 and other RP loci in our laboratory provided the impetus for the establishment of a registry for retinal degenerative disorders. In this regard, the genealogical and ophthalmological details on every individual and family member are entered into our computerised database. This bank of information has the potential to be extremely valuable for the future management and treatment of affected individuals and their families, and the Department is now recognised as a national referral centre for retinal degenerative disorders in South Africa.

In conclusion, the main findings resulting from this investigation are:

- assignment of the disease gene to a 1 cM region on 17q22
- linkage of a second SA family
- establishment of a potential predictive test for the two adRP families
- exclusion of four candidate genes
- construction of a contig which is a valuable resource for the eventual cloning of the adRP gene as well as other genes.

With the explosion of sequence and structural information available to researchers, the burgeoning field of bioinformatics, or more properly, computational biology, is playing an increasingly important role in the study of fundamental biomedical problems. The challenge facing scientists, especially in the light of the vast amount of data being generated by the Human Genome Project, will be to use the publicly-funded electronic databases [such as the Genome Database (GDB) and the National Center of Biotechnology Information (NCBI)] and computer software to help solve complex biological problems. With the tools that are available for this new age of sequence-based biology, the coming years should prove very exciting and fruitful for all projects involving the search for human disease genes such as RP17.

APPENDIX A, B & C

APPENDIX A

A.1 Department of Human Genetics request form for biological specimens.

REQUEST FOR MOLECULAR STUDIES (DNA)

Molecular Laboratory
Department of Human Genetics
1st Floor, Anatomy Building
UCT Medical School, Observatory, 7925

Tel: (021) 406 6425 Fax: (021) 477703

Please fill in all the information requested

Blood should be drawn in 3 plastic EDTA tubes (Purple top) +/- 10 mL each using a yellow barrel. Each tube should be inverted to mix.

Keep blood in fridge at 4°C until able to send to laboratory.

Please DO NOT send specimens on ice or frozen.

Surname:_____ First Name(s): _____

New Family: Yes ☐ No ☐ (If no, please fill in family name) Family name:_____

Medical Aid:_____ Medical Aid No.: _____

Sex: M ☐ F ☐ Date of Birth: Year:_____ Month:_____ Day:_____

No. of Children: _____

Ethnic Origin: Black ☐ Indian ☐ Mixed ancestry ☐ White ☐ Other ☐_____

Contact Address: _____ Town:_____ Fax: Tel: _____

Referring Doctor/Sister: _____ Town:_____ Fax: Tel: _____

Hospital or Address: _____ Town:_____ Fax: Tel: _____

Reason for Referral (Clinical diagnosis):

Affected ☐	At Risk ☐	Carrier ☐	Spouse ☐	Query ☐	Unaffected ☐
Becker Muscular Dystropy ☐		Duchenne Muscular Dys. ☐		Colonic Carcinoma	☐
Fragile X Syndrome	☐	Haemophilia A	☐	Huntington Disease	☐
Retinitis Pigmentosa	☐	Spinocerebellar Ataxia	☐	Waardenburg Syndrome	☐

Additional disorders (apparent or previously treated): _____

_____

Relevant Clinical Details:

Physical disability ☐ Mental Retardation ☐ Deafness ☐ Impaired vision ☐ Night blindness ☐

Other _____

_____

For Laboratory use only

*DNA Number:*_____ *Vol.Blood:*_____ *ml.* *Other:*_____

*Date Received: Year:*_____ *Month:*_____ *Day:*_____ *Computer Index No:* _____

CONSENT FOR DNA ANALYSIS AND STORAGE

I, Mr/Ms/Mrs/_____
request that an attempt be made to assess the probability that I, my children or my unborn child might have inherited the gene for

_____

I understand that the DNA for analysis is to be obtained from:
a) blood cells, b) skin sample, c) other: _____

The methods and risks of obtaining the specimen have been explained to me.

I also understand that:
1. The test procedure is specific to the genetic condition mentioned above and cannot determine the complete genetic makeup of an individual.
2. Lack of cooperation by key relatives in providing blood samples may decrease the accuracy of the test result for linkage analysis and/or ability to perform the test.
3. Sometimes the genetic pattern in a family renders the test results "uninformative" (not clarifying the point in question).
4. Even under the best conditions, current technology of this type is imperfect, and may lead to incorrect results.
5. I may withdraw my consent at any time without giving a reason and without this affecting my future medical care.
6. The DNA bank is under an obligation to respect medical confidentiality.

A portion of the DNA obtained may be stored to enable later testing. The following conditions apply to my DNA that is being stored: (circle any and all that you wish)

A. To be used for linkage analysis for members of my immediate family.

B. To be used for research purposes without my or my family member's knowledge, at the discretion of the Head of Human Genetics, UCT provided that any information from such research will remain confidential.

C. To be used without any restrictions.

D. Other_____

I understand that I may change my mind and withdraw my consent for DNA banking at any time.

ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY:_____ Date _____19___

_____ _____
Patient signature Witnessed consent

Pedigree Drawing

APPENDIX B

Techniques:

B.1 DNA isolation from lymphocytes

B.2 RNA isolation from transformed lymphoblasts

B.3 DNA oligonucleotides

B.4 Southern blot analysis

B.5 Single strand conformation polymorphism analysis (SSCP)

B.6 DNA sequencing

B.7 YAC agarose plugs

B.8 Pulsed-field gel electrophoresis (PFGE)

B.9 cDNA selection

B.1 DNA ISOLATION FROM LYMPHOCYTES

DNA was isolated using a modified version of the Genomix kit (Talent, Italy) as described below:

- 10 ml of frozen venous blood was thawed at 37°C and added to 2 ml of Blood Washing Solution (BWS). After centrifugation at 7000 rpm for 15 min at room temperature, the pellet was resuspended in 5 ml of BWS. The centrifugation step was repeated.
- The pellet was resuspended by vortexing in a mixture of 1 ml BWS and 1 ml of water.
- After addition of 4 ml of Lysing solution, the mixture was incubated at 68°C for 7 min.
- The lysate was transferred to a 14 ml gel barrier tube and chloroform was added with vigorous mixing in order to mix the two phases. The phases were separated by centrifugation at 5 000 rpm at 4°C for 30 min. The upper aqueous phase was poured into a 30 ml Corex glass tube, which had previously been treated with diethyl pyrocarbonate.
- 7 ml of Precipitating solution was added and mixed gently by slow inversion until a filamentous DNA precipitate was visible.
- After removal of the liquid phase, the precipitate was resuspended in 4 ml of Ionic Exchange solution and left O/N at RT.
- The following day, 8 ml of ethanol was added and the DNA precipitate was washed twice in 5 ml of 70% Ethanol and transferred to a 1.5 µl Eppendorf tube. Following centrifugation at 13 000 rpm for 1 min, all the supernatant was removed and the DNA pellet was air-dried. Finally, the DNA was resuspended in 200 µl of water and the concentration was calculated from the absorbance at 260 nm (1 OD unit = 50 µg/ml).

B.2 RNA ISOLATION FROM TRANSFORMED LYMPHOBLASTS

Where appropriate all the reagents and equipment used in this protocol were treated with diethyl pyrocarbonate (DEPC) prior to use.

- 10 ml of EBV-transformed lymphoblasts were centrifuged in a polypropylene tube at 2000 rpm for 5 min and the pellet was washed in 1 ml of phosphate buffered saline (PBS).
- The following solutions were added sequentially, with vortexing after each addition:

Solution D	1 ml
2M Na Acetate (pH 4)	0.1 ml
water saturated phenol	1 ml
chloroform:isoamyl alcohol (49:1)	0.2 ml

- The reaction was incubated on ice for 15 min and then centrifuged at 10 000 g for 20 min at 4°C.
- The RNA is in the top aqueous phase and 750 µl of this layer was removed to a microfuge tube, 750 µl of isopropanol was added and the RNA was precipitated O/N at -20°C.
- The following day, the RNA was pelleted by centrifugation at 10 000 g for 10 min, washed with 70% ethanol, airdried and finally resuspended in 20-50 µl distilled water.

i) First strand cDNA synthesis:
2.5 µg of RNA was reverse transcribed with AMV reverse transcriptase (Promega, USA) using oligo dT as a primer. The reaction was incubated for 1 hour at 42°C and in order to yield sufficient cDNA, 3 cycles of linear amplification of the RNA were performed.

ii) The reverse transcribed RNA was PCR amplified using the appropriate primers and the product was directly sequenced.

B.3 DNA OLIGONUCLEOTIDES

All DNA oligonucleotides used in this study as primers for PCR or as probes in Southern blot analysis were obtained from the following sources:

- Research Genetics (USA)
- GIBCO/BRL (USA)
- Department of Biochemistry at UCT
- overseas collaborators

B.4 SOUTHERN BLOT ANALYSIS

7.5 μg of genomic DNA was digested to completion with the appropriate restriction enzyme and separated by electrophoresis on 0.6% agarose gels O/N at 45 W.

B.4.1 Transfer to nylon membranes

The DNA was transferred O/N from the gel to Hybond N^+ membranes (Amersham, UK) using 0.4 M NaOH.

B.4.2 Hybridisation

[α^{32}P]dCTP- labelled probes were prepared by one of two methods:

(i) The Multiprime DNA labelling system (Amersham, UK)

(ii) The Rediprime DNA labelling system (Amersham, UK)

These protocols were performed according to the manufacturers' instructions. The blots were first pre-blocked in a hybridisation solution containing Salmon Sperm (Appendix C) and then hybridised to the denatured probes O/N at 65°C. The following day, the membranes were washed over three stages:

- 3X SSC at RT for 10 min
- 3X SSC/ 01% SDS at 65°C for 30 min
- 0.6X SSC/ 0.3% SDS at 65°C for 30 min

B.4.3 Autoradiography

Autoradiographs were obtained by exposure of the membrane to X-ray film (Agfa Curix, Germany) O/N at -70°C.

B.5 SINGLE STRAND CONFORMATION POLYMORPHISM ANALYSIS (SSCP)

The exonic sequences of genes were PCR amplified in 200-300 bp fragments. An equal volume of SSCP loading dye was added and the samples were loaded on MDE gels:

B.5.1 Gel matrix

The gels consisted of 0.5X MDE matrix (FMC BioProducts, USA), 0.6X TBE and either with or without 10% glycerol. The glass plates used for the gels were 380 cm long and 300 cm wide; and the combs and spaces were 0.375 mm thick.

B.5.2 Electrophoresis conditions

PCR products were denatured at 95°C for 5 min, chilled on ice for 10 min and loaded on the MDE gels. The gels were electrophoresed O/N at 2 to 6 W (depending on fragment size) on an in-house gel tank system with 0.6X TBE running buffer.

B.5.3 Silver staining

In order to visualise the bands the gels were stained over three stages in:

- 0.1% Silver Nitrate for 20 min
- 1.5% NaOH/ 0.15% formaldehyde for 10 min
- 0.75% Sodium Carbonate for 10 min

The gels were washed in distilled water between each solution.

B.6 DNA SEQUENCING

Direct DNA sequencing was performed by one of three different methods:

- The Sequenase II sequencing Kit (Amersham, USA)
- The SequiTherm EXCEL II DNA Sequencing Kit (Epicentre Technologies, USA)
- ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, USA).

In each case, the protocols were followed according to the manufacturer's instructions. In the first two methods, [α^{32}P]dCTP was incorporated into the sequencing reactions which were subsequently electrophoresed on 6% denaturing polyacrylamide gels at 60 W in 1X TBE. After the run, the gels were dried at 80°C and autoradiographed at -70°C. For the ABI Kit, the sequencing reactions were separated by automated capillary electrophoresis and analysed by an ABI PRISM Genetic Analyser (Perkin Elmer, USA).

B.7 YAC AGAROSE PLUGS

- 50 ml cultures were grown for 2 days in casein media containing Ampicillen (100 μg/ml).
- The cells were pelleted at 3000 rpm for 5 min, washed with distilled water and recentrifuged.
- The pellet was resuspended in 1 ml SCE and 7 μl beta-mercaptoethanol (BME) and incubated at 50°C.
- 3 ml of 2% InCert agarose (FMC BioProducts, USA) containing 0.01 g/ml yeast lytic enzyme (ICN) in SCE was prepared and added to the cell mixture at 50°C.
- This mixture was quickly dispensed into the slots of a plug mould (Biorad, USA) and allowed to set at 4°C for 25 min.
- The plugs were washed in:

  (i) 20 ml SCE, 140 μl BME and 10 mg/ml YLE at 37°C for 5 hrs.

  (ii) 10 ml lysis solution and 1 mg/ml proteinase K at 50°C O/N.

- The following day, the plugs were washed 3X in TE with gentle shaking at room temperature for 15min.
- The plugs were stored in 0.05 M EDTA at 4°C and were stable for up to a year under these conditions.

B.8 PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

200 ml of 1% Pulsed-field Certified agarose (Biorad, USA) gel was prepared in 0.5X TBE. Once the gel had set, the YAC agarose plugs were loaded into the wells and sealed in using 1% Pulsed-field Certified agarose. After 20 min, the gel was placed in a BIORAD CHEF DR II Mapper System, allowed to cool to 14°C for 30 min and run at the appropriate conditions in 0.5X TBE. Upon completion of the run, the gel was stained for 30 min in 400 ml of 0.5X TBE containing 20 µl of 1% Ethidium bromide. The gel was then destained at 4°C O/N and the following day, the gel was photographed under UV.

B.9 cDNA SELECTION

- 200 ng of YAC DNA was biotinylated using a Nick Translation Kit (Boehringer Mannheim, Germany) and biotin-16-2'-deoxyuridine-5'-triphosphate according to the manufacturer's instructions.
- 10 ng of the foetal brain cDNA library was amplified in ten identical 25 µl PCR reactions which were then pooled and eluted through a Sephadex G-50 spin column.
- Following elution, the starting cDNA was ethanol precipitated and resuspended in TE to a final volume of 1 µg/ µl.
- The cDNA was blocked with COT-1 DNA, AB1380 DNA and pBluescript vector in a total volume of 10 µl. The reaction was overlaid with mineral oil and the DNA was denatured at 95°C for 5 min. After addition of 10 µl of 2X hybridisation buffer, the reaction was incubated at 65°C for 4 hrs.

- 5 µl of biotinylated YAC DNA was denatured at 95°C for 5 min, mixed with 5 µl of 2X hybridisation solution and immediately added to the blocked cDNA. The hybridisation was allowed to proceed at 65°C for at least 54 hrs.
- After hybridisation, the biotinylated YAC DNA with the hybridised cDNAs was captured on streptavidin-coated paramagnetic Dynabeads (DYNAL, Norway).
- The hybrids were washed:

  i) 2X in 1 ml of wash solution 1 (1X SSC/ 0.1% SDS) for 15 min at RT

  ii) 3X in 1 ml of wash solution 2 (0.1X SSC/ 0.1% SDS) for 15 min at 65°C.

  At each washing cycle, the beads were removed from solution with a magnetic particle separator.
- After the final wash, the beads were resuspended in 50 µl of freshly-prepared 0.1 M NaOH at RT for 20 min and concentrated in a magnetic particle separator for 1 min.
- The supernatant containing the eluted cDNA was transferred to a 1.5 µl eppendorf tube, neutralised with 50 µl of 1 M Tris-HCl (pH 7.5) and passed through a Sephadex G-50 spin column. This eluted material constitutes the primary selected cDNAs.
- These cDNAs were PCR amplified and passed through a second round of selection in order to generate the secondary selected cDNAs.
- The secondary selected cDNAs were then cloned and analysed.

APPENDIX C

Media, Buffers and Solutions:

C.1 casein media

C.2 TBE (1X)

C.3 formamide loading dye

C.4 6% denaturing polyacrylamide gels

C.5 SSCP loading dye

C.6 hybridisation solution for Southern blots

C.7 cDNA selection buffers

C.8 SCE

C.9 lysis solution

C.10 Solution D

C.1 Casein media

For 1L:

6.7 g yeast nitrogen base without amino acids

10 g casamino acids

20 g Dextrose

0.07 g Adenine (hemisulphate)

Add 15g Bacto-Agar for plates.

C.2 TBE (1X)

0.089 M Tris

0.089 M Boric acid

0.002 M EDTA (pH 8.0)

C.3 Formamide loading dye

80% formamide

10 mM EDTA (pH 8.0)

0.1% xylene cyanol

0.1% bromophenol blue

C.4 6% denaturing polyacrylamide gels

5.7% acrylamide

0.3% bis-acylamide

7.65 M urea

1X TBE

C.5 SSCP loading dye

95% formamide

20 mM EDTA (pH 8.0)

10 mM NaOH

0.02% xylene cyanol

0.02% bromophenol blue

C.6 Hybridisation solution for Southern blots

1 M NaCl

1% SDS

5 % Dextran sulphate

10% PEG 6000

100 μg per ml denatured Salmon Sperm

C.7 cDNA selection buffers

Binding buffer

10 mM Tris (pH 7.5)

1 mM EDTA

1 M NaCl

2X hybridisation solution

1.5 M NaCl

40 mM Sodium phosphate (pH 7.2)

10 mM EDTA (pH 8.0)

10X Denhardt's solution

0.2% SDS

C.8 SCE

1 M sorbitol

0.01 M sodium citrate

0.06 M EDTA (pH 7.0)

C.9 Lysis solution

10 mM Tris

1% n-Lauroyl sarcosine

0.45 M EDTA (pH 7.8)

C.10 Solution D

Stock solution:	Guanidium thiocyanate	50 g
	Distilled water	58.6 ml
	0.75 M Na Citrate (pH 7)	3.5 ml
	10% Sarkosyl	5.3 ml

Solution D working solution: Add 72 µl beta-mercaptoethanol to 10 ml stock

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